INTERNATIONAL CONFERENCE ON

Harmful ALGAL BLOOMS

Ninth Conference
TASMANIA
2000

7-11 February 2000
Hobart, Tasmania, Australia

CONFERENCE PROCEEDINGS

Intergovernmental Oceanographic Commission

HARMFUL ALGAL BLOOMS 2000

Proceedings of the Ninth International Conference
on Harmful Algal Blooms
Hobart, Australia, 7-11 February 2000

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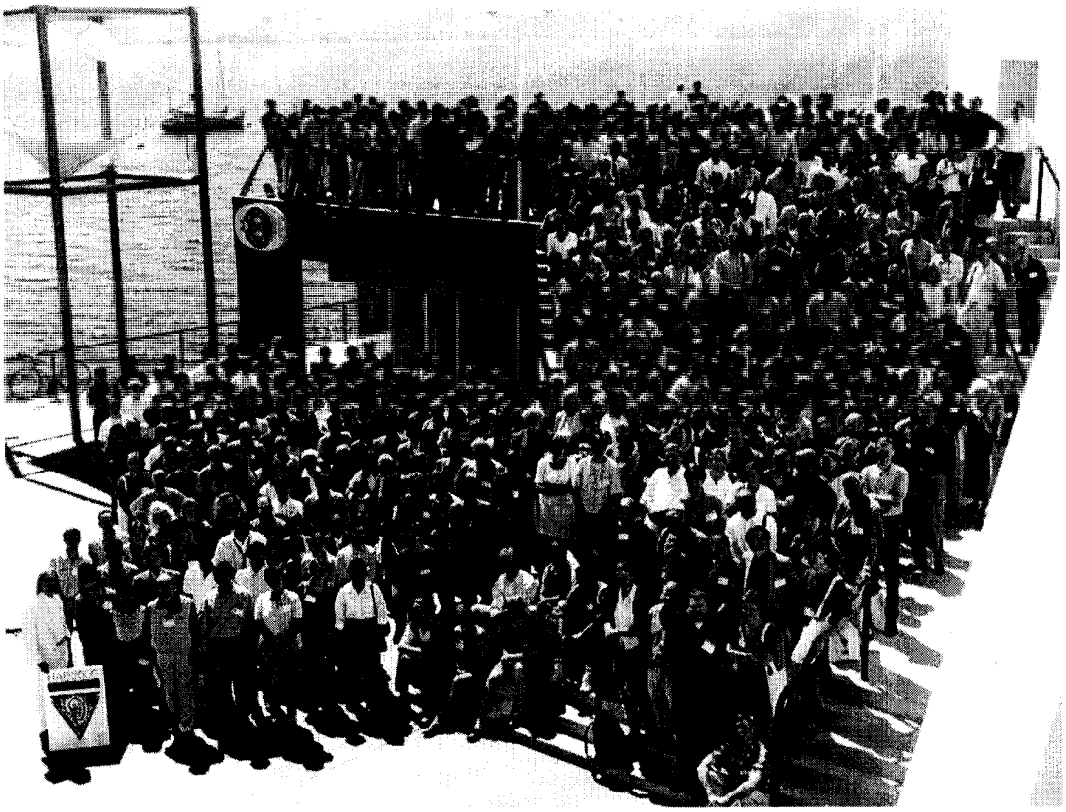
PREFACE

The 9th International Conference on Harmful Algal Blooms (HAB2000) was held in Hobart (Tasmania, Australia) from 7 to 11 February 2000. The conference venue was the Wrest Point Convention Centre located on the magnificent foreshores of the Derwent River estuary. HAB2000 brought together a record number of 526 participants (473 full and 43 day registrations, including 87 students) from 47 countries. This was the first time this prestigious conference series was scheduled in the Southern Hemisphere, but this appeared not to have deterred strong participation from Europe (122), USA (77) and South East Asia (84).



A total of 130 talks and 308 posters were presented, and where parallel sessions were scheduled the plenary introductory session featured reviews covering the full range of topics. A total of 472 abstracts, organised as a searchable database, were made available via internet during the months preceding the meeting and for registered conference participants this complete conference abstract record is also included in CD-ROM format with this Proceedings volume. The scientific programme of HAB2000 focused on new algal bloom species and new toxic phenomena, new regional bloom events, ecophysiology and bloom dynamics, cysts and sediments, eutrophication, novel toxins, ecophysiology of toxin production, exotoxins, foodchain effects, population genetics, molecular probes, immunological methods, role of bacteria, and impacts on finfish and shellfish aquaculture operations. Being held in Australia, this was the first conference in this series which prominently featured problems caused by freshwater cyanobacterial blooms, including impacts of cyanotoxins on human health and aquatic foodwebs, and aspects of drinking water treatment. A special session on international algal bloom programmes and future conferences, as well as a dedicated session on algal bloom control and management were also scheduled. The opening address was presented by Dr Meryl Williams (pp.1-2), director-general of the International Commission of Living Aquatic Resources Management (ICLARM), while the closing address was given by Prof. Max Taylor (pp.3-7), to whose

life-time pioneering achievements this conference was dedicated. Summaries of some of the conference sessions were published in *Harmful Algae News* (Intergovernmental Oceanographic Commission) no. 20, May 2000. Our thanks go to the able chairpersons of the various conference sessions : D.Anderson, D.Baden, S.Blackburn, C.Bolch, M.Bricelj, J.Burkholder, P.Busby, A.Cembella, G.Codd, B.Dale, I.Falconer, Y.Fukuyo, S.Gallacher, I.Garthwaite, E.Graneli, G.Jones, R.Lewis, M.Quilliam, J.Ramsdell, J.Rensel, C.Scholin, G.Shaw, S.Shumway, T.Smayda, C.Thomas, P.Thompson and T.Yasumoto.

The conference period was blessed with glorious summer weather and a total of 255 participants enjoyed mid-conference excursions to Tasmanian wilderness forests, wildlife park, and local shellfish and aquaculture operations. Thanks go to R. Barbour, R. Barnes, T. Brodribb, R. Brown, J. Hickey, G. Jordan, C. Lapworth, P. Lee, J. Marshall, I. Pearce, M. de Salas, L. Schimanski and J. Smith, who acted as excursion guides.

Considerable debate occurred both before and during the conference on the pros and cons of various conference publication options. While the local conference organisers proposed to raise the quality and visibility of the conference publication by seeking to publish them as a special issue(s) of the international journal *Phycologia.*, the international organising committee of this and previous meetings in this conference series expressed strong views to continue the tradition of 4 page conference papers, among others to create a publication outlet for preliminary student reports and submissions from scientists in developing countries who may have difficulty to publish in the primary literature. Accordingly, it was resolved to revert for the bulk of the conference manuscripts to the traditional 4 page IOC Proceedings format. In making our selection out of some 145 manuscript submissions, we have been guided by the referee comments of the specialists listed on the following pages.

To satisfy the wishes of authors who still preferred publication in the primary literature 17 full conference manuscripts have also been published as a special volume of the journal *Phycologia* (volume 40, part 3, 2001), edited by S. Blackburn and G. Hallegraeff as guest editors, and D.Mann as overseeing journal editor. Unfortunately, we did not have sufficient finances available to provide reprints of these *Phycologia* volumes to all HAB2000 conference participants.

At the 8th International Conference in Vigo, the offer by Karen Steidinger to host the 10th Conference in this series in St Petersburg, Florida (USA) was accepted by consensus, and this offer was confirmed during HAB2000 for scheduling in October 2002. Conference participants voted in favour of an offer by Grant Pitcher to host the 11^{th} conference in South Africa in 2005.

A meeting of the International Society for Harmful Algae (ISSHA) was chaired by the outgoing President Max Taylor, and formalised the election of Karen Steidinger, Yasukatsu Oshima, and Allan Cembella as Vice-Presidents, Stephen Bates as Secretary, and Henrik Enevoldsen as Treasurer. Following HAB2000 Karen Steidinger became the new President of ISSHA.

Thanks to the generous financial support of the Intergovernmental Oceanographic Commission of UNESCO, IOC-WESTPAC, the Scientific Commission for Oceanographic Research (SCOR), the US Environmental Protection Agency, the Mediterranean Action Plan of UNEP, the Organisation for the Prohibition of Chemical Weapons (OPCW), and the International Society for the Study of Harmful Algae (ISSHA), some 35 graduate students and scientists from developing countries were able to participate in this conference.

The logistics of organising this meeting were facilitated by the staff of Conference Design, of which Penny Archer and Ben Thiessen deserve special mention. We thank Judi Marshall and other student helpers at the University of Tasmania for organisational help, and Jeannie-Marie LeRoi and Jane Quon and co-workers for greatly enriching the conference with their theatre and art events, respectively. Steve Eastwood took the HAB2000 group picture and Jane Bailey, Valerie Dragar and Miquel de Salas provided expert help in producing the final typescript.

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Harmful
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Ninth Conference
TASMANIA
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OPENING ADDRESS

Harmful
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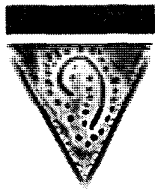

Ninth Conference
TASMANIA
2000

MANAGEMENT STRATEGIES FOR HARMFUL ALGAL BLOOMS

Opening Address, Ninth International Conference on Harmful Algal Blooms
7-11 February 2000, Tasmania, Australia

Meryl J Williams and Violeta Q. Perez-Corral

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PO Box 500, GPO 10670 Penang, MALAYSIA

The increasing incidence and consequent negative impacts of the phenomenon that is more popularly known as 'red tide' have brought all of us here together today. We all know, of course, that red tide is largely a misnomer as the events are not generally associated with tides, nor do harmful algal species necessarily reach densities that discolor our oceans and marine waters.

Harmful algal bloom (HAB) events have caused severe habitat degradation, economic losses and untold human miseries.

Although the economic losses due to HABs cannot be readily determined due to the broad range of sectors affected, many partial estimates have been made for individual cases. For instance, a 1997 bloom in southeast China cost about US$8 million; the 1998 fish kill in Hong Kong somewhere between US$10-30 million.

But, these figures do not reflect the impacts on human health and food and nutritional security, especially among the poor in developing countries. Nor do they value the long term damage to marine ecologies.

Good research on HABs is vital.

I understand that this research first emerged as a discipline at the First International Conference on Toxic Dinoflagellates held in Boston (USA) in 1974. About a decade later, new international initiatives had emerged to explore the links of HABs to human activities and environmental changes. The International Oceanographic Commission (IOC) created an international framework program on HABs in the late 1980s and an IOC HAB Programme in 1991. Now, the GEOHAB or Global Ecology and Oceanography of HABs, drawn up in 1998, presents an international plan for coordinated scientific research and cooperation.

The first regional network on harmful algae, the ASEAN-Canada Red Tide Network, was created in 1993 to link the seven ASEAN countries and Canada. The network acts as an information clearinghouse about red tide events in the region and the latest research breakthroughs.
Trade-related initiatives on HABs have also emerged in recent years. In 1995, the Asia Pacific Economic Cooperation (APEC) economies Red tide/HAB project was initiated to coordinate the monitoring and management of HABs in the Asia Pacific region. Its ultimate objective is to facilitate the free flow of traded shellfish and fish products. These efforts are similar to those of the European Union that aimed to establish import and export guidelines and 'red-tide free' certification of affected marine products.

Scientific research and trade-related endeavors converged during the First International Conference on Harmful Algae Management and Mitigation (HAMM) in Subic Bay, Philippines in May 1999.

What I would like to bring to your attention today are some management strategies currently being undertaken by governments in this part of the globe in efforts to combat the impacts of red tides and HABs. I would also like to talk about the 'people' dimension of aquatic resources management. Our research at ICLARM ultimately aims to benefit the poor majority in developing countries who depend on aquatic resources for food and livelihoods.

Several management approaches have already been identified to prevent the occurrence and mitigate the effects of HABs. Regular monitoring of red tide-affected areas and the timely banning of the harvest of shellfish products need to be done, along with public awareness campaigns and provision of alternative sources of livelihood to affected fishing communities, especially among small scale fishers, including women and children.

More pro-active strategies include the formulation of a Shellfish Sanitation Program, instituting HABs as part of the Environmental Impact Assessment or EIA process, and red tide preparedness as part of coastal zone management programs.
One important pro-active strategy is to prevent the introduction of new species or strains of the organisms that cause harmful algal blooms, such as through the control of introductions made via ships' ballast water.

As Australia well knows, algal cysts are potential cargoes of ballast water.

Ballast water management requires international cooperative action, integrated with national management approaches. In 1994, Australia adopted a national strategy for improved ballast water management. Federal

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and State Government Departments, industry and other non-government sectors actively participated in its formulation, including those from such diverse interests as international and domestic shipping, coastal communities, environment groups, ports and harbors, human health, seafood production, quarantine, fisheries and aquaculture. The Australian Quarantine and Inspection Service led development of the Strategy.

The Australian Water Ballast Management Advisory Council, which I currently chair, was formed in 1996. In mid-1998, the Federal Government with the support of the Australian shipping industry, imposed a levy on all ships arriving in Australian ports to raise $1 million a year for the next two years in support of our research and development efforts.

There are recent efforts to broaden the scope of Australian actions to prevent and manage all marine pest incursions. These actions include emergency responses and longer-term mitigation and control measures. Federal and state ministers are expected to decide on the broader scope of actions in March.

Let me now turn to the HAB situation in the Philippines, where 70% of towns are located along the coast and shellfish is a common cheap source of protein. Roughly 10% of Filipinos eat shellfish in any given day. Hence, when HAB-affected areas are quarantined during toxic red tides, many poor people are deprived of their usual sources of protein. The market is also deprived of its usual seafood products. This, in turn, causes negative impacts on the shellfish industry and on subsistence fishers, as well as consumers of seafood products.

In 1999,the inter-agency National Red Tide Task Force released the Philippine Guidebook on Toxic Red Tide Management. The Guidebook provides that a shellfish ban is issued if the level of PSP toxin is more than the allowable limit or if there is a confirmed PSP case. The Philippine allowable limit has been set at 40 μg/100 g of shellfish meat. This is just half of the WHO tolerable limit for red tide toxin, since the WHO limits are not safe for lesser built Filipinos, and especially children.

Communities and local governments must be prepared, in terms of monitoring, for rapid mobilization of resources and services, hospital capability, and training of local doctors in correct diagnosis. An effective response mechanism also entails market surveillance plans, deputization of maritime police to stop harvesting of banned products, monitoring of movement of shellfish, and alternative livelihood options. The recovery phase involves restoration to normalcy and public notification of lifting of shellfish bans.

Despite the seemingly comprehensive approach taken, however, fisherfolk groups are concerned that they are not represented on the task force and their views are not fully taken into account when decisions like shellfish bans are issued. During a blanket ban in an area, demand for all fish is low and all fisherfolk lose income. Food and nutritional security is compromised. Also, the prices for chicken, pork and other non-fish meat products may increase. First aid and medical facilities are lacking in most coastal towns. Misinformation, often caused by sensational media headlines, rather than education, reinforces the myth and ignorance about the red tide menace.

A 1997 baseline survey of opinions and knowledge showed that shellfish farmers did not have adequate knowledge about red tides. In extreme cases, the reaction of fishers to the red tide problem is outright denial. When a red tide alert was declared in Cavite town near Manila Bay two years ago, the fisherfolk organized a 'tahong' (mussel)-eating festival. Local government officials followed suit, in the full presence of media; this was considered irresponsible behavior by many sectors.

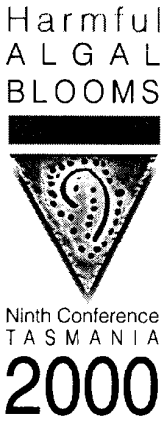
Fishers' groups would like to see that technical monitoring instruments go hand in hand with their indigenous knowledge of red tides gained from their long-time familiarity with the problem. Anecdotal evidence shows that fishers man be able to detect the impending occurrence of fish kills, though not necessarily due to red tide, from certain related events such as eels surfacing and crabs climbing out of mussel farms.

More significant, however, fisherfolk groups raise concerns about incoherent and often contradictory policies made outside the fisheries and aquaculture sector, including development plans that promote further congestion of the coastal zone. While the occurrence of HABs calls for the complete rehabilitation of bays and marine waters, many forms of development still continue to cause pollution and marine degradation.

This points to the wisdom of adopting integrated approaches to coastal zone management. These are approaches that are horizontally integrated, ensure community-based and participatory processes, provide jobs and livelihoods, help maintain healthy aquatic and land ecosystems and prevent unsustainable exploitation of our aquatic resources.

This week, at this conference, we seek a higher level of understanding of HABs phenomena. Let us remind ourselves that many poor people in Asia and Latin America depend upon our research results. And, in addition, large chunks of countries, especially in Africa and West Asia, have yet to benefit from the fruits of HAB detection and mitigation.

CONFERENCE SUMMMARY



HARMFUL ALGAL BLOOM STUDIES ENTER THE NEW MILLENNIUM

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INTRODUCTION

To summarize a HAB conference is always a difficult task but the 9th. International conference presented an even bigger challenge due to the great number of presentations and the necessary evil of parallel sessions. Thanks to excellent program organization there were usually plenary reviews of the material in both parallel sessions and so no one missed out on at least some important information from each. Here I will try to recognize some significant developments and trends without singling out any specific presentations, despite there being some clearly outstanding ones (the student awards given by ISSHA gave direct recognition of some of these, including poster presentations. Having previously given the view into the '90s [1] I now offer a personal view into the new millennium based on what was presented in Hobart in February 2000. The only explicit references will be to material published elsewhere.

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A major benefit of the wealth of material covered was that there were presentations on nearly every aspect of HABs: marine, freshwater, planktonic, benthic, taxonomic and toxicological methodologies, mitigation and management. As usual at these conferences we learned of new harmful organisms, new toxins, new phenomena and new victims. A feature of the U.S. National Program (see Programs below) was that funding encouraged the formation of large, multidisciplinary teams of twenty or more, unthinkable in other countries at this time but a hopeful indicator of the future. The study by Chris Scholin and twenty five other collaborators, published in Nature in 1999, and which I do not wish to cite because of the tediousness of naming them all, on the death of California sea lions due to domoic acid produced by a *Pseudo-nitzschia* bloom that they were monitoring, is a case in point. This led to an award, named for the late and sadly missed Maureen Keller, for Chris at this conference.
For those unfamiliar with Maureen a brief note here should not be amiss. Maureen worked at the Bigelow Laboratory of Marine Sciences in Boothbay Harbor, Maine. She collaborated with the renowned algal culturist Bob Guillard ("K medium" is named after her) and also carried valuable research into the production of dimethyl sulphide by phytoplankton, among other interests.

HAB ORGANIZATIONS AND PROGRAMS

In order to bring the appropriate people together regional, national and international organizations and programs have sprung up, four of which had meetings at the Hobart conference. An early working group on HABs was established by the North Atlantic fisheries organization ICES and a comparable one has just been established in the North Pacific as part of PICES (China, Korea, Japan, Russia, Canada and the United States). It had a brief, informal gathering to discuss initial activities. Many U.S. presentations at the conference were products of the first national funding program, ECOHAB, focussed on ecological research.

Canada established the first multinational HAB network in South East Asia in the 1990s as part of the ASEAN-Canada Co-operative Program in Marine Science (Philippines, Brunei, Malaysia, Singapore, Indonesia, Thailand and, near the end, Vietnam). Training and the design of monitoring programs were important components of this program. Although the active part of the program ended in 1998 the goal was to leave in place a communication network and this exists,

centered in the Philippines. The hope is that eventually there will be many such regional networks that can ultimately be linked into a global network. A review of knowledge gained about tropical PSP through this and other regional programs such as WESTPAC, was presented.

The Intergovernmental Oceanographic Commission (IOC) of UNESCO has been very active in promoting HAB study through sponsorship of the Harmful Algae News and through training within various regional organizations, notably WESTPAC, in which Japan is a leader. Again it is hoped that others, some of which have already started training workshops, will spread this knowledge and expertise globally. IOC sponsored publication of a Manual on Harmful Microalgae (Hallegraeff et al., 1995). An Intergovernmental Panel on Harmful Algal Blooms of UNESCO (IPHAB) has been in place for a number of years and an office to co-ordinate and expand its activities located in Copenhagen. The Scientific Committee on Oceanographic Research (SCOR) had earlier established an international Working Group (97) which culminated its efforts in a NATO-sponsored workshop in Bermuda whose proceedings have been published in a definitive volume [2].

The most recent development has been the establishment of GEOHAB, a joint international research program sponsored by IOC and SCOR. It had it's a meeting led by Patrick Gentien, chair of the Scientific Steering Committee, at the Hobart conference. This program is explicitly focussed on ecological and oceanographic aspects of HABS and should result in special funding opportunities in countries that are members of IPHAB, similar to other large projects such as JGOFS and GLOBEC. More details on this program can be obtained from the special January 2000 issue of Harmful Algae News.

Last but not least is the recent formation of the International Society for the Study of Harmful Algae (ISSHA), which had its first general meeting since its 1998 inception in Vigo, Spain, at the Hobart conference. The Society is essentially the only all-embracing organization that has an indefinite life-span and which, apart from following its members' wishes, can and should serve as a co-ordinating body to identify overlap and significant gaps in all types of HAB activities around the world, together with the Copenhagen IOC office.

CYANOBACTERIAL BLOOMS

Every conference in this series has had a special focus and in Hobart it was the problems Australia and other countries have experienced with harmful, fresh-water cyanobacterial blooms, an area generally neglected at previous conferences. These organisms, including the genera *Anabaena, Cylindrospermopsis, Microcystis* and *Nodularia*, produce neuro- and hepatotoxins, such as anatoxins, nodularins microcystins and cylindrospermopsin, that not only rapidly kill livestock or birds directly, but can have insidious low level effects on humans drinking contaminated water directly from rivers and lakes. The most alarming of these is liver cancer. Australia is not alone in this, with parts of Africa, Asia, Europe and South America also badly at risk. It seems that bodies of water in dry regions and those with agricultural-related eutrophication, are most implicated and the consumption of water directly from these water bodies riskiest. There is plainly an urgency in devising effective treatment of such water where consumption is unavoidable, as well as means to reduce the occurrence and scale of such blooms.

TAXONOMY

In the field of HABs taxonomy enjoys a currency and respectability greater than in many other fields. The reason is obvious. Not only is species identification critical but infraspecific variability must be defined and determinations of relatedness offer guidelines for organisms that should be searched for related phenomena. Not only are new species being incriminated but species and genera new to science are being found. At this conference *Pfiesteria* sp.B, several new species of *Gymnodinium* and the genera *Cryptoperidiniopsis* and *Parvilucifera* were introduced. In dinoflagellates tabulation has been shown to be a reliable guide to relationships [3] but recently increased focus has been on more difficult species where tabulation is more subtle or absent e.g. gymnodinoids, which are apparently polyphyletic. Several fish-killing gymnodinoids also share an unusual,prymnesiophyte pigment, 19'-hexanoyloxyfucoxanthin as well as chlorophyll c3, apparently acquired from a single symbiotic event in a common ancestor. This is also true for dinophysoids involved in the production of DSP toxins which have plastids undoubtedly of cryptomonad origin [refs. In 4].
These fundamental cell biological features, which also include increased awareness of genetic variation in species, are examples of the fundamental insights which are benefits of the intense focus placed on harmful species.
As aids to the identification of harmful species by non-taxonomists, or in cases where identification is difficult even for specialists, the development of molecular probes continues and grows, making use of surface antigens, variable regions in large subunit ribosomal DNA (LSU), small subunit rDNA (SSU) and even fluorescent probes for toxin presence in individual cells. The latter is particularly useful in those taxa in which toxin production is variable. An example of one of the latter that has been available for some time is that for domoic acid in *Pseudo-nitzschia* species.
The need for such molecular aids for identification is particularly urgent given the retirement of many taxonomists that has already occurred or is imminent. Although the National Science Foundation of the U.S. has recognized this impending crisis, many other countries seem oblivious to it.

BIOGEOGRAPHY

Harmful algal species, like phytoplankton in general, are apparently cosmopolitan and bihemispheric

within particular temperature boundaries, allowing, of course, for discovery. Thus it is highly unlikely that *Pfiesteria* is confined to the eastern seaboard of the United States. It is highly likely to occur, for example, in comparable estuaries in South America (Brazil, Venezuela?) and Asia. A major question at previous conferences has been whether this is due to natural or artificial spread? Ballast water introductions seem almost certain and Australia has been a world leader in taking steps to reduce this risk. Unfortunately introductions of HAB species are now taken as gospel by those concerned with other introduced species and included in lists along with much more clearly established examples. Despite the excellent pioneering work using LSU rDNA to recognize regional signatures in *Alexandrium* that has been featured at previous conferences, [e.g.5] and the great potential for ballast water introductions, no instances of artificial introduction have been established beyond reasonable doubt yet although the picture continues to grow. The use of sediments to establish prior existence has been useful in some instances to test introduction hypotheses but this is not possible for some HAB species. Biochemical markers may help.
In case one doubts the natural dispersive ability of ocean current transport over very long periods of time (thousands or even hundreds of thousands of years) consider the presence of similar benthic dinoflagellate communities not only on remote islands across the Pacific but right around the world. Drifting seaweed?

TOXINS

As usual at these conferences new toxins such as azaspiracid were introduced, indicating that we have not exhausted the witch's brew yet. More was learned about the cyanobacterial toxins and recently discovered toxins such as the spirolides produced by *Alexandrium ostenfeldii* or the yessotoxins in *Dinophysis* species. There used to be much confusion regarding the diversity of HAB toxins (those produced by *Gymnodinium breve* in particular) and there is now much greater clarity in the classes of toxins, which resolve to a surprising few, such as various types of polyethers , forms of saxitoxin, amino acids and possibly fatty acids. Notably absent are the proteins of most venoms and poisons in the toxicological world. Many presentations used highly sophisticated and sensitive methodologies allowing much finer discrimination in the past. An example was MS/MS liquid chromatography. The toxins of some such as *Pfiesteria* and the chloromonad fish killers (*Chattonella, Heterosigma*) are still under active investigation. The presence of brevetoxin in the latter has been conformed although perhaps not in quantities high enough to cause the fish kills. The role of maitotoxin, together with palytoxins considered to be the most potent marine toxins, in ciguatera fish poisoning is still an open question although, being water soluble, it is unlikely to concentrate up the food chain.
The modes of action, with the exception of phosphatase inhibitors such as microcystins, of HAB toxins are nearly all membrane related, either excessively facilitating or blocking the passage of ions such as sodium or calcium. This raises once again the question of the functions of such substances. While past experiments have supported the notion of an antipredation role for the saxitoxins and possible allelopathy by the bloom-forming chloromonads, the physiological or genetic variability of production raises doubts about them playing an essential role. Are toxin-producing strains more "successful" than non-toxic ones?

LIFE CYCLES

The importance of the benthic phase in the initiation of blooms has long been recognized and, since the species in question spend more time in this than any other phase it was argued at this conference that the benthic environment in which they sleep needs more study. The value they provide in searching for signs of earlier blooms, to check against the possibility of introductions via ballast water or other means, is still an important undertaking. Dating methods are highly precise although bioturbation in oxygenated sediments is still a problem. Factors involved in encystment and excystment were not as much a feature of this conference as earlier ones.
An outstanding mystery remains: where do non-cyst/spore species go when conditions are unfavourable for growth? *Gymnodinium breve* is a case in point. Are the cysts elsewhere and the blooms are advected into the study area? Are they still in the water column but in such low numbers as to appear absent? Is the spore too similar to the vegetative stage to be unrecognizable? For example, such a stage is unknown in the diatom Pseudo-nitzschia but its blooms do appear to sink out in coastal bays. Such large-scale sink-outs were observed in Barkley Sound, British Columbia [7]. Usually such events are seen as the usual, fatal termination of diatom blooms but, given the requirement for a solid surface for movement by pennate diatoms with a raphe, coupled with the parallel alignment of cells during sexual reproduction, normal in pennate diatoms and described in detail in a poster at the conference, it is highly likely that there is an obligate benthic phase during which sexual fusion and auxosporulation occurs, perhaps followed by dormancy. Events in such shallow sediments need to be examined following sink-outs by pennate HAB species.

ECOLOGY: HOW MANY BASIC ECOLOGIES ARE THERE?

The study of HAB species is an exercise in autecology and it could be argued that there are as many ecologies as there are species. However it does seem that some generalities can be applied. Distinctions can be readily made between planktonic and benthic; freshwater and marine, and prokaryotic versus eukaryotic blooms.
HAB studies, as their ultimate goal, attempt to predict actual, species-specific events in specific locations. Given that there are multiple species and that regulating factors can vary according to location, and that subsurface events cannot be seen by satellites, this is a much greater challenge than weather prediction.

Nevertheless HAB ecological studies have come a long way towards this goal.

Early studies were almost invariably done after the fact whereas now at least some are the results of multi-year monitoring programs set in place to watch the events unfold. Unfortunately, no matter how cutting-edge the methodology involved, many funding agencies view such monitoring as unimaginative and unsuitable for support. More than universities it is government agencies that have the capability to sustain such programs although ECOHAB, GEOHAB (see above) and the like should provide a more understanding framework in which to seek funding.

Also hampering early understanding was the application of the classical, spring bloom paradigm (small-scale mixing, upwelling, high surface nutrients, single phase) to all HAB events. While this may still serve for diatom HABs, such as *Pseudo-nitzschia*, in open coastal environments, it was clearly inappropriate for what John Cullen has referred to as "depth-regulating phytoplankton [6]. Flagellates, such as *Heterosigma*,, can make daily vertical migrations of five metres or more and thus can spend their days in well illuminated, nutrient-deplete surface waters and their nights dipping into the nutricline for nutrients, as long as shallow stratification persists. Such blooms can persist for as long as the physical structure prevails. In the Strait of Georgia, British Columbia a *Heterosigma* bloom persisted for four months in 1989. Termination of such stratification-dependant blooms is usually through turbulent mixing, diatoms replacing them when surface nutrients are renewed. At this conference it was argued that this distinction between suspension versus vertical migrators is oversimplistic and the link to stratification may be false. However, the role of ambient nutrients in favouring or impeding competitive diatom growth, was not considered an over-riding distinction and I consider this to be a major factor. An ignored question that needs to be looked at is the competition between many species that could exploit the same strategy.

The recognition that blooms have multiple phases (initiation, growth, plateau, dissipation) in which completely different factors may predominate in bloom regulation, was another step forward, the credit for this belonging largely to Karen Steidinger and her team studying blooms of *Gymnodinium breve* blooms in the Gulf of Mexico. Obviously, benthic cyst-formers can only appear in the water column once conditions for excystment have been met and these factors are quite different than those favourable for exponential growth. Aggregation by behaviour and physical structures, such as horizontal fronts and vertical clines, are yet other factors that come into play.

A critical factor in the prediction of blooms in particular localities is the determination of the role of advection. It is pointless looking for early bloom stages if the blooms originate elsewhere. The massive study of *Alexandrium* blooms in the Gulf of Maine illustrates this elegantly. Currents have the capacity to transport blooms over considerable distances and the blooms may even intensify during their transport. Earlier work on *Heterosigma* blooms in the Strait of Georgia, British Columbia (Taylor & Haigh, 1993), showed a similar advectional importance on a smaller scale. One wonders if studies on blooms of other species, such as *Gymnodinium breve* in the Gulf of Mexico, might need to be looked at on a larger scale to encompass all the stages of development.

The significance of previously neglected nutrients such as iron, believed to originate in rivers or windblown dust, or organics, including mangrove humics in the ecology of *Pyrodinium*,, is being examined more closely. It was intriguing to have back-to-back presentations on the role of iron of iron in growth and domoic acid production in *Pseudo-nitzschia* offering somewhat different conclusions. The possibility of domoic acid being a binder of iron added a new dimension to the question of toxin function.

The importance of bacteria was again a feature at this conference although the emphasis was on them as HAB destroyers rather than as sources of the toxins themselves (a major pre-occupation of past conferences). Biological control of HABs is a topic that continues to be a major concern and at this conference, in addition to the dinoflagellate parasite *Amoebophrya*, which has been receiving attention lately, a new parasite of HAB dinoflagellates, *Parvilucifera*, was introduced. It appears to be related to Perkinsus, an early branching relative to dinoflagellates.

The ecology of benthic dinoflagellates involved in Ciguatera fish poisoning, particularly *Gambierdiscus*,, was reviewed with emphasis on unanswered questions. Relatively speaking it seems to me that the picture is rather clearer than for planktonic HAB species, since it has an intense and yet indiscriminate macroalgal substrate association and a strong dislike for land runoff. These characteristics do provide a crude predictive insight into its occurrence in the coral reef environment. Important questions remaining include the reasons for these two major features of its ecology. Does algal exudate protect the dinoflagellate from harm? Is it metals, such as copper, that are the inimical components of land run-off?

MARINE FAUNA MORTALITIES: INDICATORS OF NEW OR RARE PHENOMENA?

If one takes the view that present-day HABs have been around for a long geological time i.e. hundreds of thousands of years, then one can assume that the aquatic fauna of a region must be adapted to the natural hazards that they pose. Turning this around, the imperviousness of local fauna to a particular HAB that might be expected to kill them, could be taken as an indication that they have had time to adapt to it biochemically, physiologically or behaviorally. Thus, the molecular configuration of the sodium channels in shellfish that feed on saxitoxin producers are presumably modified so that the toxin does not bind to them. When toxins are stored and concentrated they need to be sequestered in the way that is safe for the concentrator's cells. Sea otters have been shown to be able to taste saxitoxin and reject contaminated mussels and clams. Wild salmon are not harmed by *Heterosigma* blooms

that kill fish in netpens, presumably because they can sense the presence of the blooms and avoid them.
How then are we to interpret major deaths of aquatic life caused by HAB toxins? Numerous cases of fish kills have been recorded as well as seabirds killed by toxins. Whale deaths and those of manatees have been linked to HAB toxins. At the conference the latest MFM, the death of more than 100 California sea lions in 1999, was reported. Could this be taken as evidence that the contamination of their food fish by domoic acid from *Pseudo-nitzschia* is a new phenomenon off California? Given that they did not die in previous DA poisoning events in the region (seabirds did instead) and that razor clams retain the toxin in an analagous way to PSP in other clams, is this simply an evolutionarily acceptable loss, given its rarity?
Ultimately, when we know enough, it will be evolutionary interpretation that will make sense of it.

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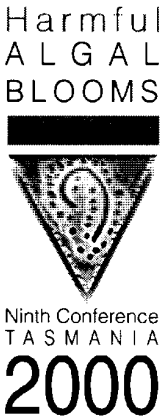
HARMFUL ALGAE EVENTS

PSP



FIRST DETECTION OF WIDESPREAD TOXIC EVENTS CAUSED BY *ALEXANDRIUM CATENELLA* IN THE MEDITERRANEAN SEA

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ABSTRACT

Two widespread toxic blooms caused by the PSP-producer *Alexandrium catenella* are documented for first time in the Mediterranean Sea. These blooms occurred the summers of 1998 and 1999 and affected confined and non-confined near-shore waters along 100 km of the Catalan coastline.

The maximum cell concentrations reached was above 10^6 cells.L^{-1} in confined waters and above 10^4 cells.L^{-1} in non-confined coastal waters. Shellfish collected for toxicity analysis revealed PSP levels of up to 983 µg PSP/100 g mussel meat.

INTRODUCTION

Alexandrium catenella has been reported from many cold-temperate areas [1,2,3]. There is a scarcity of references from the Mediterranean Sea [4, 5]. However, in August 1996 an *A. catenella* bloom was observed in the Barcelona harbour for the first time. Recurrent blooms were observed in the following years in an increasing number of stations along the coast, suggesting a progressive areal expansion in the area [6].

Here we present the first widespread toxic events caused by the dinoflagellate in the Mediterranean Sea. The occurrence of these widespread *A. catenella* blooms in Mediterranean waters is discussed in relation to their possible origin and their extension in the close coastal waters.

MATERIALS AND METHODS

Sampling strategy

Catalonia (about 400 km of coast) is situated in the NW Mediterranean Sea (NE Spain). A Monitoring of Harmful Phytoplankton Programme, based on the sampling of confined areas [7] is performed there since 1995 (Figure 1). The sampling interval being once a week from May to October, and twice a month from November to April, except for the Ebre Delta area which is sampled weekly year-round. After the *A. catenella* detection in some stations, surveillance was increased collecting additional samples in the neighbouring confined and non-confined waters. So, from 2 June until 19 July 1998, 11 confined and 30 non-confined stations were sampled for phytoplankton quantification, and 8 confined and 14 non-confined additional stations from 5 to 20 July 1999. These samples were taken with different frequencies, being the more intensively (up to every 2 days) sampled those stations where the dinoflagellate was present. The harbours were sampled in the most confined area and the non-confined stations near the coast (where the water column is 1-m depth). Samples were taken at surface.

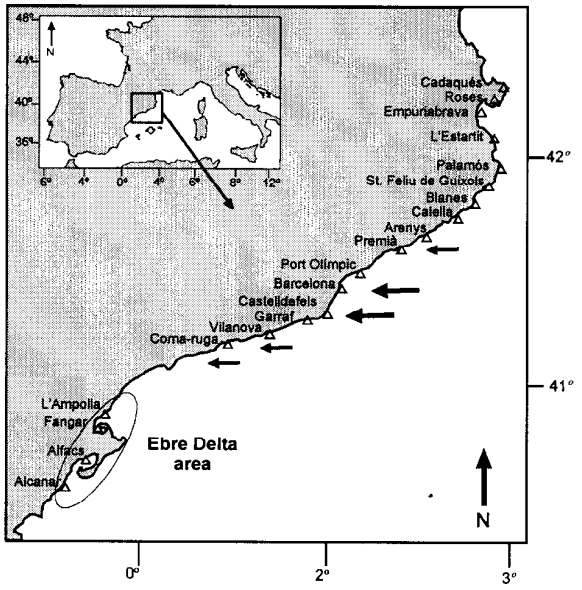

Fig. 1. Map showing the location of Catalonia in the NW Mediterranean Sea and the location of the stations sampled for the Monitoring of Harmful Phytoplankton Programme. Arrows indicate the location where transects were performed (the big arrows represent transects shown in Fig. 4).

On 6-10 July 1999 five transects (arrows in Figure 1) were performed in open near-shore waters, from the coast to 2.5 miles into the sea (located at 0, 0.5, 1, 1.5 and 2.5 miles from the coast). In the Barcelona and Castelldefels transects, CTD profiles were obtained and water samples were taken with Niskin bottles. Phytoplankton samples were preserved with formaldehyde (1% final concentration), settled and counted using an inverted microscope. *A. catenella* identification was reinforced with SEM microscopy.

Shellfish toxicity

During the events, shellfish samples were collected for toxicity analysis in some confined and many non-confined stations covering all the area affected by the dinoflagellate. The shellfish samples were collected at

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Intergovernmental Oceanographic Commission of UNESCO 2001

different stations independently of the *A. catenella* density. The objective was to delimit the toxic area and, when toxicity was recorded, to follow the toxicity pattern. Thus, 78 shellfish samples were taken during the 1998 bloom (from 27 May to 10 July) and 19 during the 1999 bloom (from 5 to 20 July). The main organisms sampled were mussels (*Mytilus galloprovincialis*). However other organisms such as wedge clams (*Donax trunculus*), striped venus (*Chamelea gallina*) and purple dye murex (*Bolinus brandaris*) were also tested for toxicity. Toxicity analysis was done following the standard mouse bioassay method [8]. The permissive toxicity level in the EU is 80 µg saxitoxin-eq./ 100g of mussel meat.

RESULTS

In the summer of 1998 a huge bloom of the dinoflagellate *A. catenella* (Figure 2) occurred affecting about 100 km of Catalan Coast and it was related to high PSP toxicity levels in this widespread area (Figure 3a). From May to the middle of June the beginning of the development phase was characterised by cell concentrations above 10^4 cells.L^{-1} in non-confined waters and up to several millions of cells.L^{-1} in some confined waters. In the Barcelona harbour the bloom started a month later than in the other stations. During the second half of June and July the bloom expanded to nearby waters. After that evenly a decay in cell abundance was observed in most stations, except in some confined areas such as the Tarragona and Barcelona harbours where the dinoflagellate was detected for longer times (e.g. end of August and mid September, respectively). The PSP toxicity sequence showed the same pattern as the cell concentration of *A. catenella* but with a delay of about a week.

The higher toxicity levels occurred between 1-7 June. The highest level (983 µg PSP/ 100 g mussel meat) was detected in the surroundings of the Tarragona harbour. But also high toxicity levels (527 and 784 µg PSP/ 100 g) were recorded in non-confined coastal waters not far away from that. Positive PSP values (higher than 80 µg PSP/ 100 g mussel meat) were detected from 1 until 21 June. During these three weeks, shellfish extraction was prohibited, this was announced in the local press and no human intoxications were recorded.

In the summer of 1999 the duration of the *A. catenella* presence, the maximum cell concentration reached, and the shellfish toxicity were lower to the previous year. Cells of *A. catenella* (Figure 3b) were detected in the confined and non-confined stations from the middle of June to the end of July (except in the Barcelona harbour where it was present until the end of August). In non-confined waters, it was only abundant (>10^3 cells.L^{-1}) from the end of June to the middle of July. Positive shellfish toxicity (> 80 µg PSP/ 100 g) was only detected in two stations at the beginning of July: one confined (149 µg PSP/ 100 g, Vallcarca harbour) and one non-confined station (109 µg PSP/ 100 g, Castelldefels beach). Shellfish extraction was prohibited for two weeks.

The transects, performed perpendicularly to the coast, showed summer stratification (data not shown). Water temperatures ranged between 20-25°C at surface, going down to 13.5°C at depth. Salinity ranged between 37.5-38.3 psu. Fluorescence levels and the cell distribution of *A. catenella* in two transects perpendicular to the coast are shown in Figure 4.

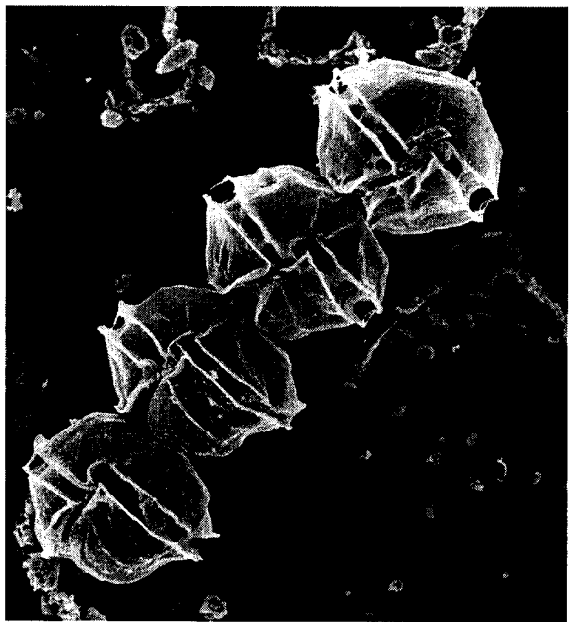

Fig. 2. Scanning electron micrograph of *Alexandrium catenella.*

Fluorescence shows two maximum levels, one in the upper inshore coastal waters, and another one below 30-40 m depth. However, the highest cell concentrations of *A. catenella* were only detected at surface and near the coast. This also happened in the other 3 transects (not shown). The phytoplankton community was dominated by dinoflagellates, mainly *Scrippsiella* spp. Other dinoflagellates observed were *Mesoporus perforatus, Heterocapsa* sp., *Prorocentrum minimum, P. triestinum, P. micans, P. compressum, Protoperidinium* spp., *P. diabolum, Dinophysis sacculus, Phalacroma. rotundatum* and *Ceratium furca.* Diatoms as *Proboscia alata, Pseudo-nitzschia* sp., and the coccolithophorid *Syracosphaera pulchra* were also present. The ratio *Alexandrium*/total dinoflagellates was 8-38% at the surface stations along the Castelldefels transect. However in the remaining 4 transects the highest values were below 5%, although values below 2% were most usual.

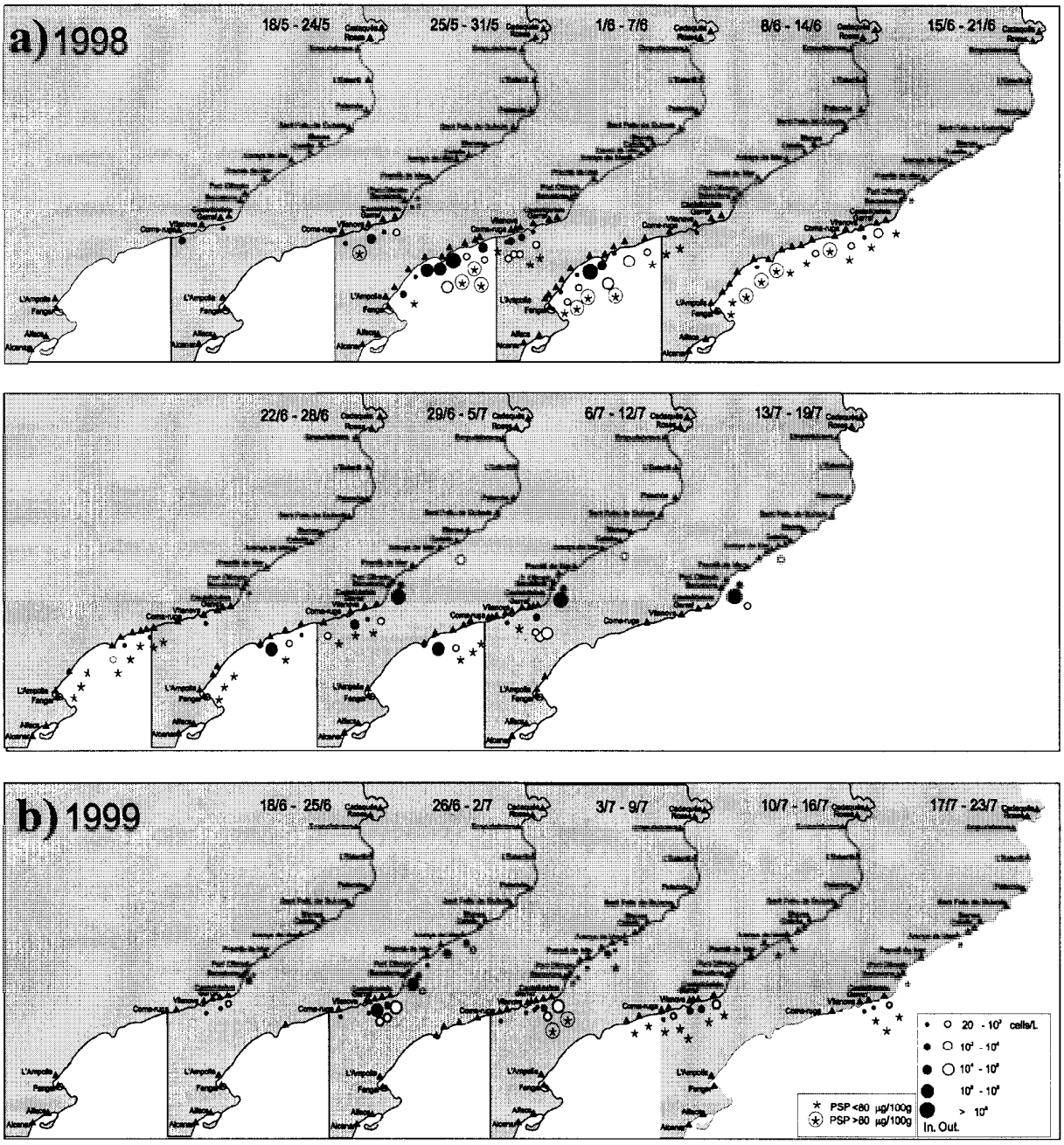

Fig. 3. Weekly distribution of *A. catenella*'s cells and toxicity during the widespread bloom in (a) summer '98 and (b) summer '99. Only the Monitoring Programme stations are named. Unnamed stations are the additional stations. (Black circles represent cell concentration inside confined areas and open circles in non-confined water; Asterisks represents shellfish toxicity).

CONCLUSIONS

While toxic events (both PSP and DSP) in the Mediterranean Sea have mainly been described in confined waters such as bays, lagoons and harbours, *Alexandrium catenella* bloomed both in confined and non-confined coastal waters. The two widespread blooms occurred during summer months, with open water temperatures of 20- 25°C.

The spatio-temporal distribution of *A. catenella* during the two events suggests three hypotheses for the origin of the bloom. The first one is the origin of

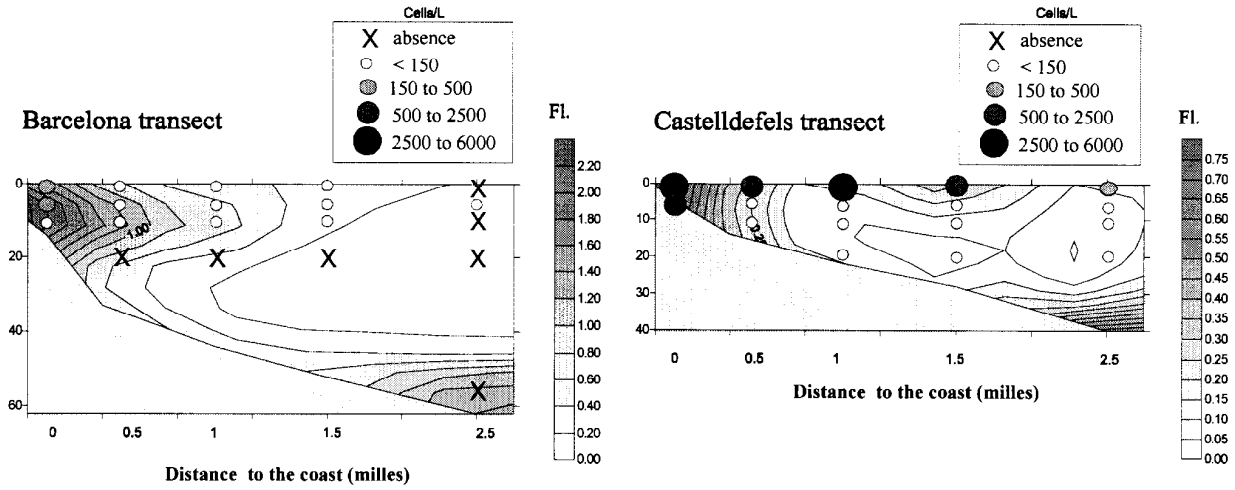

Fig. 4. Vertical cell distribution of *A. catenella* (cells.L^{-1}) and fluorescence (r.u.) at the Barcelona and Castelldefels transects during the cruise on board of "Mar Blau" on 6-10/7/99.

blooms in harbours and later expansion to open surrounding waters. Harbours may also better retain seedbed cysts than open areas, so blooms can easily begin inside harbours. The second one as originated in open coastal waters with general low concentrations of cells, which are then amplified in harbours. The differences in cell concentration must be explained by the reduced water turbulence and increased residence time in confined waters as which represent not only better conditions to grow but also reduced cellular dispersion. And the third one, a more or less simultaneous germination of *A. catenella* cysts in both open and confined waters triggered by environmental conditions. However, the data available does not give us the possibility to determine where did the bloom originate.

Cell toxicity is not a constant, it may change in different strains, environmental conditions and development phase. The minimum cell concentration of *A. catenella* thought to intoxicate shellfish is 10000 cells.L-1 [9]. In Sechlet Inlet, Taylor [1] observed toxicities up to 31000 µg PSP/100g in relation to *A. catenella* concentrations of 35000 cells.L^{-1}. In Catalonia, the maximum *A. catenella* cell concentrations was always detected inside harbours (60x10^6 cells.L^{-1} in Barcelona harbour; [6]) however the highest toxicity was always detected in open waters (e.g. 748 µg PSP/100g and was related to cell concentrations of up to 1-2.5x10^4 cells.L^{-1}). This can be explained by the shellfish filtering stopping in response to very high concentrations of toxic organisms.

The maximum toxicity detected in shellfish during the widespread blooms in Catalonia together with the results of the toxicity profile of this species (J.Franco, personal communication), indicate that *A. catenella* blooming in Catalan waters has a low toxicity as compared to the one reported by Taylor [1].

After these widespread blooms, caution should be taken in the monitoring of toxic phytoplankton in the Mediterranean area.

ACKNOWLEDGEMENTS

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SPECIES OF THE DINOFLAGELLATE GENUS *ALEXANDRIUM* (GONYAULACALES) IN THE GULF OF THAILAND

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ABSTRACT

Among 17 phytoplankton samples collected from 13 sites around the upper Gulf of Thailand, two species of dinoflagellate genus *Alexandrium* were found from 8 sites. *Alexandrium tamarense* (Lebour) Balech was found distributed widely in aquaculture ponds from 8 provinces around the upper Gulf as well as from a river mouth on the eastcoast of the Gulf where the salinity ranged from 19 to 40 PSU. *Alexandrium minutum* Halim was found in the estuarine water of the river mouth in the upper Gulf of Thailand at a salinity of 15 PSU. Clonal cultures of these *Alexandrium* exhibited specific growth rates of 0.40 to 0.65 per day. Only extracts from clonal cultures of *A. minutum* were toxic. The toxicity varied from 1.12 x 10^{-4} to 15.3 x 10^{-4} MU cell^{-1}. The toxin profile was composed of GTX_1, GTX_2, GTX_3, and GTX_4 with GTX_1 as the dominant component. However, the density of *A. minutum* found was extremely low (24 cell l^{-1}) and not at a level threatening to the environmental or human health. Mouse-bioassay as well as reverse-phase HPLC toxin analysis confirmed that *A. tamarense* found during this study was not harmful.

INTRODUCTION

Since the outbreak of PSP in Thailand in 1983 [1], four morphospecies of the PSP genus *Alexandrium*; *Alexandrium cohorticula* (Balech) Balech , *Alexandrium fraterculus* (Balech) Balech, *Alexandrium leei* Balech, and *Alexandrium tamarense* (Lebour) Balech ; had been reported from the Gulf of Thailand [2]. Later, a new species *Alexandrium tamiyavanichi* Balech was identified to be distinct from the previous *A. cohorticula* isolated from the coastal area of west part of the Gulf of Thailand [3]. Another species, *Alexandrium minutum* Halim, was recently found in a low salinity fish pond in the western part of the Upper Gulf of Thailand [4]. Among these *Alexandrium* species found in Thai waters, *A. tamiyavanichi* was reported to be toxic while *A. tamarense* was non-toxic [5,6,7]. The surveys on the distribution and abundance of harmful algae in the Gulf of Thailand and in aquaculture ponds along the coastal areas has been conducted but not on a routine basis because there has been no report on the outbreak of shellfish poisons since 1983. Due to heavy urbanization, industrialization, and agricultural activities in the coastal areas during the past two decades, it is necessary to reassess the occurrence of the harmful algae in Thai coastal environments.

METHODS

Phytoplankton samples were qualitatively collected with a 20 μ mesh-net from 17 stations in the coastal areas around the Gulf of Thailand. The sampling sites included shrimp ponds, fishpond, estuaries, and coastal waters which covered the provinces of Chanthaburi, Rayong and Chonburi on the east coast of the upper gulf, Samut prakarn, Sumut sakhon and Samut songkhram province on the upper part of the Gulf, and Phetchaburi province on the west part of the upper gulf. For each site, monoclonal cultures were established and preserved cell samples were collected for further morphological study (in either 1.5% glutaraldehyde or 4% formalin). Temperature and salinity of water from each sample site were recorded. Water samples were also collected for spectrophotometric quantification of dissolved nutrients [8].

Cultures of each isolated cell as well as the study of growth pattern were carried out with T1 medium [9] at 30±1° C under the light intensity of 3000 lux and the light:dark cycle of 12:12 hours. Either natural or cultured specimens were prepared for morphological studies by epifluorescence microscopy [10] as well as scanning electron microscopy [11]. Species were identified based on thecal plate morphology and arrangement [2,12]. Approximately 50 cells per clonal culture were used for allometric measurement of APC, 1', 6'' and S.p. plate to confirm speices identification.

The late exponential phase cultures of *Alexandrium* were harvested and extracted by 0.1% acetic acid solution. Toxicity test on these extracts was conducted using mouse bioassay [13]. Toxicity level was calculated from the standard Sommer's table for PSP and reported as MU/cell.

Aiquots of pre-filtered extracts were purified by ultrafiltration with YMI membrane. Filtrates were analyzed using a Shimadzu reverse phase HPLC system model LC-10 AD equipped with a fluorescence detector model RF-10 AXL. Mobile phases were heptanesulfonic acid and heptanesulfonic acid plus acetonitrile both in ammonium phosphate buffer for GTXs and STXs toxin, respectively. Analytical separation was performed by a LiChroCART Supersphere RP-18(e) column (250 x 4

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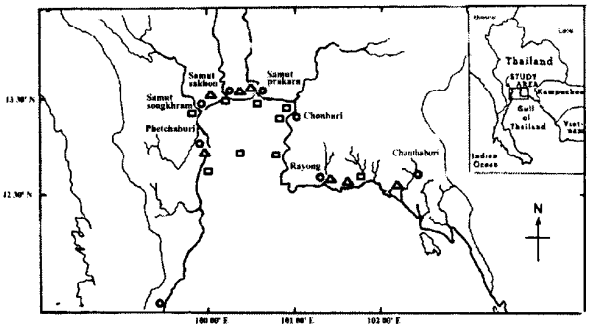
mm) under the reaction temperature of 65 °C. Eluent A was 0.05 M periodic acid and eluent B composed of 2N KOH plus 2.5 M ammonium formate plus formamide at the ratio of 20:80:100 (v:v:v). The flow rate for both eluents was 0.4 ml min^{-1} . The quantity of toxins extracted from *Alexandrium* cultures was compared with standard STXs and GTXs toxins (Sigma).

RESULTS

Distribution of Alexandrium *in the coastal areas of the upper part of the Gulf of Thailand*

From 17 samplings in 13 locations, *Alexandrium*-like cells were found in plankton samples from two river estuaries and six aquaculture ponds (Fig. 1). Water salinity in these sampling sites ranged from 15.0 to 40.0 PSU, while the temperature of water was in the range of 29.0 to 33.0°C. Dissolved oxygen in the sampling areas was not lower than 5.0 μg l^{-1}. Concentrations of nitrate were in the ranges of 0.14 to 0.58 μM and phosphate concentration ranged from 0.14 to 3.43 μM. The density of the *Alexandrium* cells ranged from less than 5 cell l^{-1} in shrimp ponds in Samut sakhon and Phetchaburi province to the highest density of to 15,000 cell l^{-1} in a shrimp pond in Rayong province (Table 1).

Fig. 1. Sample sites in the upper Gulf of Thailand.



Provinces in which the study had been conducted, (circle); sample sites(square); and sites where *Alexandrium* cells were found (triangle).

Thecal plate study indicated that only two species, *Alexandrium tamarense* and *Alexandrium minutum*, were isolated from the study areas during the period of 1996 to 1998. Size of *A. tamarense* cells ranged from 16.4 to 36.7 μm in length (av. 25.3±3.9 μm) and from 15.5 to 32.2 μm in width (av. 24.0±3.5 μm) with the length and width ratio of 0.89 to 1.46 (Fig. 2A). The length:width ratio of 6" precingular plate of this species from 0.80 to 1.20. For *A. minutum*, the cell length was from 16.1 to 30.0 μm (av. 23.1±3.1 μm) and the width ranged from 16.3 to 30.5 μm (av. 22.3±3.6 μm) with the length:width ratio of 6" precingular plate ranged from 1.31 to 1.78 (Fig. 2B). In culture, specific growth rate during exponential phase was 0.40 to 0.65 division d^{-1} for *A. tamarense* and 0.47 division d^{-1} for *A. minutum*. *A. tamarense* was distributed in shrimp ponds in Samut sakhon, Rayong, Chanthaburi and Phetchaburi provinces and in the river mouth of Rayong province while *A. minutum* was found only in the estuary of Chao Phraya river in Samut prakarn province.

Table 1. *Alexandrium* cell counts and water temperature and salinity in each sampling sites in the upper part of the Gulf of Thailand during the study period from May 1996 to March 1998.

Sample sites	Date of collection	Cell l^{-1}	Temp. (°C)	Salinity (PSU)
A. tamarense				
Samut sakhon shrimp pond	29/5/96	< 5	29.5	20.0
Samut songkhram fish pond	23/4/97	7500	-	30.0
Rayong shrimp pond	22/5/97	-	-	40.0
Rayong river estuary	6/6/97	10	31.0	20.0
Rayong shrimp pond	6/6/97	15000	33.0	35.0
Chanthaburi shrimp pond	9/9/97	50	-	22.0
Phetchaburi shrimp pond	21/2/98	<5	31.5	19.0
A. minutum				
Samut Prakarn river estuary	12/1/98	4	29.0	15.0

"-" means data not available

Toxicity of Alexandrium *extracts*

Mouse bioassay indicated no toxic substance in cultures of 16 clones of *A. tamarense* collected from 7 different sample sites. The analysis of toxin composition by HPLC technique confirmed that the extracts from *A. tamarense* contained no saxitoxin and gonyautoxin. On contrary, the extracts of 4 clonal cultures of *A. minutum* exhibited toxicity of 1.22 to 1.37 x 10^{-3} MU $cell^{-1}$. Parallel mouse bioassay test with filtrates from the culture of *A. minutum* indicated no toxin production. The toxin profiles of *A. minutum* extracts comprised of GTXs with no evidence of STXs. The most abundant component was GTX_1 (70-80%) followed by GTX_4 (20%). Trace amounts of GTX_2 and GTX_3 also occurred in the extracts of *A. minutum*. The toxicity level were equivalent to 5.7 x 10^{-8} to 1.2 x 10^{-6} MU $cell^{-1}$ and from 1.5 x 10^{-12} to 2.4 x 10^{-7} MU $cell^{-1}$ for GTX_1 and GTX_4, respectively.

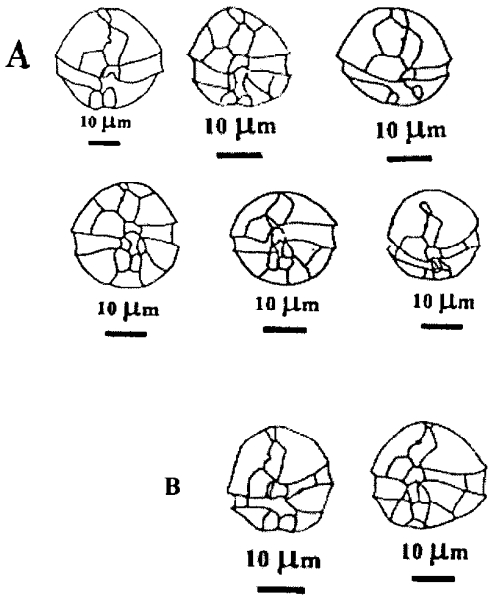


Fig. 2. Morphology of *Alexandrium* from the Gulf of Thailand. (A) *A. tamarense* from various sites; (B) *A. minutum* from Samut prakarn province

DISCUSSION

Geographic Distribution of A. tamarense *and* A. minutum

Dinoflagellate *A. tamarense* is distributed widely in the coastal areas around the upper Gulf of Thailand particularly in aquaculture ponds where the water salinity ranged from 19 to 40 PSU. Previous records of *A. tamarense* were from the river mouths and around the upper Gulf of Thailand [2] where the salinity varied between 23 and 32 PSU. This indicates the physiological adaptation of this species to a broad salinity regime both in natural and enclosed environments such as shrimp ponds and fish ponds. *A. tamarense* bloomed and caused water discoloration in the enclosed ponds in Samut songkhram and Rayong provinces during the sampling periods. It was noticed that the size of *A. tamarense* found was close to those reported from Taiwan [14]. Matsuoka *et al.* (1998) claimed that *A. tamarense* found in the crab pond (salinity 10-19 PSU) in Taiwan [18] was actually *A. minutum*. However, the density of *A. minutum* found in the estuary in our study was much lower than those from the fish pond and from Taiwanese crab pond. There were also some interspecific and intraspecific variations in thecal plate morphology particularly in the shape and size of 1', 6" precingular plate and S.p. plate.

Our study revealed the occurrence of *A. minutum* in the estuarine region in the upper Gulf of Thailand. This dinoflagellate species was first recorded in the country in a fish pond with a salinity of 4 PSU [4]. However, the clonal culture of this *A. minutum* grew in medium with 30 PSU salinity (Wisessang, personal communication). The discrepancy in *Alexandrium* densities may due to the more stable environmental conditions in the ponds in comparison to the more dynamic estuary.

Toxin production of A. tamarense *and* A. minutum

A. tamarense is well known to produce PSP toxins but the extracts of clonal cultures of *A. tamarense* from 7 sample sites did not contain paralytic shellfish toxin. This may indicate an unfavorable environment to stimulate toxin production by this species. It was suggested that *A. tamarense* grown in phosphorus deficient condition tends to increase PSP toxin production to compensate for their excess cellular nitrogen [15]. The ratio of dissolved nitrogen and dissolved phosphorus (as nitrate and phosphate, respectively) from our study was less than the Redfield ratio of 6:1, which implies that *A. tamarense* was not phosphate limited in this case. Non-toxic strains of *A. tamarense* were previously recorded from the west coast of the upper Gulf of Thailand where the first PSP event had occurred [5,6], the east coast and from the lower part of the Gulf of Thailand [5,7]. Non-toxic strains of *A. tamarense* are also known from temperate areas [16,17] and it appears that both toxic and non-toxic strains of *A. tamarense* can co-existence of in the Gulf of Thailand [7] and other parts of the world [18]. It has also been suggested that the non-PSP producing strains of *A. tamarense* isolated from Pran-buri and Ban Leam on the west coast of the upper Gulf of Thailand may be a subspecies of *A. tamarense* [12].

Previous study showed that lethal cell concentration of *A. minutum* isolated from a shrimp pond in the west coast of the upper Gulf of Thailand was 2.7×10^3 and 9.7×10^3 cell ml^{-1} for juvenile tiger prawn and sea bass, respectively [19]. In Japan, the lethal cell concentration of *Gonyaulax* spp. is 200 cell ml^{-1} [20]. These concentration levels are much higher than the concentration of *A. minutum*, 24 cell l^{-1}, found in the estuary during our study. The toxin composition found in *A. minutum* extracts is typical of the minutum complex which GTX_1 as major component followed by less abundance of GTX_4 and trace amounts of GTX_2 and GTX_3 [21].

Although the occurrence of toxic *A. minutum* reported here was too low to cause the PSP event, further studies should focus on the possibility of PSP outbreak in the future. Proper understanding of the biology and eco-physiology of this species and factors controlling the bloom in the brackish environment will be needed to prevent the outbreak. Further studies on *A. tamarense* should be conducted to investigate the factors affecting possible toxin production in Thai waters.

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PSP TOXIN CONTENT IN ALGAL BLOOMS AND MOLUSCS IN COASTAL WATERS AROUND THE ORKNEY ISLANDS AND THE EAST COAST OF SCOTLAND

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ABSTRACT

HPLC was successfully used to measure paralytic shellfish poisoning (PSP) toxins on board the research vessel "Heincke" during a cruise to the Orkney Islands and to the Firth of Forth. In May 1997, the toxin profiles, as well as the total PSP toxin content in algal cells of blooms of toxic *Alexandrium* strains were determined. Changes in the PSP toxin profile in the water column were observed and compared to the PSP toxin profiles in different marine animals.

INTRODUCTION

The main tasks of the cruise were to establish methods for the investigation of harmful algal blooms (HABs) at sea and to provide an overview regarding frequency, distribution and abundance of PSP producing algal blooms in a distinct area. The successful application of a rapid HPLC method is a powerful tool to make maximum use of expensive ship time. With this method, toxin data for each sampling site was provided within 1 h after sampling began. Fast information as to whether or not a sampling site is PSP toxin-affected, permits the effective setup of further follow-up experiments, such as strain isolation, extended sampling (e.g., depth profiles), measurement of nutrients and the determination of additional oceanographic data. During the cruise, samples from different water column depths were taken at some of sampling sites where toxicity was determined from surface plankton assemblages using our HPLC method. In these samples the PSP toxin content and its composition were determined and the results compared with the profiles of PSP toxin contamination from different shellfish species.

MATERIALS AND METHODS

Fig. 1 shows the area around the Orkney Islands and the Firth of Forth, Scotland, sampled during the 1997 cruise. The precise location of sampling sites (S) was determined using the on-board global positioning system (GPS) of the research vessel Heincke (GPS data not shown).

Samples from sampling sites (4 to 5 L, from rosette samplers taken at surface and different depths) were filtered onto a 0.45 µm filter (GFC, 50 mm diameter). Planktonic material on the filter was extracted in a 2 mL Eppendorf vial containing acetic acid (1.0 mL, 0.03 N) and sonicated (1 min) with an ultrasonic probe. The raw extracts were centrifuged for 10 min. (2980 g) and passed through a 0.45µm nylon filter. Filtered extracts were injected directly into the HPLC system.

The LC determination of PSP toxins was carried out as described recently using ion-pair elution with octane sulfonic acid in a phosphorus buffer system, post-column oxidation with periodic acid and fluorescence detection [1,2].

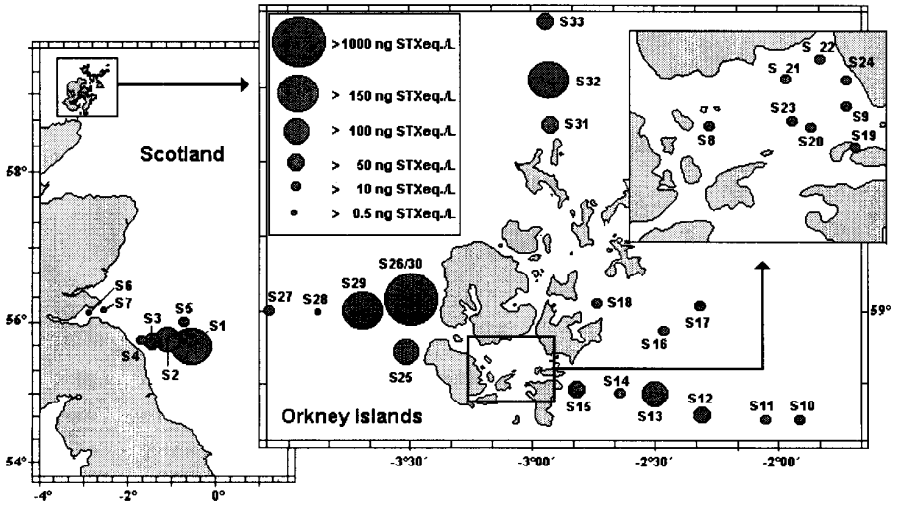

Fig. 1: Sampling area and sampling sites (S) along the Scottish coast and around the Orkney Islands in 1997

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RESULTS AND DISCUSSION

The HPLC method applied on board the research vessel "Heincke" proved to be a powerful technique for a selective and sensitive determination of PSP toxins in natural planktonic communities. Within a very short time (three weeks) a large sea area (Fig. 1) was surveyed and a detailed picture of the PSP toxin distribution was obtained. Comparison of results obtained on board with analytical data from laboratory experiments carried out 8 months later showed high stability of PSP toxin components in crude samples and extracts during storage at -20°C. Differences were within the range of the normal standard deviation of the HPLC method (< 5% for all PSP toxins analyzed). In both years (there is no indication that you have sampled for two years anywhere) the PSP toxin amount in samples from the wide area under investigation varied over a broad range (from ca. 1 ng PSP toxins in algal cells L^{-1} seawater up to more than 1300 ng PSP toxins in algal cells L^{-1}). In general, higher PSP toxin contents were found in offshore waters around the Orkney Islands (see Fig. 1).

In contrast to varying concentrations, the PSP toxin profiles found in samples from surface water over this wide sampling area were stabile. No significant differences in toxin profiles were observed. Figure 2 shows typical chromatograms obtained from four surface samples in 1997.

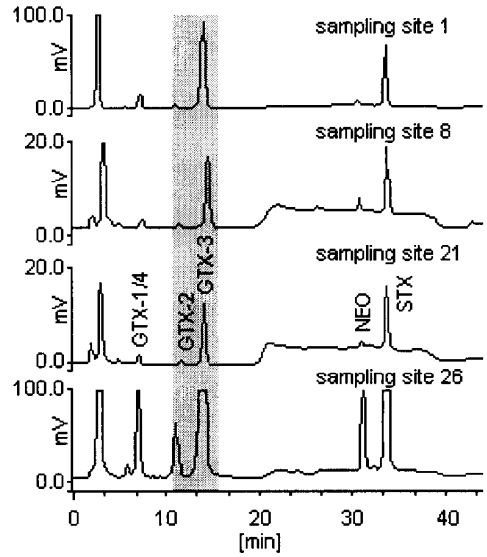

Fig. 2:Chromatograms obtained from plankton samples from surface waters

Typical PSP toxin profiles found in 1997 is presented in Fig. 3. Although Figure 3 shows a single PSP profile obtained at sampling site 1, both the PSP toxin surface profile as well as the changes in the toxin profile with depth were representative for all sampling sites analyzed in 1997.

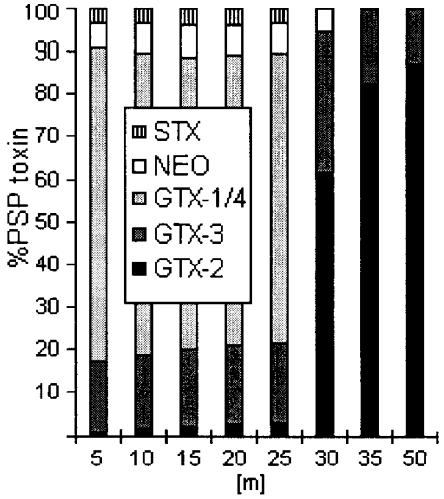

Fig. 3: PSP toxin profiles in samples from different depths in the water column at S 1

A remarkable observation regarding the PSP toxin pattern in samples from different depths in the water column was made in 1997. Figure 4 shows chromatograms from samples obtained at different depths at sampling site 26.

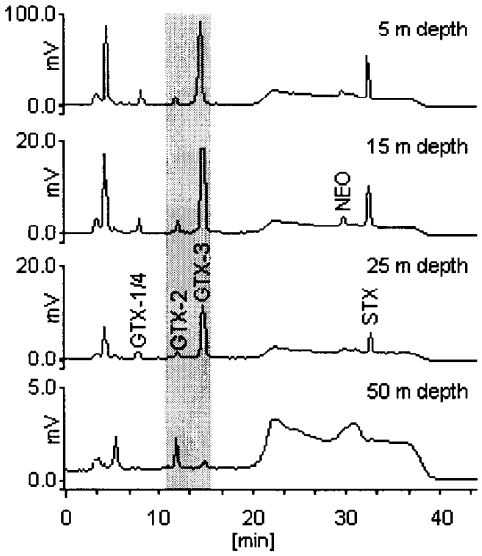

Fig. 4: PSP toxin profiles in samples from different depths in the water column at S 26

In surface samples down to 25 m the PSP toxin profile showed a high proportion of GTX-3 as compared to GTX-2 (highlighted in chromatograms) (ratio GTX-3 /GTX-2 always >> 1). These findings are in accordance to former results, e.g., in *Alexandrium excavatum* from the St. Lawrence estuary of eastern Canada [3] and in *Alexandrium tamarense* (ccmp 117) [4], where the GTX-3 concentration also exceeded the GTX-2 concentration. These findings support the assumption that 11-β-epimers (C-2, C-4, GTX-3, GTX-4) might be

the preferred products of the biosynthesis of PSP toxins in cells [5,6]. Samples taken at greater than 30 m depth showed an inverse GTX-3/GTX-2 ratio (<1) (see also Fig. 3, depth 35 to 50 m). In addition, NEO and GTX-1/4 were often not detectable whereas at least trace levels of STX were found.

As the PSP toxin content of sample material decreased with increasing sampling depth, it is most likely that the concentrations of minor toxin components, such as STX, NEO and GTX-1/4 approached the detection limit. Therefore, any conclusions regarding profile changes related to STX, NEO and GTX-1/4 must be reported with caution. However, the changing ratio GTX-3/GTX-2 is obvious and may indicate the incipient epimerization of GTX-3 into GTX-2 in unhealthy cells at this depth. A second explanation could be a changing planktonic community in the water column or the bio-transformation of PSP toxins by grazers feeding on phytoplankton or by bacteria able to transform the toxins. Whereas the surface phytoplankton was dominated by different *Alexandrium* species [7], unfortunately, there are no data about the organisms at depth in the water column. The GTX-2/GTX-3 ratios analyzed in some higher links of the marine food chain revealed a diverse picture. Although, differences in the GTX3/GTX2 ratio were found the quotient was close to 1 or even <1. Figure 5 shows typical chromatograms.

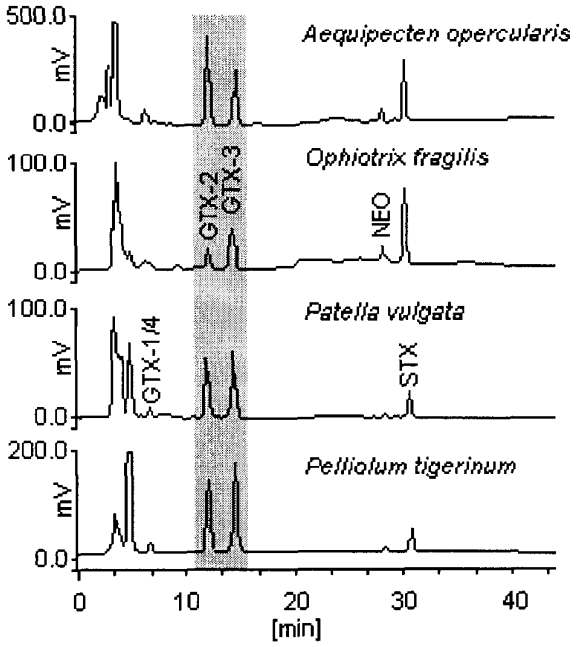

Fig. 5: PSP toxin profiles in animals from S 1

Earlier studies showed that the PSP toxin profile in mussels and scallops may not only differ between species with regard to the planktonic material upon which they are feeding but also between populations of the same species [3,4]. Nevertheless, in principle GTX-3/GTX-2 ratios from water column samples were similar to those found in animals from Scottish waters.

All results were confirmed by re-analyzing the same extracts after 8 months. No change in the GTX2/GTX3 ratios was observed. To exclude mis-interpretations due to interfering peaks especially in low contaminated samples those extracts were injected into the HPLC system without post column derivatization. These chromatograms showed no peaks and the presence of compounds with natural fluorescence activity was excluded.

CONCLUSIONS

- On-board measurements of PSP toxins maximizes the use of expensive and limited time for experiments on research vessels
- Processing toxin data directly at a sampling site allows for rapid evaluation and reaction time for further experiments, e.g., staying at "hot spots" or leaving an area without toxic algal cells
- The PSP toxin content in algal blooms around the Orkney Islands and near the Scottish coast showed large differences in concentration but little variation in the PSP toxin profiles was observed in surface samples.
- The change in PSP toxin profiles with increasing depth, i.e., increased ratio of 11-a-epimers to 11-β-epimers of PSP toxins may indicate a shift in physiological status because of unknown environmental or biological factors.

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PARALYTIC SHELLFISH POISON IN FRESHWATER PUFFER FISH (*TETRAODON CUTCUTIA*) FROM THE RIVER BURIGONGA, BANGLADESH

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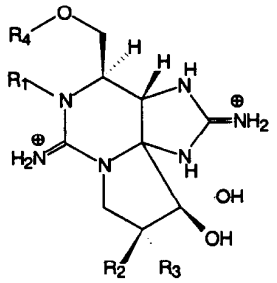
ABSTRACT

Freshwater puffer fish, *Tetraodon cutcutia*, was collected from the river Burigonga on 26 February 1999. Acetic acid extracts of skin, muscle and liver were applied to high performance liquid chromatography. Fluorescence detector HPLC demonstrated that the puffer fish contain four types of paralytic shellfish poison, viz., saxitoxin, decarbamoylsaxitoxin, gonyautoxin 4 and decarbamoylgonyautoxin 3. No N-sulfocarbamoyl toxin was detected from hydrolyzed sample of acetic acid extract of *T. cutcutia.*

INTRODUCTION

Three species of freshwater puffer fish, *Tetraodon cutcutia*, *Chelonodon patoca* and *Chelonodon fluviatilis*, are reported from Bangladesh. In spite of little economic importance in fish market, they are sometimes caught mixed up with other freshwater fish, and consumed by the poor



Toxin	R1	R2	R3	R4
STX	H	H	H	
NEO	OH	H	H	
GTX I	OH	H	OSO_3^-	H_2N-CO
GTX II	H	H	OSO_3^-	(Carbamoyl-)
GTX III	H	OSO_3^-	H	
GTX IV	OH	OSO_3^-	H	
B1	H	H	H	
B2	OH	H	H	
C3	OH	H	OSO_3^-	^-O_3S-NH-COO
C1	H	H	OSO_3^-	(N-Sulfo-
C2	H	OSO_3^-	H	carbamoyl-)
C4	OH	OSO_3^-	H	
dc-STX	H	H	H	
dc-NEO	OH	H	H	
dc-GTX I	OH	H	OSO_3^-	H
dc-GTX II	H	H	OSO_3^-	(Decarbamoyl-)
dc-GTX III	H	OSO_3^-	H	
dc-GTX IV	OH	OSO_3^-	H	

Fig. 1. Chemical structures of PSP toxins.

local people who have little knowledge on the toxicity of puffer fish, resulting in the sporadic occurrence of food poisoning incidents includes some fatal cases. However, only a few official records on the incidents have been kept, and a little information on the toxicity and toxic principle of these puffers is available in Bangladesh [1]. But no works so far been undertaken on toxicity of puffer fish, *T. cutcutia,* from the river Burigonga.

Most of the researchers demonstrated that the toxic principle of both marine and freshwater puffer fish was tetradotoxin (TTX) [2,3,4]. But recently some researchers reported that puffers contains paralytic shellfish poison (PSP) [1,5,6] (Fig. 1). The present study deals with the toxic components of freshwater puffer, T. cutcutia, form the river Burigonga, in Bangladesh.

MATERIALS AND METHODS

Puffer specimens

A total of 52 specimens of freshwater puffer fish, *Tetraodon cutcutia,* was collected from the river Burigonga on 26 February 1999. The specimens were immediately frozen, transported by air to the Institute of Nutrition and Environment, Friedrich-Schiller University of Jena, and kept frozen below -30 °C until use.

Sample treatment

Fresh sample material was homogenized with an Ultra Turrax, 1g of it was mixed with acetic acid (0.03 N 1 or 2 mL) sonicated for 10 min and kept in extraction solution for some minutes. The raw extract was centrifuged for 10 min (2980g) and the supernatant was passed through a 0.45 µm membrane filter. An aliquot of each raw extract (150 µL) was mixed with hydrochloric acid (1.0 N, 35 µL) and heated for 15 min (90°C) to convert N-sulfocarbamoyl toxins into their related carbamoyl toxins. After cooling to room temperature the reaction solutions were neutralized with sodium acetate solution (1.0 N, 75 µL). The hydrolyzed extracts were stored at -20°C until analysis within a period not exceeding 24 hours.

PSP toxin standards

Toxins STX, Neo, GTX 1-4 were purchased from the National Research Council Canada, Marine Analytical Chemistry Standards Program (NRC-PSP-1B), Halifax, NS, Canada. Standard solutions of GTX2 and GTX3 contained dcGTX2 and dcGTX3 as minor components, but the exact content of these toxins was not given. DcSTX was provided by the European Commission (BCR, The Community Bureau of Reference, Brussels) for use as standards.

Chemicals and Solvents

All chemicals used were analytical grade. Acetonitrile and tetrahydrofurane was obtained from J.T. Baker, Deventer, Holland. Octanesulfonic acid was purchased from Sigma-Aldrich, Steinheim, Germany. Periodic acid was from Merck-Schuchardt, Hohenbrunn, Germany, acetic acid (glacial) and ammonia solution (25%) from

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Merck, Darmstadt, Germany. Water was purified to HPLC-grade quality with a millipore-Q RG Ultra Pure Water System from Millipore, Milford, USA.

High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) was performed with a SIL-10A intelligent autosampler, a LC-10ATvp intelligent pump, a SCL-10Avp system controller and a RF-10AXL fluorescence detector (all from Shimadzu, Duisburg, Germany), an 1 mL CRX400 postcolumn unit (Pickering Laboratories, Mountain View, CA, USA) a LC-9A and a LC-6A pump used for delivery of postcolumn reaction solutions, and a Class-vp 5.2 software (Shimadzu, Duisburg, Germany) and a Phenomenex, Luna 5 µ RP-C18, 250mm X 4.6mm column. PSP toxins were detected by use of the excitation and emission wavelengths 333 nm and 390 nm, respectively. The temperature set for the postcolumn derivatization was 50 °C.

Theilert method [7] modified by Hummert [8] and Jaime [9] was used for determination of PSP toxins. Three eluents were used, eluent A: 98.5% 11 mM octanesulfonic acid (sodium salt), and 40 mM phosphorus acid, adjusted to pH 6.9 with NH3 1.5% THF; eluent B: 83.5% 13 mM octanesulfonic acid (sodium salt), and 50 mM phosphorus acid, adjusted to pH 6.9 with NH3 15.0% ACN, 1.5% THF and eluent C: 98.5% 40 mM phosphorus acid, adjusted to pH 6.9 with NH3 1.5% THF. The gradient used for chromatography is presented in table 1. The solution used for postcolumn derivatization contained alkaline periodic acid solution (10 mM), 550 mM NH3 flow rate 0.3 mL min-1 and acetic acid solution (1M) flow rate 0.4 mL min-1.

Table 1. Composition of the HPLC gradient.

Time (min)	Eluent A(%)	Eluent B(%)	Eluent C(%)	Flow mL min^{-1}
0.0	50	0	50	1.0
10.0	50	0	50	1.0
12.0	0	100	0	1.0
35.0	0	100	0	1.0
36.0	100	0	0	1.0
47.0	100	0	0	1.0
48.0	50	0	50	1.0
57.0	50	0	50	1.0

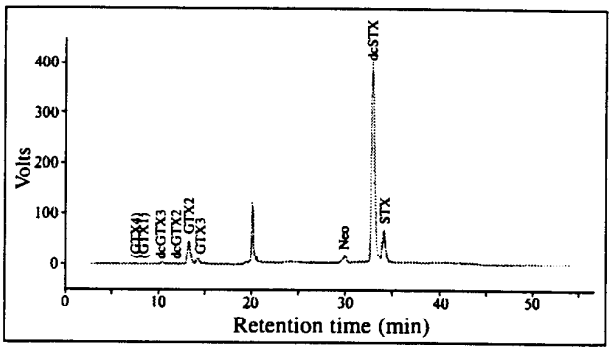

Fig. 2. HPLC chromatograms of standard PSP toxins.

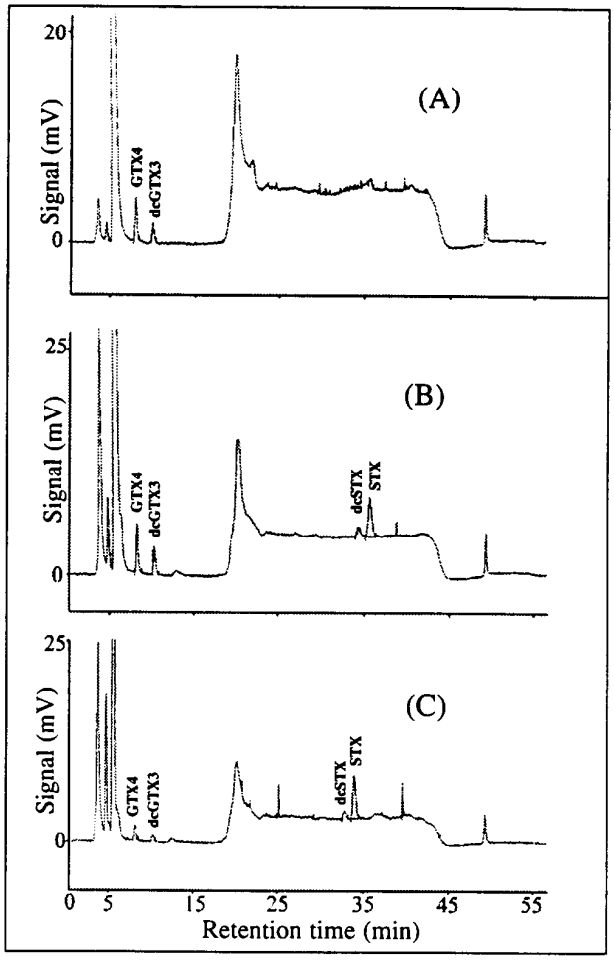

Fig. 3. Fluorescence detector HPLC chromatograms of (A) Skin, (B) Muscle, (C) Liver sample of *Tetraodon cutcutia.*

RESULTS AND DISCUSSION

The toxicity of T. cutcutia is shown in table 2. All the tissues (skin, muscle and liver) in each treatment were found to be toxic. The toxicity of skin muscle and liver were found to be 176.0±6.0, 228.33±9.50 and 99.0±6.55 MU/100g respectively. The toxicity ranged from 250 to 420 MU/100g in *T. cultcutia* from the sample of Dhaka district [1] which is little higher than Burigonga sample. Acetic acid extract of muscle and liver sample showed four peaks (Fig. 3), the retention time of which agreed well with those of GTX4, dcGTX3, dcSTX and STX (Fig. 2). The amount of different PSP components GTX4, dcGTX3, dcSTX and STX in skin, muscle and liver are shown in table 3. No dcSTX and STX was detected from the skin sample of *T. cutcutia.* The chromatograms of hydrolyzed sample did not show (data not shown) any peaks related to N-sulfocarbamyol toxin.

The toxicity of Burigonga puffer, *T. cutcutia*, ranged from 93.0 to 238.0 MU/100g (Table 2) which is much lower than those of Thai freshwater puffers which sometimes exceed several hundreds MU/g [3]. However, the recommended level of PSP toxins varied from country to country (200-400 MU/100g of shellfish or fish

products) [10]. So it is safe not to consume the puffers from the river Burigonga. The occurrence of PSP (STX) in puffer fish was first reported from two species of Japanese marine puffer fish but the Thai freshwater puffer *Tetraodon leiurus* contained PSP, consisted of STX, neoSTX and dcSTX [5,11]. In addition of common PSP components a methyl derivative of saxitoxin, N-methyl carbamoyl toxin has been isolated from Bangladeshi freshwater puffer [6]. Freshwater cyanobacteria *Anabaena* and *Lyngbya* are reported from the river Burigonga [12] and these two species are known to produce PSP toxin [13]. So, the future research should be directed towards the origin, mechanism of formation or metabolic pathway of PSP components in freshwater puffers in Bangladesh.

Table 2. Toxicity of *Tetraodon cutcutia* collected from the river Burigonga.

Date and place of collection	No. of specimens	Average total length (cm)	Average body weight (g)	Toxicity (MU/100g)		
				Skin	Muscle	Liver
26 February	10	5.2	7.3	170	219	93
1999, Dhaka	10	5.5	7.2	176	228	106
	10	6.0	7.4	182	238	98
				176.0±6.0	228.33±9.5	99.0±6.5

Table 3. Amount of different PSP components in *Tetraodon cutcutia* collected from the river Burigonga.

PSP components	Skin (ng/g)	Muscle (ng/g)	Liver (ng/g)
GTX4	1.93	2.51	0.90
dcGTX3	0.04	0.07	0.02
dcSTX	*	0.01	0.02
STX	*	0.09	0.10

* notdetected

ACKNOWLEDGEMENTS

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ALEXANDRIUM DISTRIBUTION AND TOXICITY IN THE GULF OF TRIESTE (NORTHERN ADRIATIC SEA)

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ABSTRACT

A monitoring program was carried out in the Gulf of Trieste from August 1996 to November 1997 to identify potentially toxic algae. Particular attention was devoted to the presence of dinoflagellate genus *Alexandrium,* and the PSP toxicity of laboratory cultures and blue mussels (*Mytilus galloprovincialis*) from shellfish farms in the Gulf. The biological data revealed the occurence of three *Alexandrium* species (*A. lusitanicum, A. pseudogonyaulax* and *A. tamarense*) and 19 other potentially toxic microalgal taxa, but no harmful blooms occured in the study period. Initial evidence of PSP toxicity was determined from *Alexandrium* cultures and was latter confirmed in mussels. The concentration of PSP toxins in mussels was always under the limit allowed by the Italian law, but the constant presence of PSP producers in the last years in the Northern Adriatic suggests that Harmful Algal Blooms (HAB) and human intoxications could become a future problem.

INTRODUCTION

In the Adriatic Sea, the first outbreak due to contaminated mussels by algal toxins occurred in 1976 [1] but ten years after this isolated case, the problem has become more frequent and widespread. Aquaculture activities and shellfish farms were affected with consequent heavy economical losses. Therefore, in many Italian regions, HAB projects were promoted in order to protect human welfare. In the European Union there is presently good awareness of shellfish toxicity and national laws have established health normatives to protect the production and marketing of sea products. Since 1992 in Italy two laws define the methods to analyse phytoplankton at aquaculture sites, and to determine DSP and PSP toxins in molluscs by mouse bioassay [2]. In 1995, methods for the analyses of PSP and DSP toxins were revised. DSP occurence in mussels is responsible for the selling block in many Adriatic regions every year, while only one report due to *Alexandrium minutum* and PSP contamination was recorded in 1994 along the Emilia Romagna coasts [3].

As a consequence of these episodes, in the Gulf of Trieste (Northern Adriatic Sea) a 16-month study on potentially toxic algae, in particular PSP species, was promoted by Public Health Department.

MATERIALS AND METHODS

A monitoring program was carried out monthly from August 1996 to November 1997 at three marine mussel farms A0, LBM and D0 and at another non-farm site at the Miramare Park natural oasis (C1) (Fig.1). The samples were collected at surface, 2 m and 5 m at D0; at surface, 2 m, 5 m and 10 m at A0 and LBM; at the same four depths and on the bottom (15 m) at C1. All potentially toxic dinoflagellates were analyzed by light and electron microscopy. Particular attention was devoted to the presence of the genus *Alexandrium* and to the toxicity of cultures. The PSP toxicity was checked by RP-HPLC after pre column oxidation with periodic acid of the aqueous extract of the samples [4; 5] also in blue mussels, *Mytilus galloprovincialis*, an intensively cultivated species in Italy.

Physical and chemical data were simultaneously

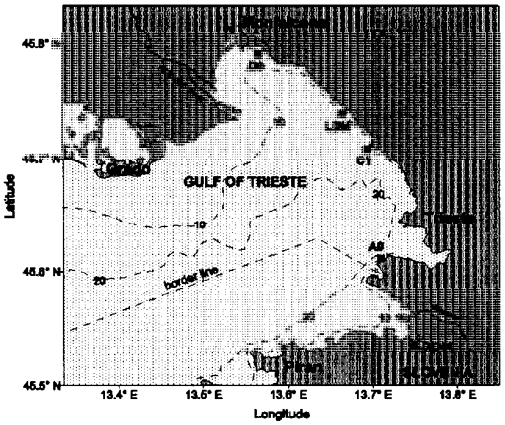

Fig. 1. Map of the sampling sites.

registered at the same stations. Dissolved nutrients were determined by means of an Alliance Autoanalyzer according to standard methods. Dissolved oxygen was determined according to the Winkler method [6]. Chlorophyll was determined in accordance to Lorenzen and Jeffrey [7].

For the biological analyses, 500 ml of water, fixed with 20 ml of buffered formaline, were sampled at each depth. Subsamples of 50 ml were sedimented and the microalgae were counted in the whole sedimentation chamber. An inverted Leitz Fluovert microscope with 400x magnification was used. Some samples with a high abundance of *Alexandrium* spp. were also washed and dehydrated for observation at a Leica Cambridge Stereoscan 430i scanning electron microscope.

Live samples for the isolation of cells for laboratory culture were taken with a phytoplankton net (0.25 μm mesh) at the various sites. Sinlge cells of potentially toxic species were isolated directly using a dissection microscope and initially incubated in medium L20 and finally grown in L1, following the recommendations of Guillard [8].

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RESULTS AND DISCUSSION

The environmental conditions

During the study period the temperature varied between 26.48°C in August 1997 and 8.97°C in January of the same year. This minimum value is high compared to the average of 6-7°C found in the Gulf of Trieste in previous years [9;10]. Salinity varied between 35‰ and 37‰ with a minimum of 24.1‰ and a maximum of 37.6 ‰. Salinity was very similar at A0, C1 and LBM, while DO was influenced by the river input and almost always showed a surface layer of freshwater. Stratification of the water column was evident at all sites from May to September 1997, except in June when bad weather caused water column mixing. The maximum stratified layer depth (between 10 and 15 m) was registered in August and September at station C1.

The geometric mean of annual nutrient concentration was 2.48 µM for dissolved inorganic nitrogen (DIN=N-NH_4^+ + N-NO_2^- + N-NO_3^-), 0.10 µM for reactive phosphorus and 2.02 µM for reactive silicate. Station D0 was most influenced by freshwater inputs from the river, as evidenced by high nitrogen and silicate concentrations (Table 1). During the summer period, the segregation of the bottom waters due to stratification caused a depletion of dissolved oxygen, reaching minimum values of 46.8% at the LBM station in July 97. The highest chlorophyll *a* concentrations all the stations were found in November 97 with a maximum of 7.67mg/l at station D0.

Table. 1. Mean annual concentration of the nutrients and chlorophyll *a* (geometric mean).

	DIN (µM)	$P\text{-}PO_4$ (µM)	$Si\text{-}Si(OH)_4$ (µM)	Chl. *a* (µg/L)
A0 (n=64)	2,15	0,12	1,58	0,53
C1 (n=80)	2,16	0,09	1,61	0,63
D0 (n=47)	5,87	0,12	4,46	0,45
LBM (n=64)	2,49	0,09	1,89	0,53

Variability of Alexandrium *species*

While there is a good awareness of DSP toxic species such as *Dinophysis* spp. in the Gulf of Trieste, the distribution and toxicity of *Alexandrium* species is less known. *Alexandrium* species were first reported in the Northern Adriatic in 1986 [11] but PSP toxins concentrations in shellfish have always remained under the legal limit and human PSP intoxication has not been reported.

The identification of *Alexandrium* cells to species level using the light microscopy (LM) is often difficult as the relevant taxonomic features of the theca are not easily resolved. However, LM methods are usually adequate to document the spatial and temporal distribution but the potential presence of more than one *Alexandrium* species, with potentially different toxicity and ecological preference, can mask seasonal patterns and correlations with chemical and physical data.

The concentration of *Alexandrium* spp. in samples was consistently low. The highest value of 820 cells l^{-1} was at 5 m depth at station A0 during April. There was no evidence of a preference for a particular depth. Average cell abundance at each site was almost always lower than 200 cells l^{-1} with the exception of 700 cells l^{-1} in September at station C1 (Fig.2).

Net samples were taken every month to identify *Alexandrium* species and to isolate and cultivate the potentially toxic species. A total of 568 dinoflagellates strains, including 34 *Alexandrium* strains, were isolated. Using calcofluor staining and SEM the 34 isolates could be identified as *A.* cf. *tamarense*, *A. lusitanicum*, *A. pseudogonyaulax*.

The finding of *A.* cf. *tamarense* was is the first report of this species from the Gulf of Trieste (Fig. 3). The ventral pore is evident in cells stained with Calcofluor. First noted in January 1997 at site C1 it was present from February to May, and again in

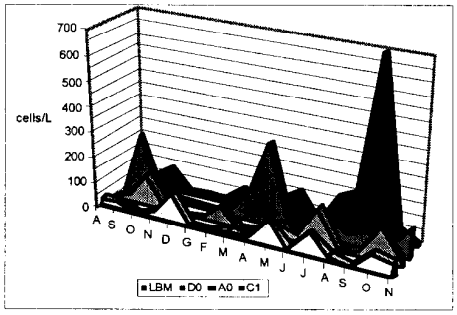

Fig. 2. Vertical distribution of the abundance of *Alexandrium* spp. in the four stations.

November 1997 all sites (Table 2). The laboratory isolates were found to not produce saxitoxins (PSP toxins) and are considered non-toxic.

A. lusitanicum (=*A. minutum*) (Fig. 4) was first reported in the Adriatic sea by Honsell et al. [11] and its sporadic occurence in this region is confirmed in this study. Based on morphological observations Franco et

Fig. 3. Epifluorescence micrograph of *Alexandrium* cf. *tamarense* stained with calcofluor in ventral view (diam=30 µm).

al. [12] proposed that *A. lusitanicum* is a synonym *of A. minutum*.

It was isolated from samples collected from March to May 1997 at sites A0 and D0 (Table 2). Some, but not all of the clones isolated were found to produce saxitoxins (Table 3).

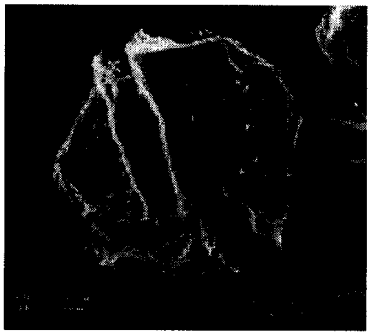

Fig. 4. Ventral view of *Alexandrium lusitanicum* cell (SEM). Scale bar = 2 μm.

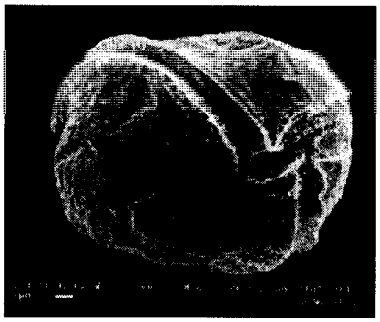

Fig. 5. *Alexandrium pseudogonyaulax* apical and partially ventral view. Scale bar = 2 μm.

A. pseudogonyaulax was prevalent from summer to autumn at all sites (Table 2) and has already been described for this area [11]. Pores are easily visible on the plates of the antapical view (Fig. 5). None of the cultures produced PSP toxins.

PSP toxins

Alexandrium cultures were tested for PSP toxins and compared with a toxic *A. minutum* strain AL1V from Vigo, Spain (Table 3). The analysis revealed the presence of gonyautoxin in culture *A. lusitanicum* AL8T Total PSP toxin content of 2.6 fmole cell^{-1} was low compared to the 7.8 fmole cell^{-1} produced by strain AL1V grown under the same culture conditions. The major toxin component was GTX4 in both cases; 90.3 mol% in ALV1 and 74.0 mol% in AL8T (Table 3). This is the first record of a toxic strain of *Alexandrium* from the Gulf of Trieste. Other clones (AL1T, AL5T) of the same species were found to be non-toxic. None of the strains of *A.* cf. *tamarense* or *A. pseudogonyalax*, produced PSP toxins. The chemical analysis confirmed

	1996					1997									
site	**A**	**S**	**O**	**N**	**D**	**J**	**F**	**M**	**A**	**M**	**J**	**J**	**A**	**S**	**O**	**N**
Alexandrium minutum																
A0								x		x						
C1																
D0										x						
LBM																
A. cf. tamarense																
A0							x	x	x							x
C1						x	x									
D0								x								
LBM							x		x	x						x
A. pseudogonyaulax																
A0									x		x		x	x	x	x
C1											x	x	x	x	x	x
D0														x		
LBM											x					

Table 2. Seasonal presence of three *Alexandrium* spp. determined by identification of cultures successfully isolated from the sampling sites.

a)	**total PSP**	Mol% PSP toxins					
	fMol/cell	GTX1	GTX2	GTX3	GTX4	B2	C2
AL8T	**2.6**	4.6	0.6	2.1	74.0	9.5	9.2
AL1V	**7.8**	5.7	0.6	3.5	90.3	-	-
b)			**total PSP**	% PSP toxins			
site	**date**		**μg/100g**	GTX1	GTX2	GTX3	GTX4
D0	April 1997		**6.7**	13.4	-	3.0	83.6
A0	April 1997		**38.5**	48.8	11.9	6.2	33.0
A0	May 1997		**32.4**	44.4	6.8	5.6	43.2

Table. 3. (a) PSP toxins of *Alexandrium minutum* strains isolated in May 1997 at site A0; (b) PSP toxin content of 100g hepatopancreas of *Mytilus galloprovincialis*.

the presence of PSP toxin (GTX1, GTX2, GTX3) in mussels as well (Table 3) coinciding with the isolation of the toxic strains of *A. lusitanicum* at sites A0 and D0. GTX1 showed the highest value with 18.8 μg/100g of hepatopancreas. This concentration is lower than the 40 μg/100g of hepatopancreas threshold according to Italian law.

CONCLUSIONS

In the Gulf of Trieste harmful algal blooms have been a continuing problem for the last ten years. The considerable biological and environmental data collected to date has been used to develop a predictive model of *Alexandrium* occurrence [13]. Previous study has shown that the highest temperature and oxygen concentrations correlate with toxic species at the offshore stations [14]. Despite new monitoring strategies and better understanding of the ecology of the toxic species the prediction of harmful blooms is still not possible [15].

Also in this study for the genus *Alexandrium* no clear prediction of occurrence is possible. The cultures have been found to produce only low level of toxins. In this case, increased toxicity may be correlated with conditions of nutrient stress rather than species

abundance [16]. Mussel PSP toxicity was not directly correlated with the presence of the PSP-producing species in the water column. It is possible that the PSP contamination was caused by an earlier bloom not detected by the monitoring program.

Over recent years, the constant presence of PSP producing species in the Northern Adriatic suggests that HAB and consequent human intoxications may be a continuing problem. In our case, PSP toxins have been completely absent in previous years and it is possible that ballast water has favoured the introduction of toxic or harmful species into the regions [17]. It may be necessary to intensify and improve monitoring to include ballast water or other sea products to limit the risk of introducing new toxic species.

To reduce economic losses the programmes should optimize the knowledge already acquired to select mussel sites unfavourable for HAB species and without extensive resting cysts of toxic species in the sediments. With more sophisticated plankton monitoring programs it should be possible to contain both the shellfish contamination and economic losses.

ACKNOWLEDGEMENTS

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PARALYTIC SHELLFISH POISONING ON THE FRENCH MEDITERRANEAN COAST IN AUTUMN 1998: *ALEXANDRIUM "TAMARENSE* COMPLEX" (DINOPHYCEAE) AS CAUSATIVE AGENT

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ABSTRACT

Although summer blooms of toxic *Alexandrium minutum* (Dinophyceae) have occurred almost yearly along the Brittany coast (N.E. Atlantic) since 1988, it was only in the autumn of 1998 that monitoring toxicity tests on shellfish collected in the Thau Lagoon (W. Mediterranean coast) showed neurotoxicity symptoms in mouse-tests. Plankton samples were nearly monospecific for a dinoflagellate subsequently identified as *Alexandrium "tamarense* complex" (Dinophyceae). Throughout the toxic event, average cell concentration seldom exceeded 5 x 10^4 cells.L^{-1}. LC/FD analysis indicated that plankton and shellfish extracts had a similar toxin profile more complex than that characteristic of *A. minutum* in France. Shellfish toxicity showed different patterns for the three species sampled. With the PSP AOAC mouse-test, maximum toxicity was found in mussels, which remained contaminated throughout the event. However, toxicity was never above the sanitary threshold for oysters and only slightly above for carpet-shells. The spatiotemporal distribution of the event in the lagoon was studied by mouse-tests on the shellfish species monitored. LC/FD results indicated that *A."tamarense* complex" was the causative agent.

INTRODUCTION

Three toxicogenic microalgae are especially monitored in France : *Dinophysis* (since 1983) [1], [2], *Alexandrium* (since 1988) and *Pseudo-nitzschia* (since 1998) [3]. When one of these algae is detected, the REPHY plankton monitoring network conducts control analyses on exposed bivalves.

Intense summer blooms of *Alexandrium minutum*, Halim are responsible for the accumulation of paralytic toxins (PSP) in shellfish along the coasts of North Brittany (N.E. Atlantic) [4], [5]. In the autumn of 1998, a bloom of another *Alexandrium* species was implicated in shellfish toxicity for the first time in France (Thau Lagoon, W. Mediterranean coast). Toxicity tests performed at the end of October as part of a routine control using the AOAC PSP method were positive, showing a toxicity of 855 µg STXeq $100g^{-1}$ of mussel meat. Plankton samples showed the nearly exclusive presence of the genus *Alexandrium*, with up to 85000 cells.L^{-1}. The bloom spread rapidly over the entire lagoon, and the subsequent two-month marketing ban had great economic impact, affecting around 10% of French shellfish production.

Contrary to observations with *A. minutum*, high mussel toxicity was generated by a relatively low number of cells. Thus, the main purpose of this study was to demonstrate that shellfish toxicity resulted from the bloom of another *Alexandrium* species.

MATERIALS AND METHODS

Shellfish samples

Three species of shellfish were sampled every three or four days: the mussel (*Mytilus edulis galloprovincialis*), the Japanese oyster (*Crassostrea gigas*), both of which are cultivated on ropes, and the carpet-shell (*Ruditapes decussatus*).

Plankton samples

From October 26 to mid-December, plankton samples were obtained in subsurface waters and studied in inverted microscopy according to the Utermöhl method. A few concentrates for LC/FD analyses were produced by direct seawater filtration using 10 µm net and then placed in acetic acid 0.1N and stored at 4°C.

Mouse bioassay for PSP toxins

Sample preparation, according to AOAC method [6] for PSP detection, consisted of a boiling acid extraction (0.1 N HCl) of 100 g of whole shellfish tissue and injection of 0.5 g tissue equivalents into each 20-g mouse.

Liquid chromatography-fluorescence detection (LC/FD)

Analysis of PSP toxins was performed using Oshima's method [7], with slight changes. Toxins were separated by RP-LC using a C_8 column (5 µm Develosil, 4.6 mm i.d. x 250 mm) with a flow rate of 0.8 mL/min. Eluent pH and/or column temperature were calibrated to optimise the separation of some gonyautoxins (dc-GTX3/B1/dc-GTX2). Toxins were quantified in duplicate using certified standards (PSP-1B) provided by the NRC (I.M.B., Halifax, Canada). For some GTXs and C toxins, identification and assay were performed indirectly after chemical hydrolysis according to Franco and Fernández-Vila [8]. Dc-toxins were only identified.

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RESULTS AND DISCUSSION

Hydroclimatic data

Thau Lagoon (fig. 1) is a large (19.5 km long and 4.5 km wide) shallow (4 m mean depth) and tideless lagoon linked to the Mediterranean Sea by two natural channels located at its two ends. At Bouzigues (N lagoon), 1998 temperatures showed little difference with the mean value over the last eight years. There was a constant increase until August, followed by a slow decrease, which in 1998 was more marked as of November 16. A slow increase in salinity is generally observed until September, after which heavy autumn rains cause a drop in lagoon salt levels. In 1998, this usual pattern did not occur since the salt level was high (39.5±0.5)‰ from September to December. A marked deficit in precipitation was observed in October.

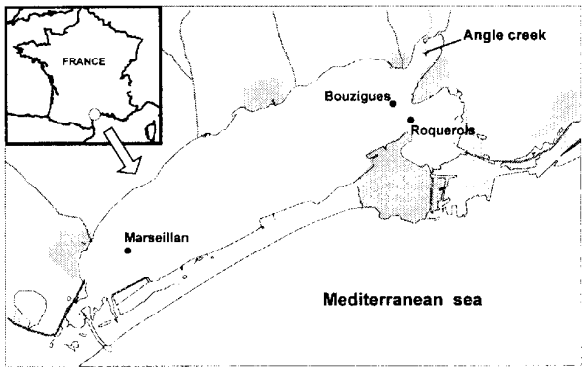

Fig.1. Thau lagoon sampling stations.

Phytoplankton observation and abundance

Microscopic studies of plankton samples collected between Nov. 3 and 12 (mid-bloom), showed the presence of cells likely to form short chains (2 to 4 cells). The theca of these cells (isolated or in chains) consisted of a collection of plates whose tabular formula corresponded to that of the genus *Alexandrium*. Cells varied from rounded to slightly flattened anteroposteriorly (fig. 2a,2b) and were heterogeneous in size (25 to 42 µm in length and width) though generally between 30 and 32 µm in length and between 30 and 35 µm in width.

Around 30 thecae (both rounded and flattened) were used to study tabulation details (notably of pores) in light microscopy. Each theca had a wide 6" plate (unlike *A. minutum*, see fig. 2c), a wide Po and a nearly of the same length and width Sp plate. Po usually had an ellipsoïdal anterior connecting pore between the right edge of the plate and the apical pore, and the Sp plate had a posterior connecting pore of variable size on its right half. A ventral pore (Vp), independently of the degree of anterioposterior flattening of the theca, was visible in some specimens on the suture between apical plates 1' and 4', only slightly indenting plate 1' (fig. 2d).

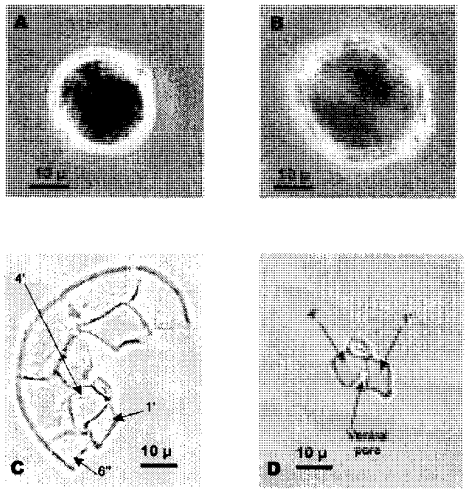

Fig 2. (a,b) *A. "tamarense* complex" cells in light microscopy. (c) Theca without a visible ventral pore on the suture between apical plates 1' and 4'. (d) Ventral pore indenting apical plate 4' more than 1'.

These details allowed us to identify these specimens as *Alexandrium* "tamarense complex", and in some cases even as *Alexandrium tamarense* because of the Vp [9]. Other specimens without a Vp may correspond to a variety of this species in French waters or an entirely distinct species (*A catenella)*. The complicated toxin profile (see below) tend to support this last hypothesis.

Figure 3 indicates the cell concentrations of *A. "tamarense* complex" at three lagoon sites. The Angle inlet area (without shellfish culture) showed the highest cell density (a maximum of 3.5 x 10^5 cells L^{-1}). Unlike other sites, Angle inlet is a nearly closed embayment with few exchange with seawater from outside. Stagnation of water masses (and their stratification?) probably accounted for the high cell concentration observed.

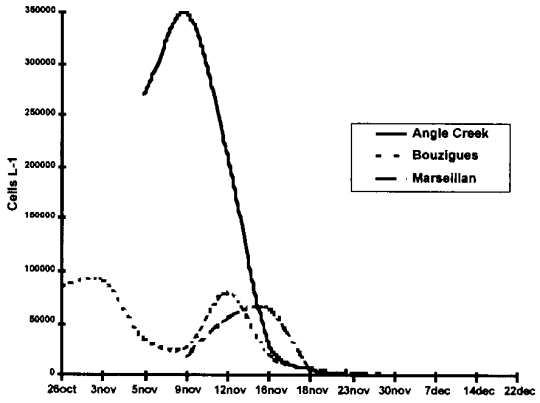

Fig. 3. "*A. tamarense* complex" cell concentrations at the three sampling stations.

Lower cell density was observed at Bouzigues station in a shellfish production zone. During the first half of November, *Alexandrium* concentrations ranged

between 9 and 2.6 x 10^4 cells L^{-1}. A decrease in cell number was parallel to the drop in water temperatures after Nov. 16. For the Marseillan station at the other end of the lagoon, proliferation (which occurred later) was also relatively lower, though shorter, and decreased in conjunction with the temperature drop. In this shallow lagoon, the main water circulation is wind-induced, which could account for the heterogeneous distribution among the three sites.

Shellfish toxicity and toxin profile

Mussel and oyster samples were collected at Bouzigues and Marseillan, whereas carpet-shells were only sampled at Roquerols. Figure 4 reports cell concentrations in water and results of the AOAC tests for three types of shellfish at Bouzigues during the toxic episode.

Mussels showed the highest rate of accumulation (855 µg STXeq. 100 g^{-1} of meat). This high level could have been enhanced in this tideless lagoon. Toxicity varied slightly around this value throughout the period of high cell concentration, then reflected changes in cell density before decreasing with the reduction of *Alexandrium* cells in the water. Though collected in the same area, oysters (a species highly sensitive to toxic algae) remained close to the detection threshold of AOAC test and below the sanitary threshold (80 µg STXeq 100 g^{-1} whole meat). These results are concordant with those of previous studies concerning PSP toxin accumulation in shellfish [10].

Although contamination was monitored in carpet-shells at a time when cell concentration was already declining, toxicity was appreciably higher (105 µg STXeq. 100g^{-1} whole meat) than the sanitary threshold. The decontamination period for carpet shells was much longer, probably because of the nature of their decontamination kinetics.

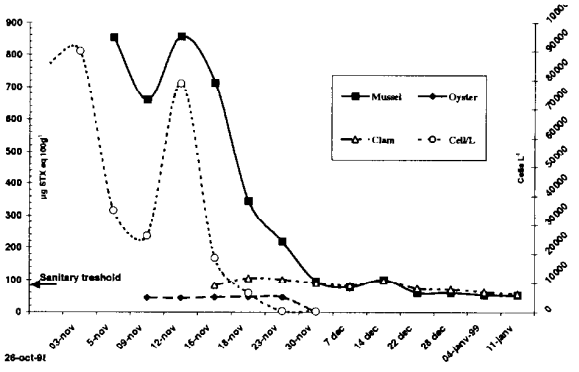

Fig 4. "*A. tamarense* complex" cell concentrations and shellfish toxicity for three species with the AOAC test.

For samples collected in Marseillan (south lagoon) mussel contamination (data not shown) was parallel to changes in cell density of *A. "tamarense* complex", although contamination maxima were lower (315 µg STXeq 100 g^{-1} mussel meat) than those for Bouzigues because of lower cell concentration. For similar reasons, oysters were not contaminated, showing results below the detection threshold of the AOAC test.

Thus, these observations indicate a coherence between results for mouse-tests and the presence of *A. "tamarense* complex" cells in the water. However, confirmation of the involvement of *A. "tamarense* complex" in the contamination of bivalves will require comparative study of the toxin profile of this species and that of extracts of exposed shellfish.

The toxin profile was determined from a concentrate of 70 litres of seawater collected on Nov. 6, 1998, at Bouzigues and filtered on 10 µ (7.1 x 10^5 cells.L^{-1}). The plankton study showed near-monospecificity for *A. "tamarense* complex".

For plankton and shellfish extracts, LC/FD analyses were performed on untreated and hydrolysed samples. As noted above, chemical hydrolysis allowed the identification and quantification of some specific toxins. Thus, the increase of GTX-3, GTX-2, STX and NeoSTX indicated the presence of C1 and C2 toxins and B1 and B2 gonyautoxins respectively. Conversely, the stability of GTX-1 and GTX-4 confirmed the lack of C3 and C4 toxins. The comparative study of the overall results of chromatographic analyses showed the complexity of the toxin profile of *A. "tamarense* complex", allowing the identification of the following toxins: toxins C1 (GTX8) and C2 (GTX8β); gonyautoxins B2 (GTX-6), GTX-4, GTX-1, dc-GTX3, B1 (GTX-5), dc-GTX2, GTX-3 and GTX-2; saxitoxin: STX, Neo-STX, and decarbamoyl-STX.

The molar fraction of the toxins is shown in Table 1. In plankton, highly toxicogenic carbamate toxins (STX, NeoSTX, GTX 1-4) represented up to 60% of total toxins, whereas poorly toxicogenic N-sulfocarbamoyl toxins were predominant (80%) in mussel extract. As expected, a major proportion of β-isomers (C2, GTX-4, GTX-3) was observed in plankton, whereas α-isomers were dominant in mussel samples. Contamination was recent since the epimerisation ratio between GTX-3 and GTX-2 only reached the final theoretical 1:3 value [11] on Nov. 30 and Dec. 7 for Bouzigues and Marseillan respectively.

	Plankton	Mussel
C1/C2	7.9	22.3
B1	18.2	59.1
B2	5.6	8.9
GTX 3/2	20.6	1.7
GTX 4/1	5.9	3.8
Neo-STX	12.1	0.8
STX	29.6	3.5

Table 1. Molar fractions of plankton and mussel samples collected on Nov. 6, 1998 at Bouzigues station.

When non-quantifiable dc-toxins were not taken into account, the toxicity of this plankton extract was estimated at 26 pg STXeq. $cells^{-1}$ (average toxicity for

an "*A. tamarense*" strain). Though toxin composition in the mussel sample was the same as that in the plankton extract, individual toxin percentages differed. Since sample extraction was different, direct comparison would not be conclusive. This difference in toxin proportions between plankton and shellfish extracts has also been already noted for a Korean *A. tamarense* species [12]. The overall results obtained allowed us to confirm the involvement of *A. "tamarense* complex" in this new episode of autumn shellfish toxicity on the Mediterranean coast.

Neurotoxic blooms in the W. Mediterranean have usually been due to *A. minutum* and *Gymnodinium catenatum* in Spain and *A. minutum* in Italy. However, non-toxic *A. tamarense* blooms were observed in Italy during the summer in 1982 and 1985 [13]. Conversely, potentially toxic species of *A. tamarense* have been found in temperate and cold coastal waters. In the last 20 years, toxic strains have been identified in North America, Asia, Argentina and New Zealand. Toxin composition of different *Alexandrium* strains studied was highly variable. As indicated by Cembella et *al.* [14], clones of *A. tamarense* from British Columbia – (Canada) can show simple profile (one or two toxins) or very complex toxin profiles similar to that described here. This variability does not seem to be related to a particular strain but to environmental conditions. Similar observations have been reported by Kim et *al.* [15] for *A. catenella*, in which two isolates entirely identical in terms of morphological criteria, isozyme analysis and monoclonal antibody, but from widely separated geographical regions, had a different toxin profile.

This variability in toxin content might also have been due to presence of two or more species that could not be differentiated morphologically, as in the case of the *A. tamarense/A. catenella* complex [16], [17]. This possibility will require further investigations.

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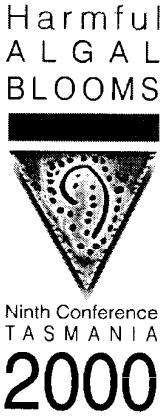
HARMFUL ALGAE EVENTS

ASP



THE OCCURRENCE OF AMNESIC SHELLFISH POISONS IN SHELLFISH FROM SCOTTISH WATERS

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ABSTRACT

Monitoring for amnesic shellfish poisons in wild and cultivated shellfish from Scottish waters commenced in 1998, following the introduction of revised legislation from the European Community. The principle toxic compound, domoic acid (DA), was detected in a variety of species, in some instances at concentrations above the regulatory limit. Although some harvesting closures were implemented in 1998, DA caused significant problems the following year. In 1999, the majority of scallop (*Pecten maximus*) fisheries from the Scottish West Coast were closed due to DA, resulting in considerable media attention and speculation on the role of nutrient inputs from local aquaculture. This paper will present data summarising the principal features of the 1999 toxic event.

INTRODUCTION

The first recorded incident of amnesic shellfish poisoning (ASP) occurred in Canada in 1987, when 107 people became ill and 3 died after consuming mussels (*Mytilus edulis*) [1]. The bivalves contained the neurotoxin domoic acid (DA), from the diatom *Pseudo-nitzschia multiseries* [1,2]. Subsequently, several other species of *Pseudo-nitzschia* were shown to produce DA [2].

Regulations stating the maximum concentration of DA permissible in shellfish, marketed in Europe, were introduced in 1997 [3,4,5]. As a consequence, monitoring for DA was implemented in Scotland in 1998 and the toxin was detected in a range of shellfish [6]. However, the implications of DA were not fully realised until 1999, when it was responsible for a harvesting ban on king scallop (*Pecten maximus*) fishing grounds over most of the Scottish West Coast. This paper describes the occurrence of DA in Scotland during 1999, and discusses some of the issues which arose.

RESULTS

Occurrence of DA in Scotland during 1999

High Performance Liquid Chromatography with strong anion exchange clean-up and diode array detection (HPLC; 6, 7), was used to analyse 2,695 samples of the major commercial shellfish species in Scotland (Table 1). DA was detected most frequently in the king scallop *Pecten maximus*, which also contained the highest concentration of the toxin (Table 1).

Occurrence of DA at concentrations greater than the regulatory limit of 20 $\mu g\ g^{-1}$ [4] in scallop gonad samples, resulted in closure of most of the Scottish scallop fishing areas with the most severe effect on the West Coast grounds (approx. 8,500 nautical miles^2; Fig. 1). Scallop gonad samples from this area contained the highest DA concentrations (Fig. 1) with the most toxic samples obtained in August. Prohibition of harvesting on the West Coast was first implemented in June 1999 and a large area was still closed to scallop fishing by April 2000.

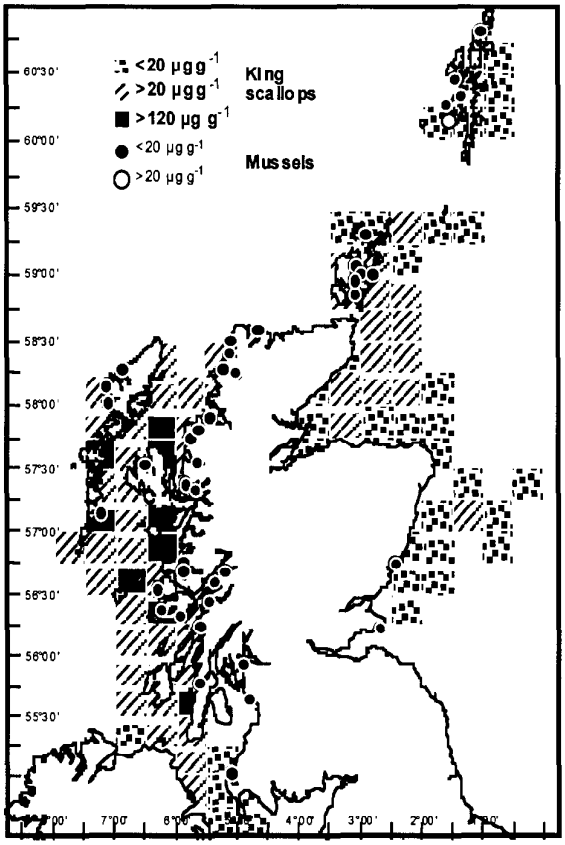

Fig. 1. Distribution of DA in Scottish waters during 1999 in *M. edulis* and *P. maximus* (gonads). Sampling areas are divided into 15 x 15 nautical miles, 1/4 ICES boxes.

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Table 1. Number of extracts containing DA from each major shellfish species analysed during 1999. nd= <2.5 μg g^{-1} minimum determination limit.

Species	Numbers analysed	Proportion of samples (%) at each DA concentration range (μg g^{-1})					Maximum [DA] (μg g^{-1})
		nd	<20	>20 <120	>120 <250	>250	
P. maximus (whole tissue)	658	0	7.8	53.2	31.8	7.2	534
P. maximus (gonad)	1195	2.2	51.4	45.2	1.1	0.1	294
M. edulis	711	81.6	18.3	0.1	0	0	23
C. opercularis	131	13	85.5	1.5	0	0	48

Routine monitoring of scallops in Scotland is based on analysis of gonad tissue. However, for comparison, whole animal extracts were also analysed for some areas. DA was detected in all whole animal extracts and at higher concentrations than in gonad samples (Table 1).

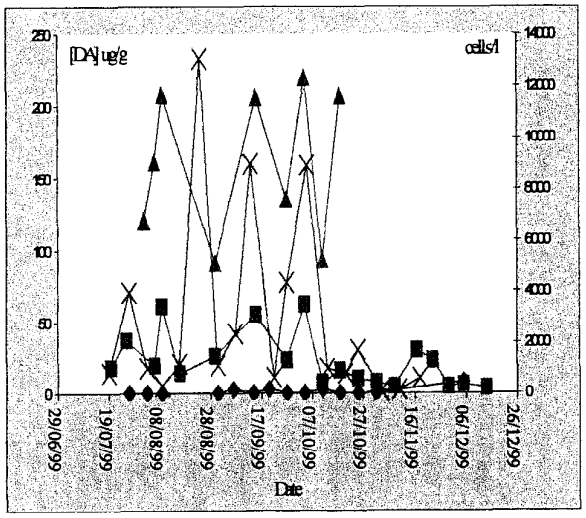

Fig. 2: DA concentrations in *P. maximus* over an eight-month period and *Pseudo-nitzschia* spp. numbers over a four-month period, from an in-shore site on the Scottish West Coast. DA concentrations (μg g^{-1}) in (■) *P. maximus* gonad, (▲) *P. maximus* whole animal and (♦) *Pseudo-nitzschia* numbers (cells l^{-1}).

Additionally, when gonad samples and whole animal samples from the same sites were compared, the amount of DA in the whole animal extract was consistently higher (e.g. Fig. 2). The second scallop species harvested in Scotland, *Chlamys opercularis* (queen scallop), also contained DA but at much lower concentrations than *P. maximus* (Table 1), even when both species were obtained from the same area.

A large proportion of mussels *(M. edulis)* analysed for DA were negative (i.e. below the limit of determination for DA by HPLC; Table 1). When DA was detected it was generally at low levels and distributed over a wide area (Table 1; Fig. 1). In the few areas where both *M. edulis* and *P. maximus* were harvested, even if the latter contained DA above closure levels, mussels were relatively unaffected. A limited number of *Cancer pagurus* (brown crabs), *Cerastoderma edule* (cockles), *Carcinus maenus* (green shore crabs), *Ostrea edulis* (native oysters), and *Ensis* spp. (razor fish) were also examined and found to contain trace levels of DA (approximately 2.5 μg DA g^{-1} or less).

Identity of Pseudo-nitzschia *species.*

Pseudo-nitzschia spp., (as determined by light microscopy from integrated water samples at 0-10 m depth) were detected at all thirty-two regular in-shore phytoplankton monitoring sites in Scotland. These diatoms were present throughout the year, although peak numbers tended to occur from April to August, depending on the site. The highest concentration of *Pseudo-nitzschia* spp. detected was 2,342,660 cells l^{-1} however, *M. edulis* from the same area, contained only trace levels of DA. *Pseudo-nitzschia* numbers rarely exceeded 500,000 cells l^{-1} with peak levels at most sites around 100,000 cells l^{-1}, even at areas where scallops contained DA (Fig. 2). The quantity of DA in the whole scallop and the gonad seemed to increase with the number of *Pseudo-nitzschia* cells, however there are too few data points to determine if a relationship exists between these two factors (Fig. 2).

Fixed plankton samples from some in-shore areas were analysed by transmission electron microscopy. Species identified were *P. australis*, *P. pungens*, *P. seriata*, *P. multiseries*, *P. fraudulenta*, *P. turgidula* and *P. delicatissima*. However, as none of these strains were isolated for culture their toxicity is unknown. Phytoplankton from off-shore areas are not examined routinely. However, limited sampling at one site resulted in the isolation and culture of *P. australis* (Bolch, unpublished data). Cell extracts from this isolate contained DA as determined by HPLC (Hess and Bolch, unpublished data.)

DISCUSSION

Shellfish toxins such as paralytic and diarrhetic shellfish poisons (PSPs and DSPs) occur regularly in bivalves harvested from Scottish waters with both toxin groups responsible for periodic harvesting bans [8]. PSPs are generally the most problematic and have been the cause of regular harvesting closures of scallop fishing grounds on the Scottish East Coast and in the Orkney Isles [8]. However, the impact of these closures were not as severe or widely publicised as the 1999 closure of Scottish fishing grounds due to DA in *P. maximus*. This resulted in disruption of a fishing industry worth approximately £15 million (based on landings of king scallops in Scotland) [9]. This figure excludes the value of support industries, which are vital to the economy of many rural communities around Scotland. Dealing with the 1999 DA event also required a large increase in resource input from regulatory authorities, with the number of samples processed increasing upto ten-fold.

Although *P. maximus* was the primary problem species, DA was also present at most mussel (*M. edulis*) harvesting sites at some point during the year, generally at low levels. Bates and colleagues [2] list information for Cardigan Bay in Canada showing that DA was below regulatory levels in mussels, if detected at all, in 7 out of 9 years examined, although *M. edulis* from this site contained sufficient DA to cause food poisoning in 1987. This emphasises requirements for stringent monitoring of mussels even in areas where DA concentrations are generally perceived to be low, as is currently the case in Scotland.

A further observation from the 1999 DA event is that benthic species such as *P. maximus* are toxic whereas rope cultured *M. edulis* harvested from adjacent areas are largely non-toxic or only slightly so, even in the presence of *Pseudo-nitzschia* spp. This suggests several things; i) the sediment may provide a constant source of DA to scallops, which is not available to rope-cultured mussels, ii) *P. maximus* concentrates more DA than mussels hence requiring lower numbers of toxic diatoms and iii) *P. maximus* retains DA for longer periods than mussels. A combination of all three factors may be involved although the latter may prove to be the most relevant. *P. maximus* is known to retain shellfish toxins such as PSPs for long periods [10], however, there is a dearth of information in relation to DA.

The Scottish phytoplankton monitoring program demonstrated that *Pseudo-nitzschia* spp., including potentially toxic strains, were present throughout in-shore areas around Scotland although the numbers did not reach the high levels associated with the 1987 food poisoning incident in Canada [2]. In off-shore Scottish waters, a DA-producing isolate of *P. australis* was obtained (Hess and Bolch, unpublished data), however, more work is required to examine its toxin-producing characteristics. Further studies are also necessary to assess the frequency and extent of its occurrence and to determine if it, and/or other diatom species, are responsible for DA in scallops in Scottish waters.

Various parties, including the media, claimed that occurrence of DA was increasing and related to outputs of nutrients from fish farms. Information is not available on the prevalence of DA in shellfish in Scotland prior to 1998, therefore it is not possible to deduce that it is increasing in occurrence. Additionally, limited information is available in the scientific literature on environmental factors which stimulate the occurrence of *Pseudo-nitzschia* spp. and subsequent DA production. In Canada, DA in mussels was traced to a bloom of *P. multiseries* which bloomed after a prolonged dry summer period, followed by an unusually rainy autumn. Highest DA values occurred when nitrate concentrations increased and phosphate levels fell. Conversely, in Monterey Bay, California, the highest concentrations of DA in a *P. australis* dominated bloom occurred when nitrate levels were low [2]. On the Scottish West Coast, insufficient information is available to draw conclusions on environmental aspects leading to scallop toxicity although certain factors can be noted. Seasonal and long term natural fluctuations in nutrient supply from offshore oceanic sources outweighs the relatively minor inputs from anthropogenic sources (approx. 1.6%) such as aquaculture, sewage, riverine and industrial waste [11, 12, W.R. Turrell & P. Gillibrand pers. comm.]. What may be more relevant is that the area is experiencing significant climate change. Winter water temperatures have increased by 4°C, over the last 6 years, resulting in a reduced annual temperature range [13]. Wind speeds are also intensifying causing increased mixing, enhanced offshore circulation, the import of more southerly water masses and enhanced offshore nutrient supply [13]. These factors will be taken into account in future research on DA in Scottish West Coast scallop fishing grounds.

A further issue, which arose during the 1999 event, was the choice of scallop organ used in DA monitoring. One study [14] demonstrated that DA concentrates in the scallop hepatopancreas, followed by the mantle, then the gonad, with the adductor muscle being the least toxic, if at all. Most of the DA was removed if the hepatopancreas was excised [14]. In Scotland during 1999, testing of whole scallops (using all organs) demonstrated that none of these bivalves were free of toxin (92.2 % of the whole scallop samples exceeded the closure limit compared to 46.4 % when the gonad tissue was used). Proposals were forwarded to the EU from various parties, suggesting that the organ tested for monitoring purposes should be based on whatever organ is marketed. Regulatory authorities are currently awaiting a decision from the EU, the outcome of which has serious implications for both the scallop industry and for regulatory authorities tasked with ensuring compliance. The data from the Scottish monitoring programme indicates that more areas would be closed to harvesting, and for longer periods, if closure action

levels were based on testing of the whole animal rather than the gonad.

In conclusion, until recently the occurrence of DA in shellfish was not considered an issue within the EU. However with increased monitoring it has now been detected in shellfish from Spain [14], Portugal [15], Ireland [16] and Scotland. The difficulties experienced in Scotland during 1999 were severe, with the long-term implications to the scallop fishing industry currently unknown. What is obvious is that research is required to determine both the environmental and physiological parameters involved in DA contamination of shellfish, both in Scotland and in other countries.

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THE COASTAL *PSEUDO-NITZSCHIA* FROM THE STATE OF RIO DE JANEIRO, BRAZIL

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ABSTRACT

On the coast of the state of Rio de Janeiro, Brazil, there are 3 environments where *Pseudo-nitzschia* spp. are a frequent component of the micro-phytoplankton (detected in 70-80% of the samples): Cabo Frio (cape with seasonal upwelling), Guanabara Bay (heavily polluted estuary), and Ribeira Bay (pristine waters). Cell density can vary from 10^2 cells.L^{-1} (common in Cabo Frio) to a maximum value of 10^5 cells.L^{-1} (constant in Guanabara Bay and often found in Ribeira Bay). In Cabo Frio (1988-90), they contributed to the first stages of phytoplankton succession triggered by upwelling, but were also well represented in nutrient-poorer, warmer waters. In Guanabara Bay (1985-87, 1998-99), the occurrence and distribution of this marine genus was mostly determined by salinity gradients: lowest abundances (at times, non detected) in the inner reaches of the bay and during the rainy season (summer). In Ribeira Bay (1987-97), the homogeneity of local hydrological features allowed for the presence of the genus year around and at high relative contributions to the micro-phytoplankton (>50% of the total cell density). At present, eight species are identified using light and/or scanning electron microscopy: *P. cuspidata*, *P. delicatissima*, *P. heimii*, *P.* cf. *multiseries*, *P. multistriata*, *P. pseudodelicatissima*, *P. pungens*, and *P.* cf. *subfraudulenta*.

INTRODUCTION

The occurrence of the diatom genus *Pseudo-nitzschia* has been recorded all along the coast of Brazil (from 2°N to 32°S), observed from close to shore (bays, estuaries, beach surf zones, sand reefs) to shelf and offshore waters [e.g., 1,2,3,4]. Most of the studies on phytoplankton distributional patterns in Brazilian waters report on the occurence of *Pseudo-nitzschia* (often referred to as *Nitzschia*) at the genus level because differentiation among species by routine quantitative methods (water sampling and settling technique) is often an impossible task. The stepped colony that moves as an entire unit is a conspicuous character of this genus that can be readily identified. Identification to species, however, requires a thorough examination of the outline and of the fine structure of the diatom cell wall.

On the coast of the State of Rio de Janeiro (270 km along an east-west coastline at 23°S), there are at least 3 environments (Cabo Frio, Guanabara Bay, and Ribeira Bay) where *Pseudo-nitzschia* spp. are a frequent component of the micro-phytoplankton (> 20 µm). These areas present distinct environmental settings (Fig.1, Tab.1). At Cabo Frio, coastal upwelling takes place during the austral summer, from October to March, when the E-NE wind prevails and sea surface temperatures are the lowest found in the year (< 18°C). Guanabara Bay is a heavily polluted estuary, especially due to eutrophication. Water quality varies from place to place because of unevenly distributed pollution sources added to a tidally induced circulation pattern. Ribeira Bay, however, has pristine waters with little freshwater input besides rainfall.

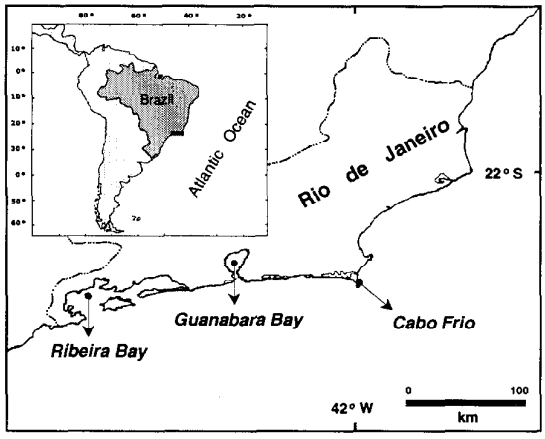

Figure 1. Study areas along the coast of the State of Rio de Janeiro, Brazil.

The objective of this research is to determine the space-time distribution of the genus *Pseudo-nitzschia* in these three contrasting environments on the coast of Rio de Janeiro. It is largely based on research projects that focus (or did in the past) on the study of the entire phytoplankton community structure, so that primary data (original counts) were used, as well as archived net samples. Although the process of data retrieval is in its infancy, it is possible to draw preliminary conclusions about the distribution and species composition of this interesting and important genus. To date, eight of the 21 described species of *Pseudo-nitzschia* have been reported to produce the neurotoxin domoic acid, which can cause Amnesic Shellfish Poisoning (ASP) [5,6].

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Intergovernmental Oceanographic Commission of UNESCO 2001

Table 1. Some hydrological aspects that differentiate the study areas: temperature (Temp, °C), salinity (Sal), nitrate (NO_3, µM), and ammonia (NH_4, µM).

Cabo Frio (1988-90)*		Temp	Sal	NO_3
	min	13.0	33.03	0.36
	max	24.5	35.81	8.54
Guanabara Bay (1980-90)[7]		Temp	Sal	NH_4
	min	19.0	13	nd
	max	31.0	37	60
Ribeira Bay (1992-98)**		Temp	Sal	NO_3
	min	20.8	33	0.07
	max	30.6	37	3.45

Data provided by (*) Instituto de Estudos do Mar Almirante Paulo Moreira and by (**) Laboratório de Monitoração Ambiental, Eletronuclear.

METHODS

Pseudo-nitzschia counts were done as part of the analysis of the whole phytoplankton community, based on water samples and the settling technique. Species composition was determined from net hauls from the study areas, which were not necessarily taken at the same time as the water samples. Cells in the concentrated net samples were cleaned of the organic material that obscure the frustule structure [8]. For light microscopy, the clean material was air dried and mounted on a microscope slide with Hyrax, and observed under oil immersion with phase contrast. For scanning electron microscopy, the cover slip with clean, dried material was affixed to aluminum stubs and coated with gold-palladium.

RESULTS

The genus *Pseudo-nitzschia* was found in 80% of all the samples investigated (total of 263 samples).

At present, eight species are identified using light and/or scanning electron microscopy: *P. delicatissima*, *P. pseudodelicatissima*, and *P. pungens*, found in all study areas; *P. cuspidata*, *P. cf. subfraudulenta* and *P. multistriata* found in Guanabara Bay; *P. cf. heimii* and *P.* cf. *subfraudulenta* found in Ribeira Bay and Cabo Frio; and *P.* cf. *multiseries* observed in Cabo Frio only. The versatility of this genus, in terms of the variety of environmental settings where it can be found, reinforces the need to understand distributional patterns at the species level, a more detailed study which is underway.

Cabo Frio (1988-90)

Pseudo-nitzschia varied from 4.8×10^2 to 6.8×10^4 cells.L^{-1} (Fig.2). The highest abundances were observed soon after pulses of nitrate due to upwelling (January-April), but they were also well represented during the rest of the period, characterized by warmer waters.

Guanabara Bay (1985, 1998-99)

Pseudo-nitzschia varied from 2.0×10^3 to 7.5×10^5 cells.L^{-1} (Fig.3). The occurrence and distribution of this marine genus was determined by salinity gradients: lowest abundances (at times, not detected) were found in the inner reaches of the bay and during the rainy season (summer). In terms of relative abundance, this genus often contributed up to 25% of the micro-phytoplankton, despite the high abundances of euglenophytes and small dinoflagellates that also thrive in the eutrophic waters of Guanabara Bay.

Ribeira Bay (1987-97)

Pseudo-nitzschia varied from 1.3×10 to 2.6×10^5 cells.L^{-1} (Fig. 4). The homogeneity of the local hydrological features allowed for the presence of the genus year around and high relative contributions to the micro-phytoplankton. The genus *Pseudo-nitzschia* often reaches 50% of a diatom-dominated community.

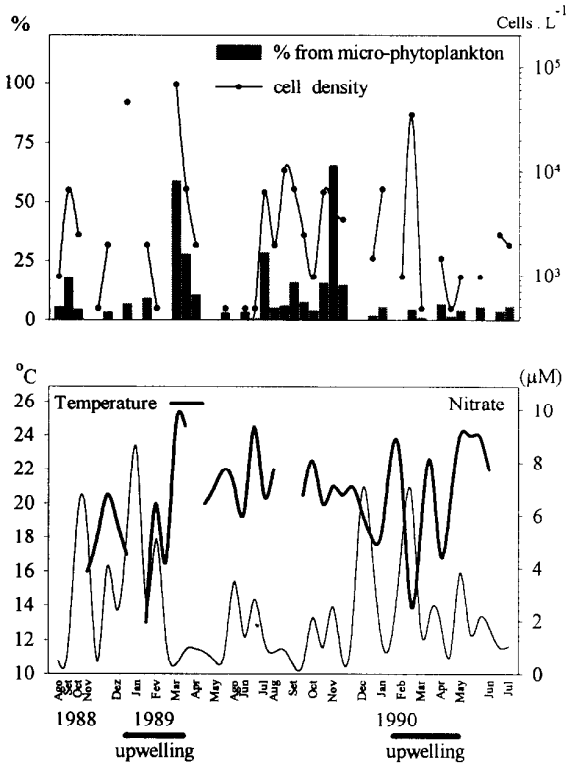

Figure 2. Data from one sampling site at Cabo Frio (site Jaconé), bi-monthly sampling at 5m depth (n=47). X-axes are the same for both graphs. The top graph shows the distribution of *Pseudo-nitzschia* (total cell number and relative abundance). The bottom graph shows the corresponding variations of temperature and nitrate, variables that indicate upwelling conditions.

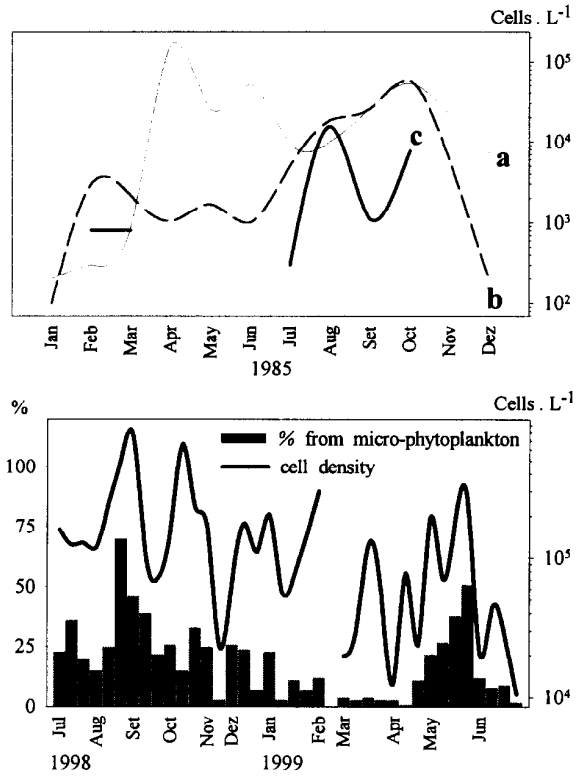

Figure 3. *Pseudo-nitzschia* from Guanabara Bay. Top graph is total cell number for 1985: average of 5 sites located (a) closer to the entrance to the bay, (b) in the middle region, and (c) in the inner reaches of the bay, visited monthly, with sampling at 5m depth (n=54). Bottom graph is total cell number and relative abundance for 1998-99: one sampling site at the entrance to the bay, with weekly sampling at sub-surface (n=38).

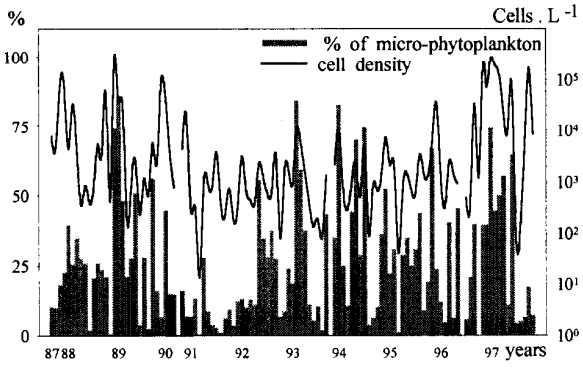

Figure 4. *Pseudo-nitzschia* (total cell number and relative abundance) from Ribeira Bay. Average of 3 sampling sites, with monthly sampling at 5m depth from September 1987 to December 1997 (n=118).

DISCUSSION

Pseudo-nitzschia distributions in these contrasting environments on the coast of Rio de Janeiro reinforce the need for a better understanding of species composition and raise hypotheses regarding life strategies and growth requirements.

In Cabo Frio, *Pseudo-nitzschia* contributed to the initial stages of phytoplankton succession (*sensu* Margalef [9]) that can be triggered by the upwelling of colder and nutrient-rich waters [e.g., 10,11,12]. On the other hand, they can be a common component of the plankton community at times of more stratified, nutrient-poor waters, as noted elsewhere [e.g., 13,14]. A better understanding of species composition could explain, in part, such differences, but other aspects can be investigated. As observed in culture [15], *Pseudo-nitzschia* can take on r-selected characters at one growth phase, when cells reproduce rapidly and form long, planktonic chains, and k-selected characters at another, when growth is slower, and chains break apart, clump, and sink. Although clumping has been observed in association with sexual reproduction of *P. subcurvata* [16], for *P. pseudodelicatissima* and *P. multiseries*, sexual reproduction takes place only when pairing cells are growing actively, not at clumping stage [17]. Long chains are commonly observed in nature, and aggregates of *Pseudo-nitzschia* cells have also been reported [18]. Such an alternation of survival strategies has been evoked to understand distributional patterns in the field [19].

The distribution in Guanabara Bay is clear: *Pseudo-nitzschia* is a marine genus with a wide salinity range for optimum growth, as determined in culture [20]. Indeed, it is reported from offshore to innermost estuarine regions [21,22,23]. Once more, a better undertanding of species composition could explain, in part, such differences. We speculate, however, that some other factor, most likely higher nutrient availability, can counteract the "negative" effects of lower salinity and allow the establishment of *Pseudo-nitzschia* populations in estuarine regions. Laboratory experiments are underway to test this hypothesis. In this highly eutrophic bay, *Pseudo-nitzschia* occurrences are not the only concern, since other potentially harmful species (e.g., *Chattonella* sp., *Dinophysis acuminata*, *Prorocentrum micans*, *Scrippsiella trochoidea*) are also abundant.

The presence of a potentially toxic *Pseudo-nitzschia* species does not necessarily imply the occurrence of an ASP event. First, as demonstrated in culture, there is a combination of factors that may control domoic acid production [24]. Second, the relative abundance of a target species with respect to micro-phytoplankton is very important, especially when one considers whether the potential vectors of domoic acid to higher trophic levels are actively swimming and/or bottom dwelling organisms [25]. In this regard, the presence of *Pseudo-nitzschia* in high relative abundances in Ribeira Bay is of great concern. The pristine waters of this region have recently attracted several mariculture projects. Monitoring and public awareness are necessary to protect consumers and the incipient mariculture industry in the area.

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IDENTIFICATION OF DOMOIC-ACID-PRODUCING *PSEUDO-NITZSCHIA* SPECIES IN AUSTRALIAN WATERS

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Abstract
Extending from an earlier taxonomic survey of *Pseudo-nitzschia* species occurring in Australian waters [1], phytoplankton samples were collected from Tasmanian, South Australian, Western Australian and Queensland marine and estuarine waters. Single cells of *Pseudo-nitzschia* spp. were isolated from samples and grown in culture. Using a combination of light microscopy and TEM, cultures were identified and then tested for domoic acid production with ELISA. In addition, preserved field samples were cleaned and observed using TEM. This study has identified for the first time the presence of *Pseudo-nitzschia australis* (a known domoic acid-producer) in Tasmanian, Victorian and New South Wales waters. The regular occurrence of *P. multiseries* in off-shore samples along the New South Wales coast was noted. Other species identified included *P. pseudodelicatissima*, *P. delicatissima*, *P. pungens*, *P. fraudulenta*, *P. subfraudulenta*, *P. subpacifica*, *P. heimii* and *P. lineola*. The present survey confirmed earlier results that the dominant bloom-forming species in Australian waters are *P. pseudodelicatissima*, *P. pungens* and *P. fraudulenta*. Preliminary analysis using ELISA has confirmed significant levels of domoic acid in *P. australis* cultures tested. Of the five *P. pseudodelicatissima* cultures tested, only one produced detectable levels of domoic acid. No toxin was detected in any of the cultures of *P. pungens* tested. Efforts to isolate cultures of *P. multiseries* have thus far been unsuccessful. The regular presence in southern Australian waters of low concentrations of *P. australis* and *P. multiseries* highlight the need for phytoplankton and domoic acid shellfish monitoring programs to be set up in marine farm areas.

Introduction

Prior to a toxic bloom event of *Pseudo-nitzschia multiseries* in Atlantic Canada in 1987 which caused three human deaths and 105 cases of acute human poisoning [2], there existed limited information on the ecophysiology, geographic distribution and complex taxonomy of this genus. It was found that *P. multiseries* had produced domoic acid, an excitatory amino acid which had subsequently been accumulated by cultured blue mussels which were consumed by the victims. Since then, many other species from this genus have been identified as potential domoic acid-producers [3] and the effects of this toxin have been observed along the entire food chain, from zooplankton, fish, birds, marine mammals and humans [4].

An earlier survey of the genus *Pseudo-nitzschia* Peragallo in Australian waters [1] identified the dominant bloom forming species as *P. fraudulenta*, *P. pungens*, and *P. pseudodelicatissima*. While *P. pseudodelicatissima* and *P.pungens* have been identified as potentially toxic species, clonal cultures of these species isolated from Australian waters were found to be non-toxic in that study. The toxin-producing species *P. multiseries* was identified as a minor component of a *P. pungens* bloom in a New South Wales estuary. The toxic species *P. australis* was never detected. Despite regular blooms of species such as *P. pseudodelicatissima* and *P. pungens* off the Australian coastline, there has been no record or documentation of a major toxic event associated with domoic acid-producing *Pseudo-nitzschia* species, with the exception of the detection of low levels of domoic acid (DA) in Lakes Entrance (Victoria) scallop gonads and viscera [5].

The mechanisms behind the formation of DA and its apparent variation between species, strains, life stage and nutrient regime are still not clearly understood. Studies have shown that predicting a toxic bloom event associated with *Pseudo-nitzschia* spp. is far more complicated than knowing species and numbers [3]. Of great concern is that different strains of the same species have varying toxin-producing abilities [3, 7]. Combining these factors with the knowledge that marine organisms from foreign ports are being continually introduced into Australian waters via ballast water [8], a continuing survey of the taxonomy and toxicology of the genus *Pseudo-nitzschia* Pergallo in Australian waters was necessary.

Methods

Collection of Phytoplankton Samples

Phytoplankton nets (20 μm) were used to collect samples from sites listed in Table 1. Live and preserved (Lugol's Iodine or 2% glutaraldehyde) samples were examined using an inverted light microscope (Axiovert 25) to confirm the presence of *Pseudo-nitzschia* spp. and to measure cell overlap in chain formations.

Isolation and Culturing

Single cells of *Pseudo-nitzschia* species were isolated from live samples using a micro-pipette and grown in modified Jorgenson's media (MJ) at 14 °C on a 12:12 Light:Dark cycle (100 $\mu E.m^{-2}.s^{-1}$). Isolates were grown in 50 mm plastic petri dishes and were transferred every 4 weeks.

Identification and Taxonomy

For identification, samples were examined with a Phillips 410 Transmission Electron Microscope (TEM). The organic matter was oxidised from samples by adding 30% H_2O_2 and heating. The samples were then mounted on formvar-coated copper grids for examination

Harmful Algal Blooms 2000
Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J. (eds)
Intergovernmental Oceanographic Commission of UNESCO 2001

Table 1. Location, date and collector of phytoplankton samples used.

Location	Date (d/m/y)	Sampler
Tasmania	1/2/99 – 1/10/00	C. Lapworth
South Australia	10/5/99	P. Christie/PIR-SA
Western Australia	19/1/97 – 5/5/99	V. Hosja
Queensland	1/8/99	A. Negri
New South Wales	1/1/97 – 20/10/99	P. Ajani/EPA-NSW
Victoria	10/3/99 – 18/10/99	A. Thorn/AWT

under the TEM. The terminology used for taxonomy follows [9].

Toxicology

Non-axenic clonal cultures of potentially toxic *Pseudo-nitzschia* species isolated from Tasmanian waters were inoculated into 125 ml Kimax glass flasks with MJ medium. For a preliminary investigation into the effects of silicate levels on the toxicity of the cultures, some representative strains were initially grown inmedium without added silicate. After two weeks, half the cultures were transferred into silicate enriched medium ($0.1 g.L^{-1}$ $Na_2SiO_3.5H_2O$).

Cultures were sampled after 30 days and then at 70 days. To test for domoic acid, 20 ml of whole culture (cells plus MJ medium) were sonicated (Braun Labsonic, small probe, 80 watts) to disrupt cell walls. The sample was then filtered (0.2µm Gelman Acrodisc filters), pH adjusted to 7-8, and frozen at –80 °C. Samples were sent to AgResearch in New Zealand for domoic acid analysis using ELISA [9].

Results

Taxonomy

Based on the results from the present survey, and combined with results of the previous study [1], a summary of the taxonomic profile and geographic distribution of *Pseudo-nitzschia* spp. in Australian waters is presented in Fig. 1. This study has identified for the first time the presence of *Pseudo-nitzschia australis* (Fig. 2a) in Tasmanian, Victorian and New South Wales waters. The occurrence of *P. multiseries* (Fig. 2b) at six sites along the New South Wales was also noted. The present survey identified the dominant bloom-forming species in Australian waters as *P. pseudodelicatissima* (Fig. 2c) and *P. pungens* (Fig. 2d).

Toxicology

Results of the toxicology tests are summarised in Table 2. Preliminary analysis using ELISA confirmed significant levels of domoic acid in extracts from all four *P. australis* cultures tested, regardless of treatment. Of the five *P. pseudodelicatissima* cultures tested, one produced detectable levels of domoic acid ($1 ng.ml^{-1}$). No domoic acid was detected in any of the cultures of *P. pungens* tested. Efforts to isolate and culture *P. multiseries* have thus far been unsuccessful.

Discussion

The present taxonomic survey confirms that Australian coastal waters have a comprehensive range of species from the genus *Pseudo-nitzschia*. The most ubiquitous and common bloom-forming species are *P. pseudodelicatissima*, *P. pungens*, and *P. fraudulenta*. Other species identified included *P. australis*, *P. multiseries*, *P. delicatissima*, *P. subpacifica*, *P. turgidula*, and *P. lineola*.

The newly identified presence of *P. australis* in Australian waters is the most important issue to arise from this study. All four cultures of *P. australis* isolated from Australian waters, regardless of treatment, produced domoic acid. While there is no record of this species blooming in Australia, the confirmed toxic nature of this species needs to be considered by phytoplankton monitoring programs in Australia.

With one of the three *P. pseudodelicatissima* strains tested showing trace levels of domoic acid, it appears that Australia could have potentially toxic and non-toxic strains of *P. pseudodelicatissima*. With this species being a dominant bloom-former in Australia, further research on its ecophysiology and comprehensive monitoring are required in order to protect both human health and susceptible aquaculture industries.

While attempts to isolate live cultures of *P. multiseries* from Australian waters have thus far been unsuccessful, it is commonly in low concentrations. Australian strains of this species must be isolated and comprehensively tested for toxin production. The other common bloom-forming species in Australia was identified as *P. pungens*. Results from this study showed all four isolated strains to be consistently non-toxic. Although it is generally considered a non-toxic species, *P. pungens* has been found to be weakly toxic in New Zealand [11] and elsewhere [3]. This should be taken into consideration when monitoring blooms of this species in high-risk areas.

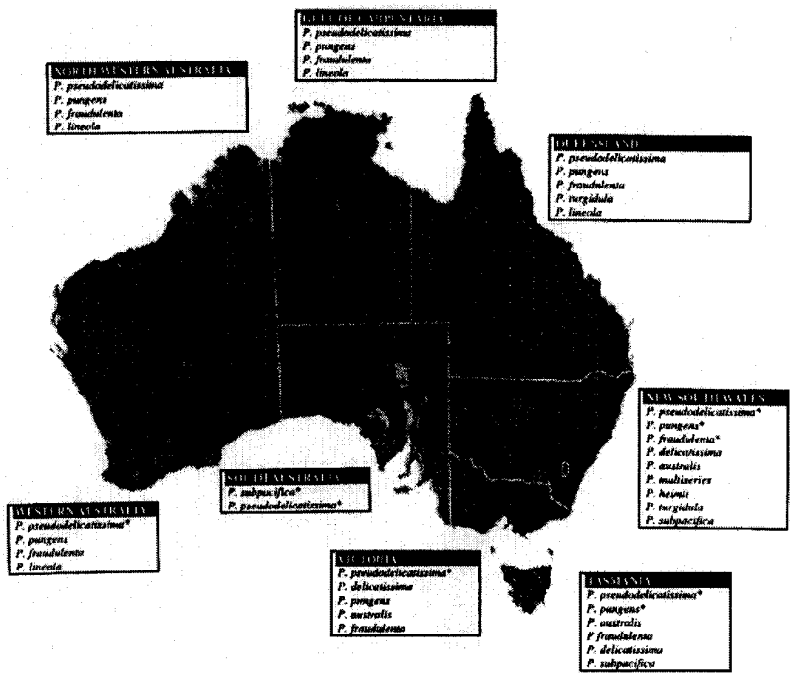

Figure 1. Summary of the distribution of *Pseudo-nitzschia* species in Australian waters. Bloom-forming species (10^4-10^6cells.L^{-1}) are indicated by * (Based on data in [1] and present work).

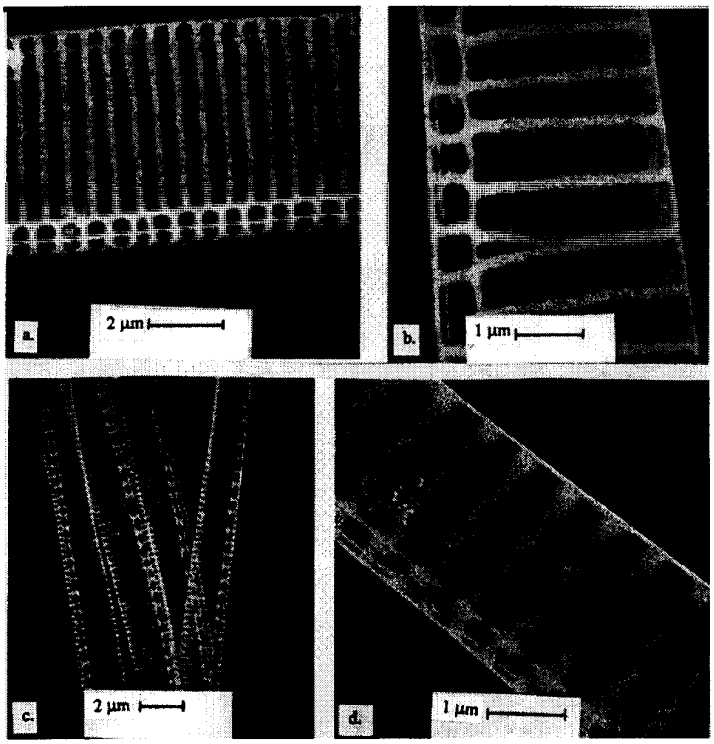

Figure 2 (a) TEM of central section of frustule of *Pseudo-nitzschia australis* from Triabunna, Tasmania; (b) TEM of central section of frustule of *P. multiseries* from Port Hacking, N.S.W.; (c) TEM of central section of several frustules of *P. pseudodelicatissima*, Berowra Creek, N.S.W.; (d) TEM of section of frustule of *P. pungens* from Derwent Estuary, Tasmania.

Table 2. Results of domoic acid analyses in cultures in Australian strains of *Pseudo-nitzschia* spp. using ELISA.

Species	Culture ID and Treatment*	Age of Culture (days)	Domoic Acid (ng/ml)
P. pungens	Tas.OC.P.pu.01.02 (-Si,+Si)	30; 70	Nd; Nd
P.pungens	Tas.OC.P.pu.01.03 (control)	30	Nd
P.pungens	Tas.OC.P.pu.01.03 (-Si,+Si)	30; 60	Nd; Nd
P.australis	Tas.TRI.P.aus.01.08 (control)	48	450
P. australis	Tas.TRI.P.aus.01.08 (-Si)	30; 70	500; 300
P. australis	Tas.TRI.P.aus.01.08(-Si,+Si)	30; 70	1,100; 80
P.fraudulenta	Tas.TRI.P.frau.01.18 (control)	30	Nd
P. fraudulenta	Tas.TRI.P.frau.01.18 (-Si,+Si)	30; 70	Nd; Nd
P.pungens	Tas.NWB.P.pu.02.02 (control)	30; 70	Nd; Nd
P.pungens	Tas.NWB.P.pu.02.12 (control)	30; 60	Nd; Nd
P.pungens	Tas.NWB.P.pu.02.13 (control)	30; 60	Nd; Nd
P.pseudodelicatissima	Tas.DE.P.pseu.01.06 (control)	30; 70	Nd; 1
P.pungens	Tas.DE.P.pu.01.07 (control)	30; 60	Nd; Nd
P.pseudodelicatissima	Tas.DE.P.pseu.01.08 (control)	30; 60	Nd; Nd

* Treatment:
"Control" = culture grown in MJ medium for duration of experiment.
"- Si" = culture grown in MJ media with no added Si for duration of experiment.
"-Si,+Si" = culture initially grown in MJ medium with no added for 1 week, followed by transfer to MJ medium with Si for duration of experiment.
"Nd" = below detection limit (1 ng/ml)

ACKNOWLEDGMENTS:

We thank the EPA (NSW) for providing many of the samples from NSW waterways and oceanic sites. Also Dr Terry Walker of AWT, Victoria and Dr Peter Christie of PIR, South Australia, for providing some of the samples used. Dr Ian Garthwaite carried out the ELISA analyses of domoic acid at AgResearch, Hamilton, New Zealand. Dr Chris Scholin provided r-RNA targeted fluorescent probes to re-confirm ID of *P. australis* and a lot of helpful advice. This study was funded by an ARC grant.

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THE DISTRIBUTION OF THE DIATOM *PSEUDO-NITZSCHIA* OFF SOUTHERN BRAZIL AND RELATIONSHIPS WITH OCEANOGRAPHIC CONDITIONS

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ABSTRACT

The temporal and spatial variability in the abundance of the diatom *Pseudo-nitzschia* spp. was investigated in shelf and slope surface waters off southern Brazil (Lat 32-34°S, Long 50-53°W). The highest cell numbers were found in spring (mean 10^4 cells l^{-1}; maximum 10^5 cells l^{-1}). In the Lagoa dos Patos estuary (32°S, 52°15'W) and the adjacent Cassino Beach, the highest abundances were observed in summer/autumn, with maxima of 10^5 cells l^{-1}. The occurrence of diatoms belonging to the genus *Pseudo-nitzschia* was associated with freshwater outflow from Río de La Plata and Lagoa dos Patos, and also with offshore fronts generated by the mixing of waters of subantarctic and tropical origin. Five *Pseudo-nitzschia* species were identified using light, scanning, and transmission electron microscopy: *Pseudo-nitzschia fraudulenta*, *P. pungens*, *P. multiseries*, *P. australis* and *P. pseudodelicatissima*. All these species are known as potentially toxic elsewhere, and are also observed off the Argentinian coast (36-48°S), confirming their widespread distribution in the SW Atlantic Ocean.

INTRODUCTION

The time and space variability of potentially toxic diatom species of the genus *Pseudo-nitzschia* is still poorly known. Extensive field data are required to improve our understanding of bloom formation mechanisms, to identify the factors triggering domoic acid production in natural populations, and to predict further occurrences [1].

In the SW Atlantic, *Pseudo-nitzschia* species are a regular phytoplankton component, identified simply as *Nitzschia* in earlier studies. Six species have been identified south of 35°S, with five of them being potential producers of domoic acid [2, 3]. Recently, potentially toxic species have also been identified in eastern Brazilian coastal waters [4].

The present work reports the spatial and temporal distribution of the genus *Pseudo-nitzschia* in the SW Atlantic (32°-34°S). The area is affected by a significant freshwater input from Río de la Plata and Lagoa dos Patos between 32°-35°S, and by the seasonal displacements of the Subtropical Convergence, generated by the confluence of subantarctic (Malvinas Current, flowing northwards) and tropical (Brazil Current, flowing southwards) waters. These currents outline the eastern boundary of a large marine ecosystem between 23°S and 55°S [5], important as nursery, feeding, and breeding ground for fishery stocks of both subtropical and Antarctic origins [6, 7]. The main objective of this work was to relate the spatial and temporal variability of the genus *Pseudo-nitzschia* to oceanographic features of shelf, estuarine and beach waters of the study area. In addition, we report the species occurring in these waters.

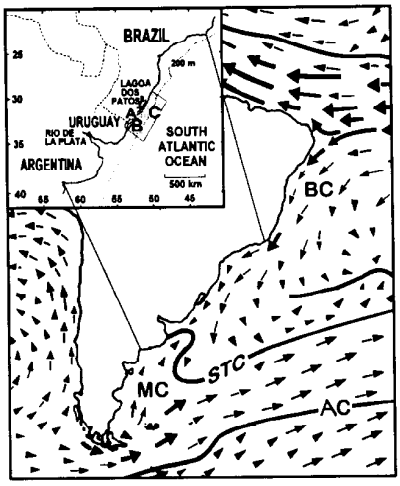

Fig. 1. Sampling area in small picture (A) Lagoa dos Patos Estuary; (B) Cassino Beach; (C) continental shelf/slope off southern Brazil. Large map showing Brazil Current (BC), Malvinas Current (MC), Subtropical Convergence (STC) and Antarctic Convergence (AC). Adapted from [7].

MATERIAL AND METHODS

Oceanographic cruises off southern Brazil (Fig. 1) were conducted in October 1987 (spring), September 1988 (late winter), February 1990 (summer), and June 1991 (autumn). Details of the study area and oceanographic conditions are described elsewhere [8]. Cells of *Pseudo-nitzschia* spp. were enumerated in surface samples. Weekly [9] or monthly samples from the Lagoa dos Patos estuary and adjacent Cassino Beach (1992-1998) were also taken. In the latter, 250 ml samples were fixed with 1% lugol's solution, while cruise samples were fixed with 0.5% formaldehyde. Cells were quantified in sedimentation chambers using an inverted Nikon microscope [10]. *Pseudo-nitzschia* species were identified using light, scanning and transmission electron microscopy techniques [11], with the microscopes ISI-DS-130 SEM and JEM-100SX TEM.

RESULTS

Following analyses of approximately 200 samples in each environment, the frequency of ocurrence of *Pseudo-nitzchia* spp. was highest at the continental shelf (80%), intermediate at Cassino Beach (40%) and lowest in Lagoa dos Patos estuary (25%). Despite these differences, the maximum concentration was the same in the different environments (10^5 cells l^{-1}, Table 1).

Table 1: Maximum concentration (MC, cells l^{-1}), occurrence frequence of *Pseudo-nitzschia* (% Psd), and number of samples (NS) analyzed in the three environments.

	Continental Shelf-slope (1987-1991)	Cassino Beach (1992-1998)	Lagoa dos Patos Estuary (1992-1998)
MC	2.56×10^5	2.99×10^5	2.10×10^5
% Psd	79.6	40.3	25.6
NS	218	193	193

At the continental shelf/slope, concentrations of *Pseudo-nitzschia* spp. were high in spring (Fig. 2), at temperature (T) and salinity (S) values of 14-17°C and 32-34 ppt, respectively (max. 10^5 cells l^{-1}; mean 10^4 cells l^{-1}). In summer, intermediate concentrations (max. 10^4 cells l^{-1}; mean 10^3 cells l^{-1}) coincided with T and S values of 20-26°C and 35-36 ppt. In autumn and winter, low concentrations (max. 10^3 cells l^{-1}; mean 10^2 cells l^{-1}) were observed in a wide TS range (12-22°C; 23-37 ppt; Fig. 2).

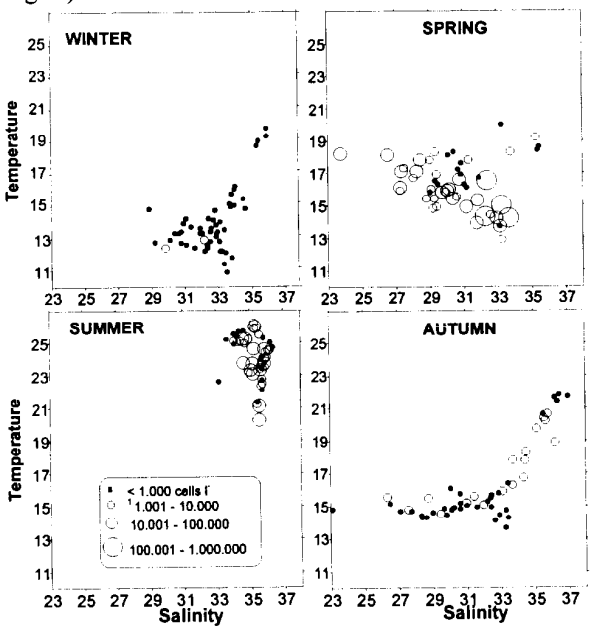

Fig. 2. Temperature and Salinity diagrams showing concentrations (cells l^{-1}) of *Pseudo-nitzschia* in surface water off southern Brazil in spring 1987, winter 1988, summer 1990 and autumn 1991.

The surface distribution of *Pseudo-nitzschia* spp. was associated with freshwater input and oceanographic fronts (Figs. 3 and 4). Cell concentrations $>10^3$ cells l^{-1} were observed near the outflow of Lagoa dos Patos, except in winter. High offshore cell concentrations in spring were associated with salinity gradients resulting from the mixing of waters from tropical, subantarctic, and terrestrial origin. In autumn, cell numbers $>10^3$ cells l^{-1} extended along the slope and near the Lagoa dos Patos mouth, while lower values prevailed on the shelf. Cells were evenly distributed in summer in a large area influenced by the influx of warm waters of the Brazil Current (>20°C; >35 ppt).

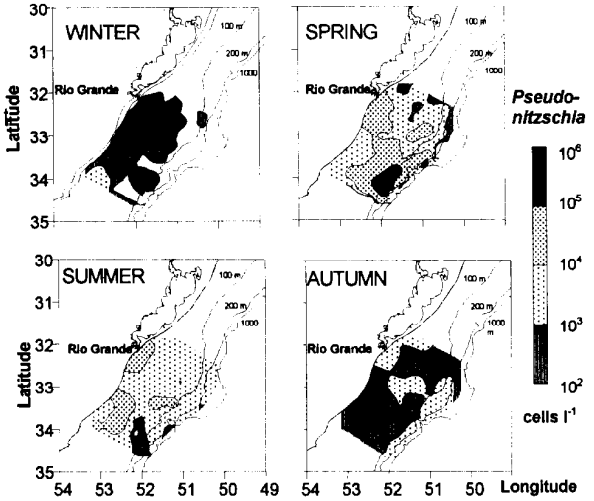

Fig. 3. *Pseudo-nitzschia* surface distribution (cells l^{-1}) off southern Brazil in winter 1987, spring 1988, summer 1990 and autumn 1991.

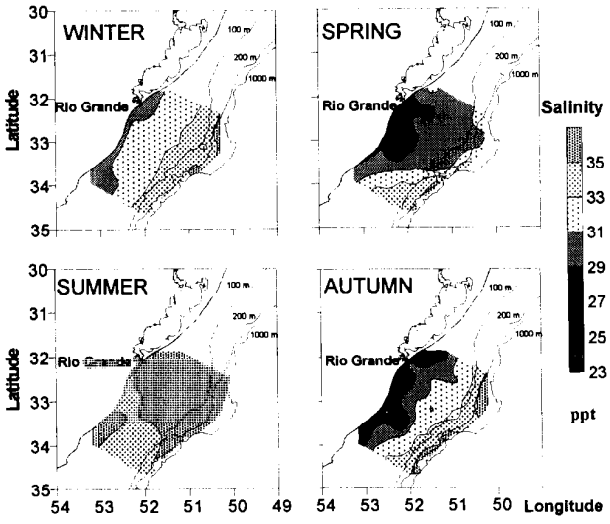

Fig. 4. Surface salinity (ppt) distribution off southern Brazil in spring 1987, winter 1988, summer 1990 and autumn 1991.

There was no significant statistical difference between the mean abundance of *Pseudo-nitzschia* cells from the Lagoa dos Patos estuary and Cassino Beach. High concentrations (10^4-10^5 cells l^{-1}) were observed in summer and autumn at the same temperature range in

both environments, but at lower salinity (18-20 ppt) in the estuary than at the beach (20-33 ppt) (Fig. 5).

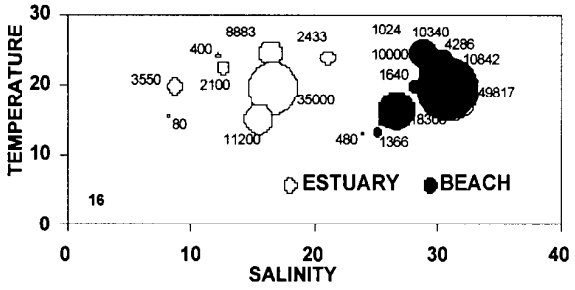

Fig. 5. *Pseudo-nitzschia* concentration (>1000 cells l^{-1}) plotted against Temperature and Salinity at Lagoa dos Patos estuary and Cassino Beach (1992-1998).

Five *Pseudo-nitzschia* species were found: *P. australis*, *P. fraudulenta*, *P. multiseries*, *P. pungens* and *P. pseudodelicatissima* (Fig. 6.1-7).

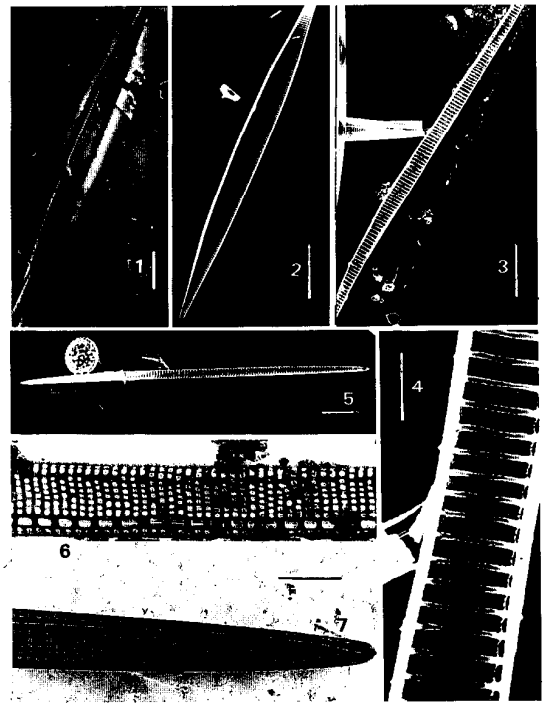

Fig. 6. *Pseudo-nitzschia australis*, chain in valve view, LM (1); *P. fraudulenta*, internal valve view, SEM (2); *P. multiseries*, internal valve view, SEM (3,4); *P. pungens*, internal valve view, SEM (5); *P. pseudodelicatissima*, TEM (6,7). Scale bar 10 μm (1,2,3,5); 2 μm (4,6,7).

DISCUSSION

Pseudo-nitzschia species show a broad range of salinity tolerance for optimum growth [1] in coastal and estuarine waters, as well as in offshore and oceanic regions of all biogeographical zones [12]. This physiological tolerance is also evident in the SW Atlantic, where *Pseudo-nitzschia* spp. occur in a wide range of environmental conditions in shelf and coastal waters. In this study, high cell concentrations were observed at salinity and temperature between 15 and 34 ppt and 15 and 25^{0}C, respectively.

Results from this study indicate that in the SW Atlantic (32-34^{0}S), the spatial and temporal distribution of *Pseudo-nitzschia* spp. is attributable to the seasonal variability of main hydrographic features. An overall low abundance (<10^3 cells l^{-1}) was observed in winter, and low cell numbers during winter/early spring in estuarine waters coincided with the period of larger freshwater outflow. Oligohaline and mesohaline conditions found in the estuary should not inhibit growth, as high cell numbers (10^3-10^6 cells l^{-1}) of *Pseudo-nitzschia* spp. were observed at salinity <15ppt in estuarine waters of the Mississipi River [13]. It is more likely that the strong runoff during winter/spring from the Patos Lagoon [14] hinders cell accumulation. On the other hand, abundance peaks in beach and estuarine waters observed in summer-autumn, after the spring bloom development offshore, suggest that the main cell inoculum for beach and inshore areas originates from shelf waters.

Inshore inocula was suggested for *P. multiseries* in eastern Canada [1]. In southern California, historical data indicate that *P. australis* blooms in spring-summer follow the intrusion of cold nutrient-rich waters from upwelling events [15]. These two examples demonstrate that it is crucial to understand how local oceanographic conditions can influence algal bloom formations.

In the SW Atlantic slope waters, the highest cell numbers were found during spring, coinciding with the presence of a front between low salinity coastal waters, subantarctic, and tropical waters. Oceanographic fronts can cause the accumulation of cells and can also enhance new primary production [e.g., 16]. The Subtropical Shelf Front is a characteristic feature in the region, as an extension of the Brazil/Malvinas Confluence over the shelf of South America [17], and its importance for the regional pelagic ecology is indicated by the development of *Pseudo-nitzschia* blooms and high chlorophyll *a* concentrations observed in the frontal area [8].

Five potentially toxic species were found in our study: *Pseudo-nitzschia australis*, *P. fraudulenta*, *P. multiseries*, *P. pungens* and *P. pseudodelicatissima*, which are also present off the Argentinian coast [2,3]. To date, domoic acid outbreaks have not been registered in the SW Atlantic. However, the presence of these species represents an environmental risk. As our results show the maximum cell concentration (10^5 cells l^{-1}) in the range of toxic events elsewhere [1], a continued monitoring of these species is worthy.

CONCLUSION

The observed spatial and temporal distribution of *Pseudo-nitzschia* spp. in the SW Atlantic Ocean leads to the conclusion that their blooming is associated with the freshwater outflow and oceanographic fronts along the continental shelf-slope. Coastal hydrology seems to favor offshore cell inoculum/source in spring extending to nearshore waters in summer/autumn. The presence of five potentially toxic species (*P. fraudulenta*, *P. pungens*, *P. multiseries*, *P. australis*, *P. pseudodelicatissima*) reaching concentrations in the range of toxic events elsewhere, represents a risk to the high productive SW Atlantic ecosystem.

ACKNOWLEDGMENT

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DOMOIC ACID-PRODUCING *PSEUDO-NITZSCHIA* SPECIES OFF THE U.S. WEST COAST ASSOCIATED WITH TOXIFICATION EVENTS

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ABSTRACT

The first confirmed death of a marine mammal due to domoic acid poisoning was reported in California, U.S.A. during May and June 1998. This sea lion (*Zalophus californianus*) mortality event was caused by their ingestion of sardines (*Sardinops sagax*) and anchovies (*Engraulis mordax*) that had accumulated high levels of the neurotoxin. A series of research cruises were motivated by the mammal deaths, resulting in the assessment of the spatial distribution of toxic *Pseudo-nitzschia* cells along the U.S. west coast. Sardines and anchovies, harvested from nearshore waters between San Francisco Bay and Monterey Bay, California, during a separate fisheries resource cruise in May, showed the highest levels of domoic acid in Monterey Bay (up to 2300 µg DA g^{-1} anchovy viscera on 24 May) around the same time when sea lions with neurological symptoms stranded. The pennate diatoms, *Pseudo-nitzschia multiseries* and *P. australis*, were the toxin-producing phytoplankton constituting algal blooms near coastal locations where sea lions with neurological symptoms had stranded, with the highest cellular toxin levels at 6 pg $cell^{-1}$ (*P. multiseries*) and 78 pg $cell^{-1}$ (*P. australis*). *P. australis* was the toxic *Pseudo-nitzschia* species in nearshore waters off the central Oregon coast during July, located within 20 km of the coast at a time when domoic acid in razor clams was reported on the beaches. Toxin-producing *P. pseudodelicatissima* cells were present in Washington State coastal waters in early August, prior to the razor clam toxification event which began in late summer. Historical records show that the sites where the greatest numbers of toxic cells were found are areas of nutrient enrichment due to upwelling and increased fluvial flow in local watersheds. These results indicate that the detection of distinct species of toxic *Pseudo-nitzschia* in key coastal areas may be used as a powerful early indicator of domoic acid toxicity in coastal regions.

INTRODUCTION

During the summer of 1998, the impacts of domoic acid (DA) poisoning were documented in several regions along the west coast of the United States. The events included sick and dead sea lions in central California due to DA poisoning and high levels of toxin measured in razor clams (*Siliqua patula*) in the Pacific Northwest. In May and June, sea lions in central California stranded from Half Moon Bay in the north to Oceano Dunes in the south [1]. These animals displayed symptoms that were consistent with domoic acid poisoning (i.e., seizures, ataxia, and head weaving; [1,2]). Anchovies that were collected from Monterey Bay, California during the period of the mammal strandings had high levels of DA in their tissues and contained frustules of the DA-producing diatom *Pseudo-nitzschia australis* in their guts [2,3,4]. In Oregon and Washington State, high levels of DA were detected in razor clams on the coast (D. Cannon, pers. comm., [5]).

During September of 1991, DA was responsible for the deaths of numerous seabirds in Monterey Bay [6]. In November of the same year, razor clams on the coast of the state of Washington became tainted with domoic acid. At the time, it was theorized that the bloom of toxic *Pseudo-nitzschia* that was responsible for the outbreak in California had moved up the coast and caused the toxification of razor clams [7]. In response to the mammal strandings in central California and in an effort to study the possibility of northward transport of a toxic bloom, five research cruises were conducted between 1 June and 31 August of 1998. Three of these cruises covered the central California region, while the other two covered an area from San Francisco Bay, California to Cape Flattery in Washington State (Fig. 1). Results from the analysis of seawater samples collected from the ocean surface are presented here.

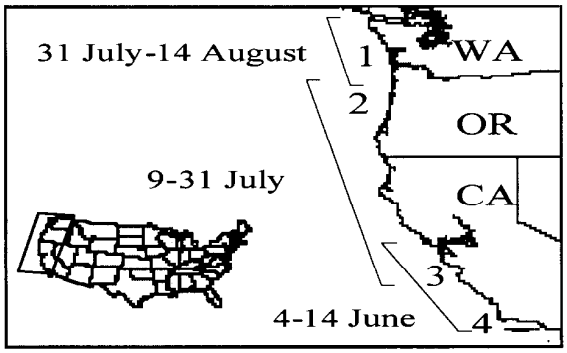

Figure 1. Area of U.S.A. coast (boxed inset) sampled during research cruises in summer and fall of 1998. Coastal areas (bracketed) were sampled during the dates shown. Locations where highest DA in seawater was measured are numbered as follows:
1 = Central Washington coast, 2 = Central Oregon coast, 3 = Monterey Bay area, 4 = Point Conception area.

METHODS

DA Analysis of Seawater

Cellular domoic acid in seawater was measured by filtering 1-liter samples through 47 mm Nucleopore HA filter. The filter was frozen then analyzed using the method described by Van Dolah et al. [8]. A glutamate decarboxylase step was used prior to analysis to remove

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endogenous glutamate in all samples. For quantification of DA in selected seawater samples, liquid chromatography-tandem mass spectroscopy (LC-MS/MS) was performed according to standard protocols [2].

DA analysis of shellfish and finfish

Levels of DA in shellfish were obtained from the Washington Department of Health (WDOH) and the Oregon Department of Agriculture. Concentrations of DA in razor clams on a given date represent a composite analysis (viscera discarded) of 6 clams. Concentrations of DA in anchovies and sardines, collected in Monterey Bay by personnel form the NMFS lab in Tiburon, CA, were determined utilizing a methanol/water extraction and analysis by High Performance Liquid Chromatography [9].

Phytoplankton cell counts and species identification

Whole seawater samples for cell counts and species identification were preserved with formaldehyde at a final concentration of 1%. A 0.1 mL subsample of unconcentrated seawater was loaded into a Palmer-Maloney counting cell, and individual *Pseudo-nitzschia* cells were enumerated at 200x using light microscopy. In order to identify cells to the species level, aliquots of samples in which *Pseudo-nitzschia* spp. were numerous were prepared using a modified $KMnO_4$/HCl oxidation method [10,11,12].

RESULTS AND DISCUSSION

Domoic acid and Pseudo-nitzschia species

Figure 1 shows the area sampled during our research cruises along the U.S. west coast and the dates of those sampling efforts. Four regions of substantial seawater toxicity are noted by the numbers 1 through 4. Cruise results, reported below in a south to north direction, are separated into the southern region that includes California (Santa Barbara to the Oregon border) and the northern region that includes Oregon and Washington. DA was widespread on the U.S. west coast during the summer of 1998 as shown in figure 2A and 3A, and the highest toxin levels in each region are attributable to different species of *Pseudo-nitzschia.*

Samples with the highest toxin levels from the Monterey Bay and Pt. Conception areas (n = 12) were analyzed also by LC-MS/MS. Comparison of values obtained using this method agreed well with those obtained by the receptor binding method (r^2 = 0.89). Sea lions and baitfish were affected by DA in areas 3 and 4 (Fig. 1), as were sea otters in the Monterey Bay area (M. Checkowitz, in preparation). The first indication of DA in seawater in Monterey Bay was in early May [2]. Toxicity peaked on 22 May and began to decline around 1 June with reports of live, sick sea lions decreasing after 5 June. Our cruise data from California were collected between 2 June and 15 June (Fig. 2A), corresponding to the declining phase of the bloom in Monterey Bay.

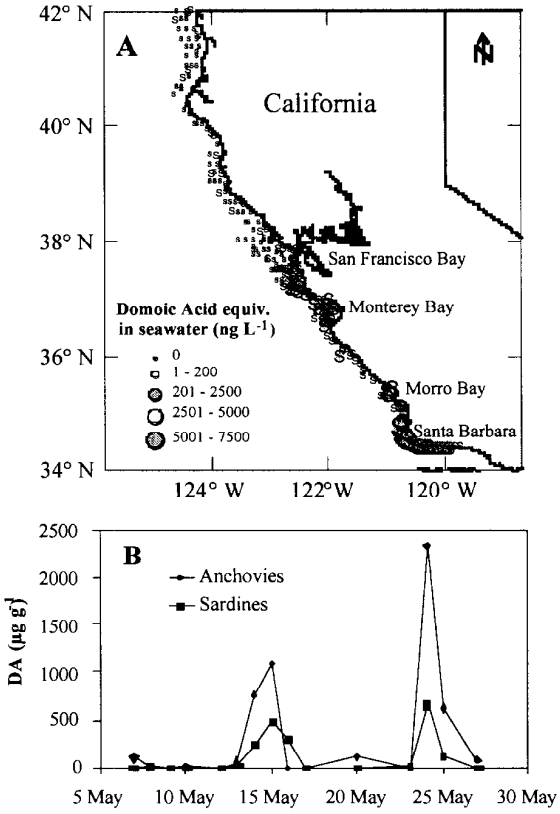

Figure 2. (A) Domoic acid equivalents in surface seawater measured off the California coast duirng the summer of 1990. (B) Domoic acid in sardines and anchovies collected from Monterey Bay in May 1998.

Whereas *P. multiseries* was seen as the dominant toxic species in the bloom north of Monterey Bay (Table 1) and *P. pseudodelicatissima* was the dominant (nontoxic) species observed inside Monterey Bay during our cruises in June (data not shown), Scholin et al. [2] observed *P. australis* as the toxic species inside Monterey Bay in May.

Sardines and anchovies collected from Monterey Bay contained the highest levels of DA, up to 2300 µg g^{-1}, on 24 May, at about the same time that high numbers of sea lion strandings were observed in the Bay (Fig. 2B). This is slightly higher than the maximum level of DA in anchovy viscera (110 µg g^{-1}) measured on 22 May [2] by Scholin et al., and demonstrates the variable levels of toxicity that will be measured in this "swimming" food source. Although the highest levels of DA in seawater were measured near Pt. Conception (Fig. 2A), this area is largely uninhabited, therefore mammal strandings that may have occurred there could have gone undetected.

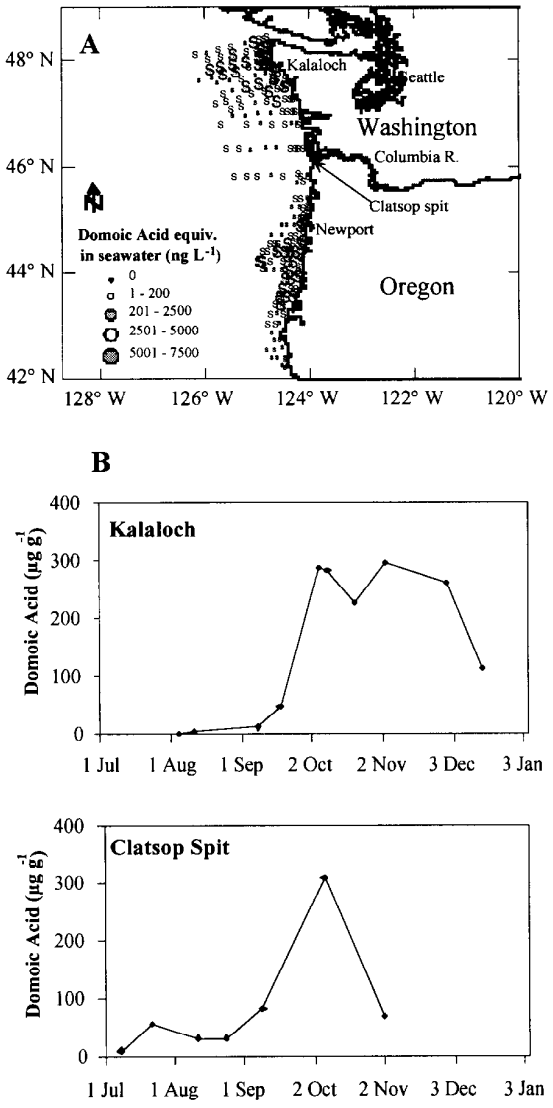

Figure 3. (A) Domoic acid equivalents in surface seawater measured off the coasts of Oregon and Washington during the summer of 1998. (B) Domoic acid in razor clams from beaches at Kalaloch, Washington and Clatsop Spit, Oregon.

The coasts of Oregon and Washington were also impacted by DA. During our cruises off the coasts of Oregon and Washington, DA was detected in seawater (Fig. 3A), at the same time that levels of DA were first detected in coastal razor clams, eventually exceeding the regulatory limit of 15 µg g^{-1} (Fig. 3B). Off the Oregon coast, *P. australis* was the toxic species, with an observed maximum DA concentration of 550 ng L^{-1} from samples taken during a cruise from 9-31 July off central Oregon (Table 1). Samples taken during a cruise from 31 July to 14 August off the Washington coast showed a maximum DA activity of 1500 ng L^{-1} with toxin attributable to a *P. pseudodelicatissima* bloom (Table 1). This is in contrast to the Monterey Bay area where *P. pseudodelicatissima* dominated as DA concentrations dropped to very low levels [2]. This suggests the possibility that the same species of *Pseudo-nitzschia* may produce vastly different levels of DA in different geographical regions.

	1. Washington Coast	2. Oregon Coast	3. Monterey Bay region	4. Morro Bay to Pt. Conception
Toxic *Pseudo-nitzschia* species	*P. pseudodelicatissima*	*P. australis*	*P. multiseries*	*P. australis*
Date of Max. DA equiv. in seawater	12 August	26 July	10 June	4 June
Max. DA equiv. in seawater (ng L^{-1})	1500	550	670	7400
Max. toxic cell number (cells L^{-1})	2.1×10^{6}	1.4×10^{4}	5.2×10^{5}	2.3×10^{5}
Max. DA (pg cell)	1.3	35	6	78
DA measured in:	Razor clams	Razor clams	Bait fish, sea lions, sea otters	Bait fish, sea lions

Table 1. Four U.S. west coast regions with high DA in the summer of 1998.

DA in upwelling zones

High levels of DA in seawater were measured in previously characterized upwelling zones, e.g., Pt. Conception, California [13], Pt. Año Nuevo, California [14], central Oregon [15], and Kalaloch Beach, Washington [5]. The highest toxin levels that were measured during all cruises were off Pt. Conception (Fig. 2A). Upwelling occurs in this area year round [13] and may provide nutrients to fuel intense blooms of *Pseudo-nitzschia*. Further north, upwelling offshore at Pt. Año Nuevo (several km north of Monterey Bay) has been suggested as a nutrient source for algal populations in Monterey Bay [14]. Huyer [15] presented evidence of an upwelling zone along the central Oregon coast, corresponding to the location where high levels of DA in seawater were measured during our cruises. Studies of the Washington coast site at Kalaloch beach during the summer and fall of 1998 indicated that it was supplied with nutrients from cool, upwelled waters during a year of anomalously low rainfall [5].

Although upwelling likely sustained the majority of domoic acid-producing blooms reported in this paper, growth of toxic *Pseudo-nitzschia* may also be fueled by nutrient loading from rivers. The appearance of toxic *Pseudo-nitzschia* after a period of heavy rainfall, resulting in freshwater nutrient runoff, has been documented in eastern Canada in 1987 [16,17], in the northern Gulf of Mexico in the early 1990s [18], on the Washington coast in 1991 [19], in Monterey Bay in 1991 [20], and in Puget Sound, Washington, in 1997 [21]. A supply of nutrient to coastal regions, whether from upwelling or fluvial sources, play an important role in fueling toxic *Pseudo-nitzschia* blooms.

Transport mechanisms

During the 1991 DA toxification event on the U.S. west coast that resulted in seabird deaths and razor clam toxicity, it was thought that currents could possibly have moved a bloom of *Pseudo-nitzschia* from central California to the Pacific [7]. However, in the fall of 1998, *P. pseudodelicatissima* was the species

responsible for razor clam toxicity on the Washington coast [5], whereas *P. australis* and *P. multiseries* were identified as the toxic species in central California [2, this paper], and *P. australis* was the dominant toxic species in central Oregon. Therefore, a northward transport of cells was probably not the mechanism of razor clam toxification in Washington State, due to the different species of *Pseudo-nitzschia* responsible for DA production in this area compared to the regions further south. On the Oregon coast, however, *P. australis* was identified as the toxic species. A portion of a bloom in central California could possibly have become entrained in the California Undercurrent, which transports water poleward [7], only to be brought to the surface off the Oregon coast during an upwelling event.

This study shows the importance of cruises in characterizing toxic *Pseudo-nitzschia* species populations in coastal locations where DA is a problem. However, because shiptime is costly, we were only able collect the large number of samples recorded in this study by "piggybacking" on several cruises of opportunity. At times, student volunteers were used to collect and filter seawater, which also cut the costs of sampling efforts. Future cruises of opportunity as well as funded efforts should be used to answer several questions. What are the physical processes that result in the transport of cells to coastal fish and shellfish populations? Are certain toxic *Pseudo-nitzschia* species routinely found in key coastal areas? Are there environmental conditions (macronutrients, micronutrients, water temperatures) that favor toxic blooms? Are there specific initiation sites along the U.S. west coast where toxic blooms originate? Only when these and other questions are answered will the forecasting of toxic *Pseudo-nitzschia* blooms be possible.

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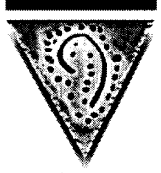
CIGUATERA AND DSP

Harmful
ALGAL
BLOOMS



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EXAMPLE OF A *GAMBIERDISCUS TOXICUS* FLARE-UP FOLLOWING THE 1998 CORAL BLEACHING EVENT IN MAYOTTE ISLAND (COMOROS, SOUTH-WEST INDIAN OCEAN)

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ABSTRACT

The dinoflagellates *Gambierdiscus* spp. are distributed world-wide in tropical and subtropical waters and form part of the natural biota of coral reefs. In the Indian ocean, populations have been identified in numerous places including the three species *G. toxicus*, *G. yasumotoi* and *G. belizeanus*. *G. toxicus* is the most commonly found and the one recognised to contaminate the food web, inducing ciguatera fish poisoning.
From April to August 1998, a severe coral bleaching event occurred in the Comoros archipelago and particularly in Mayotte, affecting almost all the coral species. More than 80% of the bleached corals died. Mass mortality offered new surfaces to multispecific algal turf colonisers which are good environmental conditions for proliferation of epiphytic microalgae such as *Gambierdiscus*, *Ostreopsis* or *Prorocentrum*.
We describe here an unusual large and monospecific bloom of *Gambierdiscus*, which was identified by scanning electron microscopy as *G. toxicus*. The density of the dinoflagellate per gram of algae support, which was one year earlier of 384 reached 60,463 in October 1998. This density is the highest ever recorded in the region and one of the highest of the world.
The question remains if the global toxic pool of the coral ecosystem may increase seriously for accumulation in the food web and cause human poisoning.

INTRODUCTION

In the Western Indian ocean, benthic dinoflagellates become problematic when populations increase and may be involved as progenitors of different seafood poisonings (ciguatera, clupeotoxism, etc.).
Coral reef species assemblage has been studied since 1990 and new species have been described [1, 2]. Special attention is focused on the *Gambierdiscus* genus due to its involvement in ciguatera fish poisoning (CFP) and three species have been confirmed to occur in the islands from the region [3]: *G. toxicus*, *G. yasumotoi* and *G. belizeanus*.
Natural and anthropogenic disturbances have been known to enhance populations in relation to new surface creation and/or nutrients. Coral bleaching is the whitening of coral colonies due to the loss of symbiotic dinoflagellates (called zooxanthellae eg. *Symbiodinium* sp.). It is a general response to environmental stress, in particular to high sea surface temperatures (SST), excessive UV radiations or bacterial diseases which may be causing whitening of coral colonies. In fact, bleaching may be a reversal process but definitive loss of photosymbiotic zooxanthellae induce death of corals. Mass bleaching offers new surfaces to multispecific algal turfs colonisation, which are good supports for proliferation of epiphytic microalgae including potentially harmful species.
First identification of *Gambierdiscus* spp. in Mayotte was done in 1979 [4]. Since that time, densities have increase but no clinical case of ciguatera has been notified in the island. To protect human health in relation with this peculiar situation, local authorities (DASS) initiated a monitoring programme in 1984. Since 1990, activities are conducted on both management and research sides on the ecotoxicological process, the food web and commercial fishes.
Here, we report on the monitoring conducted from 1992 to 1999 with special attention to the bloom observed after the 1998 coral bleaching event.

MATERIAL AND METHODS

Mayotte (12°38'-13°00S ; 44°57'-45°19'E), French island located in the Mozambique channel between Africa and Madagascar is part of the Comoros archipelago (Fig. 1A). It is one of the largest lagoons of high volcanic islands in the world, with 1500 km^2 reef formations consisting of fringing reefs, inner reefs and a large barrier reef which lies 3 to 15 km off shore (Fig. 1B). Survey of *Gambierdiscus toxicus* started in 1984 on 47 sites all around the island, a previous study being done in 1979 [4]. Since 1989, only the 13 stations showing high densities were kept for monitoring [5]. The North-East lagoon of Mayotte (Fig. 1C), which is our study area, was surveyed at the same sites from 1992 according to our methodology [1]. Since 1996, specific activities were developed to assess (i) the biodiversity of benthic dinoflagellates and (ii) the relative abundance of toxic genus.
Surveys were always carried out at the same period (late October/early November) to avoid seasonal variability. Dead corals branches covered by algal turfs were softly collected by snorkelling or scuba diving and placed underwater in plastic bags. A minimum of 10 branches from separate colonies was collected for abundance estimation. Dead corals were scrubbed in a bucket of seawater on boat or at laboratory and suspension was filtered through 850, 125, 38 and 20 µm mesh size screens and washed several times. Material retained in fractions 38 and 20 µm were fixed with 4% formalin. Fresh and dried weight of the fractions, including the highest ones, were determined. Relative abundance of

dinoflagellates (genus *Gambierdiscus*, *Prorocentrum* and *Ostreopsis*) are expressed per gram weight of fresh weight of algae retained on the 850 µm mesh sieves.
For taxonomic studies, fixed cells were isolated with a capillary pipette under a compound microscope. Cells were rinsed with distilled water to remove salt and fixatives, dehydrated in a series of graded ethanol concentrations, and critical-point dried. For scanning electron microscopy (SEM) observations, the preparations were coated with a complete layer of gold-palladium. Micrographs were taken on a Jeol 840 SEM of the Life Sciences laboratories of the Muséum National d'Histoire Naturelle.

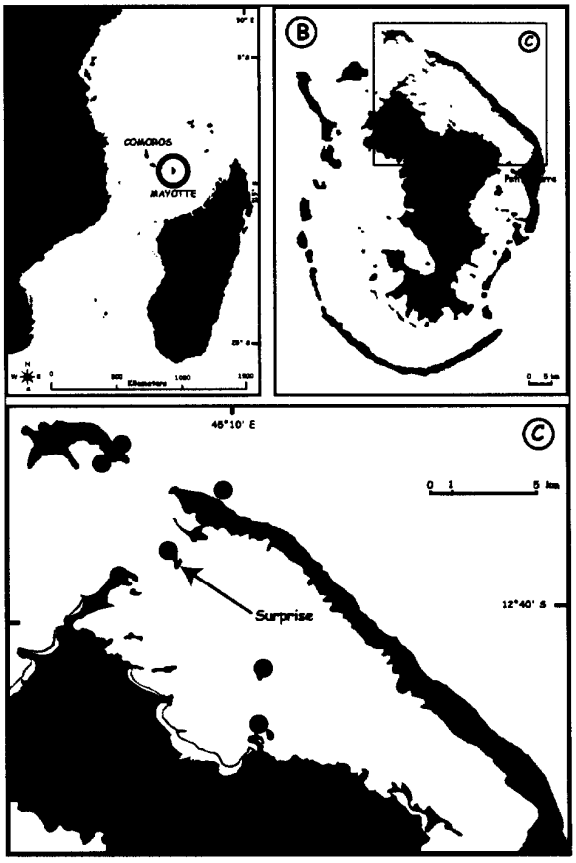

Fig. 1: (A) Mayotte in the Mozambique channel, (B) Mayotte island (C) location of Surprise inner reef and sampling locations within the NE reefs of Mayotte lagoon.

RESULTS AND DISCUSSION

Apart from *Gambierdiscus* which has been studied since its discovery and link with ciguatera in 1979, other benthic dinoflagellate taxa have only been followed since 1992.

Biodiversity of benthic dinoflagellates.

During the 1996 and 1997 surveys, 30 samples were collected from various substrates (dead branching corals, coral rubbles, macroalgae) and types of ecosystems (fringing reefs, flats, outer slopes). In these samples, 27 thecate dinoflagellates species were identified, representing 6 genera. 14 were considered toxic or potentially toxic (Table 1). Associated non-thecate dinoflagellates were also present from the following genera: *Amphidinium*, *Cochlodinium*, *Gymnodinium*, *Gyrodinium*. This inventory remains partial as analysis of samples is still undergoing. At present, biodiversity appears important and comparable to the one described for Réunion [3].

Table 1: Benthic thecate dinoflagellates from Mayotte Island * toxic, ** potentially toxic.

*Prorocentrum arenarium**	*Ostreopsis caribbeanus*
*P. belizeanum**	*O. heptagona**
*P. concavum**	*O. labens*
P. elegans	*O. lenticularis**
P. emarginatum	*O. marinus*
*P. hoffmanianum**	*O. mascarenensis**
*P. lima**	*O. ovata*
*P. maculosum**	*O. siamensis**
*P. mexicanum**	*Coolia monotis**
P. sp 1*	*C. tropicalis***
P. sp. 2	*Sinophysis microcephalus*
P. sp. 3	*S. canaliculata*
Gambierdiscus belizeanus	*Amphidiniopsis* sp.
*G. toxicus**	

Monitoring of coral reefs in relation to ciguatera risk

Focus was aimed in 1997 on the relative abundance of the three major toxic dinoflagellates genera found in all the collected samples. *Prorocentrum* is dominating the assemblage, followed respectively by *Gambierdiscus* and *Ostreopsis*. Differences observed from place to place may be explained by ecological and physicochemical conditions in the NE sector (turbidity, salinity, waves action, etc.). Grezbyk et *al.* [6] observed the same feature while Thomassin et *al.* [5], not using a 20 µm mesh sieves, underestimated the *Prorocentrum* populations.
Pattern of Mayotte is similar to those recently observed for other islands of the Indian ocean region (unpublished data) and differs from Tahiti where *Gambierdiscus* is dominant and *Prorocentrum* rare [7].
According to collected data, Surprise station was recognised as the most representative of the whole sector for *Gambierdiscus* and so retained as the reference monitoring station to detect long-term trends as part of the coral reef observatory to be set up. Table 2 shows that the first phase of the phenomenon was a large increase during the 1979-1988, linked to human disturbances and maybe to the 1983 bleaching event. During phase 2 (1989-1992) the level of contamination decreased and being stabilised to around 50 cells g^{-1} [5].
Before the 1998 observed bloom, densities were relatively stable in the 200 to 400 range in Surprise inner reef. Evidence of a previously rich coral community ended in 1996 as corals appeared massively dead this year and was attributed to local heating waters and crowns-of-thorns (COTS) invasion. Food web indicators (herbivorous and carnivorous fishes) were yearly analysed for ciguatoxins since 1992 and showed no flesh toxicity

(unpublished data). Such densities of *G.* cf. *toxicus* are similar to those described in ciguatera endemicity areas such as Mascareignes archipelago/ SW Indian ocean [1], French Polynesia/Pacific ocean [7, 8], Australia [9] or Saint Barthelemy/West Indies [10].
After the 1998 observed bloom (cf. infra), a drastic break-down in the *Gambierdiscus* population was noticed in November 1999.
Despite a global and sustainable contamination of the Mayotte's coral reefs by this potentially ciguatoxin-like producer, no clinical case of ciguatera has been notified in the island of Mayotte by health authorities to date.

Year	*Gambierdiscus* densities	Observations
1979	0.5	NE sector (in [4])
1988	300	NE sector (in [5])
1991	50	NE sector (in [5])
1992	67	Surprise bank
1993	258	Surprise bank
1994	232	Surprise bank
1995	197	Surprise bank
1996	277	Surprise bank
1997	384	Surprise bank
1998	**60,463**	Surprise bank
1999	27	Surprise bank

Table 2: Monitoring data since 1979, densities exprimed in number of cells per gram of fresh algae.

The 1998 bleaching event in Mayotte and its consequences
From February to August 1998, coral reefs from the Indian ocean have dramatically suffered from a bleaching event [11]. Due to El Niño-Southern Oscillation (ENSO) abnormalities seawater temperatures rise, the warming follow the sun, affecting in the first step coral reefs from the south of the Mozambique channel (Madagascar, Tanzania). Reefs from Réunion and Mauritius, located in open Indian ocean were less affected.
The sustainable increase in SST (up to 32°C during a few weeks) caused in Mayotte high coral bleaching first observed by local divers late March. From April to August, 80% Scleratininans but also soft corals (*Sinularia*, *Sarcophyton*) and sea anemones were affected [12]. Bleaching followed a gradient from the reef flats to the inner slopes and the seaward slope, from 0 to 35 m depth. Tabular and branching Acroporidae located on the upper part of the outer slope were the most affected (100%), while Pocilloporidae and Fungidae were less concerned.
In the last decades, Mayotte has experienced two previous coral bleaching events in 1983 [13] and 1988 but with less extension and severity.
Corals which did not recover in a few weeks died and were covered by filamentous cyanobacterian and algal turfs. During the survey carried out in November 1999, coral settlement and recovery was small (between 20 to 50 %). Algae were still dominating the population.
Consequences of the 1998 bleaching and subsequent mortality were as follow: rapid increase in algal cover of dead skeletons, growth of opportunistic species such as echinoderms and sponges, changes in the trophic chain structure (e.g. decline of species number and increase in herbivorous fishes).

The Gambierdiscus bloom
Samples of the bloom were collected on October 30, December 11 and December 18 1998. We observed that *Gambierdiscus* sp. dominated the phytoplanktonic population in October and slightly decrease in December. Cell concentration were in October of 60,463 cells g^{-1}algae, respectively in December of 13,197 and 29,389 cells g^{-1} algae. The dinoflagellate was constituting more than 95% of total biological material from October sample. Other observed dinoflagellates were *Prorocentrum* and *Ostreopsis*, with respective densities of 965 and 10 cells g^{-1} algae.
Identification of the *Gambierdiscus* species involved in the 1998 flare-up was done in March 1999 by the use of scanning electron microscopy (SEM). The species of *Gambierdiscus* involved is *G.* cf. *toxicus* according to typical morphological features of this early described species (Fig. 2). In Reunion island, 2,963 cells g^{-1}algae is the highest value obtained, in December 1998 for Saint Leu fringing reef (IOC-HAB programme). Such a high bloom of *G. toxicus* has only been reported in the Gambier islands in 1974 [14]. Highest density was 470,000 cells g^{-1} algae in one station of the archipelagos. In 1984, it was less than 10 in the same station. Increase of this epiphytic species has previously been described as a secondary consequence of coral bleaching and subsequent mortality in French Polynesia [15].

Population densities of *Gambierdiscus*, *Ostreopsis* are seasonal and show peak abundance during the springtime period [1]. Various factors are now known to promote dinoflagellates growth such as dissolved nutrients and other elements [16]. Increasing concentrations of nutrients from human sources in coral reefs are reported to promote algal growth at the expense of corals [17]. Here, it can be supposed that one of the side-effects of the bleaching event was to enhance nutrients availability in lagoonal waters. So, inorganic nutrients which were not assimilated by billions of dead zooxanthellae were free for uptake by others components of the reef ecosystem. Although no physico-chemical data are available, our hypothesis is that both the physical conditions of the springtime and the no-limits nutrients availability may have been the causes that triggered this unusual and huge bloom, specially in 1998. In future research this should be early considered to understand the patterns of such a process.

Unfortunately, no toxicity analysis of the bloom was done but toxicity controls of the food web indicators during months following this peculiar event confirmed no contamination by ciguatoxin-like compounds. Moreover, no case of CFP was notified in 1999.

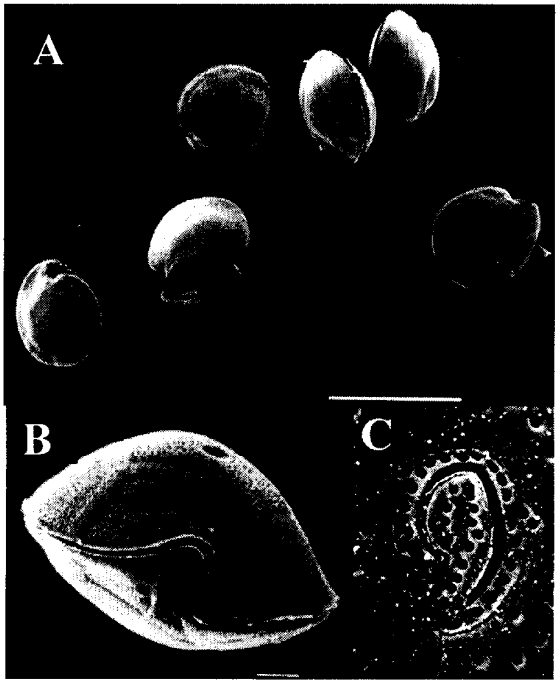

Fig. 2: SEM Pictures of *G. toxicus*. B: sulcal view; C: Po plate.

CONCLUSION

From the above elements, we can support the followings:

(i) During the last two decades, successive phases of contamination of coral reefs by *G. toxicus* and associates dinoflagellates occur due to human and natural disturbances;

(ii) Despite this fact, food web contamination by toxins remains low and no ciguatera outbreaks have been notified;

(iii) The ENSO related coral bleaching event and subsequent high mortality of coral colonies was observed from April to August and offered good substrate conditions and nutrients availability in lagoonal and confined waters;

(iv) Causes of this exceptional bloom were probably synergistic as (i) the seasonal good conditions were reached and (ii) the nutrients uptake normally by zooxanthellae was depleted by the bleaching event and was available for dinoflagellates;

(v) Taxa involved was confirmed to be *G.* cf. *toxicus* by SEM but strains were probably not producing large amounts ciguatoxin-like compounds.

The present results are in general agreements with other reports on the strain variability of ciguatoxin production by dinoflagellates and especially *Gambierdiscus*. This hypothesis requires further need of research.

ACKNOWLEDGMENTS

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TOXIC EPIPHYTIC DINOFLAGELLATES FROM EAST COAST TASMANIA, AUSTRALIA

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ABSTRACT

The dinoflagellates *Coolia* cf. *monotis* Meunier, *Ostreopsis siamensis* Schmidt, *Prorocentrum lima* Dodge and *P. mexicanum* Tafall were found to occur epiphytically on seagrass in selected temperate lagoons or bays along the Tasmanian east coast (41-43°S). The distribution of these species was limited by a preference for high salinities, low turbidity and low nutrient levels, with blooms of *O. siamensis* occurring only in warm summer months. In culture *O. siamensis* grew at temperatures between 14-25°C and salinities of 30-45psu, but was tolerant to temperatures as low as 10°C by the generation of a resting stage. *Coolia* cf. *monotis* grew under temperatures of 10-25 °C and between salinities of 15-45psu. Maximum growth rates of 0.53 and 0.58 div/day were observed for *O. siamensis* and *C.* cf. *monotis*, respectively. *O. siamensis* was found to produce ≤ 0.1pg cell^{-1} of palytoxin. Mortality in brine shrimp (*Artemia salina*) was noted upon exposure to both *O. siamensis* and *C.* cf. *monotis*. Oyster spat (*Crassostrea gigas*) was not acutely affected by exposure to high densities of cultured *O. siamensis*. Gut contents of mussels (*Mytilus edulis planulatus*) collected from the wild revealed that *O. siamensis*, *P. lima* and *C.* cf. *monotis* were commonly ingested, and contamination of commercial shellfish with dinoflagellate toxins therefore needs to be monitored.

INTRODUCTION

Ostreopsis, Prorocentrum and *Coolia* are commonly recognised as tropical, epiphytic dinoflagellates found in association with *Gambierdiscus toxicus* in ciguatera affected areas [1]. The present work reports for the first time the occurrence of this potentially toxic dinoflagellate assemblage from the temperate waters off east coast Tasmania. Tasmania is an island located at a latitude of 40° to 43°30' south, a long way removed from the tropical areas in which ciguatera is a concern. The toxic algal problems that have received attention in Tasmania in the past, have been due to pelagic phytoplankton, predominantly *Gymnodinium catenatum*, and until now epiphytic algae have not been investigated. In 1998 a suspected case of human shellfish poisoning was associated with mussels collected from Anson's Bay, Tasmania. This triggered an investigation into the phytoplankton of the area and a member of the genus *Ostreopsis* was found in high numbers in the water column. This was identified as a possible culprit and due to its typically epiphytic nature, investigations into Tasmanian epiphytic phytoplankton were initiated. In studies elsewhere, epiphytic dinoflagellate assemblages have been found to contain a number of toxic representatives, for example
O. siamensis produces a palytoxin analogue [2], *Coolia monotis* a suspected yessotoxin analogue (cooliatoxin) [3] and *Prorocentrum lima* okadaic acid [4]. Although these species are often found in association with ciguatera, none of these toxins have ever been identified from ciguateric fish, thus it is unlikely they contribute to the ciguateric fish poisoning syndrome. However, of major concern in Tasmania is the potential for problems to occur with the accumulation of toxins in both farmed and natural populations of shellfish. In Tasmania oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis planulatus*) are major shellfish industries with abalone (*Haliotis rubra*) also becoming significant in the last few years. These shellfish filter or graze on dinoflagellates, thus during a toxic dinoflagellate bloom, transfer through the food chain to the human consumer is possible.

In this study, three main issues are addressed, firstly the identification and coastal distribution of potentially toxic epiphytic dinoflagellates, secondly whether these Tasmanian strains are toxic and thirdly how the autecology of this species assemblage from a temperate area compares to that of tropical strains.

METHODS

Distribution

Thirteen sheltered, low energy lagoons, bays and estuaries were sampled along Tasmania's east coast between 41°03'S and 42°50'S. Water temperature, salinity, seagrass presence and degree of connectivity to the ocean were recorded. Phytoplankton net (20 µm mesh), seagrass and benthos samples from each site were preserved with 1-2% glutaraldehyde and selected live samples were taken for culturing. Within Anson's Bay and Little Swanport monthly samples were collected from 5 sites. Dinoflagellates were identified using light and fluorescence microscopy and SEM. Gut content of mussels (*Mytilus edulis planulatus*) collected from Anson's Bay were also examined under light microscope for dinoflagellate presence.

Growth in culture

O. siamensis and *C.* cf. *monotis* were grown at 20°C in K medium [5] and *P. mexicanum* at 17°C in GSe medium [6], under a 12:12 hr light / dark regime at 80-100µEm^{-2}s^{-1}. Temperature (range 10-30°C) , salinity (range 15-45 psu), light (range 20-500 µEm^{-2}s^{-1}) and culture medium (K, GSe, F2, ES) required for optimum growth were determined. Growth rates were measured using a sacrificial sampling technique, in which replicates were sonicated to ensure homogeneity prior to cell counts and fluorometer measurements [7].

Toxicity

Cultured *O. siamensis, C.* cf. *monotis* and *P. mexicanum* were tested for toxicity using a brine shrimp (*Artemia salina*) bioassay [8]. *O. siamensis* extracts were

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Intergovernmental Oceanographic Commission of UNESCO 2001

also analysed in a standard mouse bioassay [9] and tested for palytoxin using haemolysis neutralisation analysis [9]. In a further bioassay, oyster spat (*Crassostrea gigas*) of two size classes, 4-8mm and 10-20mm were fed *O. siamensis* and *P. mexicanum*, observed for 48hrs and gut contents examined under the light microscope.

RESULTS

Epiphytic Dinoflagellate Distribution

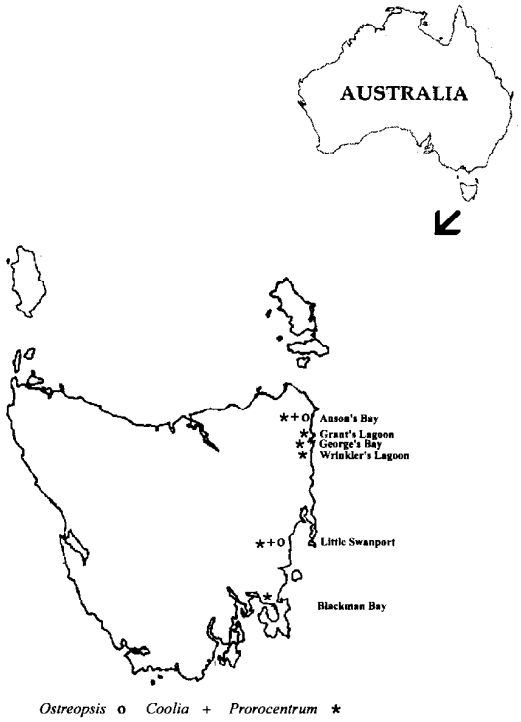


Fig. 1. Distribution of *Ostreopsis, Coolia* and *Prorocentrum* from the Tasmanian east coast, 1999

During February 1999, *Ostreopsis siamensis*, *Prorocentrum mexicanum* and *P. lima* were identified from Anson's Bay and Little Swanport. Monthly sampling of these sites also demonstrated the appearance of *Coolia* cf. *monotis* in Anson's Bay in April, 1999. The coastal distribution of these species is presented in Fig. 1. Species were associated predominantly with the seagrass *Zostera mulleri*, but also occured in sand and the water column, although in lower numbers (<50% of density found on seagrass).

At Little Swanport and Anson's Bay freshwater from the feeding rivers and oceanic water appeared to be well mixed. There was no change in salinity at any of the sites throughout each bay, nor was there any vertical temperature or salinity stratification apparent.

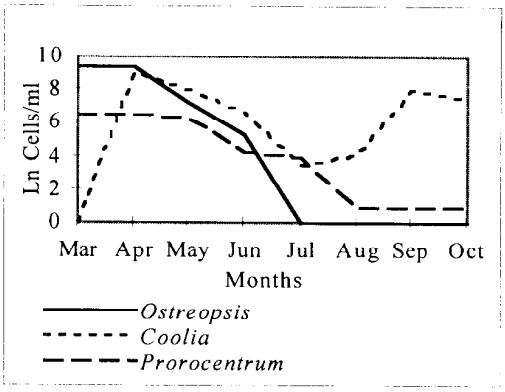


Fig. 2. Temporal distribution of epiphytic dinoflagellates at Anson's Bay, during 1999

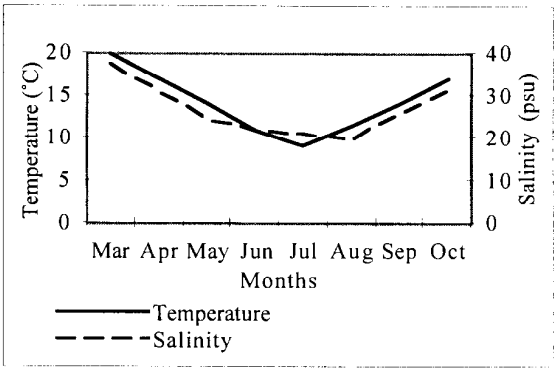


Fig. 3. Temperature and salinity profile from Anson's Bay, during 1999

Fig. 2. indicates the change in dinoflagellate density through eight months at Anson's Bay. Monthly water temperatures show a typical Tasmanian trend with highest temperatures in late summer, early autumn and lowest in winter (Fig. 3). This decrease in temperature was associated with a decline in dinoflagellate numbers through winter. A significant habitat preference ($p<0.05$) by *O. siamensis* and *C.* cf. *monotis* for shallower water between 0.5 - 1 m as opposed to depths of 2-3m was noticed.

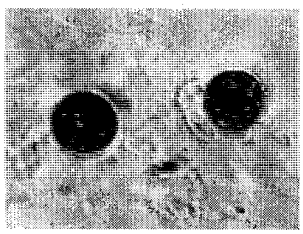
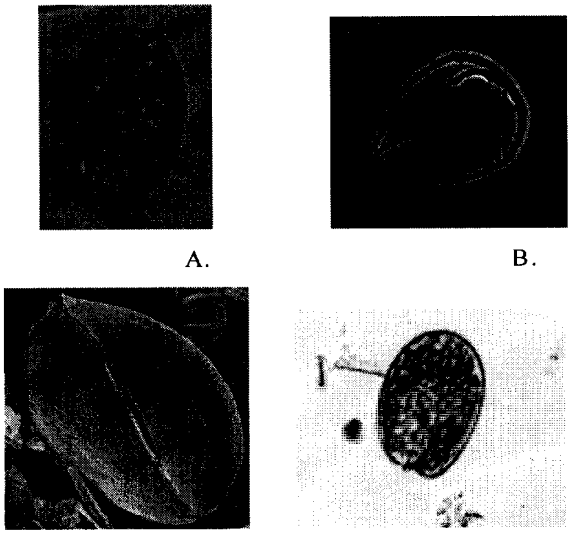


Fig. 4. *O.siamensis* (30µm) vegetative resting stage

Dinoflagellates identified from gut content of mussels collected from Anson's Bay reflected the species found in the surrounding water column: *P. lima, P. mexicanum, P. micans , Dinophysis caudata, C.* cf. *monotis* and *O. siamensis.*



A. B. C. D.

Fig. 5. Tasmanian epiphytic dinoflagellates: A. *P.lima;* B. *O. siamensis*; C. *C.* cf. *monotis;* D. *P. mexicanum*

Growth In Culture

High growth rates of *O. siamensis* and *C.* cf. *monotis* were observed in media containing low nitrogen and phosphate levels, growth was highest in K, and decreased in F2, GSe and ES. *O. siamensis* was observed in the field between temperatures of 13.5-20 °C and salinities of 25.5-37.5 psu, and grew in the laboratory between 15-25 °C and salinities of 30-45 psu. *O. siamensis* maximum growth rate (0.53 div / day) occurred at 20°C, 35ppt and 100 $\mu Em^{-2}s^{-1}$. Upon exposure to light of 500$\mu Em^{-2}s^{-1}$ a thick mucus 'pad' was observed to form at the bottom of the *O. siamensis* culture vessel, underneath which the majority of the cells were located. *O. siamensis* cultures exposed to 10°C produced spherical resting stages (Fig. 4), which upon relocation from 10°C to 20°C, regained mobility and their tear shaped cell structure.

C. cf. *monotis* was observed in the field between temperatures of 9-16.5°C and salinitites 20-31 psu, and grew in the laboratory between 10-25°C and salinitites of 15-45 psu. A maximum growth rate of 0.53 div/day was achieved at 25°C, 35ppt and 100 $\mu Em^{-2}s^{-1}$.

Toxicity

Artemia were killed within 24 hrs, upon exposure to greater than 1.2 x 10^2 *O. siamensis* cells and morbidity occurred within 24 hrs upon exposure to 800 cells. *Artemia* exhibited no clear or consistent morbid response upon exposure to *C.* cf. *monotis* and was not affected by *P. mexicanum* over 24 hrs. Oyster spat (*Crassostrea gigas*) showed no short term adverse effects over 48 hours from feeding on *O. siamensis* or *P. mexicanum.* Intraperitoneal mouse bioassay with approximately 1.6 x 10^5 cells proved lethal, symptoms were typical of palytoxins, and death occurred at 1 hr 43 min. Palytoxin was conclusively identified from both water and methanol extracts of *O. siamensis* using haemolysis neutralisation, equivalent to ≤0.1pg palytoxin / *O. siamensis* cell.

DISCUSSION

Taxonomy

The taxonomic identity of the Tasmanian *C.* cf. *monotis* requires further study. Cell size and shape is comparable to that described by Faust (1992) [17] for *C. monotis*, however, apical pore length, thecal pore distribution and size and shape of apical plate 3' are more similar to the description of *C. tropicalis* [16]. This has also been noted for New Zealand material [12].

Distribution and Autecology

This is the first report of the epiphytic dinoflagellate assemblage *Ostreopsis siamensis, Coolia* cf. *monotis, Prorocentrum mexicanum* and *Prorocentrum lima* from seagrass in truly temperate waters. It was found that *Ostreopsis* and *Coolia* are only present in sheltered open drainage habitats typified by a relatively high connectivity to the open ocean, seagrass presence and low turbidity. *Prorocentrum* rather than *Ostreopsis* dominates along the edges of the lagoons close to river runoff, whereas *Ostreopsis* dominates in the tidal exchange area. This distribution is similar to that identified in a study conducted on Mayotte Island (Mozambique Channel, Africa) [10] which found epiphytic dinoflagellates in lower abundance closer to the island shores and *Prorocentrum* rather than *Ostreopsis* dominating in the areas where dissolved organic matter was higher [10]. This preference for low nutrient habitats is also evidenced by the nutritional content of the culture medium preferred by *C.* cf. *monotis* and *O. siamensis.* The significantly higher nitrate and phosphate levels in media such as GSe and F2 prevent successful *O. siamensis* growth and cause abberant cell shape. This response is consistent with studies on Queensland isolates of *O. siamensis* which, when grown in F2 produced 'lumpy' irregular shaped cells [11].

O. siamensis presence in Anson's Bay and Little Swanport is seasonal, *O. siamensis* blooming during summer and disappearing in winter as temperature and salinity decrease. During winter *O. siamensis* appears to be replaced by *C.* cf. *monotis*. Temporal studies in French Polynesia have shown similar species succession with *O. lenticularis* numbers increasing to fill a niche left by a *Gambierdiscus toxicus* bloom [13]. Laboratory and seasonal field work suggests that *C.* cf. *monotis* survives and grows in a wider range of temperatures and salinities than *O. siamensis*, which is consistent with *C. monotis* broader biogeographic distribution worldwide [12].

Tasmanian water bodies experience a much wider temperature range than tropical areas. Over the nine

month study period at Anson's Bay the water temperature varied between 9 and 22°C. For the typically tropical *O. siamensis* to tolerate such a wide temperature range, a mechanism such as a resting stage or cyst may account for its disappearance from the water column throughout winter and reappearance in summer. No such life cycle stages were found in the field, however, a vegetative resting stage was documented in laboratory results as temperatures were lowered to 10°C (Fig. 4). This and the temperature range (15-25°C) under which the Tasmanian strain of *O. siamensis* is able to grow, indicates that this species may represent a new ecophenotype. Tropical strains grow optimally between 25-30°C [1] and a northern New Zealand strain will not grow at temperatures under 20°C [14]. Optimal growth of *C. monotis* isolated from Florida occurs at 29°C [14], a temperature at which the Tasmanian strain did not survive.

Water temperature may also account for the absence of *O. siamensis* from habitats in southern Tasmania which otherwise appear optimal. All the sites at which *O. siamensis* and *C.* cf. *monotis* have been found are within the extent of the warm East Australian Current (EAC). The EAC may be responsible for the introduction of *O. siamensis* to temperate waters. Populations of *Coolia* and *Ostreopsis* are present along the NSW and QLD coasts [11], and it is possible that the species traveled from these areas, down the EAC to suitable sites on Tasmania's east coast. We plan to pursue molecular genetic characterisation of our strains to examine this possibility.

Of additional interest is the tolerance to high light intensities displayed by both *O. siamensis* and *C.* cf. *monotis*. The ability of *O. siamensis* to cope with high light intensities has been previously speculated upon [15]; in the present study the formation of a large mucus 'pad' may effectively reduce exposure to inhibitory irradiance levels.

Toxicology

Both Anson's Bay and Little Swanport support commercial fishing operations with mussel and oyster culture in Little Swanport and pippi's harvested from Anson's Bay. As *O. siamensis* is known to bloom during summer, it is now important to determine whether or not palytoxin can be accumulated in shellfish and transmitted to human consumers. Its confirmed presence in a variety of organisms such as crabs, fish and sponges [2] and the discovery of *O. siamensis* cells in mussel guts removed from the wild, indicates that this may be a possibility.

The precise toxicity of Tasmanian isolates of *C.* cf. *monotis* is still unknown, however preliminary work suggests toxicity to *Artemia*. It is probable that *C.* cf. *monotis* contains yessotoxin analogues, which adds to our concerns of dinoflagellate toxin accumulation by commercial shellfish [3].

ACKNOWLEDGMENTS

The authors wish to thank Lyn Briggs (AgResearch NZ) for palytoxin analysis, Peter Cameron (IMVS) for mouse bioassay, Ray Brown and Lesley Rhodes for assistance throughout the year, and Shellfish Culture Pty. Ltd. for oyster spat supply.

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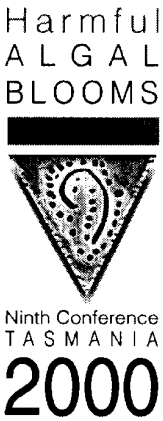
FISH KILLERS



HONG KONG'S WORST RED TIDE INDUCED FISH KILL (MARCH-APRIL 1998)

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ABSTRACT

The local press reported that the worst red tide-induced fish kill in Hong Kong's history had destroyed over 1,500 metric tons of maricultured fish stocks. The Hong Kong Government estimated the fish farmers' losses at HK$80 million (US$10.3 million), but fish farmers claimed the figure was at least HK$250 million (US$32 million). About 1,000 of Hong Kong's 1,500 fish farms were devastated by the *Gymnodinium mikimotoi* red tide. The red tide "spread out like an infectious disease," stated Hong Kong Fish Culture Association Chairman, Wong Yung-kan.

Phytoplankton samples taken near the fish mariculture cages at Mo Tat Wan on 15 April 1998 contained over one million cells L^{-1} of *Gymnodinium mikimotoi*. At high densities, the *G. mikimotoi* produced a sticky mucus. When the mucus touched plant fibers or chitin or other dinoflagellates it stuck to them. When it made contact with the gill filaments of fish or shellfish it asphyxiated up to 90% of the impacted fish within 30 minutes. This rapid mortality gave rise to speculation that the slime contained a phycotoxin but no evidence to substantiate this speculation has been published. The gills of the dead fish that were examined were coated with slime in which *G. mikimotoi* was observed.

One of the reasons that dinoflagellates produce copious amounts of a sticky mucus can be inferred from photographs taken at the peak of the 15 April bloom near Lamma Island where the Hong Kong mariculture industry was particularly hard hit. The photographs indicate that the secreted mucus sticks to inanimate objects such as plant fibers as well as to living objects such as the chitinous exoskeleton of copepods. The grazing efficiency of copepods with *G. mikimotoi* slime attached to their carapace appeared to be considerably reduced. Thus, mucus production may increase *G. mikimotoi* (dinoflagellate) survival by reducing the filtering efficiency of predatory copepods.

INTRODUCTION

Dinoflagellates are unable to move fast enough to escape most raptorial and filter feeding predators [1]. As a result, dinoflagellates have evolved an arsenal of biological compounds that suppress predation losses. Some of these substances are neurotoxic and/or psychotropic (e.g. NSP). Others are powerful paralytic toxins such as the well known paralytic shellfish poison (PSP). A third general group of toxins are diarrhetic shellfish poisons (DSP). The general effect of these biological compounds is one of predator deterrence [2]. Field observations made during a bloom of *G. mikimotoi* in April 1998 suggest that mucus can also be a major predator deterrent.

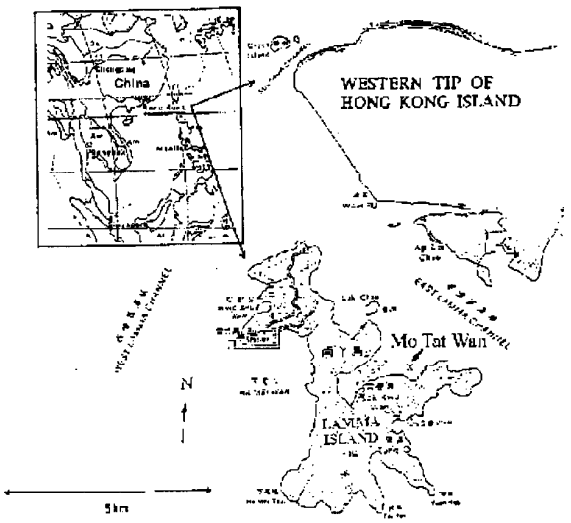

Fig. 1. The East Lamma Straits-Lamma Island sample site (X) at Mo Tat Wan with the western end of Hong Kong Island and inset indicating the locations of Manilla, Singapore, Bangkok and Hong Kong in the South China Sea.

METHODS

Weekly phytoplankton samples were taken at Mo Tat Wan in 1997 and 1998. The samples were taken near Lamma Island (Fig. 1) before, during and after the *G. mikimotoi* red tide. Phytoplankton samples were taken by passing 100 litres of surface water through a 10 micron mesh plankton net and concentrating it to a volume of 100 ml. Lugol's IKI solution [3], was added to each sample and 24 hours was allowed for sedimentation to occur. An Olympus research microscope equipped with Nomarski interference optics was used to count the organisms in the samples. A minimum of 500 individuals was counted. Copepods were categorized as nauplii or adults. Juvenile copepods, (copepodites) were treated here as adults. Predatory ciliates, primarily *Favella, Mesodinium* and tintinnids, predatory dinoflagellates, primarily *Noctiluca*, and copepods were observed during the *Gymnodinium* red tide event but only diatom and dinoflagellate phytoplankton species are reported here as these were the most abundant phytoplankton phyla observed in the weekly samples from Mo Tat Wan. Species from these two phyla were identified using the references [4], [5], [6], [7] and [8].

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RESULTS

The Mo Tat Wan phytoplankton results were plotted for the period 18 March to 21 April, 1998 (Fig. 2). Changes in the total abundance of dinoflagellates (Fig. 2a) and total diatom abundance (Fig. 2b) indicate low diatom densities (< 50,000/L) during the period that dinoflagellates were abundant and vice versa. *Gymnodinium mikimotoi* abundance (Fig. 2c) mirrors that of total dinoflagellates indicating that *G. mikimotoi* was the major component of the dinoflagellate community from mid March to mid April. The schematic diagram of *G. mikimotoi* cells and mucus trapped in the filtering appendages of an adult copepod (Fig. 3a) was based on a composite of several photographs that indicated the characteristics of mucus fouled copepods,

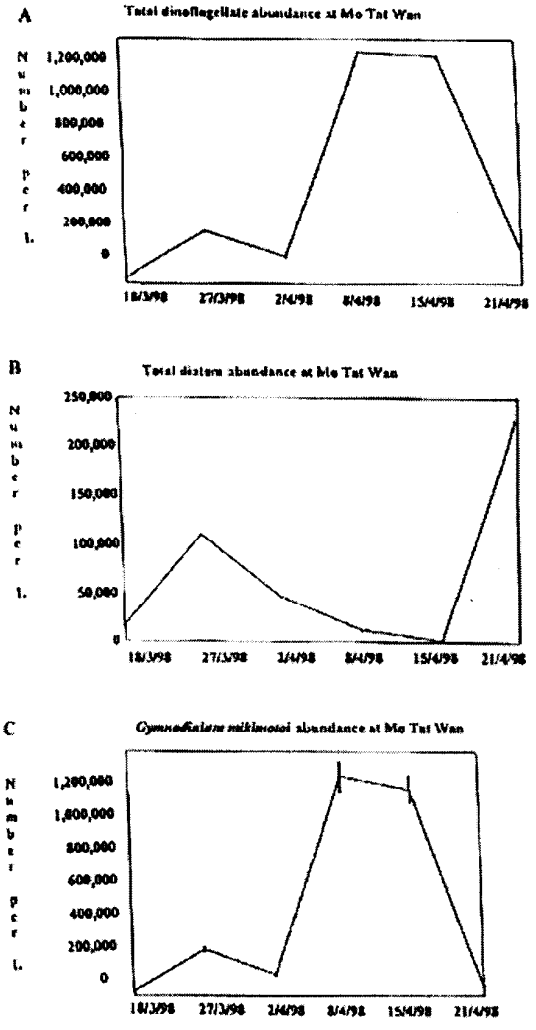

Fig.2. Abundance (number per litre) of dinoflagellates (a), diatoms (b) *Gymnodinium. mikimotoi* (c) for the period 18 March to 15 April, 1998 plus a schematic view of *G. mikimoto* (d).

In mid March, diatoms, primarily *Bacillaria paradoxa* and *Pseudonitzschia* species, dominated the surface samples and *G. Mikimotoi* was relatively rare (Fig. 2c). By 27 March, *G. mikimotoi* had increased nearly ten fold to 23,000 cells/L and the dominant diatoms were now *Chaetoceros curvisetum* (90,000/L), *C. costatum* (15,000/L) and *Pseudonitzschia* species (1,380/L). On 8 April, *G. mikimotoi* had increased to over 1 million cells/L. Diatoms declined, *Chaetoceros curvisetum* (1,800/L), *C. costatum* (<1,000/L) and *Pseudonitzschia* species (6,000/L). A second dinoflagellate, *Prorocentrum sigmoides*, reached 4,400 cells/L which was negligible compared to the abundance of *G. mikimotoi*. By 15 April, *G. mikimotoi* abundance remained relatively unchanged (1,020,400 ± 6,500 cells/L, n = 3).

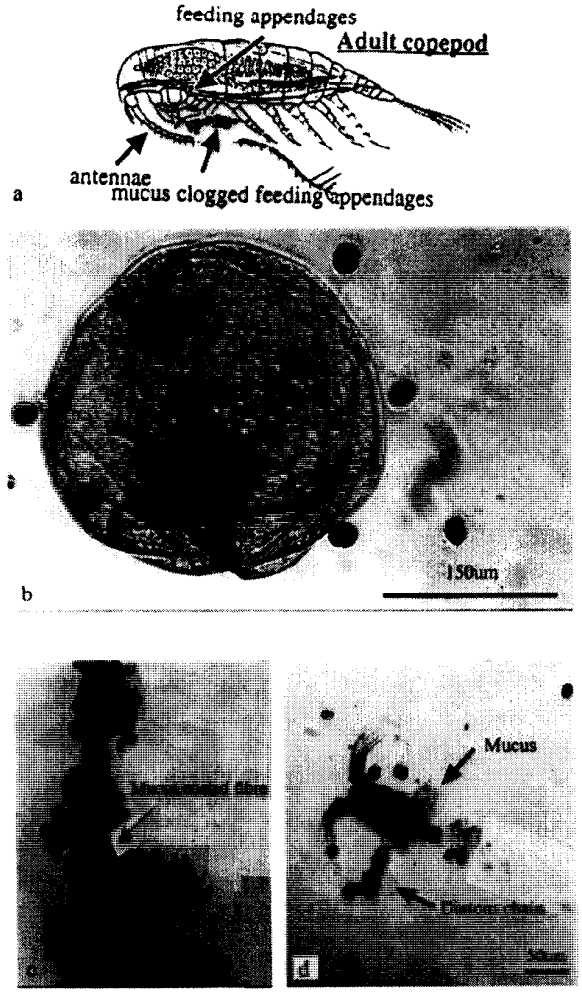

Fig. 3. A schematic view of a copepod adult with its feeding appendages clogged with mucus (a) a colony of *G. mikimotoi* enveloped in its own mucus (b) a copepod nauplius trailing *G. mikimotoi* mucus (c) a copepod nauplius surrounded by mucus secreting dinoflagellates attached to it and a plant fiber and a chain of diatoms (d).

G. mikimotoi produced copious amounts of sticky mucus during its peak in abundance, 8-15 April. The mucus secreted by this dinoflagellate caused cells of *G.*

mikimotoi to attach itself to the dinoflagellate predator, *Noctiluca scintilans* (Fig. 3b), free-floating plant fibres (Fig. 3c) and the chitin of copepod nauplii (Fig. 3d). The mucus secreted by *G. mikimotoi* allowed it to form large mucus encapsulated clumps (Fig. 4a). These clumps were observed in the gills of fish from the caged fish (mariculture) enclosures (Fig. 4b & c) from many locations in Hong Kong that reported heavy fish mortalities (Fig. 4 d) associated with the *G. mikimotoi* bloom of April 1998. News stories about the devastation of the fish in the Lamma Island area indicated that fish kills peaked on 15 April 1998. Lamma Is. fish farmers claimed that up to 90% of their fish had been asphyxiated by the dense mucilage that had coated the gills of their caged fish turning their gills white with mucus.

DISCUSSION

Several classes of phytoplankton are known to include taxa that secrete chemicals that are inhibitory or toxic to invertebrates [9]. The active agents are often secondary metabolites [10].

Mucus Production and its Impacts on Copepods

Mucus secreted by *G. mikimotoi* appears to attach to just about anything it comes in contact with making it is easy to imagine how the feeding appendages of copepods (Fig. 3a) and the gills of fish might quickly become coated with mucus. Diatoms and dinoflagellates constitute the principal food of most planktonic copepods [11]. The second antennae of a number of predatory copepods vibrate at 600 to 2,600 times per minute producing a vortex along both sides of the body, [1]. Food particles are concentrated in the center of these two vortices and come in contact with the myriad fine setae on the second maxillae. Once trapped in these setal hairs the food particles are scraped from the second maxillae by the endities of the first maxillae and passed to the mandibles and the mouth [12], [1]. If algal mucus coats the setal hairs it becomes difficult for the endites to scrape them clean. Copepods were observed in this study to stop filter feeding when they were near mucus covered *G. mikimotoi*. Presumably, this occurs to allow them to avoid fouling their feeding appendages with mucus. It is also likely that many adult copepods avoided the bloom of *G. mikimotoi* by swimming below it. Relatively few were seen at the surface during the bloom of *G. mikimotoi*. During the April 1998 bloom of *G. mikimotoi*, many juvenile and adult copepods disappeared from the surface layer when *G. mikimotoi* reached its highest concentrations (> 1,000 cells/mL). As noted above, those copepods (primarily nauplii) that remained in the surface waters were often found entangled in mucus. Gentien and Poulet observed female *Calanus helgolandicus* copepods, tangled in a "mucus web" produced by *G. mikimotoi*, [13]. The *G. mikimotoi* mucus reduced the feeding current of *Calanus helgolandicus* even when the feeding appendages were free of the mucus [13].

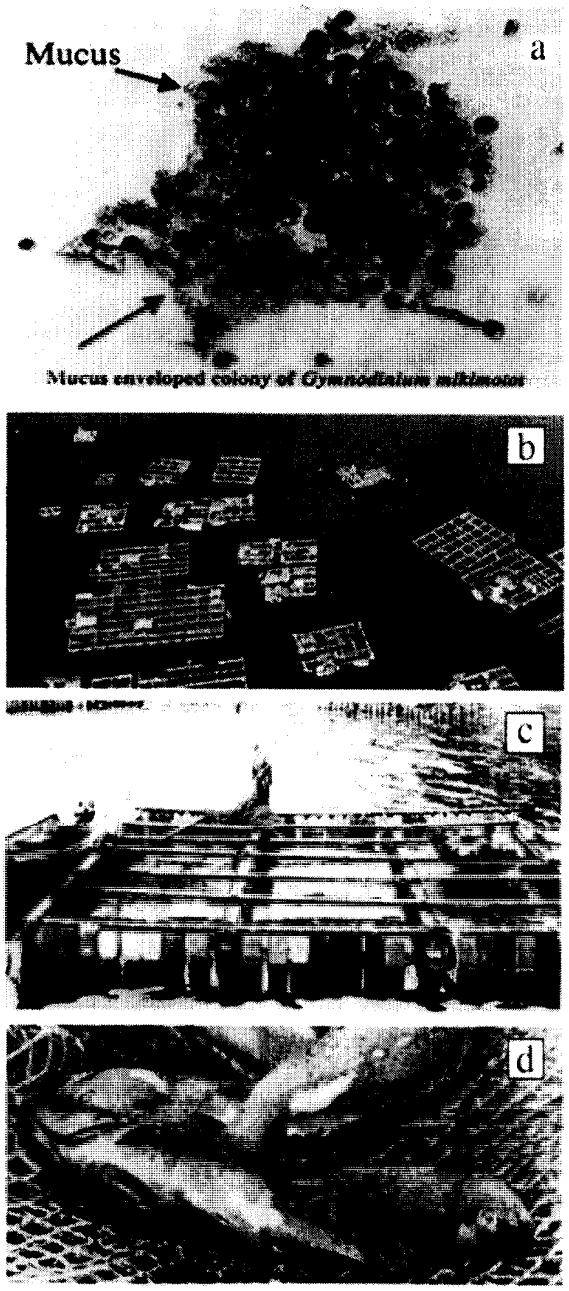

Fig. 4. Floating mariculture "cages" with dead fish on 15 April, 1999 (a), an aerial view of the fish enclosures (b) and empty enclosures (arrow) at Sok Kwu Wan following the *G. mikimotoi* bloom (c)

CONCLUSION

In conclusion, the weekly samples taken from Mo Tat Wan in the East Lamma Straits indicate three interesting patterns. As the population density of *G. mikimotoi* reached high levels 1) the abundance of diatoms declined. 2) objects such as plant fibers and

chitin that came in contact with mucus secreted by *G. mikimotoi* were often observed with *G. mikimotoi* adhering to them at the peak of the bloom. 3) few adult copepods were observed at the surface of the water.

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OTHER HARMFUL EVENTS

Harmful
ALGAL
BLOOMS

Ninth Conference
TASMANIA
2000

NEW AND DOMINANT SPECIES FROM SAM XING WAN, SAI KUNG DURING THE 1998 MASSIVE FISH KILLING RED TIDE IN HONG KONG

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ABSTRACT

A massive fish killing red tide occurred in Hong Kong from mid-March through mid-April, 1998. Weekly samples collected from a permanent station in Sam Xing Wan, Sai Kung were studied by light and scanning electron microscopy. A *Gyrodinium* sp., which is believed to have caused the fish kill, is a new species. It is morphologically closely related to *Gymnodinium mikimotoi* Miyaka & Kominami ex Oda, a fish killer firstly described from Japanese waters. Its highest cell density recorded from this station reached $2x10^5$ cell/L of water. One other species, which was revealed by scanning electron microscopy, is probably an unidentified dinoflagellate species. During a 16 week period from 1 January to 27 May, dominance shifted between dinoflagellate and diatom species. *Alexandrium* spp. , *Ceratium furca* (Ehrenberg) Claparède & Lachmann, *Gymnodinium catenatum* Graham, *Gymnodinium sanguineum* Hirasaka, *Prorocentrum dentatum* Stein, *Prorocentrum triestinum* Schiller, *Scrippsiella trochoidea* (Stein) Loeblich III, *Chaetoceros* spp., *Pseudo-nitzschia pseudodelicatissima* (Hasle) Hasle, *Skeletonema costatum* (Greville) Cleve, and *Thalassiosira* spp. were all dominant or relatively abundant species during this period.

INTRODUCTION

For the first half of the year 1998, totally 29 red tides occurred, and at least 16 species were involved in these events (Data from Agriculture and Fisheries Department, Hong Kong). The most serious events occurred from mid-March to mid-April, and caused a massive fish kill [1]. During this period, 22 of the 26 fish culture zones (1,501 farms) were affected and 3,546 tons of fish were killed with an estimated loss of 315 million HK$ (US$ 40) [2]. The causative species is believed to be an undescribed *Gyrodinium* species similar to *Gymnodinium mikimotoi* complex. The red tide and fish kill were firstly reported to occur in the northeast of Hong Kong waters on 18 March 1998, and at the same time, it was also detected at our permanent station, Sam Xing Wan, Sai Kung, a little more to the south. This report presents some of the dominant or co-dominant species revealed by either light microscopy or scanning electron microscopy.

MATERIAL AND METHODS

Sampling

From mid-March to mid-April 1998 surface water samples (1 liter water) were collected for phytoplankton analysis at a permanent station located in Sam Xing Wan, Sai Kung, in the eastern waters of Hong Kong. Net plankton samples were collected by passing 20 liters of surface water through a net of 10 μm mesh size (Wildlife Supply Company). Live cells from the concentrate (50 mL) in f/2 medium were used for further observation.

Light microscopy

Bottle samples (1 liter) were fixed with acidified Lugol's iodine solution [3]. They were concentrated to 10 ml and then 25 μL of the concentrate were transferred to a glass slide using a micro-pipette and covered with a 18x18 mm cover slip. The entire slide was counted. Net live samples were also prepared in the same way and observed. Photos were taken using an Olympus BX-60 light microscope.

Scanning electron microscopy

Another 50 mL concentrate was fixed with acidified Lugol's iodine solution for scanning electron microscopy. For dinoflagellate species, a drop of well mixed preserved net sample was filtered using a polycarbonate membrane filter (1 μm pore size). It was then dehydrated with an alcohol series (30, 40, 50, 70, 80, 85, 90, 95, 100%), and critical point dried. For diatoms, equal volumes of well mixed preserved net sample and 69% nitric acid were transferred to a beaker. The mixed sample was placed on a hot plate and heated at 80°C for 2 hours. The sample was then washed again with distilled water until the pH value reached about 7. A drop of the cleaned sample was filtered using a polycarbonate membrane filter (1 μm pore size) and washed 3 times with distilled water. Both the dried dinoflagellate and diatom samples were sputter coated with Au/Pd, and finally examined with a Leica Cambridge S360 scanning electron microscope.

RESULTS AND DISCUSSION

Phytoplankton samples collected during the first few months of the year revealed several dinoflagellate species including the undescribed *Gyrodinium* species, some being toxic or potentially toxic. A bloom dynamic study of the samples collected during this period showed that several species, including both diatoms and dinoflagellates, bloomed and dominated the phytoplankton assemblage.

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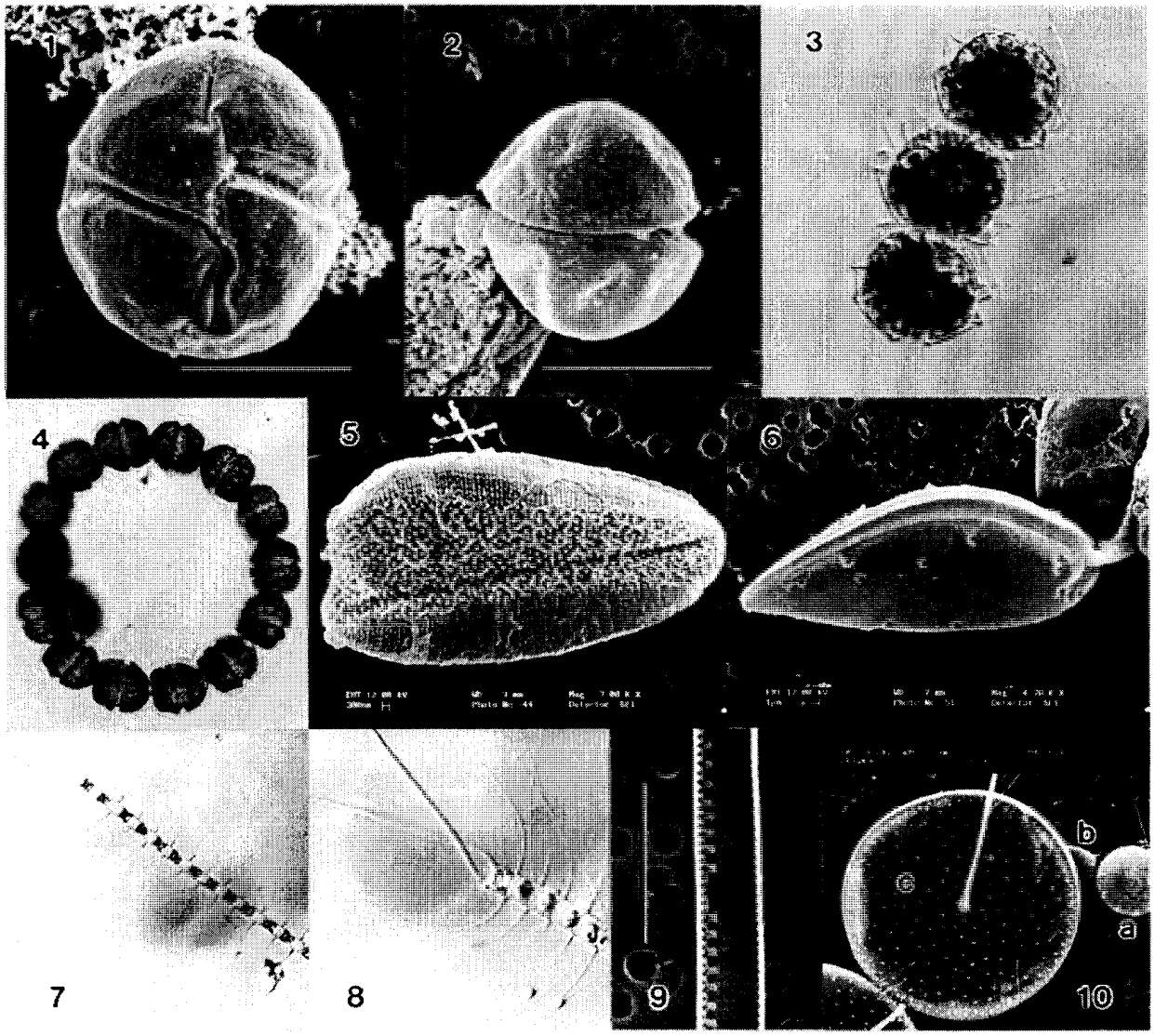
1. *Gyrodininum* sp. (Figs 1-2)

This species is believed to be responsible for the massive fish killing noted and the highest cell density recorded reached $2x10^5$ cell/L of water on 18 Mar. when water temperature was 18.8°C and salinity was about 33‰. When it first appeared different people identified it as *Gyrodinium aureolum* Hulburt or *Gymnodinium mikimotoi*. However detailed study showed that it is different from both of them and description as a new species has been submitted [4].



Figs 1-2 Ventral and dorsal view of *Gyrodinium* sp. (SEM). Scale bar = 10 μm.
Fig. 3 *Alexandrium* sp. (LM). Magnification = 200.
Fig. 4 *Gymnodinium catenatum* (LM). Magnification = 200.
Fig. 5 *Prorocentrum dentatum* (SEM).
Fig. 6 *Prorocentrum triestinum* (SEM).
Fig. 7 *Chaetoceros distans* (LM). Magnification = 100.
Fig. 8 *Chaetoceros diadema* (LM). Magnification = 100.
Fig. 9 *Pseudo-nitzschia pseudodelicatissima* (SEM). Scale bar = 5 μm.
Fig. 10 *Thalassiosira tealata* (a), *Thalasiosira conferta* (b), *Thalassiosira rotula* (c).

2. *Alexandrium* spp. (Fig.3)

There were probably 3 different *Alexanduium* spp. present during the first half of the year. The total abundance was highest on 25 March, 1998, when water temperature was about 20°C and salinity was about 33‰, with a total cell number of $2.8x10^4$ cell/L. *A. catanella* and *A. excavatum* have been previously reported to cause red tides in Hong Kong. *A. catanella* also causes paralytic shellfish poisoning [5]. Laboratory study of a Hong Kong strain of A. catenella revealed an optimal growth conditions were pH 8.5, salinity 30-35‰, temperature 20-25°C [6].

3. *Ceratium furca*

This species started to build up in early February and reached its highest density (1.8×10^4 cells/L) on 17 February. This species often bloomed in Hong Kong coastal waters. From 1975 to 1997, it was involved in 24 red tide events and 17 were formed by this species alone. However, it has never been reported to cause any fish kills or other human impact.

4. *Gymnodinium catenatum* (Fig.4)

This species was present in almost all samples during the first half of the year, but was relatively more abundant in January and February than the rest of the sampling period. The highest density reached $6x10^3$ cells/L. This species is a PSP producer, often reported to cause problems worldwide [7,8]. However, it has not yet been reported to have any harmful effects in Hong Kong.

5. *Gymnodinium sanguineum*

This species was the dominant species in the sample collected on 18 March 1998, the cell density reached $3x10^5$ cell/L, which is more than the density of the undescribed *Gyrodinium* sp., the second most dominant species at $2x10^5$ cell/L. Based on historical records, this species has often caused red tides in Hong Kong, but has never been reported to cause harmful effects in Hong Kong, although it has been found to produce toxins and cause fish kills in other places [9,10,11].

6. *Prorocentrum dentatum* (Fig.5)

This species reached its peak concentration in late April, which lies in the normal period for it to bloom according to the historical record. It formed 4 red tides during the period 1975-1997, and a fish kill was claimed to be caused by a bloom which occurred from 2-24 May, 1984. Its killing effect is believed to be due to induced hypoxia-anoxia events.

7. *Prorocentrum triestinum* (Fig.6)

This species reached its highest concentration of $6x10^5$ cells/L during the first week of March 1998. Between 1975 and 1997, this species was involved in forming red tides 29 times in Hong Kong, of which 22 were caused by it alone, and it was once implicated in fish kills. However it has not been reported to cause problems elsewhere.

8. *Scrippsiella trochoidea*

This is a very common species in Hong Kong waters. In the last two weeks of March 1998, this species was very abundant, reaching $8x10^4$ cell/L and $6x10^4$ cell/L respectively, ranking third and first in the phytoplankton assemblages. It caused red tide once in 1988, together with *Gymnodinium mikimotoi,* but has never been reported to have harmful effects.

9. *Chaetoceros* spp. (Figs7-8)

This was the second group of species to appear after the massive fish kill event. The total abundance was $2x10^5$ cell/L. Historically, several species of *Chaetoceros* have caused 7 red tides in Hong Kong waters, but they have never been reported to result in any problems. *Chaetoceros concavicornis,* which was reported to cause fish kills elsewhere [12], has never formed dense blooms in Hong Kong waters.

10. *Pseudo-nitzschia pseudodelicatissima* (Fig.9)

This species bloomed in early March, two weeks before the massive fish kill event when it reached its highest concentration of $6x10^5$ cell/L. This species often blooms in Hong Kong waters, but has never been reported to cause harmful effects. However, it was reported to produce domoic acid and cause shellfish contamination in the field and in the laboratory elsewhere [13,14].

11. *Skeletonema costatum*

This species dominated the plankton assemblages immediately before and after the massive red tide. The highest density occurred on 10 March, 1998, and was about $1.1x10^6$ cells/L. This species has caused 38 red tides from 1975 to 1997 in Hong Kong. Red tide formed by *S. costatum* together with *Gonyaulax polygramma* caused fish kills in 1988 in Hong Kong (AFD database). It was also reported to cause fish kills or gill injury in other regions [15].

12. *Thalassiosira* spp. (Fig.10)

Species of *Thalassiosira* started to bloom from early April and the bloom lasted for 2 months, e.g. *Thalassiosira rotula* Meunier, *Thalassiosira conferta* Hasle and *Thalassiosira tealata* Takano. The total

concentration reached as high as $1.7x10^5$ cell/L. Several species of *Thalassiosira* have been reported to cause red tides on 15 occasions in Hong Kong, but have never been reported to have harmful effects. However, it is one of the diatoms which has been shown to cause fish kills or gill injury [15].

In summary, 1998 was an unusual year in the history of red tide events in Hong Kong in that there was an increased number of algal blooms compared to the average after 1988. An undescribed species, which was believed to be toxic formed massive red tide and caused fish kills, which resulted in great economic loss. This study also found other unknown species, which will be discussed elsewhere.

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WIDESPREAD OUTBREAK OF A HAEMOLYTIC, ICHTHYOTOXIC *GYMNODINIUM* SP. IN SOUTHERN CHILE.

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ABSTRACT

A large-scale fish kill occurred in the waters around Chiloe Archipelago, Chile, during March and April 1999. Water samples revealed the near-monospecific bloom of a gymnodinioid dinoflagellate which reached concentrations of 4 - 8 x 10^6 cells L^{-1} in brown patches. Airborne observations and an intense temporal and spatial phytoplankton monitoring program strongly suggested that this bloom was of coastal oceanic origin. One possible cause could be the unusual climatic anomalies in the precipitation pattern during the last 14 months in the area, with a long period of reduced rainfall and bright sunshine. Surface water temperatures at 15°C, were 1.5°C higher than normal in the area. Many caged salmon died as a consequence of this bloom, probably due to the haemolytic activity induced by the algae. Laboratory tests carried out with diluted seawater samples showed an 18% haemolytic activity with an algal cell concentration of 250 cell mL^{-1} against red blood cells (RBC) at a concentration of 40 million mL^{-1}. In addition, analysis of the sea water samples revealed a marked allelopathic property which may explain the almost monospecific nature of the bloom. The dinoflagellate *Alexandrium catenella* was not inhibited in growth by *Gymnodinium* excretions. In contrast, the development of the diatom *Leptocylindrus minimus* was completely repressed. Both species were observed in the area of the bloom.

INTRODUCTION

An unusual dinoflagellate bloom was observed in the South-Eastern Pacific Ocean near the Chiloé Archipelago, in March-April 1999. The phenomenon also affected the southermost area of the fjords and channels of the Magellan region during the first two weeks of April. Fish and invertebrate kills were associated with the bloom of an unidentified *Gymnodinium* species. Precipitation in 1998 and 1999, in Southern Chile was lower than normal (Meteorological Service of Chile), and we suggest that this condition contributed to the magnitude of the bloom. The area affected has considerable wild fisheries, fauna and intensive fish farming activities. The progression of the bloom was monitored almost daily and water samples from the discoloured patches around Chiloé Archipelago were tested *in vitro* to examine the ecological effect of the bloom, and toxicity as regards marine animals.

MATERIALS AND METHODS

The phytoplankton population was examined prior to, during and after the event thanks to the action of the monitorig program along extensive areas of Southern Chile (Figure 1): This program has been in progress for more than 10 years [1].

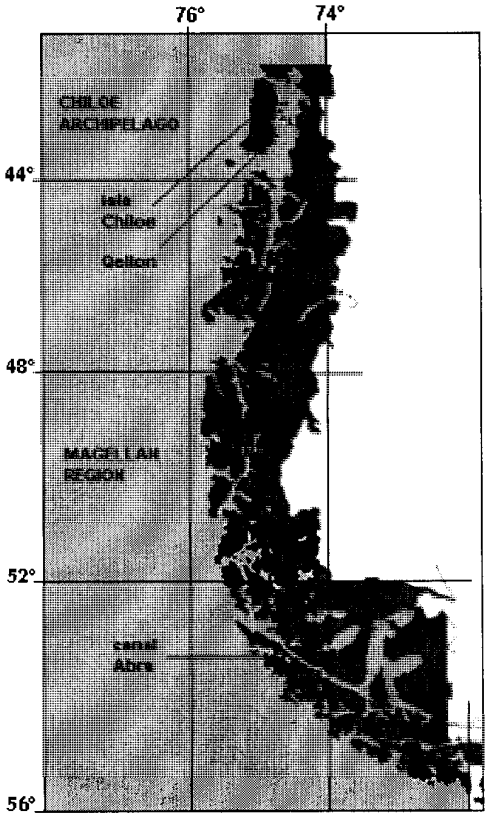

Fig. 1 : The studied area.

The fish farmers involved in the monitoring network contributed with information about the losses of both wild animals and farmed fish. Airborne observations around Chiloé Archipelago were part of the contingency plan, as well as Niskin bottle sampling of coloured patches, (variable in size) including coastal-oceanic and inland marine waters respectively. Samples from the Magellan region (Canal Abra) were collected using a house pipe up to 10 m depth. Algal cells were maintained in a temperature-controlled room at 13°C, with a light-dark cycle of 16 : 8 at 40 µE m^{-2} s^{-1}. The alga (a species of the genus *Gymnodinium*) found in

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these seawater samples was isolated and its culture was intended in the seawater enriched with 100% f/2 medium [2] in the conditions described above.

The allelopathic effect of *Gymnodinium* sp. culture was tested on the diatom *Leptocylindrus minimus* (strain LM01), and the dinoflagellate *Alexandrium catenella* (strain ACC08). Both strains were isolated during blooms in this part of Chile, in 1998 and 1995 respectively, and cultivated in 100% f/2 medium in the laboratory. Seawater samples taken from the *Gymnodinium* sp. culture were passed through Whatman GF/F filters to remove the phytoplankton cells, and the filtrate was called A. A fraction of A was passed through C18 and Florisil Sep-Pack cartridges (Waters) to extract the dissolved organic substances [3] [4] and the treated fraction was called B. After the addition of f/2 enrichment in A and B, and filter sterilization (Sterivex 0.22 µm) both were inoculated for monospecific bioassays with *L. minimus* , and *A. catenella.* Their growth was examined until the stationary phase, cell counts, and compared to a control (C) cultivated in sea water from a non impacted area. Logistic growth curves were obtained in each bioassay by applying the NLREG© analysis program [5], and the exponential growth rates (µ) were compared.

The haemolytic assays were performed according to the method of [6] using human red blood cells (RBC), and freshly collected field samples (sea water + *Gymnodinium* sp. cells) and cultures of the *Gymnodinium* sp.
Mineral nutrients were analysed following the standard methods [7], and the concentration of chlorophylls according to [8].

RESULTS AND DISCUSSION

Historical, geographical and climatic description

The area impacted by *Gymnodinium* sp. extended from 42° to 54 °S, over 1330 km long (Fig. 1). Along the Chiloé Archipelago the first mortalities of marine fauna were reported near Quellon on 13th March, co-occurring with brown patches. The patches were sharply delimited. In the following days, airborne observations showed the presence of well-defined brown patches which accompanied salmon kills at aquaculture sites in the inland waters of the Chiloe Archipelago. In most of the areas discoloured water caused by *Gymnodinium* sp. was accompanied by mortalities of wild animals (sea urchins, wild fish, worms, mollusc). The bloom was more intense during the period of neap tides, sunny and calm weather. Spring tides starting on 27th March, coincided with the disappearance of the patches and hardly any mortalities were recorded, but during the following period of neap tides, patches and salmon mortalities occurred again on 9th April. Along the Magellan region the phenomenon (discoloration associated with the death of sea stars, conger eels, scallops, octopus, marine snails and sea urchins) was detected at Canal Abra on 9th April.

Phytoplankton observations of bloom water from Chiloé showed that *Gymnodinium* sp. constituted 99 % of the population, and the maximum concentration in the patches were between 4 and 8 x 10^6 cells L^{-1}. Concentrations in the Magellan region were 3 - 43 x 10^3 cells L^{-1} and constituted 55-99% of total phytoplankton, but poor in species (up to 12) being *Pseudo-nitzschia australis* and *Thalassiosira* cf. *delicatula* the more important species. This was the first record of *Gymnodinium* sp. in this area.
Day and night sampling results suggest vertical distribution of the cells. During bright sunshine days, cell concentrations were higher at the surface forming sharply defined brown patches. At night the cells were located in the first 10 m of the water column.
One factor suggests that the blooms were of offshore origin. The first cells were detected (10 cells mL^{-1}) in samples collected in oceanic coastal water during late February and during the same period no cells were observed in the inland sea. Airborne observations showed brown patches in the coastal oceanic waters which were sample specific showing mono specific abundance of *Gymnodinium* sp.. Information from the Magellan region showed that fish and invertebrate mortalities occurred in channels directly connected to the Pacific Ocean, and *Gymnodinium* sp. was absent in sampling sites located in the inner waters. This feature suggests that this bloom originated outside the fjord and channels area and probably in tidal fronts between open (salinity above 30 p.s.u.) and inner waters (15-25 p.s.u.).
In the Chiloé Archipelago seawater surface temperatures were 1.5°C higher in the area (15°C) than normally expected [1]. The bloom may have been stimulated by a long period of strong sunshine. Furthermore, the period of low fresh-water run-off, may allow oceanic water (salinity 33 p.s.u.) to penetrate the inland sea area providing conditions more suited to oceanic species. The bloom of *Gymnodinium* sp. appeared to be strongly associated with the frontal structure of water masses. The chlorophyllian biomass was of 150 µg Chl a L^{-1}, and 90 µg phaeophytin a L^{-1} on 26th March, in a sample from the bloom containing 4 x10^6 cell L^{-1}.

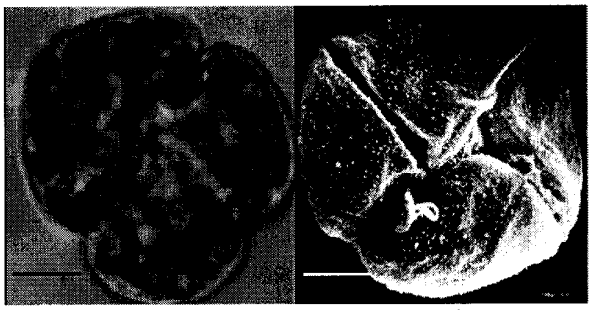
Effects on fauna and Aquaculture

The relationship between animal mortality and the presence of coloured water was first established at fish-farm sites. Affected salmon showed abnormal behaviour and abundant mucus around the gills. Mortalities of sipunculids, echinoderms (sea urchin), worms and molluscs were also noted.
In only one month an estimated 500 000 marketable salmon (about 1 500 ton) were lost. Although this is less than 1% of gross annual salmon production in Chile, it constituted a severe and sudden loss to the individual farmers concerned.

The alga

The algal cell responsible for the bloom is a *Gymnodinium* species, approximately 30-40 µm long and 20-35 µm wide, with a large nucleus (10 µm)

located in the lower central part of the hypocone (Fig. 2a). Numerous small chloroplasts are visible around the cell. An apical groove is present in the epicone (Fig. 2b). After a period of growth in the culture, smaller cells (10-15 µm) could be seen. Large cells were particularly fragile and may not have survived transport to the laboratory.



a b

Fig. 2 : *Gymnodinium* sp. cell (scale bars = 10 µm), a : microphotonic picture, b : electronic microscopy.

The main pigment contents of the *Gymnodinium* sp. are fucoxanthins and 19'-acyloxyfucoxanthins (Carreto, per. com.). At the time of the fish kill event, the algal cells were fragile and did not keep their morphological features after formaldehyde fixation, and this was an obstacle in obtaining a more complete characterization.

The allelopathic property

The experiments with *Alexandrium catenella* and *Leptocylindrus minimus* indicate that *Gymnodinium* sp. may produce allelopathic exudates which could contribute to the formation of monospecific blooms [9] [3].

Table 1 : Phytoplankton growth rates (μ d^{-1}) characteristic of the logistic growth functions, corresponding to the bioassays in C (control seawater), A (bloom seawater) and B (bloom seawater filtered through Sep-Pack cartridges) with the different algal species.

	Leptocylindrus minimus	*Alexandrium catenella*
C	1.42±0.17	0.26±0.04
A	0.31±0.08	0.25±0.03
B	1.81±0.24	0.47±0.11

Our tests showed that *Leptocylindrus minimus* growth was totally inhibited in the seawater fraction A (Table 1) in where *Gymnodinium* sp. had grown, while the growth of a dinoflagellate culture, *Alexandrium catenella*, was practically unaffected. The organic-free fraction B, did not differ from the control (C), indicating that the active allelopathic substances produced by the blooming cells were removed by the treatment of seawater and are likely to be dissolved organic substances.

Haemolytic property

A marked haemolytic activity became evident after 30 minutes' incubation at 18°C with field samples containing 4 000 cell mL^{-1}. Tests carried out on field samples diluted to 250 cell mL^{-1}, showed a significant activity, with 18% haemolysis in a 40 x 10^6 mL^{-1} RBC suspension (Table 2).

Table 2 : Influence of *Gymnodinium* sp. cell concentration on the haemolytic activity (% of total lysis) in the bloom seawater.

Cell mL^{-1}	500	1000	2000	3500
% lysis	7	57	81	94

CONCLUSION

This has been the first outbreak of an ichthyotoxic *Gymnodinium* bloom in Southern Chile, at least since the beginning of the phytoplankton monitoring program (1989). The airborne observations and large number of water sample (in space and time) results demonstrate that this *Gymnodinium* sp. bloom originated outside the fjord and along channels of a vast area covering from 41° to 54°S during a period of less precipitation than normal.
The *Gymnodinium* sp. presented several similarities to other *Gymnodinium* species associated with toxic events. The location of *Gyrodinium aureolum* in a frontal zone was described by [10], and the allelopathic property pointed out by [4] in the case of *G. mikimotoi* [11]. The mode of action of the toxins on the cell membrane, resembles that of *G.* cf. *aureolum* detected in the Ushant front [12] and in the Atlantic coastal waters of France [13]. In this case, polyunsaturated fatty acids, identified as the toxin precursors by [14], were detected in the lipid composition of the cells [15], and their allelopathic and haemolytic properties demonstrated. The pigment composition including fucoxanthin derivatives draws *Gymnodinium* sp. close to other ichthyotoxic dinoflagellates : *G. catenatum, G. galatheanum, G. veneficum, G. breve* and *Gyrodinium aureolum* [16].

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EARLY 1998 MASSIVE FISH KILLS AND ASSOCIATED PHYTOPLANKTON IN PORT SHELTER WATERS, HONG KONG

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ABSTRACT

In March through April 1998, a massive red tide occurred in south China, including Hong Kong, coastal waters. This red tide covered a huge area and lasted for more than one month, killing various caged fishes and causing a large economic loss. By 20 April, it was reported to be declining and was predicted to have no further momentum to distribute on a large scale. It was found that from February to May 1998 there was a continuous algal bloom in Port Shelter, an inlet of an enclosed bay in Hong Kong's northeast coastal waters. Phytoplankton data collected from a permanent station located in this area were analysed. Diatoms and dinoflagellates were the two main groups which dominated the phytoplankton. In general, when there was an increase in the density of diatoms there was a decline in the density of dinoflagellates, and vice versa. Dinoflagellate species, which included the fish killer, a new *Gyrodinium sp.*, started to bloom in late February and reached their highest density on March 18, when fish kills were first reported at Crooked Island. During the 16 weeks period, dinoflagellate species dominated three times, in mid-February, mid-March and mid-May.

INTRODUCTION

1998 was an unusual year in terms of red tide and harmful algal bloom events in Hong Kong. It was the second highest year for the number of red tides and harmful algal blooms (Red tide database, Agriculture, Fisheries and Conservation Department, Hong Kong Government) since the first recorded red tide event in June 1971 [1] and the heaviest in terms of the damage caused to the maricultural industry [2].

The most massive red tide or harmful algal bloom occurred from mid-March to mid-April, lasting for about one month and causing very serious economic and ecological problems [3]. The causative species was suspected to be the most dominant species, a new *Gyrodinium* species (its description will be published elsewhere). This bloom was reported to start from Nanao, which is located in the Shenzhen Special Economic Region of mainland China on the eastern coast of Mirs Bay. The killing effect was noted in Hong Kong waters on 18 March at Crooked Island, and then moved southward and then westward (Fig.1).

Fig.1 Area covered by the massive red tide from March through April, 1998 in south China coastal waters around Hong Kong (arrow indicator is the permanent sampling station; other arrows show direction of movement of the red tide).

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In Hong Kong coastal waters, a similar species, *Gymnodinium mikimotoi* Miyaka & Kominami ex Oda (=*G. nagasakiense* Takayama & Adachi) [4,5], was previously reported to have caused red tides, but never on such a large temporal and spatial scale, and causing such a massive fish kill. This report presents the succession of the fish killing species and other dominant algal species over the 5 months period from before to after the massive red tide event at a permanent station located in Port Shelter, where red tides occurrences have ranked second in Hong Kong over the past two decades. The causes of the fish kills will also be discussed.

MATERIALS AND METHODS

Sampling station

Ma Nam Wat (22°21.20'N, 114° 16.08'E) is located in the Port Shelter area (Arrow indicator in Fig.1), at a transition between a semi-enclosed Bay, Hebe Haven, and Ngau Mei Hoi, a bay which opens to the more oceanic sea, in the eastern waters of Hong Kong. Within Ma Nam Wat, a fish farm is located, and Ngau Mei Hoi is surrounded by several fish farms. Two rivers, the Ho Chung River and Tai Chung Hau stream both discharge freshwater into Hebe Haven. The Ho Chung River runs 20 km through the Ho Chung Valley, collecting water from a 7.2 km^2 catchment. The Tai Chung Hau Stream originates in the Ma On Shan Country Park and drains into the bay [6].

Samples and Sampling

Water samples were collected on a weekly basis from Feb. 1 to May 27, 1998 using 1 liter plastic bottles for quantitative plankton analysis. Preservation involved adding 2 ml of acidified Lugol's solution to 1 liter of sample. Net samples were collected by passing 20 L surface water through 10 μm mesh size plankton net. One part of the net sample was kept for live cell observation by adding f/2 medium, and the other part was Lugol preserved. Samples were examined and counts made under an Olympus BX-50 compound light microscope. Hydrographic data including salinity, temperature, turbidity and dissolved oxygen were measured using a U-10 Horiba water quality checker (Horiba Ltd., Miyanohigashi, Kisshoin, Minami-ku, Kyoto, Japan). Water transparency was measured using a standard secchi disc. Meteorological data, including daily air temperature and daily rainfall, were purchased from the Hong Kong Observatory, and/or were downloaded from its homepage [7].

RESULTS

Phytoplankton dynamics

Phytoplankton bloomed during almost the entire period of sampling. Five weeks' samples had a total cell number which exceeded 1.5 million per liter of water, and one of them reached nearly 4 million (27 May, 1998, Fig.2). The plankton assemblage was mainly composed of two groups, diatoms and dinoflagellates (Fig.3), and dominance shifted between these two groups. Of the 16 weeks' samples collected from 1 February to 27 May, 1998, 5 were dominated by dinoflagellates, representing 3 distinct time periods and 11 were dominated by diatoms representing 4 distinct time periods (Fig.3). The causative species of the massive red tide, a *Gyrodinium* sp., appeared in all 16 samples. It peaked on 18 March 1998 (Fig.2), when this red tide started to show up at Crooked Island. On the same day, other mostly dinoflagellate species also appeared to be very abundant, with *Gymnodinium sanguineum* Hirasaka being denser than the *Gyrodinium* sp. Both before and after this sample, several other species were also found to bloom, including both diatoms and dinoflagellates. The dinoflagellate species that bloomed during this period of time included *Alexandrium* spp. ($2.8x10^4$ cell/L), *Gymnodinium catenatum* Graham ($6x10^3$ cell/L), *Gymnodinium sanguineum* ($3X10^5$ cell/L), *Prorocentrum triestinum* Schiller ($6x10^5$ cell/L), *Scrippsiella trochoidea* (Stein) Balech ($8x10^4$ cell/L), *Ceratium furca* (Ehrenberg) Claparède & Lachmann ($1.8x10^4$ cell/L), and *Prorocentrum dentatum* Stein ($2x10^4$ cell/L).

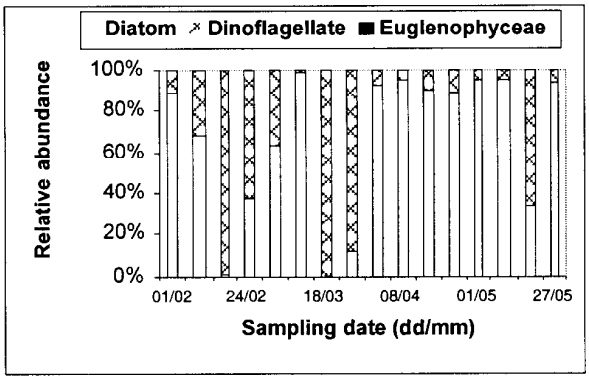

Fig. 2 Succession of the *Gyrodinium* sp. in comparison with total phytoplankton (1 Feb.-27 May, 1998)

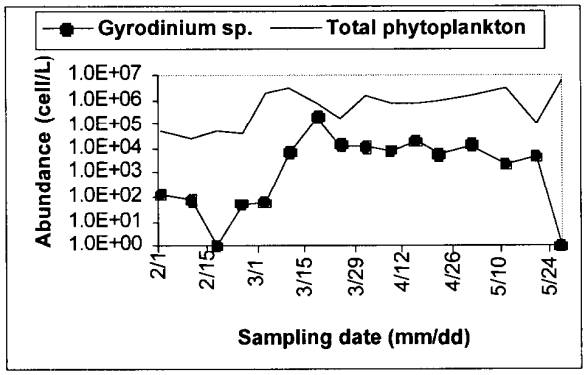

Fig.3 Relative abundance of different groups of algae over time (1 Feb. -27 May, 1998)

Fish kills

Wherever the red tide passed it killed fishes. The fish kills were claimed to show no discrimination, including, as claimed by the farmers, coral-trout, coral cod, grouper, giant grouper, yellow grouper, brown spotted grouper, mud grouper, tiger grouper, mangrove snapper, red snapper, russell's snapper, sea-perch, Japanese sea-perch, seabream, black seabream, gold-lined sea bream, head grunt, eel, red pargo, yellow croaker, purple amberjack, and pampano. The red tide killed not only various kinds of caged fishes but was also reported to affect nearly all kinds of natural coral fishes and it killed some of them. Some fishes were claimed to have disappeared from the area they used to inhabit; butterfly fish for instance.

By 17 April, about one month from the start of the bloom, the red tide had invaded nearly all corners of the coastal waters of Hong Kong, including 22 of the 26 fish farms and 5 swimming beaches. In Hong Kong alone, 1,260 fishery households were affected, 2,500 tonnes of fishes were killed, and a direct economic loss of 250 million HK$ was estimated by the fish farmers. In Mainland China's Guangdong waters, it was reported that by 17 April, this red tide had killed more than 260 tonnes of fishes, causing a direct economic loss of about 40 million Yuan RMB.

DISCUSSION

During the past two decades, red tide data analysis showed that the major causative species of red tides were *Noctiluca scintillans* (Macartney) Kofoid & Swezy, *Prorocentrum* species, and *Gymnodinium* spp. in the eastern and northeastern waters of Hong Kong, including Tolo Harbour and Port Shelter where our sampling station is located [4,5]. Red tides caused by these species occurred throughout the year and peaked in March and April [4,5], and autumn [4]. *Prorocentrum* species which were dominant in several samples collected from our station during the early months of 1998 were found to bloom often during periods when the weather was unstable [5]. *Gymnodinium mikimotoi* which is a close relative of the *Gyrodinium* sp., the causative species of the massive red tide of early 1998, was often found to bloom under particular climatic conditions. Thus, "in September, 5-10 days before the bloom there were usually tropical depressions with strong winds and heavy rain followed by warm sunny weather for a few days. In December, the weather pattern usually involved a cold front with strong wind followed by some rain and then a fine sunny period" [5]. This is also the weather pattern observed in early 1998, when dinoflagellate blooms were again detected, whilst higher dinoflagellate cell density was observed always during lower wind speed periods. Similar weather conditions seemed to also favour *G. mikimotoi* bloom formation in the Japanese Seto Inland Sea [8].

Gymnodinium mikimotoi and one other *Gymnodiniium* species have caused fish kills in Hong Kong coastal waters, and standard mouse bioassay showed positive results with the water and killed fish [5]. In 1998, fish which were killed by blooms of the *Gyrodinium* sp. had their scales erect and their skin whitened. Areas of the skin and gills were severely damaged and showed reddened patches. Such symptoms were not seen during blooms of *G. mikimotoi*. Dead fish gill examination showed a large amount of mucus around the gills, however, it is unknown whether the mucus was produced by the causative species or by the fish gills due to irritation by the *Gyrodinium*. Fish kills caused by oxygen depletion occurred in the past in Hong Kong coastal waters, but this was unlikely to be the reason for the 1998 fish kills, because fish were seen to be killed during the day time when oxygen concentrations were still high at locations where fish kills were noticed (Minna Wong, AFCD, per commu.). It was suggested that this species could produce a strong ichthyotoxin. Fish kept in aquaria in restaurants were also killed, and it was reported that the local seawater from where the algal bloom occurred has been used. The same species also bloomed in Japan [9]. It was reported that as fishing boats came into Shimonoseki Port, captured fish such as red sea bream, bastard halibut and sea bass held in cages by the boats, suddenly began to struggle and were killed before they even entered the high cell density area. However, the ecotoxicology of this species needs further study.

The causative species of the 1998 massive red tide in Hong Kong and surrounding coastal waters is believed to be a new species and its identity will be reported elsewhere. It is also suggested to be a local species. It occurred during a typical time period of the year when dinoflagellate dominated phytoplankton blooms often occur. The typical climatic and weather conditions may be the determining factors for dinoflagellate blooms in this period of the year. Turbulence helped disturb the bottom sediment, mixing the water column, and providing nutrients to the water. It was also found that during this period in 1998, surface water temperature and salinity from our permanent station ranged from 18.8-26.5°C and 32.8-33.1‰ respectively, which were suitable for related species blooms. This condition is suggested to be determined by many factors, e.g. rainfall, and warm water intrusion from the South China Sea [10]. The causative species might be able to produce strong fish killing toxins. However, more work should be carried out in terms of the ecotoxicology of the species, and the triggering factors for bloom formation.

ACKNOWLEDGEMENTS

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SPATIAL AND TEMPORAL DISTRIBUTION OF RED TIDES IN HONG KONG WATERS DURING 1983-1998

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ABSTRACT

Red tides have frequently occurred in Hong Kong waters with 740 cases in total during 1983-1998. We used the Geographic Information System (GIS) to map all occurrences of red tides and examined the spatial and temporal distribution of these red tides in the Hong Kong territorial waters. Red tide occurred frequently in shallow waters (<10 m), including fish farm zones and the proximity of public beaches. Most red tides (74%) occurred in sheltered waters (the northeastern waters) such as Tolo Harbour, Mirs Bay and Porter Shelter to the east of Hong Kong; and 70% of the total red tide cases occurred between December and May and the lowest frequency was during July-November, with < 5% each month. There was a spatial-temporal progression of red tides: red tides during winter occurred in the east and spread to the west during spring. These spatial and temporal patterns appear to be related to the seasonal monsoon events: the prevailing northeast winds in winter and spring and the dominant southwest winds in summer.

The 10 most frequently-occurring species were (in descending order) *Noctiluca scintillans*, *Gonyaulax polygramma*, *Skeletonema costatum*, *Mesodinium rubrum*, *Prorocentrum minimum*, *Ceratium furca*, and *P. triestinum*, *Gyrodinium splendens*, *Thalassiosira* spp., *P. sigmoidens*. The first 4 species occurred in many regions whereas the next 6 species appeared to confide in the northeastern waters where oceanic waters dominate most of the time. *N. scintillans* occurred most frequently (199 cases) and its blooms occurred mostly during December to May. *Gonyaulax polygramma* blooms occurred only between February and May when oceanic conditions prevail. *Skeletonema costatum* blooms occurred mostly between May and September during which salinity started to decrease due to the influences of the Pearl River estuarine plume and rainfall.

INTRODUCTION

Harmful algal blooms have caused large damages to aquaculture and poisoning threats to human health [1]. Numerous mechanisms operating over various spatial scales have been proposed for being responsible for the formation of red tides. For example, El Niño has been suggested to be responsible for outbreak of harmful algal blooms in the western Pacific Ocean [2, 3] and near south China coastal waters [4]. Other large-scale harmful algal blooms have been reported to be connected with mesoscale climatic or hydrographic events [5, 6].

Red Tides have frequently occurred in the Hong Kong territorial waters in the past 20 years as the coastal population and economic growth have rapidly increased in southern China. Red tides not only impose a serious threat to fish farms, but also force the closure of public beaches in Hong Kong [7, 8]. In 1988 there were large numbers of red tides [9] and in 1998 there were massive fish kills during spring due to harmful algal blooms [4]. The frequency of Red tides in these waters has been suggested to be related to an increase in the human population [10], nutrient inputs [11, 8], and N:P ratios [12]. However, there has been a controversy [13] over these reports. In this study, we used GIS to map the spatial and temporal distribution of red tides in Hong Kong waters during 1983-98 and attempted to relate their distribution patterns to atmospheric and oceanographic environments.

Hong Kong is situated in the south coast of China, facing the northern part of the South China Sea and lying to the west coast is the Pearl River estuary. Thus, its coastal environments are profoundly influenced by three water regimes: estuarine waters in the Pearl River estuary, oceanic waters from the South China Sea and coastal waters from the South China Coastal Current to the east. These influences are strongly subject to two seasonal monsoons. The objectives of this study were to examine the monthly spatial distribution of total red tides using the Geographic Information System (GIS) and to examine if there a general pattern of red tides related to the monsoon events and coastal oceanographic factors and processes.

MATERIALS AND METHODS

Data on reports of red tides were obtained from the Department of Agriculture and Fisheries (AFD), Hong Kong Government. AFD organizes a Red Tide Reporting Network among different government agencies and collected data on sightings of red tides using a standarized form: Sighting of Red Tide/Discolored Water Report Form. The data provided to this study include locations, dates, period of red tide outbreaks and major causative species. As the monitoring program focuses on water discoloration, the records might have missed Red tides that were not visible in color or present in subsurface layers. Therefore, we will use the term "red tides" in this study instead of harmful algal blooms.

In a single entry in the database, there were records of more than one location or more than one causative species. In these cases, the records were separated into multiple entries by locations or species. Therefore, the number of red tides in the GIS database is larger than that that in the original records by AFD and in the EPD's reports (EPD 1997). The coordinates of the location of red tides were found in the map and added to

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Intergovernmental Oceanographic Commission of UNESCO 2001

the data, which were entered into the GIS system (Archview® GIS). Using GIS software, we were able to map all cases of red tides and displayed the monthly spatial distribution of total red tides and the most frequently occurring species.

In this study, we divided the Hong Kong territorial waters into 7 water zones based on geographic features (Fig. 1), which is different from the 10 water control zones defined legislatively by the Hong Kong government. Zone T includes Tolo Harbour and Tolo Channel; outside zone T is M which includes Mirs Bay; P designates Port Shelter; V designates the Victoria Harbour; H is the zone south of Hong Kong Island; L is the zone south of Lantau Island and West Lamma Channel between Lantau and Hong Kong Islands; and W is the zone between the western New Territories and Lantau Island.

RESULTS

There were a total of 740 red tides during the period from 1983 to 98 in the whole region (Fig. 1). Red tides occurred most frequently in the eastern territorial waters in Zones T (288), M (126) and P (122). The other zone where red tides frequently occurred was Zone S (108). Comparatively few red tides occurred in the waters of Zone W (29) and L (36).

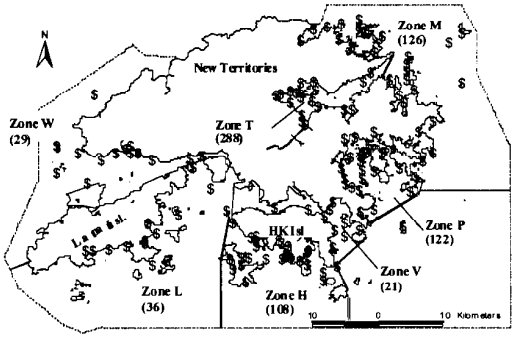

Fig. 1. Spatial distribution of total red tides (red tides) in Hong Kong during 1983-98. Black solid triangles mark the sites of red tide occurrences. The numbers in parenthesis are the total of red tides in each zone.

The majority of red tides (70%) occurred between winter and spring (December-May) (Fig. 2). The frequency of red tides was 51 in January, increased during February (75) and March (109) and peaked in April (124). The number of red tides was still high during May (93), but decreased in June (56). The lowest number was recorded in September (24). The temporal distribution held true for the monthly average and the number of years in which there were red tides for a particular month (Fig. 2).

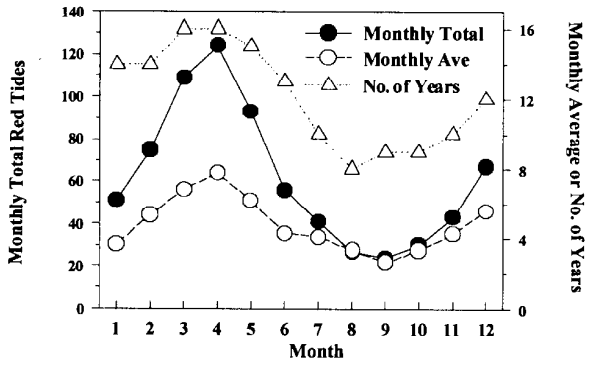

Figure 2. Monthly distribution of total red tides in Hong Kong during 1983-98. Monthly averages (Monthly Ave) over the number of years (No. of Years) are also shown.

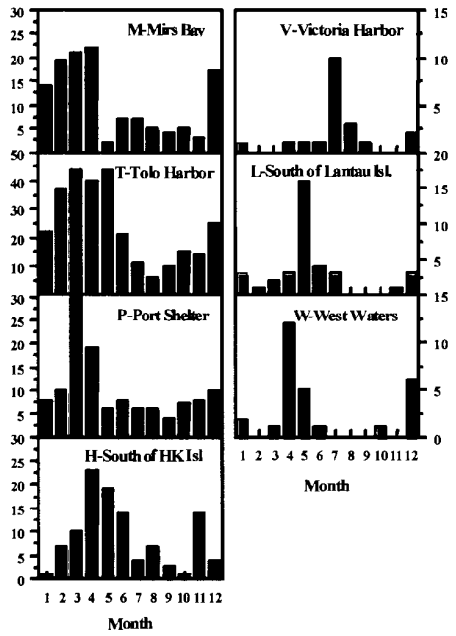

Figure 3. Monthly distribution of red tides in different zones of Hong Kong during 1983-98. Note the scale on T is different from the rest.

There appeared to be a temporal-spatial progression of red tides around the territorial waters. In winter (December-January), red tides occurred mostly in the eastern waters such as Zones T and M, and spread to waters in Zones P and H during February-April (Fig. 3). By May, red tides spread further west to waters in Zone L. Red tides retreated from Zone L during June and from Zone H during July, but invaded Zone V during July (Fig. 3). The occurrence of red tides showed strong seasonal changes. The monthly frequency of red tides in

Zones M and T increased from winter to spring, while in Zone P, it remained almost constant (ca. 7 cases) except for March and April during which the number of red tides tripled and doubled, respectively (Fig. 3). In Zone H, the frequency increased from winter to spring, reaching the maximum (23) during April and then decreased in May and June (Fig. 3). Red tides occurred much less frequently in Zone H during August-October. The exceptional high frequency (14) in November was due to 1998 alone, which counted for 9 cases. In Zones W and L, the frequency of red tides showed a single peak in April (12) and May (16), respectively (Fig. 3).

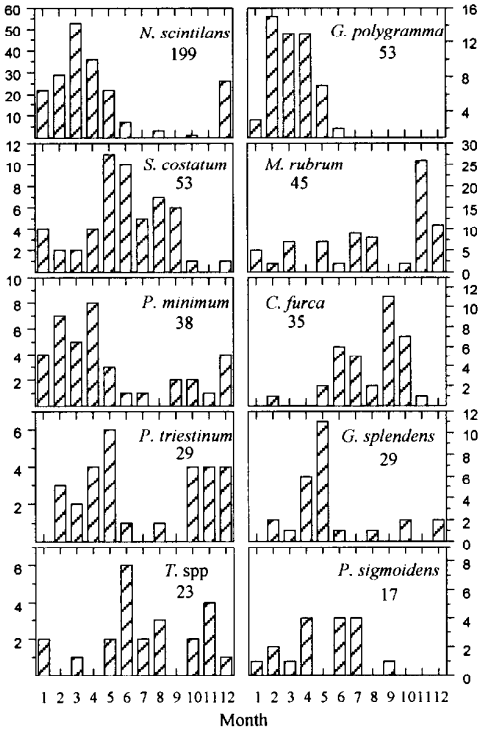

Figure 4. Monthly distribution of the 10 most frequently occurring red tide species in Hong Kong during 1983-98.

The most frequently occurring 10 red tide species were, in descending order, *Noctiluca scintillans* (dinoflagellates), *Gonyaulax polygramma* (dinoflagellates), *Skeletonema costatum*, (diatoms), *Mesodinium rubrum* (Cryptophyta), *Prorocentrum minimum* (dinoflagellates), *Ceratium furca* (dinoflagellates), *P. triestinum* (dinoflagellates), *Gymnodinium splendens* (dinoflagellates), *Gyrodinium aureolum* (dinoflagellates), and *Thalassiosira* spp. (diatoms). A total of 199 cases of *N. scintillans* occurred during the period from December to May with over 20 cases each month, and the highest frequency was during March (Fig. 4). *G. polygramma* blooms (53 cases) occurred mostly in the eastern waters during February-June with few blooms in Zones L and W (Fig. 4). In contrast, *S. costatum* blooms peaked during May and frequently occurred during June-September in Zones T, H and V. Dinoflagellates including *P. minimum*, *P. triestinum*, *G. splendens* and *Gyrodinium auroaulum* (Fig. 4) bloomed mostly during winter and spring. While the other dinoflagellate *C. furca* bloomed mostly in June-October, only in Zones T, M and P. However, most dinoflagellate blooms occurred only in Zones T, M and P (Fig. 4). In contrast, *Mesodinium rubrum* appeared to occur in all regions and all year around, particularly more frequently in 1998.

DISCUSSION

Most red tides occurred within a 6 month period from December-May (70%), and in the waters east of Hong Kong (74%). These temporal and spatial patterns can be explained when considering seasonal monsoon events and oceanographic conditions. During the period from December to April, the northeast monsoon prevails and the China Coastal Current that originates from the north dominates the Hong Kong coastal water circulation [14, 15, 16, 17, 4]. Due to the Corriolis force, the China Coastal Current turns towards the shore and results in a downwelling against the shore. Because of this, the water exchange or flushing rate is reduced during this period in a coastal embayment or semi-enclosed bay opening to the south. The similar process has been reported for Alfacs Bay (Ebro Delta, NW Mediterranean Sea), in which *Gyrodinium corsicum* Paulmier formed massive blooms from December 1994 to April 1995, a period characterized by an initial period of water stability and low outward flux of water during subsequent months [18]. Mirs Bay (Zone M) and Port Shelter (Zone P) are all semi-enclosed and Tolo Harbor and Channel are sheltered within Mirs Bay (M, Fig. 1). Temperature is usually above 15 ^{o}C in all three areas, which is almost an optimum temperature for many temperate algal species. This suggests that these red tide species might have originated from temperate regions via the China Coastal Current, or ballast waters [19, 20]. The sites of red tides were all shallow (<10 m), possibly within the euphotic zone. The long residence time, plus suitable environmental conditions (temperature, salinity, and shallow waters) may have contributed to more red tides in these regions. Starting in April/May, the wind shifts due to the transition from the northeast monsoon to the southwest monsoon. Due to the Corriolis force, the surface waters are turned away from the coast; they are replaced from beneath by deep oceanic waters. Rainfall is maximal in Hong Kong during June-August. The addition of freshwater from rainfall and runoff increases the outward flow at the surface layer and entrains deep waters inwards (similar to an estuarine circulation). The other immediate effect of rainfall is to reduce the salinity in the surface layer. The salinity near the bottom is even higher in July than in January while the surface salinity decreased from January to July [4], indicating the invasion of oceanic waters from the South China Sea [17]. As a consequence, the residence time of the surface layer is shorter. The lower salinity and more rapid flushing of these waters may contribute to fewer occurrences of red tides in late spring and summer.

The Zone W are part of the Pearl River Estuary and hence they have estuarine characteristics: variable salinity, high turbidity and high turbulence. These environmental conditions are not suitable for dinoflagellates which can not tolerate high concentrations of suspended particles and turbulence. In Zone W, relatively few blooms occurred, and none occurred during July-September. The exception is *Noctiluca scintillans* that can tolerate turbulent water [21]. Similar estuarine conditions apply to Zone L.

The Zone H south of Hong Kong Island are transient between oceanic and estuarine conditions depending on the season. The open waters in Zone H are flushed relatively quickly because the deep East Lamma Channel between Hong Kong Island and Lamma Island allows waters to move freely during tidal cycles. There are a few sewage outfalls located in the south shore of Hong Kong Island. There are many sheltered areas in Zone H and all the red tides occurred in these areas. The salinity of two sheltered both bays to the east of Zone H is above 30 at the surface during winter and early spring, and throughout year at the bottom. The water column becomes stratified as salinity decreases at the surface in April [9]. Rainfalls are maximal during summer. Rainwater contains high concentrations of nitrogen. The added nitrogen can lead to the limitation of Si in July [22]. These physical conditions and nutrient regimes appeared to explain the temporal distribution of red tides in Zone H. In April, the monsoon shifts from the northeast to the southwest and the water can be slack during the shift. This could explain the sudden increase of red tides in Zone H to its maximal level in April.

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ON A BLOOM OF *CHATTONELLA* IN THE NORTH SEA/SKAGERRAK IN APRIL-MAY 1998

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ABSTRACT

In April-May a mass occurrence of *Chattonella* aff. *verruculosa* (Rhaphidophyceae) caused brownish water discoloration in parts of the Skagerrak and the North Sea. Both wild fish and fish in pens were killed during the bloom. On April 12 the bloom was common outside the Limfjord on the west coast of Denmark. About ten days later dense populations of *Chattonella* were recorded in the Skagerrak in an area north of Skagen. In the beginning of May (1-3) significant mortality among large penned Atlantic salmon was reported at the southwest coast of Norway. Later in May (7-19) very high concentrations of *Chattonella* were recorded all along the west coast of Denmark, south to at least the Esbjerg area. This could partly be spreading from the area north of Skagen via an unusual, temporary southwest current, but *in situ* growth of algae seen along the west coast of Denmark in April might have contributed as well. The bloom developed in waters rich in nitrate, most probably brought to the Skagen area from the southern North Sea. High N:P atomic ratios in particulate material, mainly consisting of *Chattonella,* collected by the end of the bloom pointed to phosphorus-limitation of the bloom. Zooplankton grazing was likely low as the number of common zooplankton collected by net (180 µm) in area of the bloom was small, perhaps due to zooplankton avoiding the *Chattonella* bloom and low grazing-pressure. This was, to our knowledge, the first record of this species in European waters, and world-wide, the first report on fish mortality associated with this species.

INTRODUCTION

Because of observations of very high nitrate concentrations and N:P ratios along the west coast of Denmark and in eastern Skagerrak during a routine cruise in April 1998, the Institute of Marine Research released a general warning on the potential risk of harmful algal blooms. In the beginning of May 1998 a dense bloom of *Chattonella* aff. *verruculosa* (Rhaphidophyceae) impacted the southwest coast of Norway where several fish farms are located. Fish exposed to the bloom were killed and the Directorate of Fisheries was warned. The research vessels "G.M. Dannevig" and "Håkon Mosby" were directed to the area for sampling beginning on 9 May. At that time, the acute mortality in the fish farms was over, and the algae concentrations had declined. Simultaneously there were reports on some mortality among wild fish along the west coast of Denmark. As blooms along the Danish coast occasionally may propagate to Norwegian waters we decided to study the situation along the Danish coast. Since the sixties the southern coast of Norway has experienced many algal blooms harmful to fish and wild biota, e.g. *Gyrodinium* cf. *aureolum* (*Gymnodinium mikimotoi*) [see 1] and *Chrysochromulina polylepis* [see 2]. The growth of the fish farming industry since the seventies has resulted in some regular monitoring of possible harmful algae, with increased monitoring and mapping activities when large blooms occur. Increased activities serve two purposes; providing fish farmers and management authorities better information on the situation, and more and better data for documentation and understanding of the blooms. In this communication new insights gained during the bloom of *Chattonella* aff. *verruculosa* in May 1998 is provided.

Chattonella verruculosa was described from Japanese waters [3]. It belongs to the class Raphidophyceae, with many species known to cause fish mortality. The killing mechanism is poorly understood, but may be due to clogging of gills by mucus excretion or production of some toxic substances [see 4].

MATERIALS AND METHODS

Studied area

The Skagerrak can be considered a fjord-like branch of the North Sea with a deep trench along the Norwegian coast and shallow water along the Danish coast. In general, the circulation in the Skagerrak is anti-clockwise, and the distribution of water masses is mainly regulated by the in- and outflowing water to and from the North Sea and the steep local bottom topography, which is of special importance for the behaviour of the water masses [5, 6]. About 70% of the water entering the North Sea is assumed to pass through the Skagerrak before it leaves the North Sea. In addition, there is a large amount of fresh-water supplied to the Skagerrak from the Baltic Sea, via Kattegat, as well as from local and continental rivers, the latter discharging to the southern North Sea [7]. The distribution of the relatively fresh surface waters in the Skagerrak is strongly influenced by varying weather conditions, but during weak, local winds the surface waters mainly follow the general anti-clockwise circulation; hydrographical conditions in the Skagerrak are complicated and may change rapidly. The Jutland Coastal Current appears to constitute a major link between the eutrophicated waters of the southern North Sea and the water of the Skagerrak.

Data collection

Data on the bloom are partly from routine activities and partly form the "Chattonella"-cruise on 9-19 May. The areas studied and sampling stations are shown in Figs 1 and 3. Two routine activities contributed useful data for documentation of the bloom: One, a regular cruise in the Skagerrak area, performed in April each year, to look for unusual nutrient conditions in the area after the spring bloom [8] (Fig. 1), the other an every second day sampling of algae in the Flødevigen Bay on the southern coast of Norway. The observations of algae in the Flødevigen Bay roughly reflect the situation for large parts of the Skagerrak [9]).

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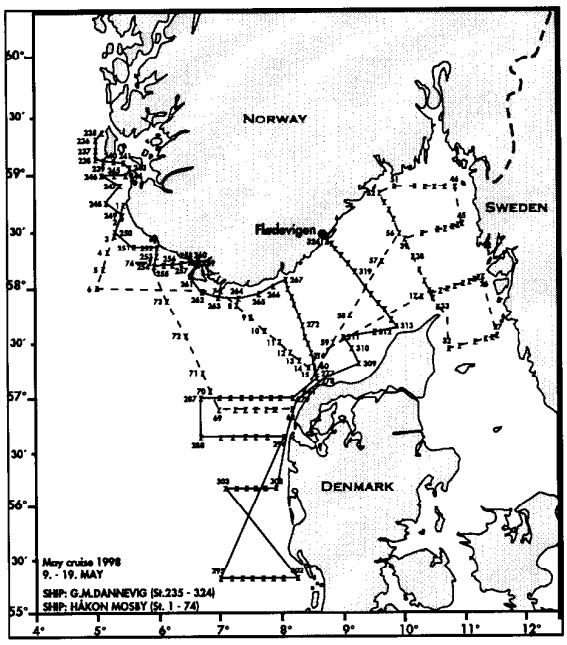

Fig. 1. Stations grid for the *Chattonella*-cruise", 9-19 May 1998.

Temperature, salinity, fluorescence, nutrients, particulate material and chlorophyll *a* were measured by standard methods. Phytoplankton samples were counted either living or fixed with iodine in Palmer-Maloney chambers. From 8-27 April counts were made for one integrated (0-30 m) fixed sample from each station, and an integrated (0-3 m) living or fixed sample from the Flødevigen Bay. While, during 9-19 May the cruise, counts were obtained on living or fixed samples from 0, 5 and 10 m, plus one extra for depths with a pronounced fluorescence maximum not reflected in the samples from the 0, 5 and 10 m depths. *Chattonella* aff. *verruculosa* (Fig. 2) was identified at the University of Oslo, Department of Marine Botany by professor Jahn Throndsen.

Fig. 2. A typical *Chattonella* aff. *verruculosa* cell, with length 30 μm, the length varied from 10 to 40 μm.

RESULTS AND DISCUSSION

During a few days in the beginning of May fish farmers in the Flekkefjord area on the southwest coast of Norway lost 350 tonnes of Atlantic salmon. The extent of the fish mortality varied considerably among the fish farms. This was probably due to patchy distributions of algae and different physiological status and power of resistance of the fish. Mainly fish approaching 2 kg and more were killed, with lower mortalities when fish were not fed. Small fish survived even if exposed to the same amounts of algae. Unfortunately water samples were not collected during the acute bloom phase, so the concentrations of algae killing the fish are not known. However, in samples collected shortly after the most acute situation *Chattonella* aff. *verruculosa* was common. Other algae were also present, including some *Heterosigma*-like cells.

The pre-bloom situation

The routine April cruise provided some background data on the bloom. Large amounts of nitrate were noted along the west coast of Denmark and further to the north of Skagen. High concentrations of *Chattonella*, up to 2 million cells l^{-1} (mean for the 0-30 m sample), were found on 23 April in an area north of Skagen (Fig. 3). As *Chattonella* was the dominating species the profiles of

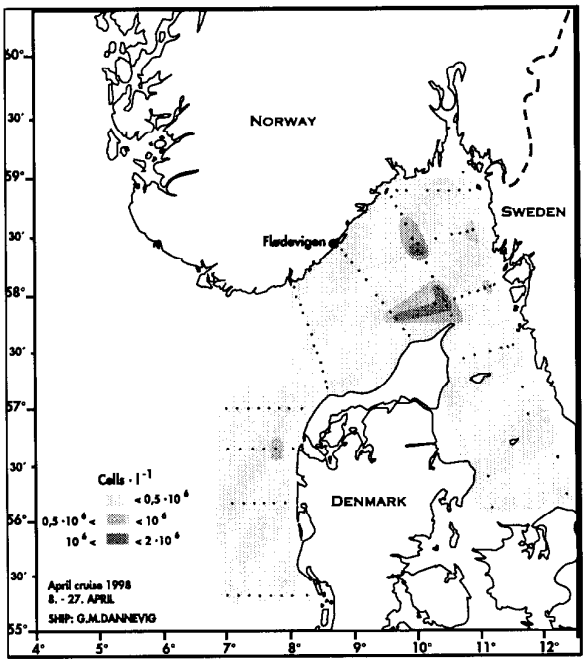

Fig. 3. The distribution of *Chattonella* during a routine cruise, 8-27 April 1998.

chlorophyll reflected its presence mainly in the upper 10 m with salinities of about 34. From the Skagen area, we suppose, the bloom propagated to the west, and hit the southwest coast of Norway. It is not the common current pattern in the area, but the unique wind conditions in April-May may temporarily have caused unusual currents [10]. From the Skagen area, the bloom also spread northwards along the Swedish west coast, mainly as a sub-surface population according to the profiles of chlorophyll.

From the middle of April to the middle of May, wind-conditions were quite variable. Initially winds were blowing from southeast to southwest, approximately 15 $m\,s^{-1}$. During the last days of April the wind changed north-easterly approximately 10 $m\,s^{-1}$. Winds in the first days of

May were still northerly, then turned easterly at low speed before the wind velocity increased to vary between southeast and southwest. Around 15 May, the wind direction changed to northerly and the wind velocity increased again towards the end of May [10].

The bloom

As mentioned above, we did not obtained much data from the southwest coast of Norway in early May, but monitoring in the Flødevigen Bay noted a peak of *Chattonella* on 5-8 May when up to 3 million cells l^{-1} were recorded in the 0-3 m sample (Fig. 4); levels of chlorophyll in the same samples were 5-7 $\mu g\, l^{-1}$. The 0-3m peak in the Flødevigen Bay was accompanied by salinities of 20-22 during a few days with strong stratification (Fig. 4). In Flødevigen the temperature at 1 m rose from about 8 to 13°C during May and the salinity varied between 17 and 30 (Fig. 4). On 9 May *Chattonella* was transported off the coast with off-shore winds causing near-shore up-welling, and did not recur on the coast (Fig. 4). The episode demonstrated clearly that large amounts of algae may appear and disappear rapidly, closely associated with meteorological and hydrographical conditions.

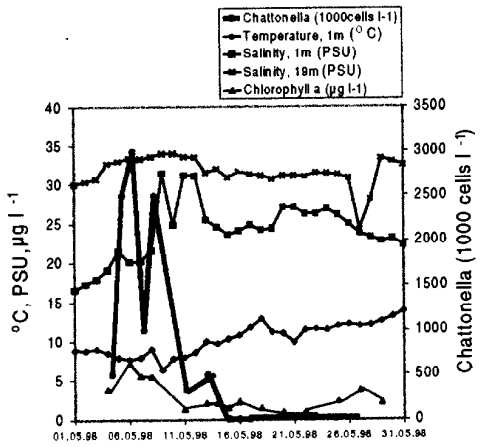

Fig. 4. Recordings of temperature (1 m), salinity (1 and 19 m), chlorophyll *a* (0-3 m) and *Chattonella* cells (0-3 m, bold curve) in the Flødevigen Bay during May 1989.

However, along the west coast of Denmark large amounts of *Chattonella* remained for a longer period and over a large area (Fig. 5). Fish mortalities were noted in the large bloom area mapped on 12-16 May, with dead wild fish including garfish (*Belone belone*), herring (*Clupea harengus*), sand eel (*Ammodytes* spp.) and mackerel (*Scomber scombrus*). This bloom could also have originated north of Skagen in April and propagated westwards, but *Chattonella* cells was also recorded in samples collected outside the west coast of Denmark in April, suggesting this could have been the inoculum for the bloom in May. The highest concentrations of *Chattonella*-like cells recorded along the west coast of Denmark in May were 24 million cells l^{-1} in 0 and 5 m; levels of nitrate were still rather high in the bloom area, 3-15 $\mu mol\, l^{-1}$. The horizontal distribution (Fig. 5) fit well with the distribution of chlorophyll and was also observed from satellite (SeaWiFS) [10]. The High N:P atomic ratios (about 40-60) in

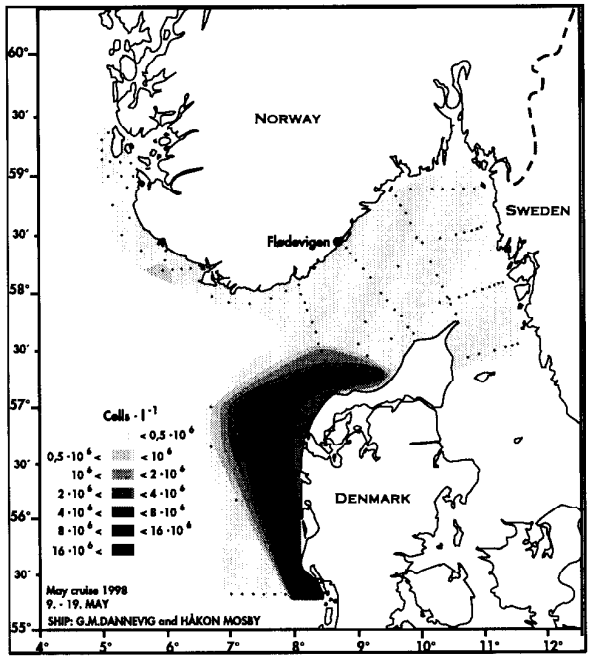

Fig. 5. The distribution of *Chattonella* during the "*Chattonella*-cruise" 9-19 May 1998.

particulate material, principally *Chattonella,* collected at the end of the bloom (station 293 and 307) suggested phosphorus-limitation of the bloom. The change and variation of wind direction and speed in the last part of April and the beginning of May was suggested as one of the reasons that the bloom collapsed in the Skagerrak area at that time, and also outside the west coast of Denmark [see 10].

Occasionally phototaxis of *Chattonella* was observed. Brownish patches on the wall in the shade was seen in a bucket with surface water left in sunshine on the ship's deck. The patch contained large amounts of *Chattonella.* Following rotation of the bucket 180 degrees, the brownish patch rapidly disappeared from the wall which was now exposed to direct sunshine, and gradually appeared again on the wall in the shade. In addition to indicating that *Chattonella* preferred lower light to full sunlight, an estimated of swimming speed was made: with 10 minutes for the bulk of the enclosed population to swim across the 25cm bucket, this equals to a swimming speed of about 2.5 cm per minute or 1.5 m per h.

The surface water collected north of Esbjerg was delivered to Danish colleagues the same day for pigment analyses and toxicity testing of the water. Pigments indicated that representatives from the Dictyochophyceae, ("naked stage" of *Dictyocha speculum*?) were common with *Chattonella*, while toxins were not recorded with the methods applied (Hanne Kaas, Vandkvalitetsinstitutet pers. communication, 1998). Nor was any toxicity found

associated with the *Chattonella* when Norwegian experts screened meat from mussels and fish exposed to the bloom for toxicity using mouse bioassays (Tore Aune, Department of Pharmacology, Microbiology and Food hygiene, Norwegian School of Veterinary Science, pers. communication, 1998). An alternative explanation for the harmful effects of *Chattonella* aff. *verruculosa* is then proposed as high mucus excretion: A mixture of algae and mucus may simply have caused clogging of the fish-gills. Such a mechanism is consistent with the lower mortality among small salmon compared to large.

Zooplankton grazing did not appear to have a major impact on the bloom. Numbers of common zooplankton collected by net (180 µm) at station 277 in the bloom area were small, perhaps reflecting zooplankton avoidance of the *Chattonella* bloom or high death rates of zooplankton exposed to the bloom. Low numbers of zooplankton may have led to a low grazing pressure on *Chattonella*.

This was, to our knowledge, the first record of *Chattonella* aff. *verruculosa* in European waters, but close relatives have been recorded earlier in the North Sea [11]. *Chattonella* aff. *verruculosa* has so far not been associated with fish mortality; however, its harmful effects on fish are not unexpected, due to other known harmful representatives (e.g. *Chattonella marina* (12), *Chattonella antiqua* (13), *Heterosigma akashiwo* (14)) within the class Raphidophyceae.

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ENVIRONMENTAL CONDITIONS DURING THE *CHATTONELLA* BLOOM IN THE NORTH SEA AND SKAGERRAK IN MAY 1998

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ABSTRACT

In late April 1998 *Chattonella* aff. *verruculosa* was registered for the first time in European waters. High concentrations of *Chattonella* were observed off the north coast of Denmark. Anthropogenic nutrients from the southern part of the North Sea most probably stimulated the alga bloom. Early in May 1998 *Chattonella* caused death in fish farms in an area close to the southern tip of Norway. After that *Chattonella* disappeared, and was not observed blooming along the Norwegian coast. However, it was observed in very high concentrations on the west coast of Denmark, from the Jammerbugt in the north to Esbjerg in the south, resulting in dead garfish, herring, sandeel and mackerel. It was feared that a new wave of *Chattonella* might follow the prevailing cyclonic circulation in the Skagerrak and reappear on the Norwegian coast. Due to the potential danger to fish farms, the situation was closely monitored, and in addition to *in situ* measurements from ships and satellite data, an operational ecological model (NORWECOM) was used. A change in the normal circulation in the Skagerrak, together with a continued blocking of inflow to the Skagerrak along the northwest coast of Denmark and a depletion of nutrients, prevented a return of *Chattonella* to the Skagerrak. This change along the Danish west coast, due to prevailing northerly winds, was probably a main reason that the bloom collapsed in that area.

INTRODUCTION

In the Skagerrak there exists a general cyclonic circulation, and the distribution of water masses is mainly regulated by the in- and outflowing water to and from the North Sea and the steep local bottom topography which is of special importance for the behaviour of the water masses [see 12, 9, 4]. Since about 70% of the water entering the North Sea is assumed to pass through the Skagerrak before it leaves the North Sea, many of the hydrographic events taking place in the North Sea will be reflected in this area. In addition there is a large freshwater supply to the Skagerrak from the Baltic Sea, Kattegat, local rivers and continental river discharge to the southern North Sea [5]. The Jutland Coastal Current appears to constitute a major link between the eutrophicated waters of the southern North Sea and the waters of the Skagerrak and Kattegat. The distribution of the relatively fresh surface waters in the Skagerrak is strongly influenced by varying weather conditions, but during weak local wind situations the surface waters mainly follow the general circulation. From 1988 and onwards, environmental surveys have been performed in April in the Skagerrak, Kattegat and in the North Sea along the western Danish coast down to the Esbjerg area to monitor nutrient levels after the spring bloom [2]. At that time, depending on the size of the continental river discharge to the German Bight, large water masses with high nitrate concentrations and unusual high N:P-ratios will under southerly wind conditions be transported with the Jutland Coastal Current to the Skagerrak area. During 1988-1996, the variation in nitrate in the Jutland Coastal water on the Danish west coast in April ranged from 20 000 tonnes in a dry year (1996) to more than 100 000 tonnes in a wet year (1994) [2]. Similar to nitrate, the highest concentrations of silicate were observed in 1994 and the lowest in 1996. The variation in phosphate, however, was rather small in the same years, from about 2300 tonnes in 1996 to 4300 tonnes in 1994. An environmental situation with extremely high nitrate concentrations is thought to be a good background for harmful algae blooms in this area, and such water masses can then be transported with the Norwegian Coastal Current to the southwestern Norwegian coast with its large Atlantic salmon production in pens.
This paper tries to combine *in situ* observations with model simulations and satellite observations showing the bloom situation and possible reasons for its termination.

MATERIALS AND METHODS

The area covered during the cruise in April is indicated by dots in Fig. 1, the same area covered during the bloom period of *Chattonella* aff. *verruculosa* between 9 and 19 May, is also shown in Fig. 6. Temperature, salinity and fluorescence were observed by CTD, and water samples were obtained at standard depths. Inorganic nutrients and chlorophyll *a* were measured by standard methods onboard. An integrated phytoplankton sample between 0-30 m was taken at every station in April together with one sample at 10 m depth and fixed with iodine. In addition, a sample was taken if there was any pronounced fluorescence maximum. On the cruise in May phytoplankton samples were taken from 0, 5 and 10 m in addition to a possible subsurface maximum. The NORWegian ECOlogical Model system (NORWECOM), a coupled physical, chemical and biological model system applied to study primary production and dispersion of particles, fish larvae and pollution [10, 1, 11], was run with a fine resolution (4x4 km grid) domain covering the Skagerrak embedded in a coarse resolution (20x20 km grid) model covering the

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extended North Sea. The depth-integrated chlorophyll *a* concentration was derived from SeaWiFS data that had been acquired in near real-time in April and May 1998. SeaWiFS data were acquired and processed by the Plymouth Marine Laboratory (PML, UK), which has implemented a specific scheme for the retrieval of chlorophyll *a* in the North Sea [6]. The PML algorithm showed the same level of accuracy in the North Sea (case 2) waters as the NASA algorithm [7] in open ocean (case 1) waters [8].

RESULTS AND DISCUSSION

The pre-bloom situation

During the monitoring cruise on 8-23 April, extremely high nitrate concentrations and high N:P ratios were recorded along the west coast of Denmark stretching into the Skagerrak along the Danish coast (Figs 1, 2). The nitrate concentrations were among the highest that have been observed since this monitoring started in 1988. The high chlorophyll values along the Danish west coast in the beginning of the cruise (10-13 April) were, according to the phytoplankton composition, a spring bloom situation (Fig. 3). Only small amounts of *Chattonella* were recorded in this area. The first observation of large amounts of *Chattonella* was recorded [see 3] in the Skagerrak north of Skagen on 23 April. Very high chlorophyll concentrations were observed in the uppermost 10 meters in a large part of the section from Skagen to the Norwegian coast. Based on this unusual situation with very high nitrate concentrations and extremely high N:P, the Institute of Marine Research issued a general warning against the risk of an algal bloom (possibly harmful) in the area.

Bloom and break-down period

In a two week period following the monitoring cruise no field data were available. However, both modelling results and satellite images help to explain the development of the bloom in this period. NORWECOM showed that on 26 April, there was a flow of water into the Skagerrak along the Danish west coast (Fig. 4), the expected circulation during periods of southerly winds. However, in the inner Skagerrak the circulation was anti-clockwise. The highest flagellate concentrations were, according to the model, along the west coast of Denmark, and in the southern part of the Skagerrak, extending toward the south coast of Norway (Fig. 5). This circulation may be the reason that the first observations, on 2-3 May, of *Chattonella* on the Norwegian coast were made close to the southern tip of Norway. In this area *Chattonella* caused the death of 350 tons of farmed salmon. The satellite image from 1 May shows that the highest chlorophyll *a* values were found on the west coast of Denmark extending into the Skagerrak, but the inner parts are covered by clouds and thus no information is available from that area. The model showed that on 8 May, northerly winds had blocked the inflow of nutrient-rich Continental coastal water, and the inflowing water originated in the central North Sea. On 12 May, high concentrations of *Chattonella* were observed on the northwest coast of Denmark and extended about 20 n.m. away from the coast. In the following days a detailed survey showed that the waters west of Denmark had extremely high concentrations of *Chattonella* [see 3], giving large chlorophyll concentrations in the area (Fig. 6). This area was characterised by high nitrate concentrations and high N:P ratios. NORWECOM showed that on 15 May (Fig. 4), continental coastal water was still blocked from flowing into the Skagerrak and that there was instead an outflow along the Danish north coast. In accordance with this the modelled flagellate concentration on the north coast of Denmark decreased from 15 to 19 May (Fig. 7). Thus the outflow of water pushed *Chattonella* out of the Skagerrak. A reduction is also seen in chlorophyll *a* concentrations in this area in the satellite image from 15 to 17 May (Fig. 8).

On a survey of the inner parts of the Skagerrak and the northern Kattegat on 16-18 May, *Chattonella* was not detected. On 18 May, observations on a section on the northwest coast of Denmark where high concentrations of *Chattonella* had been observed a week earlier, showed highly variable concentrations from station to station, indicating that the bloom was in its break-down phase. North-westerly winds in the last 10 days of May caused a continued blocking of inflow of continental coastal water to the Skagerrak. The model showed this and it was confirmed by a drifting buoy launched on 18 May off the west coast of Denmark. The buoy remained in the area and was picked up close to the launch position on 3 June.

SUMMARY

Due to the blocking of inflow of nutrient rich *Chattonella* infested continental coastal water to the Skagerrak, the late stage of the bloom remained in the waters west of Denmark, and was diluted because of the south-going current west of Denmark and starvation due to low nutrient concentrations. The situation, however, had the potential to harm fish farms on the Norwegian coast if the flow had followed the prevailing anti clockwise circulation pattern in the Skagerrak. The use of a 3 dimensional model and satellite images in addition to field observations has shown to be very valuable in forecasting the development of an algae bloom.

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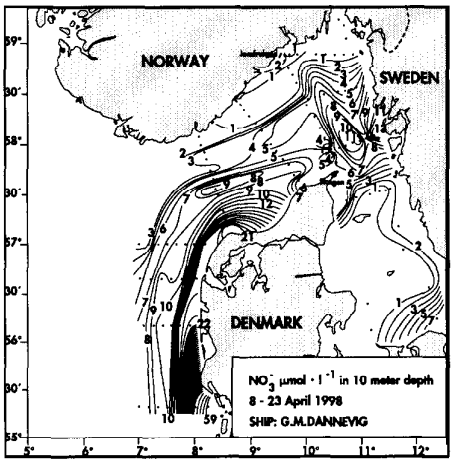

Fig. 1. Horizontal distribution of nitrate (µmol·l^{-1}) in 10 m depth in the North Sea, Skagerrak and Kattegat in April 1998.

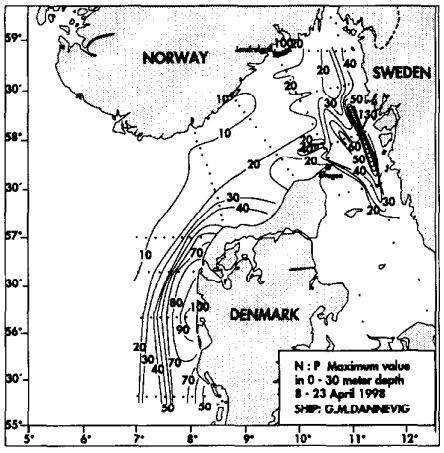

Fig. 2. Horizontal distribution of maximum N:P ratio in 0-30 m depth in the North Sea, Skagerrak and Kattegat in April 1998.

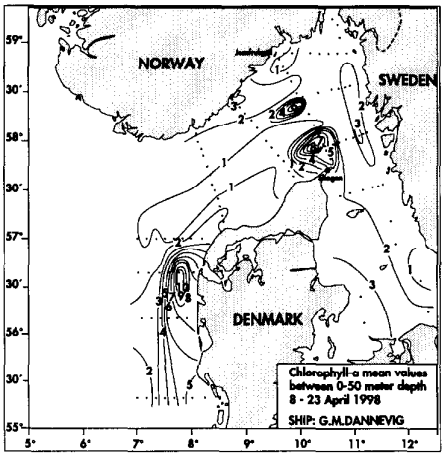

Fig. 3. Horizontal distribution of mean chlorophyll *a* values (µg·l^{-1}) between 50 and 0 m depth in the North Sea, Skagerrak and Kattegat in April 1998.

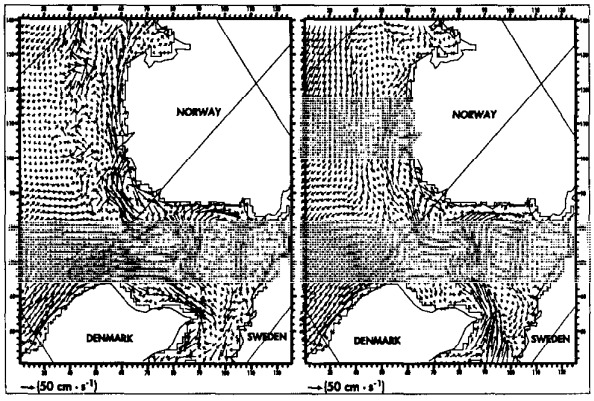

Fig. 4. Modelled current velocity in 10 m depth on 26 April 1998 (left) and modelled current velocity in 10 m depth on 15 May 1998 (right).

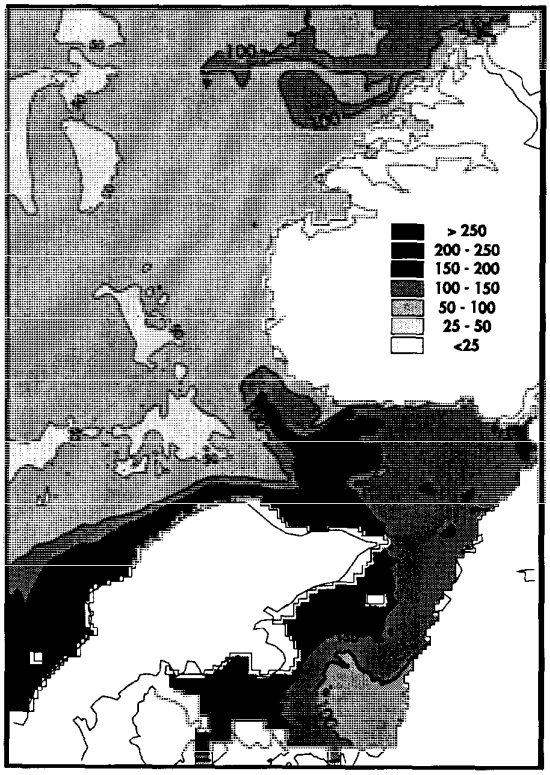

Fig. 5. Modelled flagellate concentration ($mgC \cdot m^{-3}$) in 5 m depth on 26 April 1998.

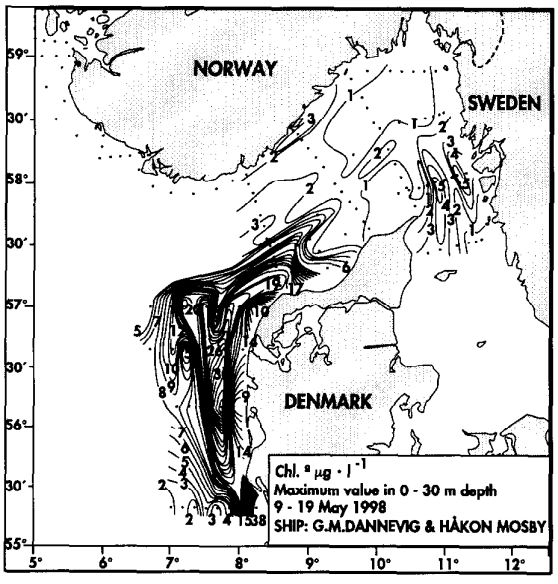

Fig. 6. Horizontal distribution of the maximum chlorophyll *a* value ($\mu g \cdot l^{-1}$) in 0-30 m depth 9-19 May 1998.

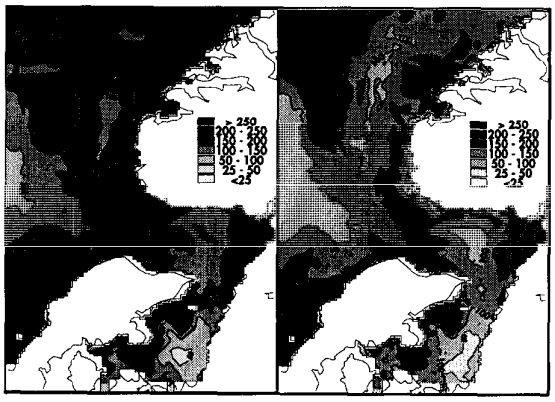

Fig. 7. Modelled flagellate concentration ($mgC \cdot m^{-3}$) in 5 m depth on 15 May (left) and 19 May (right) 1998.

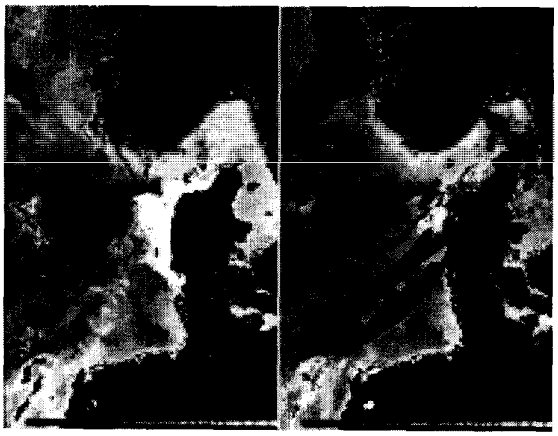

Fig. 8. The SeaWiFS chlorophyll concentration ($mg \cdot m^{-3}$) on 15 May (left) and 17 May (right) 1998. Source: S. Groom, CCMS-PMI, UK, Copyright: NASA SeaWiFS project/OrbImage Corp.

RANGE OF *HETEROSIGMA AKASHIWO* EXPANDED TO INCLUDE CALIFORNIA, USA

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ABSTRACT

Heterosigma akashiwo is a raphidophyte known to produce red tides which can result in fish mortality. During the spring of 1998 a red tide prevailed in San Pedro Bay, within the Los Angeles and Long Beach Harbors complex, centered at the mouth of the Los Angeles River. Based on light microscopy of living cells, this red tide organism was isolated and tentatively identified as being *H. akashiwo*. This algae had never been documented previously in San Pedro Bay. To provide confirmation that *H. akashiwo* had become established in San Pedro Bay, the isolate identity was determined by sequencing of the nuclear ribosomal internal transcribed spacer (ITS) regions and 5.8S gene. Species determination was made by direct comparison to previously identified *H. akashiwo* ITS sequence (isolate NIES 6; GenBank accession number AF157381). The result of this work was that there was complete sequence identity between the San Pedro Bay isolate and *H. akashiwo* isolate NIES 6. This is the first documentation of *H. akashiwo* in Californian waters, USA.

INTRODUCTION

Heterosigma akashiwo (Hada) Sournia (akashiwo = red water in Japanese) is a small (10-25μ) fragile Raphidophyceae alga which has often been confused with other flagellates [1]. Blooms of *H. akashiwo* are known to be associated with massive fish mortality events worldwide [2]. This alga often blooms in protected embayments enriched with nitrogen, phosphorus, vitamin B12, iron and magnesium, at temperatures between 15° and 30°C, and at salinities between 10 and 40 ppt [2-4]. Identification of raphidophytes in plankton collections is problematic. Visual identification requires live cells since preservation via formalin or Lugol's iodine disrupts the cell membrane [5].

METHODS AND OBSERVATIONS

On May 13, 1998 a red colored algal bloom was observed in the estuary of the Los Angeles River. This was immediately after a rain storm, with increased urban runoff outflow from the river. Surface salinity was very low, at 10 ppt. Nutrient measurements made in the estuary (sampled at 1 meter depth) at that time were as follows: ammonia nitrogen was 22.8 μgatoms/liter, nitrate nitrogen was 25.7 μgatoms/liter, and phosphate phosphorus was 2.3 μgatoms/liter. Ammonia nitrogen was measured using the Salicylate Method [6]. Nitrate nitrogen was determined using a modified Cadmium Reduction Method [7]. Orthophosphates were tested using the Ascorbic Acid Method [7].

By May 15, 1998 the red tide had spread throughout the eastern half of the Los Angeles and Long Beach Harbors complex in San Pedro Bay. This bloom was centered at the mouth of the Los Angeles River, extending out to the Middle and Long Beach Breakwaters, and to Belmont Pier, covering an approximate area of over 15 km^2. The Secchi depth ranged from 0.25 to 0.50 meters within the bloom and the color of the water was a brick red (Forel Ule color XVI - XVIII).

Surface water samples were analyzed via light microscopy and the dominant organisms were found to be raphidophytes, numbering 38 million per liter. Phytoplankton net samples also contained *Ceratium spp.* at considerably lower densities than the raphidophytes. Efforts were made to preserve the raphidophyte cells in an intact state, using either 5% formalin or Lugol's iodine, which were both unsuccessful. Live cultures were maintained for 24 hours at the Southern California Marine Institute (SCMI) Fish Harbor Laboratory and then sent to the Provasoli-Guillard Center for Culture of Marine Phytoplankton (CCMP) at the Bigelow Laboratory for Ocean Science in West Boothbay Harbor, Maine.

After consultation between SCMI and CCMP, and based on light microscopy of living cells, this organism was tentatively identified as being in the genus *Heterosigma*, possibly *H. akashiwo*. This culture was then isolated, and maintained in the collection, referred to hereafter as CCMP 1870.

In April of 1999 isolate CCMP 1870 was sent to the National Marine Fishery Service (NMFS), Northwest Fisheries Science Center in Seattle, Washington for positive identification. The isolate identity was determined by sequencing of the nuclear ribosomal internal transcribed spacer (ITS) regions and 5.8S gene. The procedure used is described as follows:

Five ml of the culture (approximately 1 x 10^4 cell ml^{-1}) was filtered directly onto a Millipore DVPP 25 mm filter (0.65 μm pore size), air dried, and stored at room temperature. To reduce the chance of cross contamination, barrier pipette tips were used, all buffers and disposable-ware were UV irradiated for 10 minutes [8], and all surfaces were washed with DNA-Off™ (CGC Inc., Lincoln Park, NJ, USA) before use to destroy potentially contaminating DNA. The entire ITS region, containing ITS-1, 5.8S and ITS-2, was amplified from one 3mm disk DVPP filter per reaction using two eukaryotic ITS "universal primers" (ITS-4 and ITS-5) derived from fungal sequence [9]. The possibility for potential sequence errors, generated by polymerase base misincorportation during PCR were reduced by using a proofreading DNA polymerase mixture DyNAzyme

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EXT (Finnzymes ØY, Espoo, Finland). In addition, buffers were employed using standard Finnzymes $MgCl_2$ (F-511) protocol, with 0.1 mM for each primer (50 µl reactions). A PTC-100 thermocycler (MJ Research, Watertown, MA, USA) was used for amplification at 94°C (1 min.); 45°C (1 min.); and 74°C (1 min.) for 25 cycles. To insure that the correct DNA sequence was obtained, in addition to the proofreading DNA polymerase mixture, multiple (> 8) separate PCR reactions were carried out and fractions were pooled before DNA purification and sequencing. DNA from PCR reactions was isolated by separation on a 1.8% I.D.NA™ agarose gel (FMC, Rockland, ME, USA) in 1x TAE buffer (40mM Tris-acetate, 1mM EDTA) with ethidium bromide/UV visualization, and the DNA fragments were excised. DNA was extracted from the gel slices with Qiagen gel-isolation kit (Qiagen Inc., Santa Clarita, Ca, USA) and quantification of DNA was determined with a TKO 100 fluorometer (Hoefer Scientific, Piscataway, NJ, USA). The resulting DNA was used as double stranded sequencing templates with universal primers [9]. The complete sequence was obtained for both strands of each fragment individually using Big Dye Terminator Cycle Sequencing with an ABI 377 autosequencer (Applied Biosystems Inc., Foster City, CA, USA). Species determination was made by direct comparison to a previously identified *Heterosigma akashiwo* ITS sequence from isolate NIES 6, GenBank accession number AF157381 [10]. There was complete sequence identity between *H. akashiwo* isolates NIES 6 and CCMP 1870 (ITS GenBank accession number AF157383), confirming that the May 15, 1998 sample from the Los Angeles River mouth was indeed *H. akashiwo*. Subsequently, on May 10, 1999, *Heterosigma* was again observed at the Los Angeles River estuary, co-dominant with a bloom of *Pseudo-nitzchia spp.* This observation was made in dry weather, with the salinity being 30 ppt. The nutrient measurements (sampled at 1 meter depth) were as follows: ammonia nitrogen was 6.4 µgatoms/liter, nitrate nitrogen was 17.8 µgatoms/liter, and phosphate phosphorus was 1.0 µgatoms/liter. A sample of *Heterosigma* was collected on Millipore DVPP filter paper and was once again confirmed to be *H. akashiwo* according to the method described above.

DISCUSSION

The two blooms of *Heterosigma akashiwo* reported here occurred approximately one year apart and in quite different oceanographic conditions. The first bloom was associated with high-nutrient stormwater-runoff (low salinity) and the second bloom occurred during dry weather (high salinity) with much lower nutrient concentrations. These diverse conditions point out just how flexible *H. akashiwo* can be in its bloom requirements, demonstrating it's ability to take advantage of a broad range of physical conditions, making bloom-prediction models much more difficult to generate.

Previously, *Heterosigma akashiwo* had never been fully documented in California waters. San Pedro Bay, in the vicinity of the Los Angeles River estuary, fits the suitable environmental requirements for *Heterosigma akashiwo*. The question arises as to how *H. akashiwo* arrived in San Pedro Bay. *H. akashiwo* cysts have been shown to remain viable for up to 30 weeks in the dark (Imai, 1996), but both benthic and motile cells lyse at 0°C and pelagic cells lose motility below 10°C, especially in lower salinity waters (Tomas, 1978). These qualities reduce its ability to survive passive transport through northern seas between oceanic regions. Although it is possible to explain dispersal by current drift, another likely mechanism would be that resting benthic cells or cysts have been spread through ship ballast water [11-13]. *Heterosigma akashiwo* is known to have a benthic cyst stage capable of overwintering [2], and therefore potentially surviving in the dark for extended periods. Viable *H. akashiwo* cells were not only identified from vessel ballast water after a seven day cruise from Tokyo, Japan to Seattle WA, USA, but were the dominant species recovered [13]. If the environmental conditions were right at the time of a ballast exchange in San Pedro Bay, a bloom may have immediately occurred, thereby "seeding" the area.

While it is impossible to absolutely state that *Heterosigma akashiwo* was introduced by ship ballast water, nevertheless, it is now established in San Pedro Bay. Previously, the only populations of *H. akashiwo* documented on the west coast of the United States have been in Washington State, with blooms reported in Puget Sound, Strait of Juan de Fuca, and coastal regions. In 1964 *Olisthodiscus luteus* was reported in San Diego Bay [14]. *Olisthodiscus luteus* was originally classified as a Chrysophyte flagellate, but has since been placed in the Raphidophyceae and has been confused in the past with *H. akashiwo*. An identifying characteristic of *H. akashiwo* is that it spins in a distinctive barrel-roll motion when swimming, while *O. luteus* does not [5]. In addition, *O. luteus* has a siliceous benthic cyst, and the benthic resting spore of *H. akashiwo* is naked, surrounded by mucoid material [15]. Blooms of *H. akashiwo* are typically pelagic, but the organism identified as *O. luteus* from San Diego Bay was associated with the sediment interface typical of *O. luteus* [5]. It was presumed that the 1964 occurrence of *O. luteus* in San Diego Bay may have been *H. akashiwo* because of the confusion over nomenclature changes, however the description given by Lackney and Clendenning [14] is more consistent with *O. luteus* than with *H. akashiwo*. Therefore the present occurrence of *H. akashiwo* in San Pedro Bay is the first confirmed record of this alga in California waters.

ACKNOWLEDGMENTS

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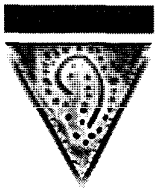
PFIESTERIA

Harmful
ALGAL
BLOOMS



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DISTRIBUTION OF *PFIESTERIA* SP. AND AN ASSOCIATED DINOFLAGELLATE ALONG THE US EAST COAST DURING THE ACTIVE SEASON IN 1998 AND 1999.

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ABSTRACT

Over 800 water and sediment samples from estuarine sites of the US Atlantic coast were assayed for the presence of *Pfiesteria* sp. and a cryptoperidiniopsoid by PCR probing of extracted DNA. Positive results were found in about 2% of samples derived from routine monitoring of coastal waters. In general, the geographic ranges of the species tested were the same, and extended from New York to Florida.

INTRODUCTION

The toxic dinoflagellate *Pfiesteria piscicida* has been implicated as the causative agent of some fish kill and fish lesion events along the eastern seaboard since its discovery [1, 2, 3]. Recently, a second species, *P. shumwayae*, has been described that also exhibits toxin production and fish-killing activity [4]. Toxicity associated with *Pfiesteria* sp. is known to be a human health hazard, based on both laboratory and field observations [5, 6, 7].

The distribution of *Pfiesteria* sp. through 1997 was determined by a "standard" method for identification of which consists of three sequential steps: 1) identification of "*Pfiesteria*-like" organisms by light microscope examination of water samples; 2) fish bioassays to test for toxin production; and 3) positive identification by thecal plate tabulation of cells by scanning electron microscopy after membrane-stripping or suture swelling [8, 9]. Using these methods, Burkholder and colleagues [2, 8] found *Pfiesteria* species in estuarine water from the Indian River, Delaware to Mobile Bay, Alabama on the US east and gulf coasts. These results were primarily determined by samples taken in response to reported fish kill or fish lesion events.

The recent development of molecular probes to *Pfiesteria piscicida* provides a powerful new tool for determining its distribution in natural systems [10, 11, 12]. The gene probe approach has the advantages of lower cost and providing results in a shorter time (<48 hr) than the light microscope – bioassay – SEM method. Thus, it is amenable for monitoring since numerous water or sediment samples can be taken and analyzed on a routine basis. A drawback to the probes currently available, however, is that they do not determine whether the populations detected are producing or capable of producing toxins since their target is ribosomal DNA. Thus, in efforts to determine the distribution of toxic *Pfiesteria* complex species, a combination of molecular probes followed by fish bioassays is necessary.

Here we report our use of PCR probes to determine the distribution of *Pfiesteria* species and a cryptoperidiniopsoid dinoflagellate, from sites along the US East Coast during 1998 and 1999.

METHODS

Surface water samples were concentrated for DNA extraction by vacuum filtration of 30-300 ml through a 25 mm GF/C glass microfibre filters. The filter was then placed in 1 ml of CTAB buffer in a 2 ml microfuge tube and stored at room temperature until extraction [13]. Sediment samples (≈ 0.25 g) were extracted using a commercial method (UltraClean™ Soil DNA Kit, Mo Bio Laboratories, Inc.) as soon as possible after collection, usually within one week. Extracted DNA was resuspended in 20 µl of sterile TE buffer, and stored at -20°C until use.

The extracted DNA was first probed by PCR with 18S rDNA primers to assure that the sample contained amplifiable DNA. PCR reactions for detection of *Pfiesteria piscicida*, *P. shumwayae*, and a cryptoperidiniopsoid dinoflagellate utilized the primers and followed the protocols reported previously [11, 12]. Briefly, 50 µl reactions contained 50 mM KCl, 20mM Tris-HCl (pH 8.4), 4.0 mM MgCl, 200 µM nucleotides, 1 U *Taq* DNA polymerase, (PCR Supermix, Gibco/Life Technologies, Gaithersburg, MD), 0.8µM of each primer, and 0.1 – 1 µl of the sample DNA. PCR reaction conditions were: 95°Cx5min; then 40cycles (95°Cx30s / 60°Cx30s / 72°Cx40s); then 72°Cx5min followed by cooling to 4°C until reaction products were analyzed.

RESULTS AND DISCUSSION

Prior to 1998, *Pfiesteria* species had been reported to occur from Delaware to Mississippi on the east and gulf coasts of the United States [2, 8], primarily as a result of investigating fish kill or fish lesion events. In 1998, a year in which there were few fish kills attributed to *Pfiesteria*, 21% of 170 samples probed by PCR tested positive for *P. piscicida* [11]. Again, however, most samples were from sites of fish health problems. In the current study 53 samples were positive for *P. piscicida*, 14 were positive for *P. shumwayae*, and 18 for the cryptoperidiniopsoid (Table 1, Fig. 1). With the exception of water samples from Maryland, and

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sediment samples from New Jersey and South Carolina, the samples are from routine monitoring, rather than sites of fish health impacts as in previous studies. Excluding the sites that have a current or historical fish health concern, about 1.7% of the samples tested positive for *P. piscicida*, 1.4% for *P. shumwayae*, and 1.7% for the cryptoperidiniopsoid. Although this frequency may seem low, there was only one fish kill event that was definitively attributed to *Pfiesteria* in 1998; in 1999 no fish kills were clearly attributed to *Pfiesteria.*

Table 1. Incidence of *Pfiesteria* and a cryptoperidiniopsoid in estuarine water samples from the U.S. east coast, 1998-1999, as determined by PCR probes.

State	Type	N	# Positive Samples		
			P. pisc.	*P. shum.*	Crypto.
NY	Water	163	5	3	1
NJ	Water	38	0	0	0
	Sediment	18	4	0	0
DE	Water	253	2	3	4
	Sediment	25	4	0	0
MD	Water	79	27	4	6
VA	Water	36	1	0	0
NC	Water	14	3	0	0
SC	Water	20	2	2	1
	Sediment	37	4	0	0
GA	Water	20	0	0	0
FL	Water	128	1	2	6
	Sediment	12	0	0	0
Total	Water	751	41	14	18
	Sed	92	12	0	0

Although the number of sediment samples assayed in this study was much lower than the number of water samples, we found positive assays for *P. piscicida* with a higher frequency (13%) than in water samples (5%). This result is consistent with the ephemeral nature of *Pfiesteria* in the water column, and the predominance of benthic life stages [1, 2]. Although we did not find any positive indication of *P. shumwayae* or the cryptoperidiniopsoid in sediment samples reported in this study, we have found positive indications of both taxa in samples after 1999 (data not reported here). Thus, routine monitoring of water is not likely to be the optimal method to detect *Pfiesteria* sp., except during conditions conducive to fish lesion or fish kill events. Routine monitoring should emphasize sediment sampling in future studies, although a disadvantage of sediment sampling is that we do not yet have suitable method for preservation of sediments, and therefore sediment samples need to be analyzed soon after collection.

The distribution of positive samples in this study and others [2, 8] suggests that the geographic range of these taxa overlap (Fig. 1). Through 1999, sampling remained based predominantly as a response to fish health problems (except in Maryland and Delaware), and little information is available on either fine scale spatial or temporal distribution or on very broad scale distribution. Overall, we believe that *Pfiesteria* sp. are common, but normally benign inhabitants of estuarine systems, and some of our preliminary data (not shown here) suggests the geographic range will be world wide. Given the potential for both fish and human health problems, continued study of both fine and broad scale distribution of these organisms is prudent.

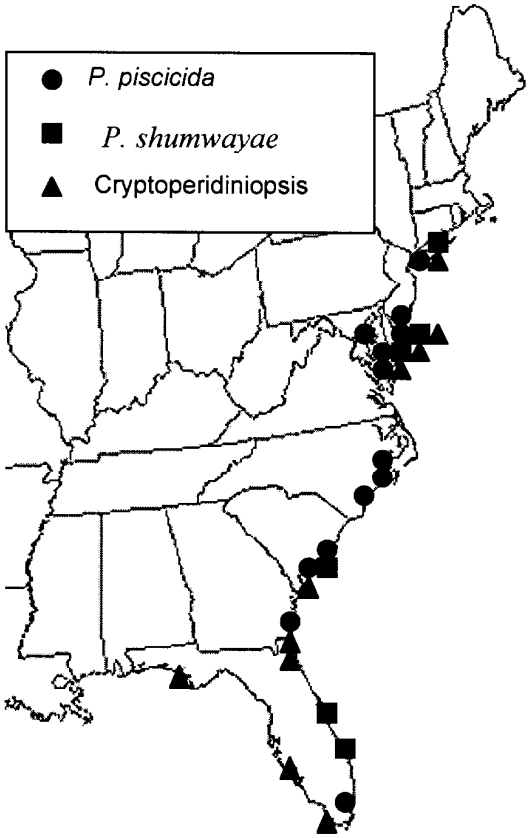

Fig 1. Approximate location of cryptoperidiniopsis and *Pfiesteria* sp. on the US east coast detected by PCR during 1998 and 1999.

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GROWTH AND TOXICITY STUDIES OF THE DINOFLAGELLATES *CRYPTOPERIDINIOPSIS* SP., *GYRODINIUM GALATHEANUM* AND *PFIESTERIA PISCICIDA*

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ABSTRACT

Cryptoperidiniopsis sp. and *Gyrodinium galatheanum* were isolated from sediment and water samples collected in Chesapeake Bay tributaries for growth study comparisons with a virulent strain of *Pfiesteria piscicida*. *Cryptoperidiniopsis* sp. and *P. piscicida* were observed to have complex life histories that include amoeboid, cyst, gamete, planozygote, and zoospore stages. The *Cryptoperidiniopsis* sp., *Gyrodinium galatheanum*, and *P. piscicida* were each placed into culture flasks containing f/2-Si medium with *Cryptomonas* sp. (CCMP #767 Provasoli-Guillard) as a food source. The inoculum of dinoflagellates was adjusted so that the initial concentration was always 500 mL^{-1}. Factors investigated include prey concentration, prey preference, and fish toxicity. The growth rate of *Cryptoperidiniopsis* sp. was found to be high (_ = 1.43) when compared to other dinoflagellates, including *P. piscicida* (_ = 0.84) and *G. galatheanum* (_ = 0.88). All three dinoflagellates can feed on a wide variety of algae, but cryptophytes were preferred as a food source by each. In each species, the maximum dinoflagellate abundance was related to increased concentration of algal prey. In preliminary bioassay experiments, *P. piscicida* was found to be positive for fish toxicity, while *Cryptoperidiniopsis* sp. and *Gyrodinium galatheanum* were negative for fish toxicity having killed no fish over an 8-10 week period under the same conditions. SEM examination of the *Cryptoperidiniopsis* sp. by Karen Steidinger, and gene sequencing analysis by David Oldach, indicate this species is distinct from *C. brodyi*.

INTRODUCTION

Heterotrophic dinoflagellates are common in many estuarine systems [1]. Since the discovery of the widespread occurrence of the fish killing dinoflagellate, *Pfiesteria piscicida*, in estuarine waters [2,3], increased attention has been placed on this category of dinoflagellates. Because *P. piscicida* can be maintained in culture on algal prey, many related dinoflagellate species may be able to be cultured in a similar fashion. Attempts at culturing some dinoflagellates may have failed because they were presupposed to be autotrophs. Because of this, Stoecker [4] states, many cases of mixotrophy in dinoflagellates probably go undocumented. By focusing on sediment isolations, we hope to show there are several dinoflagellates indigenous to Chesapeake Bay, which share similar affinities to *P. piscicida*, such as feeding mechanisms and preferences. Although Schnepf and Elbrachter [5] state tube feeding is common among both naked and thecate dinoflagellates, few studies have examined the grazing of phytoplankton by dinoflagellates equipped with a peduncle [1,6]. Since it is known that the nontoxic stages of *P. piscicida* can consume algae [7] and heterotrophic dinoflagellates are often abundant in estuarine systems, it may be possible to show *Pfiesteria* and especially *Pfiesteria*-like dinoflagellates can be significant grazers of phytoplankton in estuarine systems. The possibility of other shared characteristics, such as toxin production, was also investigated.

During 1998 and 1999 the Old Dominion University phytoplankton analysis laboratory monitored 130 stations in the Virginia tidal waters and the lower Chesapeake Bay for the presence of *Pfiesteria piscicida* and other *Pfiesteria*-like dinoflagellates. Whenever high concentrations occurred, both water and sediment samples were incubated for subsequent clonal isolation of the dinoflagellate, culturing of these cells, and further examination under SEM and with fish bioassays. Presented here are the results of growth and preliminary toxicity studies for two common dinoflagellates isolated from the sediment and a virulent strain of *P. piscicida* provided by Dr. JoAnn Burkholder.

METHODS

Representative sediment and water samples were taken from 130 different stations in Virginia estuaries in 1998 and 1999 (Fig. 1). Many of these stations were sampled monthly between May and October. All stations were sampled at least once each year. Sediment sub-samples from 80 of these sites were incubated to encourage the growth of encysted dinoflagellates. Two dinoflagellates commonly came from these cultures and were subsequently identified as *Gyrodinium galatheanum* and a species of *Cryptoperidiniopsis*. Karen Steidinger independently verified species identifications with SEM. David Oldach also confirmed the *Cryptoperidiniopsis* sp. through gene sequencing analysis.

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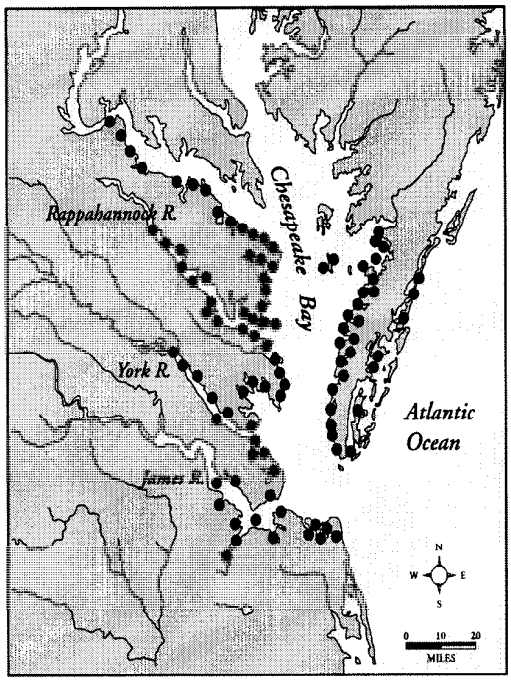

Fig. 1. Dinoflagellate sampling station locations in Chesapeake Bay, USA.

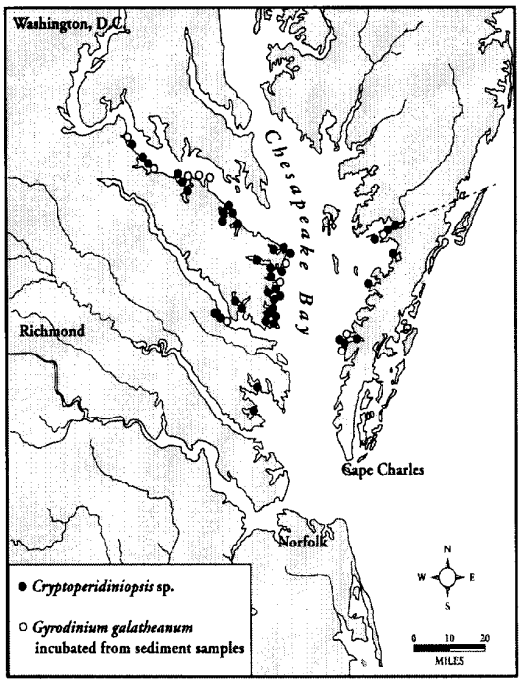

Fig. 2. Locations from which pfiesteria-like dinoflagellates were isolated.

For growth studies, each of the three dinoflagellates were placed in separate 250 mL culture flasks containing f/2-Si medium at 15 psu with an 'initial batch' of *Cryptomonas* sp. (CCMP #767 Provasoli-Guillard) as a food source. In the prey preference studies, three phylogenetically diverse phytoplankton species were used. *Cryptomonas* was one of the three algal prey along with *Skeletonema costatum* and *Dunaliella*. The same amount (by abundance) of each prey type was added to every flask. All sample and control flasks were in triplicate. Cultures were maintained under a 12 h: 12 h light: dark cycle at 22 °C. In all cases the initial inoculum of dinoflagellates was 500 mL^{-1}. Control and experimental flasks were sampled at a fixed point daily samples were preserved with Lugol's solution and enumerated using a Palmer-Maloney counting chamber. In determining dinoflagellate concentrations, only the motile zoospore stage was counted. Initial prey density was determined using a hemacytometer with a depth of 0.5 mm.

For toxic fish bioassays, the dinoflagellates were placed in culture vessels containing Tilapia. Sample and control vessels were run in duplicate, using artificial seawater at a salinity of 15 psu as per Burkholder and Glasgow [7]. All cultures with fish were maintained in a biohazard level III facility.

Bioassay experiments were conducted over an 8-10 week period. Fish were checked at least twice daily for signs of stress or death.

RESULTS

Cryptoperidiniopsis sp. was isolated from 52.5% of the incubated sediment samples, while *Gyrodinium galatheanum* was cultured from 13.8% of the samples (Fig. 2). Other *Pfiesteria*-like species, such as *Cryptoperidiniopsis brodyi,* were also found. *G. galatheanum* appears less morphologically similar to *P. piscicida*, but was included in these studies because of its common occurrence and previous reported toxicity in relation to its occurrence during fish mortality events [8,9]. The genus *Cryptoperidiniopsis* currently contains one species, *C. brodyi*, which has been found previously from North Carolina to Florida. Here we report the occurrence of *C. brodyi* in Chesapeake Bay waters, which was confirmed through gene sequencing analysis by Dr. David Oldach. *C. brodyi* was much less common than the other, undescribed species of *Cryptoperidiniopsis*. Based on independent SEM examination by Karen Steidinger and gene sequencing analysis by David Oldach, this *Cryptoperidiniopsis* strain is different morphologically and genetically from *C. brodyi*. Due to the prevalence of this *Cryptoperidiniopsis* sp., it was included in this study.

All three dinoflagellates were observed feeding on the algal prey. Swarming response due to the introduction of algal prey was noted in similar fashion to Glasgow et al. [10] and Lewitus et al. [11] for *Cryptoperidiniopsis* sp. and *P. piscicida*. These dinoflagellates were both observed feeding myzocytotically by means of a peduncle. The ingestion of an individual cell was very quick, taking only a few seconds. Although the prey can be quite large in

comparison, Hansen and Calado [12] state these types of dinoflagellates have adapted feeding mechanisms that allow for this raptorial feeding. *G. galatheanum* moved much more slowly and fed on individual algal cells much more slowly (~ 4 minutes on average). Growth rates were calculated for the three dinoflagellates based on the portion of time when their concentration was increasing. The growth rate of *Cryptoperidiniopsis* sp. was found to be high (_ = 1.43) when compared to other dinoflagellates, including *Gyrodinium galatheanum* (_ = 0.88) and *Pfiesteria piscicida* (_ = 0.84) (Fig. 3). The growth rate for *P. piscicida* feeding on a cryptophyte fell within its previous reported range of 0.83-1.11 [10]. The growth rates of all three are higher than the mean dinoflagellate growth rate of 0.57 calculated by Tang [13] and *Cryptoperidiniopsis* sp. is particularly high, surpassing any of the 35 dinoflagellate growth rates she recorded.

Cryptophytes are clearly an important food source for heterotrophic dinoflagellates. The maximum dinoflagellate abundance (yield) was related to increased concentration of cryptophytes in all three dinoflagellates (Fig. 4). The three dinoflagellates can feed on a wide variety of algae, but the cryptophyte, *Cryptomonas*, was optimal as a food source by each dinoflagellate over *Skeletonema costatum* and *Dunaliella* (Fig. 5). Optimal growth of *Pfiesteria* in the presence of cryptophytes over other algal food sources is similar to the finding of Glasgow et al. [10]. Stoecker et al. [14] found the ingestion material of the dinoflagellate *Prorocentrum minimum* to be correlated with cryptophyte abundance as well. Potential prey selection has been proposed before for peduncle feeding dinoflagellates [6,15].

The three different dinoflagellates were most commonly observed as motile zoospores while feeding on the phytoplankton prey. *Cryptoperidiniopsis* sp. and *P. piscicida* also formed amoeboid and cyst stages in association with prey depletion, as noted for *P. piscicida* in Glasgow et al. [10]. The amoeboid stages represented a very small portion of the total cells observed during the period used for calculating the growth rates and were not included in the enumeration. No amoeboid or cyst forms were observed for *G. galatheanum* at any time during the study.

In fish bioassays, using Tilapia, this particular strain of *Cryptoperidiniopsis* sp. and the isolated *Gyrodinium galatheanum* did not produce fish kills over a 10 week period, but in tanks inoculated with *P. piscicida* fish mortality was observed. No deaths occurred in control tanks. More testing on these and additional strains of dinoflagellates is necessary to confirm the preliminary results for the various *Cryptoperidiniopsis* spp. and other *Pfiesteria*-like dinoflagellates we have isolated from Chesapeake Bay.

Heterotrophic feeding behavior was also noted with these other dinoflagellates derived from the sediment.

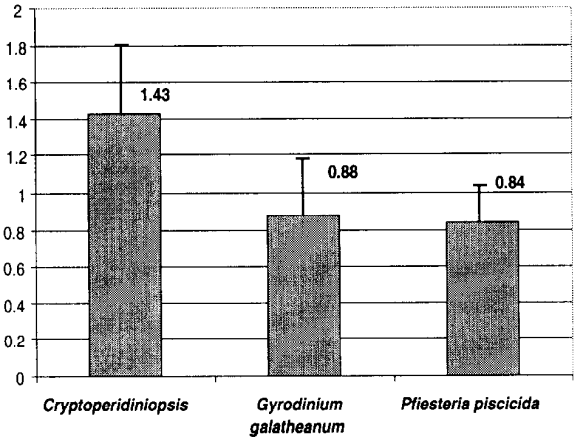

Fig. 3. Growth Rates of Dinos on *Cryptomonas* Prey

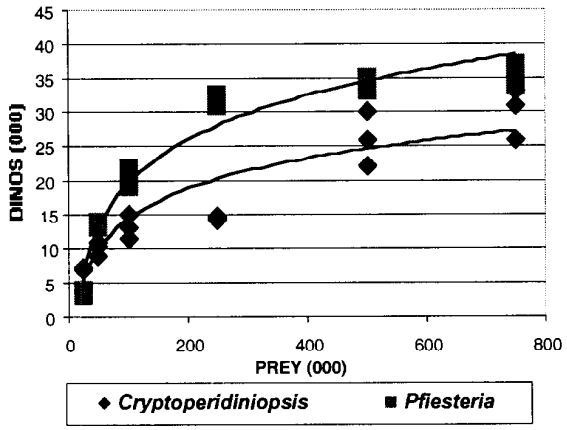

Fig. 4. Predicting dinoflagellate maxima from initial prey.

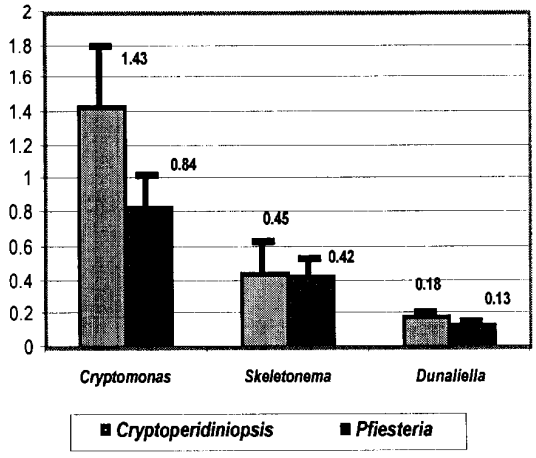

Fig. 5. Growth rates of *Cryptoperidiniopsis* and *Pfiesteria* on various Algal Prey

SUMMARY

There are several species of *Pfiesteria*-like dinoflagellates present in estuarine systems. Most research attention has understandably been placed on one species, *Pfiesteria piscicida* [11]. Among the general grouping of heterotrophic dinoflagellates, *Pfiesteria*-like dinoflagellates were common representatives of the phytoplankton community in Chesapeake Bay. These include *Cryptoperidiniopsis* sp., *C. brodyi*, and *Gyrodinium galatheanum*. Many other dinoflagellates were found in the water and sediment samples which were very different from *Pfiesteria* such as *Amphidinium* sp. and *Heterocapsa triquetra*, *Prorocentrum minimum*, and *Protoperidinium brevipes*. Several dinoflagellates in Chesapeake Bay have been shown by Li et al. [16] to ingest cryptophytes including *Ceratium furca*, several *Gymnodinium* species, *Prorocentrum minimum* (by [14] as well), and *Protoperidinium brevipes*. Mixotrophy has also been noted in *Gymnodinium sanguineum* and *Gyrodinium uncatenum* by Bockstahler and Coats [17].

Heterotrophic dinoflagellates similar to *Pfiesteria* can have a significant grazing effect on other phytoplankton in estuarine systems. Growth studies indicated among the *Pfiesteria*-like dinoflagellates, *Cryptoperidiniopsis* sp. had greater feeding and growth rates than *P. piscicida* and *G. galatheanum*, although all three dinoflagellates possessed growth rate values considered high for other heterotrophic dinoflagellates [10,13,18,19,]. Cryptophytes were grazed at a higher rate than the other two prey algae. Glasgow et al. [10] discuss the problems tube feeding dinoflagellates might have penetrating diatom frustules. We noted the dinoflagellates consistently fed on damaged *Skeletonema* cells or where breaks had occurred in the chains. Perhaps this was a chemotactic response by the dinoflagellates to the breaks or it was simply easier for the dinoflagellates to penetrate the frustule in those situations.

Although all three dinoflagellates achieved high growth rates while feeding on *Cryptomonas*, *Cryptoperidiniopsis* sp. had rates notably higher than the other two. In fact, *Cryptoperidiniopsis* sp. possesses one of the highest growth rates ever recorded for a dinoflagellate. *Pfiesteria piscicida* non-toxic zoospore abundances have been correlated with cryptophyte abundance in North Carolina [10]. In Chesapeake Bay, cryptophyte densities commonly surpass 10^6 cells L^{-1}. This may be one reason why *Cryptoperidiniopsis* is quite common in Chesapeake Bay. It may be able to achieve high densities more quickly than other dinoflagellates, even *Pfiesteria*, by feeding on a common category of phytoplankton. This type of rapid feeding on cryptophytes may allow these *Pfiesteria*-like dinoflagellates to compete against other dinoflagellates and algae.

Cryptoperidiniopsis sp. is clearly distinct and separate from *Pfiesteria piscicida* based on SEM analysis of its external morphology, genetic analysis, and possibly the lack of toxin production. However, there are several similarities which *Cryptoperidiniopsis* sp. shares with *P. piscicida* including myzocytotic feeding with a peduncle, preference for cryptophytes as food, the ability to graze on other types of algae if forced, a complex life cycle with several life stages including amoeboid forms, and a cyst stage, which was widely distributed in Virginia estuaries. This study shows there may be several heterotrophic dinoflagellates, which have adapted modes of feeding and flexibility similar to *Pfiesteria piscicida* in order to survive and proliferate in estuarine systems.

In preliminary testing, the two most common dinoflagellates raised from sediment incubations, *Cryptoperidiniopsis* sp. and *G. galatheanum*, were not shown to be toxic to fish. No fish (tilapia) died over a 10 week time period while being exposed to the presence of these two dinoflagellates. However, more testing is needed on several different strains of these dinoflagellates to be definitive.

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FISH-KILLING ACTIVITY AND NUTRIENT STIMULATION OF A SECOND TOXIC *PFIESTERIA* SPECIES

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ABSTRACT

Toxic strains of a second *Pfiesteria* species, being formally named as *Pfiesteria shumwayae* sp. nov. within the family Pfiesteriaceae (Dinophyceae, Dinamoebales), have been verified from the mid-Atlantic and southeastern United States, Scandinavia and New Zealand. A total of 64 toxic clones were tested for attraction to fresh fish tissues, excreta and secreta, separated from the live animals for < 2 hours. They also were tested for lethal activity toward fish; and toxic strains of *P. shumwayae* and *P. piscicida* were com-pared for their response to inorganic N and P enrichments. All clones showed strong attraction to fresh fish materials. More importantly, all clones become toxic in the presence of live fish, and caused fish death in bioassays with live cells at similar densities as those encountered during fish kills. Klep-tochloroplastidic *P. shumwayae* sp. nov. was strongly stimu-lated by nitrate whereas *P. piscicida* was more stimulated by phosphate, partly mediated through algal prey abundance.

INTRODUCTION

At least 30 toxic dinoflagellate species have been newly recognized within the past 15 years [1]. One of these was the ichthyotoxic dinoflagellate, *Pfiesteria piscicida* Steidinger & Burkholder, which was first implicated as a causative agent of major fish kills in the largest and second largest estuaries on the U.S. mainland, Chesapeake Bay and the Albemarle-Pamlico Estuarine System [2-7]. In 1995 a second toxic *Pfiesteria* species was detected along with *P. piscicida* at an in-progress fish kill in a North Carolina estuary [8]. The species is being named as *Pfiesteria shumwayae* sp. nov. [9]. Like *P. piscicida, P. shumwayae* sp. nov. zoospores are stim-ulated to become toxic in the presence of live fish or their fresh tissues, and they become temporarily nontoxic without live fish [9, 10]. Clonal populations may be toxic or non-inducible [9-11]. Zoospores feed primarily by myzocytosis and osmotrophy, and exhibit swarming behavior as prey are depleted [as in 12]. Our objectives here were to **(a)** test multi-ple strains of this species from various estuaries for attraction to live fish and ichthyotoxicity; and **(b)** compare zoospore production by kleptochloroplastidic *Pfiesteria* spp. in re-sponse to inorganic N and P enrichments.

MATERIALS AND METHODS

Cultures. The toxic isolates of *P. shumwayae* sp. nov. used in this study were obtained from estuaries along the east/southeast coasts of the United States from Delaware south into Florida (Table 1, Fig. 1). They were transported to the laboratory in a shaded container at ambient temperature, and were maintained in a biohazard III facility at 50 µmol m^{-2} s^{-1} (12:12-hr light : dark cycle) prior to being tested for ichthyotoxic behavior. They were verified as ichthyotoxic us-ing our standardized fish bioassay process [6, 9, 10, 13, 14].

Pfiesteria spp. must be given a prey source for growth in culture [2, 3, 6, 9, 10, 12], and live fish must be provided in biohazard III facilities (to protect laboratory workers from neurotoxic aerosols [17]) to detect and grow toxic strains (TOX-A functional type [6, 10, 13, 14]). *Pfiesteria* spp. Were cloned from positive fish bioassays as in [9, 10]. Popula-tions were grown for 2 weeks with cryptomonad prey, and then were inoculated into fish bioassays and monitored at least daily to confirm toxicity and to grow toxic clones. Cultures were maintained at 23°C, 12:12 L:D cycle, and 80 µmol m^{-2} s^{-1}, in 15 psu f/2-Si media [18] made with sterile-filtered synthetic seawater (0.2 µm-porosity; deionized water + CORALIFE® Scientific Grade Marine Salts). Each isolate was tested separately (fish bioassays, below; n = 2-3).

Attraction to Fish Tissue, and Ichthyotoxicity. We tested attraction of sub-populations of zoospores from each of the 64 TOX-A and recently toxic TOX-B isolates of *P. shumwayae* sp. nov. to fresh fish epidermis, excreta and mucus (from *Oreochromis mossambicus* Peters; total length 7-9 cm; separated from live animals for <2 hours). Swim-ming speed and direction of movement (net-to-gross dis-placement index [NGD] as in [6], with 1.00 indicating a straight line and ≤ 0.50 indicating random swimming) were quantified over 2-minute trials ± fresh fish epidermis (video microscopy; n=20 zoospores / isolate). Micro-capillary tube assays ([11]; aperture diameter, 30 ± 3 µm, means ± 1 SD) were conducted to further evaluate the chemosensory attrac-tion. We quantified the number of cells entering tubes filled with an aqueous mixture of sterile-filtered, fresh fish mucus and excreta over a 10-minute period (n = 4 clonal cultures as replicates, $>10^3$ zoospores/replicate). In both experiments controls (n=same 4 clonal cultures, > 10^3 zoospores/replicate) were treated similarly except that fish materials were absent (micro-capillary tubes filled with sterile-filtered medium).

The fish bioassay process of [6, 7, 10, 13, 14] was used to separately test for toxicity of all 64 isolates of *P. shumwayae* sp. nov. (Table 1). The isolation and toxicity testing proce-dure involved **(a)** adding natural samples to fish in bioassays; **(b)** isolating clones from fish-killing bioassays using flow cytometry [9, 10]; **(c)** growing clonal cultures with algal prey [13]; and **(d)** retesting

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clones for ichthyotoxicity in additional fish bioassays [7, 9, 10, 13, 14]. Algal prey were *Cryptomonas* sp., cloned from the UTEX commercial source. Once the second set of fish bioassays exhibited ichthyotoxici-ty, the dinoflagellate species was re-cloned and re-confirmed with SEM [4, 9] (identification and toxicity also cross-confirmed by independent laboratories with demonstrated expertise [9, 10, 15, 16]). Fish bioassays were conducted for toxic *Pfiesteria*; negative controls were fish bioassays conducted identically without toxic *Pfiesteria* [6, 9, 10, 13, 14]. This fish bioassay process is the 'gold standard' to detect/grow toxic *Pfiesteria* spp. [6, 10 13, 14, 19]. It must be used be-cause reliable toxin assays for natural samples cannot be de-veloped until purified toxin standards are available [20, 21]. Data were compared to fish bioassays with toxic clones of *P. piscicida* ('positive controls') that we continuously maintain.

Table 1. Field locations that have yielded toxic *Pfiesteria shumwayae* sp. nov., with toxicity verified by positive fish bioassays and species identified by SEM and/or PCR.[a] The samples were not col-lected during fish kill/disease events unless noted (kill). Dates and locations represent a partial list, intended to demonstrate the pro-gression of discovery of this toxic species in mesohaline estuaries, coastal embayments and tidal creeks of the southeastern U.S.[b]

Locations Positive for *P. shumwayae* sp. nov. (new isolates/year)	Field Samples[c]	Time to 1st Fish Death (days)	Zoospores/mL (1st Fish Death)	Species Verified
1995 – 1 Isolate				
NC–New River (kill)	W	35[d]	3,000	SEM
1996 – 3 Isolates				
NC– Neuse	W	30 – 39	500 – 15,000	SEM
1997 – 3 Isolates				
MD–Pocomoke (kill)	W	9 – 25	900 – 18,000	SEM
NC–Neuse (2)	W	31	14,000	SEM
1998 – 12 Isolates[e]				
FL–St.Johns, Tampa Bay[f]	S	45 – 68	1,200 – 25,000	SEM, PCR
MD–Big Annemessex, Manokin, Patuxent, Pocomoke, Trappe Cr., Wicomico	S	30 – 49	600 – 26,000	SEM, PCR
NC–Neuse (2)	W	25 – 40	800 – 28,000	SEM, PCR
Neuse (kill)	W			
1999 – 32 Isolates[e]				
DE--Inland Bays: Indian R, (3), L. Assawoman Bay, (4), Rehoboth Bay (3)	W, S	37 – 73	1,000 – 22,000	SEM, PCR
MD–Big Annemessex (2), Manokin (3), Pocomoke (5), Patuxent (2), Trappe Cr. (3), Wicomico (3)	S	33 – 72	500 – 28,000	SEM, PCR
NC– Neuse (4)	W,S	25 – 71	400 – 37,000	SEM, PCR
2000 – 11 Isolates				
AL–Mobile Bay	S	53	5,700	SEM, PCR
DE–Pepper Cr., Arnell Cr.	W	30 – 31	3,900 – 7,800	SEM, PCR
FL–Pensacola Bay, St. Johns R.[f]	S	33 – 62	1,300 – 23,000	SEM, PCR
MD–Chesapeake (5)	W	26 - 56	590 – 24,000	SEM, PCR
NC–North Bay R.	W	73	2,500	SEM, PCR

[a] These isolates were confirmed as toxic strains using the standardized fish bio-assay process [6, 13, 14]. One isolate was obtained unless otherwise indicated (number in parentheses after estuary). SEM was conducted (membrane-stripped or suture-swollen zoospores [4, 9]; PCR was first available in 1998 [15, 16].

[b] Isolates from kills were sampled during in-progress fish kills that involved > 1,000 fish (see [5-7, 10] for more information.

[c] Water (W) = samples collected from depth 0.5 m. Sediment (S) = the upper 2 cm of the bottom sediments mixed with overlying water.

[d] The fish bioassay was initiated 5 days after collection. Had it been initiated after 1-3 days, we would have expected fish death within $\leq$ 21 days [13].

[e] The relatively high number of isolates from Maryland reflects the high federal/ state funding that was available for Maryland, relative to other states.

[f] Toxic strains of a second pfiesteria-like species previously had been verified with positive fish bioassays [6, 8] in 1993 from the Florida locations listed, but the identity of the second species detected earlier remains uncertain.

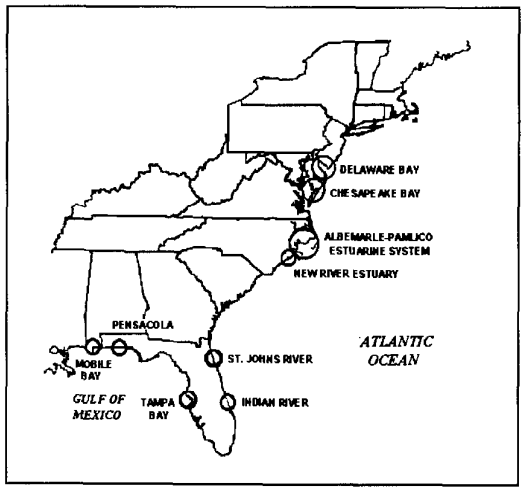

Fig. 1. Geographic range of *Pfiesteria shumwayae* sp. nov. in the U.S.A. to date, with the species identification confirmed using SEM [3, 4, 9] and PCR [15, 16]. Open circles designate locations where toxic strains (specifically, potentially toxic populations, the TOX-B functional type) of *P. shumwayae* sp. nov. have been confirmed. This range overlaps that of *Pfiesteria piscicida*, and covers much of the U.S. Atlantic and Gulf Coasts.

Nutrient Experiments. Batch cultures of *P. piscicida* (Neuse River) and *P. shumwayae* sp. nov. TOX-B zoospores were maintained with cryptomonad in f/100-Si medium [18]. Prior to experiments, algal prey were grazed to <10 cells/mL. Zoospores of each *Pfiesteria* species (separate trials; initially at 150 cells/mL) were inoculated into P_i or N_i – enriched media (0, 100, 500, or 1000 µg PO_4^{-3} or NO_3^-/L) 50-mL cul-ture flasks containing pre-acclimated prey at a prey : zoo-spore ratio of 15:1 (n=4; experiments repeated twice). Sam-ples were collected initially and daily for 4 days. Controls consisted of prey alone $\pm$ nutrients, and each *Pfiesteria* spe-cies $\pm$ nutrients. Zoospores and prey were quantified as in [5, 6]. Descriptive and univariate analyses were completed; 1-way ANOVA with PROC GLM was used to test for similari-ties among treatments and to describe response curves. Pro-bability values were considered significant at $\alpha < 0.05$ [22].

RESULTS

Attraction to Fish and Ichthyotoxicity. All 64 toxic isolates of *P. shumwayae* sp. nov. demonstrated strong, dire-cted attraction to fresh fish epidermis, excreta and mucus. Mean swimming velocity of toxic zoospores was 640$\pm$90 µm s^{-1} in the presence of fish materials, versus 290$\pm$140 µm s^{-1} without fish materials. The NGD index

was ca. 1.00 (mean ± 1 SD, 0.96±0.03) for zoospores exposed to fresh fish materi-als, vs. 0.41±0.12 (random path) without fish materials. Toxic zoospores entered micro-capillary tubes filled with sterile-filtered fish mucus/excreta at a rate of 82±5 cells/ mL (mean ± 1 standard error [SE]), vs. zoospore rate of entry into control tubes at 3±1 cell/mL. All 64 isolates additionally showed ichthyotoxicity and promoted fish death (Table 1), but ranged from weakly to highly toxic depending on the cell density and the isolate. Fish death in cultures with weakly toxic isolates required > 4 to 48 hours, at cell densities of > 2,500–30,000 zoospores/mL. However, fish death with high-ly toxic isolates ranged from 2 to 4 hours, at cell densities of 300-2,500 TOX-A zoospores/mL. Observed toxic activity in *P. shumwayae* sp. nov. was within the range reported for *P. piscicida* at similar cell densities [5, 6, 13]. By comparison, all control fish without *Pfiesteria* exposure remained alive and apparently healthy without signs of distress or disease.

Response of *P. shumwayae* sp. nov. and *P. piscicida* to Inorganic N and P. All N and P additions, ± algal prey, stimulated cell production of *P. shumwayae* sp. nov. and *P. piscicida* to significantly higher abundances than initial den-sities of 150 zoospores/mL ($P < 0.05$; Figs. 2, 3). In contrast, control *Pfiesteria* spp. without nutrients and cryptomonad prey mostly encysted, and control populations of *Pfiesteria* spp. + cryptomonad prey but without nutrients yielded signif-icantly lower zoospore production (< 10^3 cells/ mL than N- or P-enriched *Pfiesteria* spp. (at all nutrient additions + cryptomonad prey. Cryptomonads without *Pfiesteria* increased exponentially, with maximal densities at the highest nutrient levels (3.08×10^5 cells/mL and 3.61×10^5 cells/ mL at 1000 µg NO_3^-N/L and 1000 µg PO_4^{-3} P /L, respectively).

Zoospore densities across all N and P concentrations + cryptomonad prey remained at < 4.0×10^4 cells/mL. *P. shumwayae* sp. nov. showed higher stimulation by N_i enrichment + cryptomonad prey ($P < 0.05$; Figs. 2, 3). *P. shumwayae* sp. nov. attained significantly higher zoospore production than *P. piscicida* at each nitrate enrichment level, with the stimulatory effect apparently mediated by algal prey abundance (significant correlation between *P. shumwayae* sp. nov. zoospore densities and cryptomonad abundance; $P < 0.05$). Highest zoospore densities ($1.17 \pm 0.08 \times 10^4$ cells/ mL) were observed at the intermediate N_i concentration + cryptomonad prey, coinciding with a 15:1 zoospore : crypto-monad ratio. This ratio was the highest observed among the controls and treatments (zoospore : prey ratio < 11:1). High *Pfiesteria* grazing activity and zoospore production, together with a noticeably longer lag period (> 1 day) than in other treatments before cryptomonad cells began to reproduce, led to prey depletion by *P. shumwayae* sp. nov. and swarming activity [30] under moderate N_i enrichment. Prey depletion and zoospore swarming behavior did not occur at other N_i or P_i levels. *P. shumwayae* sp. nov. zoospores rapidly repro-duced at the highest N_i enrichment, and they apparently grazed the cryptomonad population before prey cell densities were sufficient to allow for the high zoospore production that was observed at the moderate N_i level. In contrast, *P. pisci-cida* zoospore production were more strongly enhanced by P_i additions; highest densities occurred at $2.70 \pm 1.5 \times 10^4$ zoo-spores/mL [mean ± 1 SE]), corresponding to the highest P_i level (Fig. 3). There was a trend toward higher production by *P. piscicida* than by *P. shumwayae* sp. nov. at each P_i level ($r^2 = 0.88$ [*P. shumwayae*] to 0.98 [*P. piscicida*]; $P < 0.05$). The stimulatory effect of P_i was correlated with prey abundance for both *Pfiesteria* spp. cell production ($P < 0.05$).

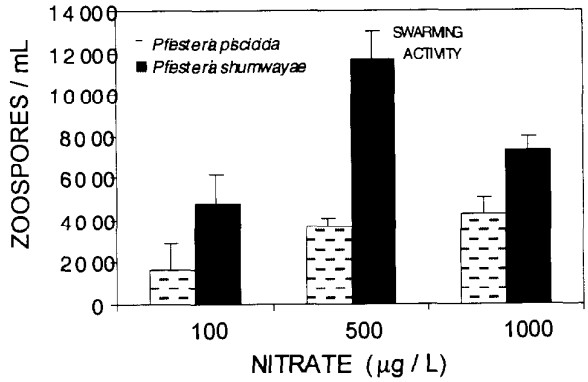

Figure 2. Response of *Pfiesteria piscicida* and *Pfiesteria shumwayae* spp. nov. TOX-B zoospores to N_i enrichment + (N, P-limited) cryptomonad prey in batch cultures. Zoospore production for each species was higher + N_i than in un-enriched controls (at 10 ± 3 µg NO_3^-N/L; <500 cells/mL; $P < 0.05$; means ± 1 SE, n=4; repeated twice with similar results).

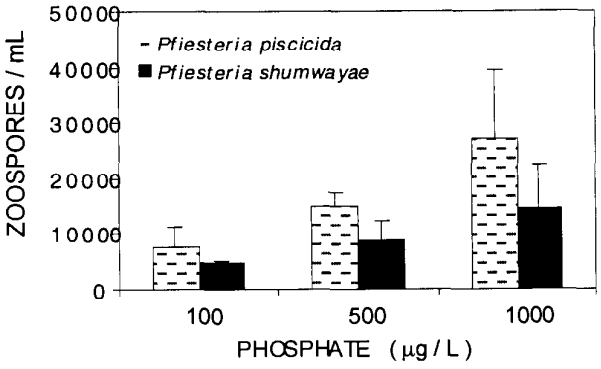

Figure 3. Response of *Pfiesteria* spp. TOX-B zoospores to P_i enrichment + (N, P-limited) cryptomonads in batch cultures. Zoospore production was higher + P_i than in un-enriched controls (at 5 ± 2 µg $PO_4^{-3}P$/L; < 500 cells/mL; $P < 0.05$; means ± 1 SE, n=4; repeated twice with similar results).

DISCUSSION

In the decade since we first tracked toxic *Pfiesteria* in certain major estuarine fish kills in the southeastern U.S., much attention has been directed toward cryptic dinoflagel-lates in estuaries. The small 'gymnodinioid-'appearing (actu-ally thinly armored, peridinioid [4, 9,

23]), heterotrophic zoospores of these species had been overlooked because of their size (diameter commonly $\leq$ 10 μm); lack of pigmenta-tion; tendency to lyse, encyst, or adsorb to sediment particles during standard phytoplankton collection/preservation proce-dures [24], and lack of growth in defined culture media [2, 3, 6, 14]. The recent focus on small estuarine dinoflagellates has revealed other 'pfiesteria-like' species with complex life cycles [9, 25], and/or ambush-predator behavior [26]. Yet, thus far only two ***toxic*** *Pfiesteria*/'pfiesteria-like' species have been verified (neither clonal status nor toxicity were verified in [27]) – indicating that complex life cycles and attack behavior are more common than toxicity [9, 10, 14].

Like *P. piscicida,* the second known toxic *Pfiesteria* species thrives in eutrophic, poorly flushed, shallow estuar-ies [6, 8, 19]. In this study, higher production of toxic strains of *P. shumwayae* sp. nov. in temporarily nontoxic mode occurred with N_i than with P_i enrichment. The effect appar-ently was partly mediated through algal prey abundance. Thus far, general nutrition of the two species appears similar, with prey ranging from bacteria to mammalian tissues [6, 9]. But subtle distinctions in comparative responses of *Pfiesteria* spp., such as effects of N vs. P additions in this study, may provide insights about long-term changes in abundance and toxicity. In the Neuse Estuary we observed ca. 40% increase in N_i and 15% decrease in TP in the 1990s [10, 28]. These trends have coincided with an apparent increase in *P. shumwayae* sp. nov. over time, based on PCR analyses [15, 16] of archived water samples from fish kills [5, 6, 7, 10].

Recently available molecular probes [15, 16], together with our standardized fish bioassay process [6, 9, 13, 14, 19], have been used to detect and verify toxicity of *P. piscicida* and/or *P. shumwayae* sp. nov. in the mid-Atlantic and south-eastern U.S., Scandinavia and New Zealand [29]. Such find-ings exemplify the rapid progress that is being contributed by many researchers on the biology, biogeography, nutrition, and impacts of species within the toxic *Pfiesteria* complex.

ACKNOWLEDGMENTS

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COMPARATIVE RESPONSE TO ALGAL PREY BY *PFIESTERIA PISCICIDA, PFIESTERIA SHUMWAYAE* AND AN ESTUARINE 'LOOKALIKE' SPECIES

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ABSTRACT

Zoospore production was examined during grazing on algal prey (*Rhodomonas* sp., in single-species batch-culture trials) by Neuse Estuary clonal isolates of *Pfiesteria pisci-cida, Pfiesteria shumwayae* sp. nov., and a benign cryptoperi-diniopsoid species. Sub-cultures of *P. piscicida* from the same clone had been maintained for 4 months in fish-killing mode (TOX-A functional type, actively toxic) or, separately, in temporarily nontoxic mode (TOX-B, tested as capable of killing fish but grown on cryptomonad prey without fish for 3 weeks). An older clone of *P. piscicida* was non-inducible (NON-IND, having lost its fish-killing ability over time in culture; grown for 6 months on cryptomonads). *P. shum-wayae* sp. nov., TOX-A and TOX-B cultures (same clone of origin) had been maintained similarly as TOX-A and TOX-B *P. piscicida*, respectively. The cryptoperidiniopsoid had been fed cryptomonads as had TOX-B *Pfiesteria* spp. NON-IND *P. piscicida* and the cryptoperidiniopsoid had the highest zoospore production, appeared to retain kleptochloroplasts for longer periods, and exerted the lowest grazing pressure on algal prey, suggesting higher reliance on photosynthesis. Zoospore production was intermediate by TOX-B *Pfiesteria* spp., which were comparable in grazing to TOX-A *Pfiesteria* spp. TOX-A *P. piscicida* zoospore production (weakly toxic, based on zoospore densities and time to fish death) was significantly lower than that by TOX-B or NON-IND cultures, or by the cryptoperidiniopsoid. Lowest production was shown by the (highly toxic) TOX-A *P. shumwayae* strain which mostly encysted when forced to change to an algal diet. Thus, zoospore production on algal prey differed signif-icantly among these dinoflagellates, depending on history of toxic activity (*Pfiesteria* spp.) and proclivity for mixotrophy.

INTRODUCTION

In the presence of live fish or their fresh tissues and excreta, dinoflagellates within the toxic *Pfiesteria* complex are stimulated to produce toxins or bioactive compounds that cause fish stress, disease, and death [1, 2]. The two toxic *Pfiesteria* species known thus far include *P. piscicida* and the *P. shumwayae* sp. nov. [3, 4, 5, 6]. Toxic strains of these spe-cies are strongly attracted to live fish prey [1, 7]. These char-acteristics – strong attraction to live fish, toxicity stimulated by the presence of live fish, ***and*** production of ichthyotoxins (as in [8]) that cause fish death and disease under ecologi-cally relevant conditions [8, 9] – are required for species placement within the toxic *Pfiesteria* complex [1, 6, 8, 9].

Pfiesteria spp. have small, cryptic peridinoid zoospores [4, 6] that closely resemble the zoospores of various other, mostly undescribed 'gymnodinioid'-like estuarine dinoflagel-late species [1, 3, 10, 11]. Some of these, such as *Cryptoperi-diniopsis* spp. (gen. ined.) spp. [12], have a complex life cycle [13] similar to that previously described for *P. pisci-cida* [4, 14] and *P. shumwayae* sp. nov. [6], with flagellated, amoeboid and cyst stages. However, all *Pfiesteria* 'looka-like' species examined thus far, such as cryptoperidiniopsoid species and a species informally called 'shepherd's crook [12], have shown no ability to cause fish disease and death in the standardized fish bioassay process (1, 6. 8, 9, 15) via toxin production by live cells at densities within field range [8, 9, 15, 16]. These replicated, repeated fish bioassays (dura-tion 10-12 weeks) included multiple strains, tested separ-ately, from estuaries in different regions [6].

The above information on *Pfiesteria* spp. refers to toxic strains [1, 7, 8, 9, 15]. Many well known, so-called "toxic" algae (here, including toxic heterotrophic dinoflagellates as in [5]; e.g., toxic cyanobacteria [17], toxic prymnesiophytes [18], the toxic *Pseudo-nitzschia* complex [19], and the toxic *Alexandrium* complex [20]), as well as *Pfiesteria* spp., have naturally occurring strains that apparently are non-inducible (incapable of producing detectable toxin [9]). Moreover, as an apparent artifact of laboratory conditions, many toxic strains of 'toxic algae' lose their ability to produce toxin over time in culture [19, 20, 21]. Toxic *Pfiesteria* strains have become non-inducible over time in culture with fish (in 98% of the clones, after 8-10 months; in the remaining 2% of the clones, after 1-3 years), more quickly if fed non-fish prey (after 2-6 months [8, 9, 15]).

Pfiesteria spp. zoospores have proficient grazing ability, particularly on some algal prey species [1, 22]. Zoospores typically feed myzocytotically by attaching peduncle to the prey cell and suctioning the contents into a food vacuole for digestion. Cryptoperidiniopsoid species consume algal prey by the same mechanism [13]. In addition, *Pfiesteria* spp. and cryptoperidiniopsoid species can engage in limited pho-totrophy through acquisition of kleptochloroplasts from algal prey [6, 23]. Our observations have indicated, however, that important differences exist between and within individual species with respect to algal grazing activity.

The objective of the present study was to evaluate the zoospore production, grazing, and potential for mixotrophy of different functional types of *Pfiesteria piscicida, P. shum-wayae* sp. nov., and the cryptoperidiniopsoid species when given cryptomonad prey. This work represents one facet of the research being conducted in our laboratory to further understanding of the complex nutritional strategies employed by toxic *Pfiesteria* complex dinoflagellates and certain spe-cies of close morphological resemblance ('pfiesteria-like' species

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Intergovernmental Oceanographic Commission of UNESCO 2001

as in [8]; note that [1] used this term in reference only to ichthyotoxic species), and to strengthen insights about trophic controls on stage abundance and toxicity.

MATERIALS AND METHODS

For this research, we defined a strain as a group of organisms within a species that is characterized by some particular quality [24]. We additionally considered the following functional types for *Pfiesteria* spp., within and/or among strains: (1) actively toxic (TOX-A [8, 9], referred to as toxic in [1]), in fish-killing mode immediately before the experiment; (2) temporarily nontoxic (TOX-B [8, 9], referred to as nontoxic in [1]), capable of ichthyotoxic activity but presently in a nontoxic mode – given algal prey but not live fish); and (3) non-inducible (NON-IND in [8]), incapable of producing bioactive substances that cause fish distress, disease and death, based on present understanding of induc-tion of toxicity [9]. Within the same strain, the TOX-B func-tional type serves as the 'seed' population for TOX-A *Pfies-teria*. Neuse Estuary clonal cultures were obtained using flow cytometry as in [6, 9]. Species were identified from SEM (suture-swollen cells as in [1, 6]) and PCR molecular probes ([25, 26]). Toxicity of *Pfiesteria* spp. was verified by fish bioassays [1, 8, 9, 15]. *Pfiesteria* spp. have not been grown successfully without addition of algae, fish or other prey [1, 9]. Thus, clonal *Pfiesteria* was considered as uni-dinoflagel-late-species culture grown from 1 cell, with endosymbiont bacteria and algal prey (as in [8]).

The three dinoflagellate taxa were tested separately with algal prey in single-species trials. Six treatments were estab-lished with an initial cell density of 150 dinoflagellate zoo-spores/mL (n=3): (1) Actively toxic *P. piscicida* (*P.pisc* TOX-A functional type, Center for Applied Aquatic Ecology [CAAE] clone #271A-1; weakly toxic, causing fish death in the standardized fish bioassay process [1, 8, 15] in 12 to 24 hours at ca. 5,000 toxic zoospores [TZs]/mL); (2) Temporari-ly nontoxic *P. piscicida* (*P.pisc* TOX-B functional type, CAAE clone #271A-1; tested as capable of killing fish in the fish bioassay process, then grown on cryptomonad prey [*Rhodomonas* sp., cloned from commercial CCMP 757], without fish for 3 weeks); (3) Non-inducible *P. piscicida* (*P.pisc* NON-IND functional type, CAAE clone #114-1-6, an older clone that had lost its ability to cause fish disease and death; had not been toxic upon repeated testing over 2 years in the fish bioassay process [1, 9, 15]; had previously been grown for 6 months with cryptomonads; (4) Actively toxic *Pfiesteria shumwayae* sp. nov. (*P.shum* TOX-A, CAAE clone #104T; a highly toxic isolate, causing fish death in standardized fish bioassays [1, 8, 9, 15] in < 1-2 hours at ≥ 800 TZs/mL); (5) Temporarily nontoxic *P. shumwayae* sp. nov., (TOX-B, CAAE clone #104T; tested as capable of killing fish, then grown on cryptomonads without fish for 3 weeks); and (6) a cryptoperidiniopsoid species (CAAE Neuse clone #7B-2) fed cryptomonads for the previous 6 months. Note that an isolate of the NON-IND functional type of *P. shumwayae* sp. nov., similar in history to the NON-IND *P. piscicida* isolate, was not available for this study.

In the experiments, the dinoflagellates were given algal prey as *Rhodomonas* sp. (CCMP757, Bigelow Laboratory, Boothbay Harbor, Maine, U.S.A.) in a 1:15 predator : prey ratio, in batch cultures with both the predators and the prey added initially. This prey was selected because of known pre-ference by *Pfiesteria* spp. for cryptomonads among algal prey species [14, 23]. The *Rhodomonas* sp. was cloned fol-lowing its arrival from the commercial source [6, 9]. Algal prey controls (no dinoflagellates) and dinoflagellate controls (no algal prey) were also established (n = 3) for each species and functional type. The cultures were maintained in 15 psu, sterile-filtered f/1000 medium, at 20°C and 50 μmol m^{-2} s^{-1}, on a 12:12 hour L:D cycle. They were gently mixed and sub-sampled at 12-hour intervals for 6 days. Subsamples were preserved with acidic Lugol's solution [27], and were ana-lyzed for cell abundance and species using light microscopy (200x, phase contrast, Olympus BH2 microscope, Olympus Corporation, Melville, NY; Palmer chambers as in [27]). The data were corroborated with a Coulter Multisizer IIe particle analyzer, Coulter Corp., Miami, FL. One-way ANOVA was used to test for differences between controls and treatments, and among treatment means. Probability values were consi-dered significant at $p < 0.05$ [28].

Mixotrophic potential was assessed for controls and each treatment at the end of the experiment (day 6) using light and epifluorescence microscopy (600x, phase contrast, Olympus BH2 microscope with fluorescence attachment) on samples that had been preserved with 2% buffered formalin [29]. Zoospores containing kleptochloroplasts were readily dis-cerned from those without, since the pigmented inclusions autofluoresced reddish-orange under UV excitation [6, 23]. Starch bodies were easily distinguished, as well, in phase contrast [6, 23]. The percentage of zoospores containing typical chloroplast inclusions and starch bodies indicative of kleptochloroplastidy in each treatment was determined for at least 100 cells $sample^{-1}$, and was inferred to be directly related to mixotrophic potential [23]. Growth and ingestion rates were estimated for the time interval when the zoospore populations increased (days 1-5, after which some treatments became prey-limited) following the equations of [29, 30, 31].

RESULTS

All dinoflagellate controls (without algal prey) demon-strated negligible cell production. The dinoflagellate species and functional types differed significantly in response to algal prey (Table 1). TOX-A *Pfiesteria* spp. (from fish-killing cultures; term used here in reference to functional type status initially in experiments) had significantly lower cell production with algal prey than TOX-B *Pfiesteria* spp. and the cryptoperidiniopsoid (Fig. 1). Among treatments with (recently) actively toxic cultures, NON-IND *P. piscicida* attained highest cell production, followed by the cryptoperi-diniopsoid (day 6). (Weakly toxic) TOX-A *Pfiesteria pisci-cida* had significantly higher cell production on algal prey than (highly toxic) TOX-A *P. shumwayae*, which had lowest cell production. Thus, zoospore production on cryptomonads was directly related to history of toxicity (or lack thereof, for the cryptoperidiniopsoid and the NON-IND *Pfiesteria* [9]):

NON-IND > Cryptoperi- >> TOX-B >> weakly > highly
P.piscicida diniopsoid sp. *Pfiesteria* spp. toxic toxic
TOX-A TOX-A
P. piscicida *P. shumwayae*

After 6 days, the treatments with highest zoospore pro-duction (*P. piscicida* NON-IND and the crytpoperidiniop-soid) had exerted low to intermediate grazing pressure on their algal prey, in comparison to other treatments and algal prey controls (Table 1). This apparent contradiction resulted from the increased proclivity for mixotrophy that was shown by nontoxic (NON-IND) *P. piscicida* and the cryptoperidini-opsoid and, to a lesser extent, by the temporarily nontoxic (TOX-B) *Pfiesteria* spp., relative to toxic (i.e., recently TOX-A) *Pfiesteria* spp. Most NON-IND zoospores had functional kleptochloroplasts, and apparently achieved higher cell pro-duction via mixotrophy relative to TOX-A and TOX-B *Pfies-teria* spp. and the cryptoperidiniopsoid. Cryptoperidiniop-soid zoospores also engaged in mixotrophy (ca. 70% of the cells with viable kleptochloroplasts and darkly staining starch bodies). TOX-B *Pfiesteria* spp. zoospores were intermediate in kleptochloroplast retention (8-10% with viable klepto-chloroplasts). In contrast, TOX-A *Pfiesteria* spp. did not re-tain kleptochloroplasts (in < 1% of the zoospores), and likely depended solely on heterotrophy for nutrition.

The potential for partial reliance on photosynthesis (mix-otrophy) was also inferred from grazing activity on crypto- monad prey among the dinoflagellate species and functional types. Highest ingestion rates were demonstrated by toxic *Pfiesteria piscicida* (TOX-A and TOX-B, also indicated by prey depletion). NON-IND *P. piscicida* and the cryptoperi-diniopsoid showed significantly lower grazing activity. Thus, the higher growth rate and cell production achieved by NON-IND *P. piscicida* with lower algal grazing activity, in com-parison to the lower growth rate and cell production by toxic *Pfiesteria* spp. with higher grazing activity, apparently oc-curred via more substantial reliance on photosynthesis from kleptochloroplasts. TOX-A *P. piscicida* showed tight coupl-ing in predator-prey oscillations relative to NON-IND *P. piscicida*, indicating higher (heterotrophic) reliance on the prey for zoospore production than by kleptochloroplastidic (mixotrophic) NON-IND *P. piscicida* [23, 29, 30, 31] (Fig.2).

DISCUSSION

The nutritional strategies of these dinoflagellate species ranged from heterotrophy (actively toxic [TOX-A] *Pfiester-ia* spp.) to increased reliance on functional mixotrophy (tem-porarily nontoxic [TOX-B] *Pfiesteria* spp., the cryptoperidin-iopsoid, and NON-IND *Pfiesteria*). These differences in trophic strategy promoted significant differences in popula-tion dynamics among the species and strains. Even within a *Pfiesteria* species, the three functional types showed distinct population dynamics with algal prey. Recent TOX-A *Pfies- teria* spp. maintained low growth on algal prey relative to other functional types (also in [9]). But the most tightly coupled Lotka-Volterra fluctuations occurred for TOX-A *P. piscicida* zoospores and their algal prey [31]. Species and strains with kleptochloroplasts engaged in photosynthesis and, thus, likely were less reliant on algal prey for nutrition.

Mixotrophy (with kleptochloroplasts) apparently enabled substantial increase in growth rates and cell production for the strains and functional types of *Pfiesteria* spp. and crypto-peridiniopsoid species that were capable of this form of photosynthetic carbon acquisition.

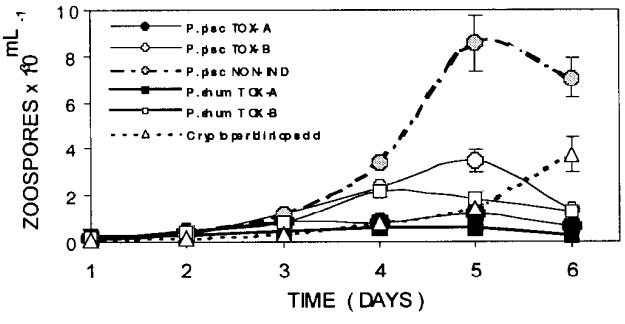
Table 1. Mean zoospore specific growth rates, division times, maximum yields, ingestion rates, and percentage with mixotrophic inclusions by the end of the 6-day experiment, for each species and strain tested. Note that specific growth (k) and ingestion rages were calculated for the time interval of exponential zoospore production (days 1-5), excluding influence of other life stages (e.g., amoebae).[1]

Treatment	Sp.Gr.Rate (k, div. day^{-1})	M.D.T. (T_D) (hours)	Max.Yield (cells mL^{-1})	Ingest. Rate (I)	Mixotrophy (% cells)
P. piscicida					
NON-IND	1.43 ± 0.04	16.8 ± 0.5	8540 ± 1200	9.4 ± 0.3	95
TOX-B	1.42 ± 0.05	17.0 ± 0.6	3470 ± 450	54.5 ± 3.4	8
TOX-A	0.75 ± 0.04	32.1 ± 1.8	1230 ± 140	55.8 ± 3.9	< 1
P. shumwayae					
TOX-B	0.84 ± 0.10	29.2 ± 3.2	1820 ± 340	37.3 ± 1.9	10
TOX-A[2]	0.22 ± 0.07	127.0 ± 31.2	590 ± 120	8.7 ± 0.4	< 1
Cryptoper.	1.15 ± 0.05	20.9 ± 0.85	3750 ± 780	21.0 ± 2.4	70

[1] Data include sp. gr. rate (specific growth rate , k, divisions day^{-1}), M.D.T. (mean division time), max yield (maximum yield of zoospores), ingestion rate (I, prey cells zoospore^{-1} day^{-1}), and 'mixotrophy' (percentage of zoospores with autofluorescent pigmented inclusions and localized starch bodies on day 6 – determined with light microscopy and epifluorescence, for ≥ 100 zoospores sample^{-1}). Rates were calculated following equations of [29, 30].

[2] Values for the TOX-A functional type of *P. shumwayae* sp. nov. were based on cell production estimates from clone #104T only. Other clonal *P. shumwayae* cultures (n=3) that were treated similarly had negligible zoospore production during the experimental period.

Figure 1. Response of different functional types of *P. piscicida*, *P. shumwayae* sp. nov., and the cryptoperidiniopsoid zoospores to *Rhodomonas* sp. prey in 6-day trials. NON-IND *P. piscicida* and the cryptoperidiniopsoid attained highest zoospore production on algal prey, with temporarily nontoxic (TOX-B) and actively toxic (TOX-A) *Pfiesteria* spp. attaining less production, respectively.

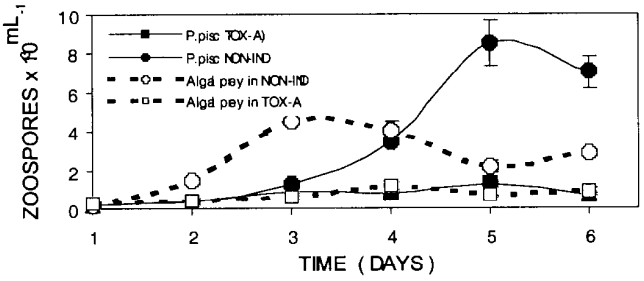


Although (weakly) TOX-A *P. piscicida* maintained higher grazing activity on algal prey, its growth rate and cell production were low relative to that of other *P. piscicida* functional types fed the same prey, suggesting that recently toxic populations are not as adept at retaining and utilizing photosynthesis from kleptochloro-plasts in supplying carbon and energy for cell production.

The (highly) TOX-A *P. shumwayae* zoospores had both the lowest growth and lowest ingestion rates on algal prey

among the taxa tested, with negligible kleptochloroplast retention. The data indicate that the cytological switch from heterotrophy to mixotrophy is not instantaneous but,

Figure 2. Variable population dynamics between toxic (TOX-A) *P. piscicida* with *Rhodomonas* sp. prey and NON-IND *P. piscicida* with *Rhodomonas* sp. prey in 6-day trials. The TOX-A functional type demonstrated tight apparent coupling in predator-prey oscilla-tions relative to the NON-IND *P. piscicida*.



rather, is regulated by certain biochemical and/or environmental con-trols. Similar trends were reported for functional types of *P. piscicida* given algal prey with nutrient additions [9]. *Pfies-teria* functional types also have shown distinct responses to fresh fish tissues, secreta and excreta [7]. We are continuing to investigate the efficiencies and cytology involved in al-tered reliance on heterotrophy vs. mixotrophy in the nutrition of the toxic *Pfiesteria* complex and closely related species.

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CYANOBACTERIA BLOOMS IN DAMMED RESERVOIRS, THE DAUGAVA RIVER, LATVIA

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ABSTRACT

High water temperatures (> 27°C) in July and August 1999 was associated with the extensive cyanobacterial bloom, conquer of *Microcystis* species (mainly *Microcystis aeruginosa* Kütz., *Microcystis wesenbergii* Kom., *Microcystis viridis* (A.Braun) Lemm. The maximum phytoplankton biomass, > 85 mg L^{-1}, was recorded in August 1999 in the lower Reservoir of Cascade. In September 1999, *Microcystis* species were partly replaced by *Anabaena* spp. and *Oscillatoria* spp. In the Riga Reservoir conspicuous large phytoplankton biomass, more than 92% of *M.* aeruginosa, formed dense surface scums and poor water quality. Observations indicated, that *Microcystis* spp. settled to the bottom of reservoirs in October, after having dominated surface waters for about three months. By September 1999, colonies of *Microcystis* associated with *Anabaena* spp., *Oscillatoria* spp., *Spirulina* sp. and unicellular diatoms formed a 1.5-3.0 cm thick green layer on sediments. High amounts of *Microcystis* in sediments would favour high microbial activity ultimately leading to phosphorus efflux and potentially increasing cyanobacteria in overlying water. It would be hazard for Riga drinking water.

INTRODUCTION

The first phytoplankton studies on Daugava River, Latvia were undertaken in 1924, when the Riga sewage collection system was expanded and phycological studies were expanded to the Riga Canal and the lower Daugava [1]. In total 480 species of algae (65 blue-greens) were observed [2, 3, 4]. First reports of cyanobacteria blooms in the cascade of hydroelectrical power plant were noted in 1977 in the Riga reservoir following filling in 1975 [5]. An increasing percentage of cyanobacteria was noted in lower Daugava [6]. The aim of present study is to show, however, the extensive cyanobacteria blooms are associated with highest observed water temperatures.

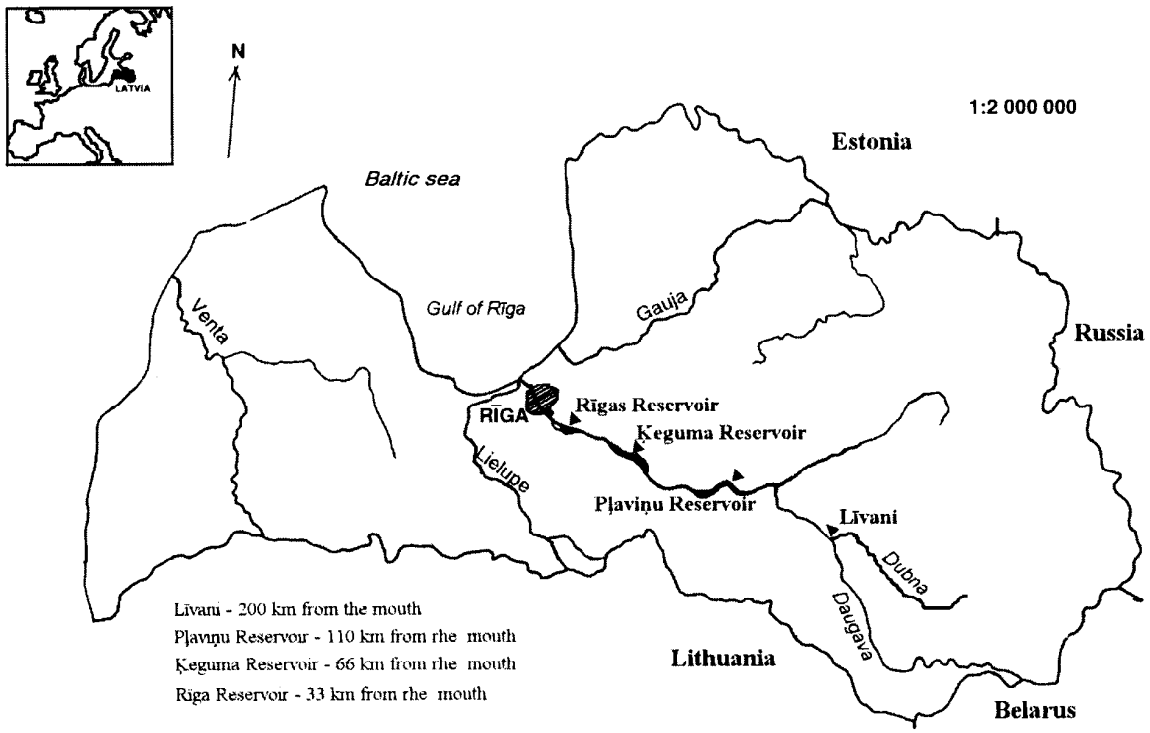

Fig. 1. Sampling sites of the Daugava River.

Harmful Algal Blooms 2000
Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J. (eds)
Intergovernmental Oceanographic Commission of UNESCO 2001

MATERIALS AND METHODS

The Daugava River is among the largest rivers (1005 km) of the Baltic Sea basin (Fig.1); the catchment area of the Daugava River is 87900 km^2 and involves portions of the Daugava River lies on the territories of five countries (Russia, Belarus, Lithuania, Estonia and Latvia); 24700 km^2 or 39% of its total area is Latvian. The runoff of the river is affected by the operation of a cascade of 3 reservoirs constructed in 1939 (Keguma HPP), 1962 (Plavinu HPP) and 1975 (Riga HPP) (table 1). Mean July – August temperature for Daugava reservoirs is 21-22.2°C.

Table 1. Characteristics of the Daugava Reservoirs

Daugava Dammed Reservoirs	Area, km^2	Mean depthm	Max depth M	Volume, mlnm^3	Retention time, years
Plavinu	34.9	14.6	42	509	33
Keguma	24.9	9.5	16.5	157	27
Riga	42.4	7.5	18	339	19

Phytoplankton samples were collected by Ruttner type water sampler for quantitative analysis. Samples were immediately fixed with aqueous formaldehyde (40 ml/l). Phytoplankton species biomass was determined by counting cell numbers and measuring cell volumes. Algal volume was converted to biomass by assuming the specific density of the cells to be equal to that of water. Live samples used for qualitative species identification were taken with a 10 μm plankton net by vertical hauls through the water column. Cell numbers were determined with a Zeiss microscope using magnifications of 100x, 200x , 400x,and 600x [7].

RESULTS AND DISCUSSION

There first phycologic studies over the length the Daugava River were done from 1959 to 1964 [2]. Before dam construction up epiphytes like *Meridion circulare* Ag., *Diatoma vulgare* Bory., *Rhoicosphaenia curvata* Grun. and, *Cocconeis pediculus* Ehr. dominated the phytoplankton. A potamophilous complex of phytoplankton species typified by diatoms and greens has been completely replaced by limnophilous diatoms *Aulacoseira italica* (Ehr.) Simonsen, *Aulacoseira italica* var. *tenuissima* (Grun.) Simonsen and colony forming blue – green algae species *Microcystis aeruginosa* Kütz., *Microcystis pulverea* (Wood) Forti, *Gomphosphaeria* spp., *Aphanizomenon flos-aquae* (L.) Ralfs.
Phytoplankton succession in the Daugava Reservoirs is comprised of a spring diatom bloom, followed by a period with relatively low phytoplankton biomass dominated by chlorophytes and small numbers of diatoms. For example, investigations on phytoplankton dynamics (by athor) from 1978-1980 in Riga Reservoir - the last reservoir formed in 1975, show a typical development of phytoplankton divisions for river-type reservoirs (Fig. 2).

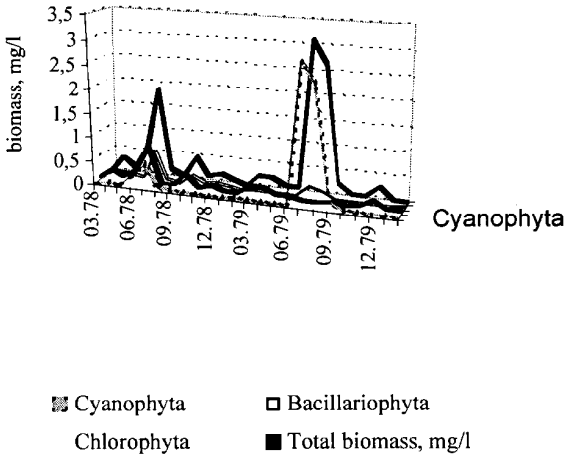

Fig.2. Dynamics of phytoplankton biomass in Riga Reservoir, 1978-1980.

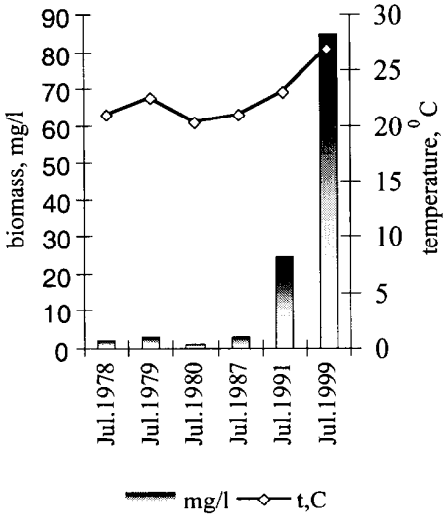

Fig.3. Dynamics of Cyanobacteria biomass (mg/l) and temperature C° in Riga Reservoir, 1978-1999.

Enhanced temperatures above 27°C in July and August 1999 coincided with the extensive cyanobacterial bloom of mixed composition dominated by *Microcystis* species (mainly *M. aeruginosa* Kütz., *Microcystis wesenbergii* Kom., *Microcystis viridis* (A.Br.) Lemm. The maximum phytoplankton biomass was more than 85

mgL^{-1}, recorded in August 1999 24.7 mgL^{-1} in July 1991 in the lower Reservoir of Cascade - Riga Reservoir. In July 1999, there was an increase of cyanobacteria biomass in the cascade of Reservoirs downstream in the Daugava River (Fig.4). In September 1999, *Microcystis* species were partly replaced by *Anabaena* spp. and *Oscillatoria* spp. The *Microcystis* spp. settled to the bottom of the reservoirs in October, after having dominated for about three months. This conspicuous large phytoplankton biomass was constituted, of > 92% *Microcystis aeruginosa* Kütz. and dense surface scums of cyanobacteria have resulted in poorer water quality not only in Riga Reservoir but in the lower Daugava and Riga canal. In contrast, in July and September 2000 (water temperature 18-20 C°) practically there were no blooms of Cyanobacteria in Daugava Reservoirs.

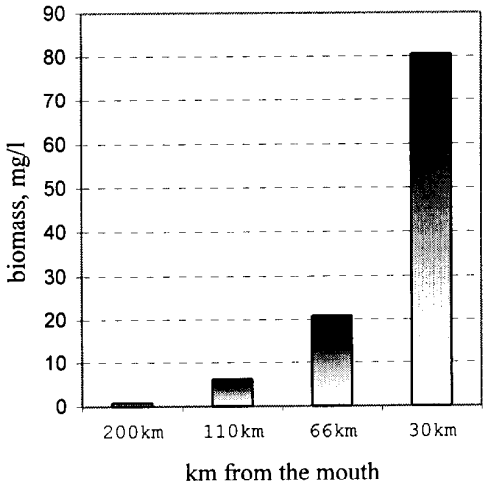

Fig. 4. Dynamics of Cyanobacteria biomass downstream the Daugava River, July 1999.

The hydrochemical composition of water and sediment of the lower Daugava are mainly determined by the operation of the Riga hydroelectrical power plant, by periodic inflow of brackish waters from the Riga Gulf, and as well by intensity of hydrodynamic processes due to the impact of the Riga city [4]. These processes control the Daugava estuary area and Riga canal. For September 1999, colonies of *Microcystis* spp. associated with *Anabaena* spp., *Oscillatoria* spp., *Spirulina* sp. and unicellular diatoms formed a 1.5-3.0 cm thick green layer on the sediment surface. These data suggest that high amounts of *Microcystis* in the sediments of Riga reservoir and *Aphanizomenon* in the sediments of Daugava estuary and Riga canal should increase microbial activity, ultimately leading to phosphorus efflux from the sediments thereby potentially increasing hazards arriving from additional toxin forming species.

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CO-OCCURRENCE OF PSP TOXINS AND MICROCYSTINS IN MONTARGIL FRESHWATER RESERVOIR, PORTUGAL.

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ABSTRACT

Since 1996, regular phytoplankton quantifications were carried out in several Portuguese freshwater reservoirs, in order to point out the most problematic regions in terms of cyanobacteria blooms and toxic occurrences. In Montargil reservoir (39°N, 8°W) an intensive bloom of phytoplankton was detected in May, with algal material accumulating on the water surface. Two predominant cyanobacteria, *Aphanizomenon flos-aquae* (L.) Ralfs and *Microcystis aeruginosa* Küetzing, were identified after microscopical examination and the extracts of the samples collected during the bloom period revealed high toxicity by mouse bioassay. In order to distinguish, characterize and identify the organisms and the toxins responsible for the observed toxicity, different strains of cyanobacteria were isolated and established in culture. The presence of both toxic and non toxic strains of *M. aeruginosa* during the bloom, occurring simultaneously with a PSP producer *A. flos-aquae* was confirmed by ELISA and HPLC analysis of the cultured strains. The complexity of cyanobacteria community and the co-occurrence of different types of toxins in freshwater environments are probably frequent situations and demand careful attention in assessing risk for human health.

INTRODUCTION

Cyanobacterial toxins are a diverse series of organic compounds, which can be classified into different groups according to their targets and toxic actions [1,2]. Hepatotoxins and neurotoxins are the types most commonly found in surface freshwater and have been related to several incidents of animal and human illness [3,4].

The most prevalent of the recognised hepatotoxins are the microcystins (MCYST), a family of more than 50 structurally similar monocyclic heptapeptides, commonly found in *Microcystis*, but also reported in other cyanobacteria, such as *Anabaena*, *Oscillatoria* and *Nostoc* [5]. These compounds have severe acute and long-term toxic effects in animal and human health [1,6,7].

Saxitoxin and related toxins are potent neurotoxins responsible for the human poisoning syndrome called paralytic shellfish poison (PSP). PSP toxins are not considered as widespread in freshwater supplies and they do not appear to pose the same degree of risk from chronic exposure as microcystins [8,9]. However several species of cyanobacteria, including *Aphanizomenon flos-aquae, Anabaena circinalis, Lyngbya wollei* and *Cylindrospermopsis raciborskii* have been reported as PSP producers in freshwater environments [10,11,12,13] and a significant number of animal deaths have been reported from neurotoxic freshwater bloom occurrences [4,11,13].

In Portugal, like in many other areas of the world, toxic cyanobacteria blooms are very common events [14,15]. Montargil reservoir is one of the problematic reservoirs in terms of cyanobacteria. During monitoring tests in 1996, an intensive bloom of cyanobacteria was detected and bioassays carried out with the concentrated bloom material showed evident signs of mixed toxicity. Hepatotoxicity was observed during *M. aeruginosa* dominant period but clear signs of neurointoxication were also reported, associated with high densities of *A. flos-aquae* [16].

To determine the specific involvement of the different species on the overall natural toxicity, strains of *A. flos-aquae, M. aeruginosa* and *Anabaena spiroides* were isolated from the natural samples and established in culture. The production of PSP toxins by *A. flos-aquae* was recently confirmed [16]. Here we present evidences of simultaneous occurrence of PSP toxins and microcystins in the natural bloom, being produced by different strains isolated from the naturally occurring population in Montargil.

MATERIAL AND METHODS

Cyanobacteria cultures

The natural bloom detected in Montargil reservoir during the spring and summer of 1996 was the source for the cultures of the cyanobacteria here studied. Their taxonomic classification was based on morphological characteristics, as described by Baker [17,18]. The isolation method and culturing conditions are described in Pereira *et al.* [16]. Mass cultures were produced in duplicate in 4-liter bulks. During culture growth, cell density was measured under the inverted microscope using either Sedgewick-Rafter or Palmer-Maloney chambers [19]. Aliquots were taken periodically for microcystins detection by ELISA. For HPLC analysis of toxins, cultures were harvested at the end of the exponential phase and concentrated either by decantation under natural light or by centrifugation before freeze-drying.

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ELISA analysis for microcystins

Competitive ELISA was performed for microcystins analysis during cultures growth using a commercially available plate kit for detection of microcystins in water (Envirogard, Strategic Diagnostics Inc., Newark, USA). Culture samples (15ml) were treated twice by freeze thawing followed by filtration over non-sterile 0.45µm filter (Millex HV13, Millipore, USA). The extracts were diluted to fall within the range of the kit standards (0.1 to 1.6 ppb microcystin-LR) and added in duplicate to the 96 well microtiter plate. Spectrophotometric measurements at 450 nm were performed with a Dinatech MR 5000 plate reader.

Extraction and HPLC-UV analysis for microcystins

Lyophilized cultured material was extracted using the procedure described by Krishnamurthy, et al. [20] with slight modifications. Briefly, the cells (100 mg) were extracted in 10 ml of butanol-methanol-water solution (5:20:75, v/v/v), for one hour at room temperature by constant magnetic stirring. After homogenization the extracts were centrifuged, the supernatants were kept and the cell pellets were re-extracted. Supernatants were combined and concentrated in a Speed Vac (AES1000, Savant, NY, USA). The extracts were subjected to a C_{18} Sep-Pak solid-phase extraction, the toxin-containing fractions eluted with methanol 80% (v/v) and filtered through 0.45 µm membrane prior to HPLC analysis.

Reversed phase HPLC-UV was carried out with a Shimadzu HPLC pump (Model LC-6A) connected with a silica-base reversed phase C18 column (Hypersil ODS 5um, 150X4,6mm, Supelco Inc., Bellefonte, PA, USA). The UV detection was performed at 238 nm with a tunable absorbance detector (Waters mod. 486). As mobile phase, 0.05M Phosphate buffer and Methanol (58:42) pH 3 was used with a flow rate of 1,0 ml/min [21]. Injections of 20 µl were done using a sample injector (Rheodyne model 7125) with a 20 µl loop. All chemicals and solvents used were HPLC or analytical grade. The presence of microcystin-LR was confirmed by comparing peak retention times with those of the standard purchase from SIGMA Chemicals (St. Louis, MO, USA).

Extraction and HPLC-FLD analysis for PSP toxins

The lyophilised culture cells (10 mg) were extracted with one volume (4 ml) of 0.5 M acetic acid and two volumes of methanol : chloroform (1:1) solution. The cell suspension was sonicated in an ultra-sound bath for 5 min, stirred for 30 min at room temperature, and then centrifuged. The lower organic phase was reextracted similarly and the upper aqueous phases were combined and concentrated in a Speed Vac (AES1000, Savant, NY, USA). Prior to analyses of PSP toxins, the extracts were subjected to a C_{18} Sep-Pak solid-phase extraction and filtrated through a 5,000 Dalton cut-off filter (Millipore Co., MA, USA) [22].

The post-column HPLC-FLD analysis was carried out using a Shimazu HPLC system according to the method of Oshima [23]. Toxin separations were performed on a silica-base reversed phase column (Hypersil MOS 5um, 150X4,6mm, Supelco Inc., Bellefonte, PA, USA). Toxin concentrations were determined by comparing peak areas for each toxin with those of the standards. All chemicals and solvents used were HPLC or analytical grade.

RESULTS

Cyanobacteria strains

Six strains of the predominant cyanobacteria occurring in Montargil reservoir in 1996 were successfully isolated from the natural toxic samples, as indicated in Table 1.

Strain code	Species	Isolate	Date isolation and toxicity of natural samples
LMECYA 1	*Microcystis aeruginosa*	Single cell	May-8 neurotoxic
LMECYA 3	*M. aeruginosa*	Single colony	June-11 neurotoxic
LMECYA 7	*M. aeruginosa*	Single colony	June-11 neurotoxic
LMECYA 13	*M. aeruginosa*	Single colony	July-21 hepatotoxic
LMECYA 17	*Anabaena spiroides*	Single trichome	May-8 neurotoxic
LMECYA 31	*Aphanizomenon flos-aquae*	Single trichome	June-11 neurotoxic

Table 1: Cyanobacteria strains isolated from Montargil reservoir during the bloom period of 1996

Microcystins analysis

Fig. 1 shows the HPLC-UV chromatograms of the extracts prepared from the lyophilized LMECYA 1-17 culture material. Mcyst-LR was easily identified in the extracts from cultures LMECYA 3 (fig. 1.2) and

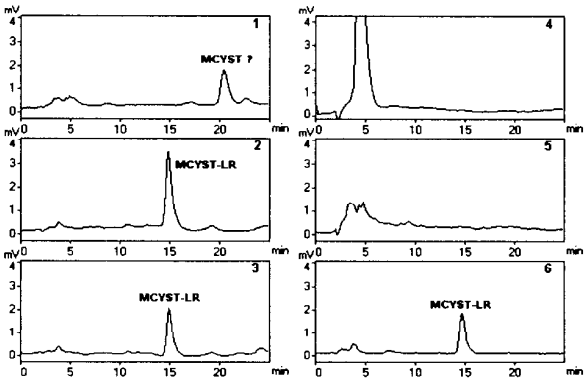

Fig. 1. HPLC-UV chromatograms for microcystins analysis of strains LMECYA 1 (1), LMECYA 3 (2), LMECYA 7 (3), LMECYA 13 (4) and LMECYA 17 (5) and microcystin-LR standard (6).

LMECYA 7 (fig. 1.3) by comparison with the corresponding standard mcyst eluting at 14,6 min (fig. 1.6). Mcyst-LR was the only microcystin detected in both culture extracts and the toxin content of the cultured LMECYA 3 (2,28 µg/mg dry weight) is more than twice the toxin content detected in LMECYA 7 (1,02 µg/mg d.w.).

The analysis of the extracts obtained from the cultures LMECYA 1, 13 and 17 showed no peaks with retention times close to the standard mcyst-LR. However LMECYA 1 showed one major peak eluting at 20,3 min. (fig. 1.1). The compound could not be identified as a certain microcystin but by the UV spectrum may be assigned as a microcystin. Moreover, when the production of mcyst of this culture was analyzed by ELISA the results were comparable with those of LMECYA 3 and 7. On the contrary, mcyst were not detected by ELISA for LMECYA 13 and for LMECYA17.

Figure 2 shows the growth curves of strain LMECYA 1 and LMECYA 7 and the mcyst concentrations in the cultures (µg/L) detected by ELISA. Although the presence of mcyst was detected in both strains, the detailed analysis of these cultures shows that the patterns of toxin production seem to be quite different along the growth cycle of each strain. Whereas culture LMECYA 1 shows a steady increase in mcyst concentration (µg/L) during the exponential and stationary phases, the culture LMECYA 7 shows a pronounced increase in mcyst concentration during the exponential phase followed by a marked decreased during the stationary phase.

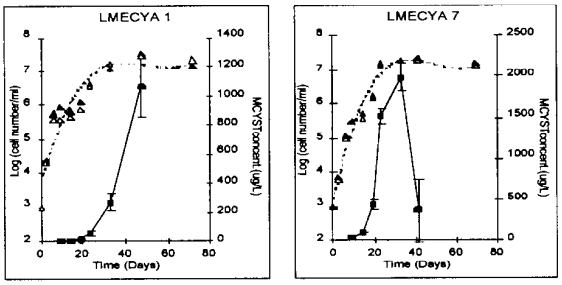

Fig. 2. Growth curves (dashed lines) and microcystins concentrations (mg/L) detected by ELISA for LMECYA 1 and 7 (bars=±std.dev.).

It is important to notice that the ELISA procedure here used measures total mcyst content in the cultures, including both mcyst cell contents and mcyst dissolved in the medium after cell lysis. Assuming cell lysis is low during initial and exponential growth phases, we estimated the mcyst content per cell (fentomole/cell) for each strain throughout these growth phases based on mcyst concentrations (µg/L) in the culture and its cell densities (cells/L) (fig. 3).

The graphs show marked differences between the two strains with respect to mcyst content per cell (10 to 20 times higher in LMECYA 7). However the differences found could be due to the low cross reactivity of the antibodies supplied with the ELISA kit (which are raised against mcyst-LR) with microcystins other than mcyst-LR. These differences reduce substantially after the sudden decrease of mcyst content per cell in LMECYA 7 and the sustained increase observed in LMECYA 1.

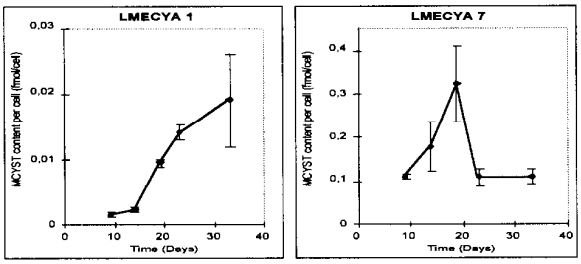

Fig. 3. Microcystins contents per cell (fentomole/cell) for LMECYA 1 and LMECYA 7 during exponential growth. Estimations were based on mcyst concentrations in culture detected by ELISA and total number of cells (bars=±std.dev.).

HPLC-FLD analysis for PSP toxins

PSP toxins were detected only in strain LMECYA 31. HPLC-FLD chromatograms of the cultured *A. flos-aquae* cells extract showed four PSP toxins: neoSTX, dcSTX, STX, and GTX5 (Fig. 4), confirming the sustained PSP production previously described [16]. The toxin content of the LMECYA 31 was 3.1 nanomole/mg dry cells, being GTX5 the major component (56.3 mole %) followed by neoSTX (16.8%), STX (12.4 %) and dcSTX (10.1 %). However, as showed by Dias *et al.* (in this conference), toxins contents and relative proportion in this strain may change along its growth cycle.

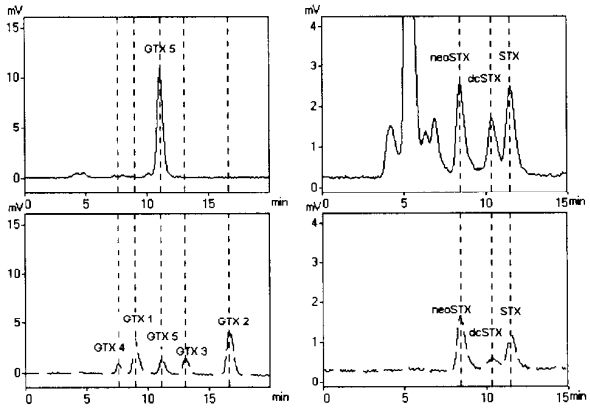

Fig. 4. HPLC-FLD chromatograms of LMECYA 31 for GTX group and for STX group (upper graphs) and for correspondent standard mixtures (lower graphs).

DISCUSSION

Cyanobacterial blooms composed of both toxic and non-toxic strains have been reported before [24] and the proportion of toxic versus non-toxic strains within the bloom community is often pointed out as a key factor influencing the overall toxicity occurring in natural environments [2].

In this study, PSP and microcystins production were detected, respectively, in separate cultured strains of *A. flos-aquae* and *M. aeruginosa* isolated from the same natural bloom sample colleted in June 11th. (Table

I), showing that both types of toxins were being simultaneously produced during the bloom. Despite the presence of microcystins, mouse bioassays performed with this sample showed only typical signs of neuro-intoxication. In fact, *A. flos-aquae* was the dominant species [16] and, given its high toxin content, the prevalence of PSP toxins over microcystins masked the later ones, which escaped one's notice when the sample was tested by mouse bioassay.

Marked differences in toxicity were observed among the *M. aeruginosa* strains isolated during the bloom period, with both mcyst producing and non-producing strains being present. Analysis by HPLC-UV revealed the production of mcyst-LR in two of the four *M. aeruginosa* isolates and the toxin content detected for LMECYA 3 (2,28 μg/mg dry weight) approximately doubles the content found in LMECYA 7 (1,02 μg/mg). Moreover, variations in the quantity of mcyst produced per cell (fentomole/cell) were also detected within the same strain during culture growth. In culture LMECYA 7, namely, the mcyst content per cell, as detected by ELISA, varied strongly along the growth cycle, increasing until mid-exponential phase and decreasing remarkably thereafter. The same analysis carried out along the growth curve of LMECYA 1 revealed a different pattern in toxin variation with a steady increase of toxin concentration throughout the all period of exponential growth. Mcyst-LR was not detected by HPLC-UV in this strain but an unidentified peak was observed with a retention time of 20,3 min. Although the nature of this compound has yet to be determined, it was deduced to be a member of the microcystins family since no other peaks were observed in the extracts that could explain the detection of microcystins by ELISA.

Toxic variations, inter and intra strains of cyanobacteria, seems to be a common feature in natural blooms [2,25]. In this study, the establishment of isolated cyanobacteria cultures from a natural bloom and its toxicological study showed evidences on the complexity of the bloom communities, which can be formed by a cocktail of cyanobacteria producing simultaneously different types of toxins, in different quantities and at different rates along the growth cycle, with some strains being non toxigenic. These characteristics support the unpredictable nature of the blooms in what respects to the overall toxicity occurring in the natural reservoir.

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HARMFUL ALGAE IN RUSSIAN EUROPEAN COASTAL WATERS.

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ABSTRACT

Nineteen species of *Dinophysis* are known from the N-E Black sea. At the Caucasian Black Sea coast - the only region of mussel farming in Russia – *D.rotundata* and *D.caudata* occur at concentrations up to 600 cellsl^{-1}, and cases of DSP poisonings were observed there during the past 4 years, and DTX2 was found in farmed mussel in 1999. Massive noxious macroalgal bloom of several *Cladophora* species develop in summer at Anapa (biggest children resort in Russia). *Pseudo-nitzschia pseudodelicatissima* and *P.seriata* form winter-spring blooms in Black Sea, and concentrations reach over 10^6cellsl^{-1}. In the N.Caspian Sea influenced by Volga discharge cyanobacterial blooms are a frequent phenomenon. In the Baltic toxic cyanobacterial blooms are recurrent in the Gulf of Finland and Kursha Gulf. *Dinophysis* species also occure there but local molluscs are not used as food. In Kandalaksha Gulf of theWhite sea, where mussel banks are abundant, and at the Kola coast of Barents Sea *D.norvegica* occurs up to 1000cellsl^{-1} in July-August. *Pseudo-nitzschia seriata* is one of the dominant species of spring bloom at Kola coast. In Pechora estuary bays (Eastern Barents coast) *Aphanizomenon/Anabaena* late summer blooms cause fish mortality every year.

INTRODUCTION

Many known potentially toxic algae inhabit Russian European coastal waters, among them species of *Dinophysis, Prorocentrum, Pseudo-nitzschia* and cyanobacteria; their ability to produce toxins has not been tested in many cases.

Dinophysis species seem to be the most important for public health since they occur at the regions where mussel farming (Black Sea) or subsistence and recreational harvesting exists (Black Sea, less at White and Barents Seas). No data on PSP-causative *Alexandrium* species in the Russian European Seas is available to date.

There is no legal regulation of the algal toxins' control in seafood in the Russian Federation, and no official statistics on the phycotoxin poisonings is available. Some cases may have gone unrecognized due to the misdiagnosis.

Here we present the summary of results of our research on harmful algae in coastal waters of the Black, White, Barents, Baltic Seas in 1996-1999, and review earlier data on HAB species biogeography.

RESULTS

Macroalgae

Blooms of the green filamentous algae of the genus *Cladophora (C.albida, C.glomerata, C.vagabunda)* were first observed at Anapa coast in the early 80s, and during the last decade they became annual. The cause of the blooms is the heavy discharge of organic and inorganic nutrients from i) the town sewerage ii) agricultural fields iii) mountain soil. The bloom usually starts in February-March and continues through to November; storms interrupt blooms throwing the seaweed on shore. Fine sandy sediments prevent the growth of other macroalgae, whereas *C.vagabunda* – the major bloom forming species - grows unattached, forming tangles that cover up to 100% of the bottom, with biomass up to 400-800 g/m^2. *C.vagabunda* standing crop in July 1999 in the bloom area of 15 km^2 was 7500 tons. Due to the very shallow waters at Anapa *Cladophora* is found 2000m offshore where depth is less then 20 m. Our study showed that the main source of nutrients for the bloom is city sewerage; submarine wastewaters piped through discharges 2 km off-shore, right into the longshore current, so that nutrient-enriched water mass is carried back to the city and further north along the beach where children summer camps are situated. Waves drive seaweed to beach, which results in the formation of brown jelly mass near the surfline (**Fig.1**); at the central beach of Anapa bulldozers are employed to remove decaying *Cladophora* mass. The bloom became a concern for those involved in tourism business because of the tourists' complaints.

Fig.1 *Cladophora* at Anapa bay beach.

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Dinophysis

First reports of *Dinophysis* in the Northern and Eastern Black Sea are from the late 19th century and to this date the list has grown to 19 species [1-7], all of them occur at Southern Crimean coast (**Table 1**). Phytoplankton at the Caucasian coast generally is poorer and updated lists contain 11 species of *Dinophysis*. The most common species are *D.rotundata, D.caudata, D.acuminata.*

Species of the genus *Dinophysis*, a minor component of phytoplankton, received little attention in the studies of Black Sea microalgae. Cell counts seldom exceeded 500 cells·l^{-1}, usually less then 100 cells·l^{-1}, both near and off shore. Many species were reported only once and a long time ago, identification sometimes was tentative. In summer-autumn 1996-1999 at the Northern Caucasian (Sochi-Anapa) coast we observed *D.caudata* and *D.rotundata* at concentrations up to 600 cells·l^{-1}, *D.caudata* was more abundant; rare cells of *D.tripos* were found in winter samples. *Prorocentrum lima* was found at less then 100 cells·l^{-1}. At Southern Crimean (Sevastopol-Laspi) coast *D.rotundata, D.ovatum, D.tripos* and *D.acuminata* were found; peak concentrations of *D.rotundata* were 500 cell·l^{-1} (August 1996), and another DSP-causative species *Prorocentrum lima* was found at the same samples at 3200 cell·l^{-1}. *Dinophysis* counts were always less then 1% of total phytoplankton.

The Caucasian coast of The Black Sea is the most important area for *Dinophysis* monitoring since it is the only region of shellfish farming in Russia, including several mussel (*Mytilus galloprovincialis*) and oyster (*Ostrea edulis*, *Crassostrea gigas*) farms. In Anapa region we observed cases of poisonings with DSP symptoms after mussel consumption in 1996-1999. In August 1999 DTX2 at 1160-2544 ng/g hepatopancreas was found in local farmed mussels (HPLC analyses made by M.L.Fernandez, EU Community Reference Lab.) when *D.caudata* and *D.rotundata* were 200-600 cells·l^{-1} and 3 DSP cases observed by us happened at the same time. More poisoning cases associated with mussel consumption were reported by local people at the same period. That was the first confirmed DSP toxicity in Black Sea.

It should be noted that although local people know of the sickness that may follow consumption of shellfish, they are unaware of its possible phycotoxic origin and of the problem of toxic phytoplankton in general. Food poisonings are common phenomena during the hot summers, and symptoms of poisoning by bad fish or shrimp are similar to those of DSP. And according to our data the abundance of *Dinophysis* in coastal waters of the Black Sea strongly correlates with water temperature, so that we observe more DSP cases during the hot time when bacterial poisonings are more probable as well.

The primary role of high water temperature in *Dinophysis* development and consequent DSP cases in Black Sea shows when we compare the two past summers: during the hot summer 1999 *D.caudata* concentration at Anapa coast reached 600 cells·l^{-1}, and cases of DSP happened from June to August; on the other hand, during relatively cold summer 2000 (surface water temperature at the main shellfish farm less then 25°C) we observed *D.caudata* at 50 cells·l^{-1} at best, and no DSP cases were observed by us or reported by local people.

Concentration of *Dinophysis* at Caucasian coast can be temporarily raised due to the South-Westerly storms bringing water masses from the central part of the Sea where *Dinophysis* is always more abundant [4,5,7]. We observed that phenomenon during two storms in the end of June and in the end of August 2000: both times after the storms there were 20 to 60 *Dinophysis* cells (including *D.tripos*, a species unusual for the region) per mussel stomach at the Utrish shellfish farm near Anapa, although no *Dinophysis* was detectable in the water; 3 days later *Dinophysis* disappeared from mussels.

Several potential DSP-causative *Dinophysis* species (*D.acuminata, D.acuta, D.norvegica, D.rotundata,*) inhabit Russian coastal waters of the **Baltic Sea** [8], but local molluscs are not used as food and no shellfish farming exists there.

In the Kandalaksha Gulf of the **White Sea**, where mussel banks are abundant, and at Kola coast of **Barents Sea** species of *Dinophysis* sometimes occure at up to 10^2-10^3 cells·l^{-1} in summer [9-12]. Local mussels is only an occasional component of the diet and shellfish farming has a poor future there. No *Dinophysis* species were found in the **Caspian Sea** [13-16].

Pseudo-nitzschia

In all Russian seas (with the exception of **Caspian Sea**) potentially ASP causative diatoms of the genus *Pseudo-nitzschia* can be found. *Pseudo-nitzschia pseudodelicatissima* (earlier referred to as *Nitzschia delicatissima*) and *Pseudo-nitzschia seriata* develop winter-spring blooms in **Black Sea**, peak concentration over 10^6cells·l^{-1} [7]. These blooms cover vast areas in the central part of the Sea and coasts. Although sometimes during mussel harvesting period *P.pseudodelicatissima* and *P.seriata* concentrations at Caucasian coast were high (20000 cells·l^{-1} in late August 2000), we have not observed cases of ASP.

Pseudo-nitzschia delicatissima and *P.seriata* are listed for the **Baltic** coastal waters of Kaliningrad region, but no blooms were registered.

Potentially ASP-toxic *Pseudo-nitzschia seriata* is one of the dominants of the Spring bloom at the Kola coast of the **Barents Sea** [9-12].

Haptophytes

Haptophytes *Chrisochromulina* sp. and *Prymnesium* sp. that can produce ichthyotoxins occur in the Russian Baltic but they were not identified to species, were not blooming and no fish death events were associated with them.

Cyanobacteria

The effect of phytoplankton bloom on marine fauna clearly shows in bays of the Arctic estuary of the

Pechora river (eastern **Barents** sea) where blooming cyanobacteria (*Aphanizomenon flos-aqua* dominant species with concomittant hepatotoxic *Anabaena* spp.) cause massive fish mortality each year; although *Anabaena* provided hepatotoxicity of about 10 mkg microcystin/g dry weight of net sample measured by Microtox test in 1997, the main cause of fish death apparently is oxygen depletion during the bloom; the bloom sometimes causes gelatinous masses. This is important case because Pechora is the spawning river for *Salmo salar* salmon.

In the brackish waters of the Northern **Caspian Sea** cyanobacterial blooms are a recurrent phenomena, but their impact on the marine fauna of that highly productive area is poorly investigated. Cyanobacterial blooms in the Northern Caspian result from nutrient-enriched discharge of Volga water [14-17].

This same situation occur in the **Baltic** (Gulf of Finland and Kursha Bay) with *Nodularia spumigena, Aphanizomenon flos-aqua, Microcystis aeruginosa*; toxicity of those species has been well established elsewhere, but the influence of the blooms on the fauna requires investigation; no marine fauna mortality cases connected with them were reported in the Baltic as yet.

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TABLE 1

TOXIC AND POTENTIALLY TOXIC ALGAE IN RUSSIAN EUROPEAN COASTAL WATERS
(only blooming cyanobacteria are listed)

	BARENTS SEA	BLACK SEA				CASPIAN SEA	BALTIC SEA	WHITE SEA	
		South Crimean Coast [1,2,4,5,7]	South Crimean Coast'96-97	Caucasian Coast [1-7]	Caucasian Coast'96-2000	[13-17]	[8, our data]	[10,11, our data]	[10,12, our data]
Aphanizomenon flos-aquae L. (Ralfs.)						+	+		+
Anabaena variabilis Kutz.									+*
Dinophysis acuminata Clap. et Lachm.		+	+	+			+		+
D.acuta Ehr.		+		+			+		+
D.apiculata Meunier		+							
D.arctica Mer.		+		+-				+	+
D.baltica (Pauls.)Kof.et Skogsberg		+-							
D.caudata Kent		+		+	+*				
D.dentata Schill.				+-					
D.fortii Pav.				+-					
D.hastata Stein		+	+						
D.levanderi Wolosz.		+							
D.minutum Cl.		+							
D.norvegica Clap. et Lachm.				+-				+	+
D.ovatum (Clap. et Lachm.) Jorg.		+	+						
D.ovum Schutt		+		+					
D.paulsenii Wolosz.		+							
D.pulchellum Lebour		+							
D.rotundata (Clap. et Lachm.) Kof.et Michener		+	+	+	+*		+		
D.rudgei Murr. et Witt.		+							
D.sacculus Stein		+							
D.similis Kof. et Skogsberg		+							
D.sphaeroideum Schill.		+							
D.tripos Gour.		+	+		+-				
Microcystis aeruginosa Kutz.						+	+*		
Nodularia spumigena Mertens						+	+*		
Pseudo-nitzschia delicatissima Cleve							+[1]		
P. seriata Cleve		+[1]	+[1]	+[1]	+[1]		+[1]		+[1]
P.pseudodelicatissima (Hasle) Hasle		+	+	+	+				
Prorocentrum lima (Ehr.) Dodge		+	+	+	+				

* - confirmed toxic; [1] -light microscopy identification; +- -single observation or doubtful identification

SEASONAL DYNAMICS OF DINOFLAGELLATES AND RAPHIDOPHYTES AND DISTRIBUTION OF THEIR RESTING CYSTS IN KAMAK BAY, KOREA

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ABSTRACT

Seasonal dynamics of dinoflagellates and Raphidophytes in the water column and distribution of their resting cysts in sediments were investigated in Kamak Bay, Korea, from May 1997 to June 1998. A total of 58 species were identified including 56 Dinophytes and 2 Raphidophytes. Standing crops of dinoflagellates exhibited a maximum of $2x10^5$ cells/l in June 1997. Dominant species comprised *Prorocentrum dentatum* in June 1997, and *Prorocentrum minimum* and *Ceratium furca* from May to June 1998. Raphidophytes appeared with very low standing crops only in the summer season.

Resting cysts included 31 taxa of dinoflagellate cysts as well as *Chattonella* (Raphidophyte) cyst. Cyst abundance varied spatially and temporally; total cysts ranged from $4.6x10^2$ to $1.0x10^3$ cysts/cm^3, and living cysts from $1.4x10^2$ to $5.6x10^2$ cysts/cm^3. Living cysts constituted 25-56% of total abundance and increased in winter. The abundance of living cysts exhibited a significant relationship with water temperature ($r=-0.66$, $p=0.02$) as well as seasonal succession of planktonic cells in the water column. Seventeen heterotrophic dinoflagellate cysts comprised 55% of total number of cyst species and their abundance comprised 50-55% of total cyst abundance in inshore waters and 29-36% in offshore waters.

INTRODUCTION

About 300 species of phytoplankton are known to cause red-tides and most belong to the flagellates. The scale and frequency of red-tides has increased year by year, apparently due to eutrophication of coastal waters [1]. Red-tide species include flagellates, which produce a cyst during the life history and then sink in the bottom to be dormant for some period [2]. Flagellates produce their cysts usually in unfavorable conditions, and in some waters they play an important role as seed population for the next red-tide [3] as well as are involved in transfer by the ballast water of intercontinental ships [4].

Kamak Bay is a semi-enclosed bay in Korea where red-tide occurs frequently from late spring to early autumn. The bay has become eutrophic due to an increase of sewage from land. It has been a farming ground for shellfish such as oysters for a long time [5]. However the inner bay has been polluted by industrial complex establishment and thus red-tides occur frequently. Previous studies in this bay were concerned with oyster farming and plankton [6, 7], but few studies were done on the benthic environment. The southern coast of Korea has a high loading of organic material in the sediment, which relates closely to red-tide occurrence in the water column as well as to the increase of resting cysts. So far the study of resting cysts has focused on Masan-Chinhae Bay [8, 9, 10] and a part of southern Korean waters [11, 12, 13]. However, more field data concerning the resting cysts of red-tide causative organisms are necessary to clarify the presence of seed populations in Korean coastal waters. This study was done in order to understand the dynamics of flagellates and their resting cysts in terms of spatial and temporal distribution, and the relationship with environmental condition.

MATERIALS AND METHODS

Sampling was done monthly at Kamag Bay in the southern coast of Korea from May 1997 to June 1998. Both plankton samples and sediment samples were taken in station 1 (inner bay), station 2 (center of shellfish farming), and stations 3, 4, 5 located in the mouth of the bay (Fig. 1). One liter of water was collected for plankton analysis and then fixed with Lugol's iodine solution. Plankton cells and benthic cysts were counted quantitatively using a Sedgwick-Rafter counting chamber under an inverted light microscope (Olympus CK-2) and expresses as standing crop of cells/l. Species were identified based on Steidinger & Tangen [14] and Chihara & Murano [15].

The resting cysts were collected from surface sediments using a TFO hand core sampler and isolated by the biological process modified from Matsuoka *et al.* [16]. One ml of subsample was used for counting and then calculated as a unit of cysts/cm^3. Cyst counts were differentiated into living cysts and empty cysts according to all content.

RESULTS

Plankton dynamics

A total of 58 species were identified representing 22 genera. *Scrippsiella trochoidea* occurred every month. Species which occurred frequently for more than 10 months included *Ceratium furca, Gyrodinium spirale, Noctiluca scintillans, Protoperidinium bipes, P. pellucidum, P. steinii* and *Gymnodinium* sp. Among the Raphidophytes, *Chattonella marina* occurred at station 5 at the mouth of the bay and *Fibrocapsa japonica* at stations 1 and 2 in the inner bay. The number of species at each station ranged from 0 to 21. The number of species increased starting in spring and then decreased after autumn. The species number showed minima in winter and started to increase again in

Harmful Algal Blooms 2000
Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J. (eds)
Intergovernmental Oceanographic Commission of UNESCO 2001

spring.

Standing crop of dinoflagellates in the surface layer ranged from 60 to $2x10^5$ cells/l with an annual average of $8x10^3$-$3x10^4$ cells/l. The maximum occurred in June, and the minimum in January. The inner bay showed a higher standing crop than the outer bay. Monthly mean standing crop of dinoflagellates ranged from $2x10^2$ to $1.2x10^5$ cells/l, being high through May to August and low from November to February. Raphidophyte's abundance ranged from $1x10^2$ to $1x10^3$ cells/l, increasing in the summer like the dinoflagellates, but being less abundant.

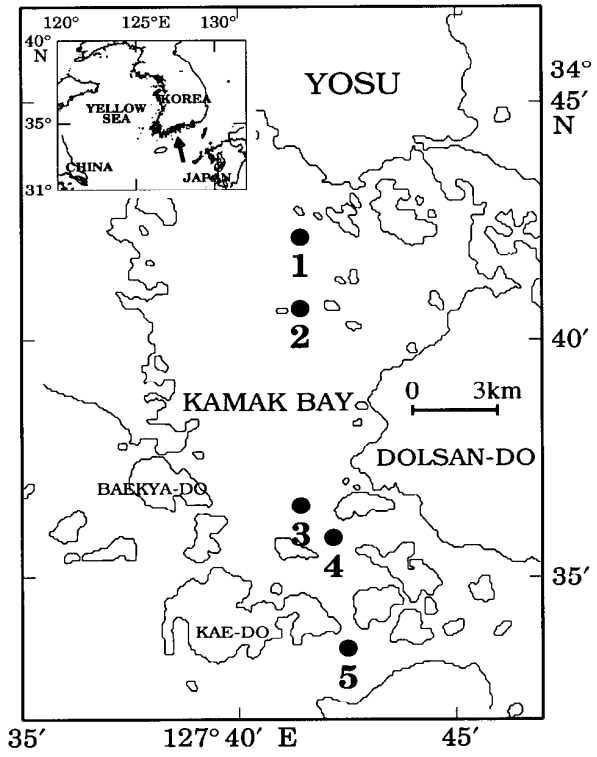

Fig. 1. Map showing sampling stations in Kamak Bay, Korea

Prorocentrum dentatum predominated at all stations in June 1997 with 45-66% abundance, *Ceratium furca* in July 1997 with 21-25%, *Prorocentrum minimum* in July 1998 at the inner bay with 84-40% and *Ceratium furca* in July 1998 at the outer bay with 29-81%. *Scrippsiella trochoidea* dominated station 1 in June with 36.0%, but *Ceratium furca* predominated at stations 2 to 5 with over 80% in June 1998. Raphidophytes showed a low abundance except for June 1998 at station 1 with 15% abundance and no dominant species.

Dynamics and distribution of resting cysts

A total of 31 dinoflagellate cysts, representing 13 genera and *Chattonella* (Raphidophyte) cyst were identified. (Table 1). *Protoperidinium* cysts were most predominant and comprised 13 species. *Gonyaulax* cysts included 5 species and the remaining genera represented by 1 or 2 species. Monthly occurrence patterns showed that *Gonyaulax spinifera*, *Gymnodinium* type, *Protoperidinium minutum*, *Protoperidinium* type cyst occurred every month. *Alexandrium* cf. *affine*, *Pheopolykrikos hartmanni*, *Protoceratium reticulum*, *Protoperidinium americanum*, *P. conicum*, *S. trochoidea* were found at 6 or 8 times per year. On the other hand, *Chattonella* cysts were distributed from July to August and from January to April. The number of cysts at each station was in the range 12 to 16 being highest in September and lowest in November.

Table 1. Species list of dinoflagellates and Raphidophytes cysts at 5 stations in Kamak Bay from May 1997 to April 1998

Species	1997 M	J	J	A	S	O	N	D	1998 J	F	M	A
Dinophyte												
Autotrophic												
Alexandrium cf. *affine*			*	*		*	*	*	*		*	*
Alexandrium tamarense			*		*	*			*		*	*
Cochlodinium sp.					*		*					*
Gonyaulax digitale			*									
Gonyaulax scrippsae				*				*			*	*
Gonyaulax spinifera	*	*	*	*	*	*	*	*	*	*	*	*
Gonyaulax verior						*				*	*	
Gymnodinium sp.	*	*	*	*	*	*	*	*	*	*	*	*
Gyrodinium sp.	*	*	*	*	*	*		*				
Lingulodinium polyedrum		*					*		*	*	*	*
Pheopolykrikos hartmanii	*	*	*	*	*		*	*	*		*	*
Protoceratium reticulum				*	*		*	*	*	*	*	*
Pyrophacus steinii								*		*		
Scrippsiella trochoidea	*	*	*	*	*		*	*	*	*	*	*
Heterotrophic												
Diplopsalis lenticula		*						*		*		
Polykrikos kofoidii	*			*			*		*	*	*	*
Polykrikos schwartzii		*										
Preperidinium meunieri	*			*	*	*				*		
Protoperidinum americanum	*		*			*	*		*	*	*	*
Protoperidinium avellanum	*											
Protoperidinium claudicans	*	*										
Protoperidinium compressum	*			*		*						
Protoperidinium conicoides	*						*		*			
Protoperidinium conicum			*	*	*		*	*	*	*		
Protoperidinium latissimum								*				
Protoperidinium leonis				*								
Protoperidinium minutum	*	*	*	*	*	*	*	*	*	*	*	*
Protoperidinium oblongum	*	*	*	*		*				*		*
Protoperidinium pentagonum							*			*		
Protoperidinium subinerme					*	*	*					
Protoperidinium sp.	*	*	*	*	*	*	*	*	*	*	*	*
Raphidophyte												
Chattonella sp.				*	*					*	*	*

Cyst abundance ranged from $2x10^2$ to $1x10^3$ cysts/cm^3 including living and empty cysts. Living cyst abundance ranged from 50 to $8x10^2$ cysts/cm^3 and

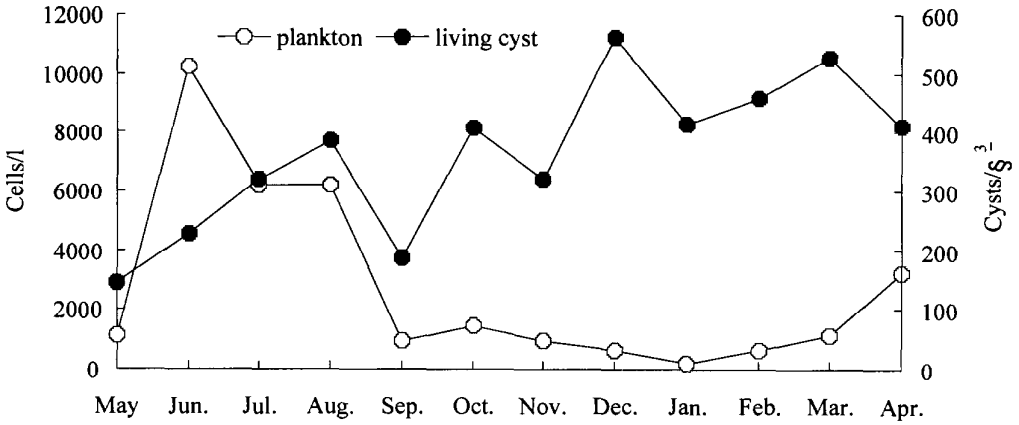

Fig. 2. Monthly variation of plankton standing crop and living sediment cyst abundance of dinoflagellates in Kamak Bay from May 1997 to April 1998.

empty cysts from $1x10^2$ to $9x10^2$ cysts/cm^3, implying that empty cysts prevailed in the sediment rather than living ones.

Dinoflagellates can be generally divided into trophic types: autotrophic, heterotrophic, and mixotrophic. Heterotrophic dinoflagellates can be more abundant in eutrophic waters rather than in clean waters. Heterotrophic dinoflagellates in Kamak Bay include *Diplopsalis, Polykrikos, Preperidinium*, and *Protoperidinium* (Table 1). The number of these was 17 species and comprised 55% of total species number. The total abundance of these ranged from $2x10^2$ to $6x10^2$ cysts/cm^3, living cyst from 80 to $3.5x10^2$ cysts/cm^3, and empty cyst from $1.4x10^2$ to $4.1x10^2$ cysts/cm^3. The abundance of the heterotrophic species ranged from 23-46% for living cysts and 35-70% for empty cysts. They were predominant at stations 1 and 2 in the inner bay, showing a higher abundance at the inner station (stations 1, 2) than at the outer station (stations 3, 4, 5).

We analyzed the vertical distribution of resting cyst from top to 20 cm depth at 1 cm intervals. Cyst abundance showed an increasing trend from the bottom to the top of sediment. The layer of 17-20 cm showed a very low abundance, while the upper layer showed an increased abundance. The abundance continued to increase and decrease at regular intervals with the highest value of $1.1x10^2$-$1.4x10^2$ cysts/cm^3 at the 0-5 cm layer.

Raphidophyte cysts ranged from 0 to 30 cysts/cm^3 for total cysts, comprising 0 to 25 cysts/cm^3 for living cysts, and 0 to 16 cysts/cm^3 for empty ones. Raphidophyte cysts were found at a very low abundance compared to dinoflagellate cysts. Living cyst existed only from July to August and from January to February. Empty ones were found to be more abundant than living ones.

Relationship between cyst abundance and environmental factors

Water temperature was in the range of 7.6-25.9°C and showed the high value from May to September with an average of 19.9°C. Living cyst abundance and water temperature showed a significant negative correlation ($r=-0.66$; $p=0.02$). However the total cysts to water temperature showed $r=-0.38$ and the empty cysts to water temperature $r=0.15$, which is not significant ($p>0.05$). On the other hand, salinity ranged from 28.0 to 33.9‰ showing a maximum in February and then decreased from May to a minimum in August. The correlation coefficient between cyst abundance and salinity is not significant ($p>0.05$); total cyst ($r=0.27$), living cyst ($r=0.48$) and empty cyst ($r=-0.13$). Thus it suggested that only the correlation between living cysts and water temperature is significant in Kamak Bay.

Relationship between plankton and resting cysts

Of the 13 genera of dinoflagellate sediment cysts, 11 genera were coupled with plankton cells in the water column, but *Lingulodinium* and *Pheopolykrikos* genera were not. Figure 2 shows the relationship between the abundance of living cysts and the plankton cells of dinoflagellates in the water column. The abundance fluctuation of living cysts showed a reverse relation to that of planktonic forms. This was more apparent in the spring. However both showed similar variation in summer. The cyst-plankton relationship thus exhibits a different pattern according to season.

DISCUSSION

The plankton dynamics in the water column and the resting cyst patterns in the sediment varied spatially and temporally. The number of dinoflagellates identified totaled 53 and was lower than 63 species reported previously in Kamak Bay, but higher than 42 species in Chinhae Bay in southern Korean waters [10].

Dinoflagellate abundance increases frequently in summer, when water temperature rises. In this study area diatoms were predominant throughout the year, whereas dinoflagellates bloomed only at high water

temperatures. *Prorocentrum minimum* especially predominated at stations 1 and 2 at the inner bay. While Raphidophytes are known to bloom for a short periods in summer [17] and were also abundant in May and June 1998, but were not found in other seasons. The Raphidophyte *Fibrocapsa japonica* is known to occur in the southern Korean waters in recent years.

Dinoflagellate cysts included a total of 31 species, comprising 25 thecate species and 6 unarmored species. This is a high diversity compared with other results: 17 species in Masan Bay [8], 20 in southern Korean waters [11], 22 in Korean coastal waters [12], and 27 in Chinhae Bay [10]. Total cyst abundance mainly reflected abundance of empty cysts, but living cysts depended significantly on water temperature as well as plankton variations. Cyst abundance was highest at station 1, the most inner station, and lowest at station 2, in the middle bay with lowest depth. This suggests that the topography of bottom affects the distribution of cysts [18]. On the other hand, stations 3, 4, and 5 showed very similar abundance patterns and lower abundance than station 1. These stations are located at the mouth of bay when water velocity is faster to prevent settling to the bottom [19].

The heterotrophic dinoflagellate cysts were more abundant at the inner station than at the mouth of the bay. This indicates that the inner bay is more eutrophic than any other part of the bay, which confirmed the result of Omura Bay in Japan [20].

Seasonal succession of dinoflagellates can be explained mainly by water temperature, which also affects formation and germination of cysts [21]. In this study, living cyst abundance was related more closely to water temperature rather than empty cysts. The abundance of living cysts increased in winter, whereas dinoflagellates were predominant in summer. The standing crops of motile cells affect the abundance of living cysts. The motile and the living cyst showed a negative correlation in winter, but this relation was not clear in summer.

Raphidophytes showed a different pattern between plankton and cysts. *Chattonella marina* and *Fibrocapsa japonica* were found in the plankton, but were not observed in cyst assemblage. We observed only *Chattonella* type cyst from July to August and from January to April. Thus a monitoring of both plankton and cyst should be considered. Furthermore, studies on the excystment and encystment of the flagellates are necessary to understand their seasonal succession in this bay.

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BLOOM OF THE DINOFLAGELLATE *ALEXANDRIUM AFFINE* (INOUE AND FUKUYO) BALECH, IN TROPICAL AMBON BAY, INDONESIA

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ABSTRACT

A bloom of *Alexandrium affine* was observed for the first time in Ambon Bay, Indonesia during November 1997. The water became reddish brown and covered a portion of the inner part of Ambon Bay. The average abundance of *A. affine* was 2 x 10^6 cells l^{-1}. This outbreak of *A. affine* was unique since this is the first occurrence of an *Alexandrium* bloom reported in Ambon Bay. The influence of river runoff and the amount of rainfall prior to the bloom are suspected to be important factors in maintaining the nutrient level, especially nitrate and ammonium, in inner Ambon Bay.

INTRODUCTION

The genus *Alexandrium* has more than 30 species and many can produce toxins [1]. In Southeast Asia, the species *A. tamiyavanichi* is considered the only known *Alexandrium* species to produce toxin [1]. *Alexandrium affine* has been known to inhabit a wide range of geographical areas and has been found in temperate waters, as well as in the tropics [1]. In 1974, 1975 and 1977 Fukuyo et al. [2, 3] reported a bloom of *Protogonyaulax affinis* in several parts of Japan. However, there were no reports on the toxicity of this species at the time of those blooms.

The first reported case of a HAB in Ambon Bay was in 1994, caused by a paralytic shellfish poisoning species, *Pyrodinium bahamense* var. *compressum*. During that outbreak, three children died and several more people had to be hospitalised after consuming shellfish from the bay [4].

Ambon Bay is a small embayment in Ambon Island in the eastern part of Indonesia (Fig. 1). Ambon Bay is divided into two parts, the inner and outer bays, which are separated by a shallow sill at the narrow channel (300 m wide and 15 m deep) between the two bays.

Several studies have reported nutrient concentrations in Ambon Bay, although the reports are lacking in continuity. Sutomo [5] measured Chl-a in Ambon Bay and hypothesized that the increase in NO_3 in this area was highly correlated with the amount of rainfall during the rainy season (April – September). Other studies [6, 7] reported that in 1987 the concentration of NO_3 and PO_4 in two rivers emptying into the inner Ambon Bay ranged from 14.8 – 22.7 µM and 16.4 – 21.0 µM for nitrate and phosphate, respectively. Previously, there have been no reports on NH_4 and SiO_4 concentrations for Ambon Bay.

In this paper we will discuss the variations of some environmental parameters associated with the bloom of *Alexandrium affine* from October – November 1997. The results presented here are part of the Ambon Bay phytoplankton study conducted from August 1996 - July 1998. This study is critical in understanding the dynamics of HABs, especially in a small tropical embayment like inner Ambon Bay.

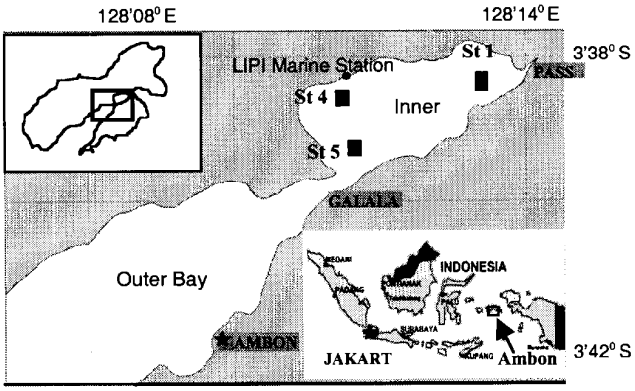

Fig. 1. Map of Ambon Bay and sampling stations. Insets: Ambon Island and map of Indonesia

METHODS

As part of a continuous two week sampling schedule from August 1996 - August 1998, water samples were collected at stations in the inner and outer parts of Ambon Bay. When the bloom of *A. affine* was reported, sampling was conducted every day for the first 5 days and then every 2 days until November 18, 1997. Three stations were chosen to represent the inner Ambon Bay (Figure 1), since the bloom only occurred in this area.

Water samples were collected at three different depths (0, 5 and 20 m) with a 2.5 L hand-held Van Dorn bottle. Temperature and salinity were determined by means of a thermometer and a Beckman salinometer. Chlorophyll-a was measured spectrophotometrically [8], with a phaeopigment correction [9]. Nutrient samples were frozen immediately and concentrations of nitrate, phosphate, ammonium, and silicic acid were measured in the laboratory following the IOC Methods [10, 11]. A few drops of Lugol's iodine solution were used as a fixative and subsamples of phytoplankton were settled in a 50 ml chamber and counted on an inverted microscope. For more precise identification of *A. affine*, scanning electron microscopy was used. In this study we consider only the dominant species that were found in the water samples.

Upon receiving a bloom report from the local people, a water sample was taken from the location near the "LIPI" Marine Station (see Figure 1) on November 5, 1997. Subsequent sampling was conducted on November 7, 8, 10, 14 and 18 to monitor the bloom as it progressed

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in Ambon Bay. Earlier sampling data from October 15, 1997 were used to compare to the conditions before the bloom.

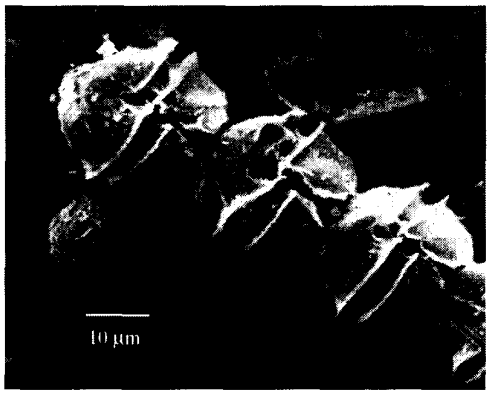

Fig. 2. Micrograph of *Alexandrium affine* from Ambon Bay

RESULTS

On November 5th 1997, a very noticeable phytoplankton bloom occurred in Ambon Bay, Indonesia. The water turned reddish brown, which attracted the attention of the local people since red tides have not been common in this area. This water discoloration covered the inner portion of Ambon Bay, which is about 500 m wide and 2 km long. The bloom continued for at least two weeks (until mid November 1997) before it disappeared. From surface visual observations, the outbreak was patchy and moved counter-clockwise with the current. An intensive examination of this event, including microscopic analysis determined that the cause of the water discoloration was a dinoflagellate *Alexandrium affine* (Inoue and Fukuyo) Balech (Figure 2).

Figure 3 shows the cell abundance (dominant species) and Chl-a concentrations from each station. The majority of the phytoplankton cells were found at 5 m. At station 5, on Nov. 7, cells were also found at the surface layer. This probably coincides with the initiation process of the bloom.

In general, the characteristics of the nutrient profile (Figure 4) are as follows; a clear reduction of nitrate during the developmental stage of the bloom, followed by the increase of ammonium a few days after the peak in *A. affine*. There was a sharp decline of silicic acid on Nov. 7, indicating possible water exchange between the outer and inner bay. Phosphate concentrations during the bloom were less variable, but showed a slight decrease during the bloom. Phosphate concentrations ranged from 0.2 _M at the surface to 0.9 _M at the bottom. There were no reports of PSP poisoning of humans after the outbreak of *A. affine* in Ambon and its surrounding areas.

Inter-station variation

Station 1 is located at the head of the inner Ambon Bay, and marked by a fairly large mangrove

stand. The highest concentration of *A. affine* was 4.4 x 10^6 cells L^{-1} (0 and 5 m), recorded at this station on November 8, 1997. The presence of another *Alexandrium* was also high at this station reaching more than 1 million cells L^{-1}. This potentially new species has several characteristics such as; non chain-forming, produces temporary cysts and belongs to the *Gessnerium* group. Nutrients at this station were relatively high in NO_3 and PO_4 in the bottom layer. Silicic acid was the highest at this station compared to the other stations.

Station 4 is located on the westside of the inner Ambon Bay. This area is relatively calm during the dry season (south-west monsoon). The bloom of *A. affine* was spotted at Stn. 4 due to the circulation pattern in the inner bay being counter-clockwise, resulting in the accumulation of cells in the western part of the inner Bay. The highest abundance of *A. affine* (0 and 5 m) at this station was found on Nov. 8, 1997 (4.35 x10^6 cells L^{-1}). However, there was an increased abundance on Nov. 7, 1997. The profiles of Chl-a and NO_3 at this station have a similar pattern to that at Station 1, which is in agreement with the cell abundance pattern. The concentrations of NH_4 and SiO_4 were similar at all depths.

Station 5, is at the mouth of the inner bay and connects the inner to the outer bay, and thus has the strongest influence from currents. The highest abundance of *A. affine* was on Nov. 7, 1997. However, this station has the lowest number of *A. affine* compared to Station 1 and 4. NO_3 and PO_4 were relatively similar at all depths indicating fairly strong mixing in this area.

DISCUSSION

The *Alexandrium affine* bloom in Ambon Bay occurred in a relatively short period (1–2 weeks). The highest cell abundance was found in the 5 m depth layer, reaching almost 5 millions cells L^{-1}. The bloom appeared to start in the area close to station 5 and moved counter-clockwise with the current to station 1 and accumulated at station 4. Other species also found in relatively high abundance during the bloom were *Thalassionema nitzschioides, Planktoniela sol, Trichodesmium* sp., and *Alexandrium* sp.

The high phytoplankton abundance in Ambon Bay is rather typical of a coastal system, where it has a high uptake of NO_3 derived from run-off and mixing processes. It is also obvious that as the phytoplankton abundance started to decrease, the concentration of ammonium (NH_4) started to increase. Part of the ammonium input is believed to be due to grazing activity by zooplankton. The influence of domestic sewage from the population surrounding the inner Ambon Bay is also critical in determining the level of ammonium in the bay.

The co-existence of some large diatoms (e.g. *Planktoniela sol*) during the *A. affine* bloom is believed to contribute to the draw down of silicic acid on Nov. 7. This condition, however, changes rapidly when water from the outer bay, through tidal circulation, replaces a portion of the water in the inner bay, resulting in an increased of silicic acid concentration in the inner bay. The water from the outer bay is highly influenced by the

open ocean and contains a relatively high concentration of silicic acid, up to 60 μM (Wagey, unpublished data).

The influence of river run-off and rainfall are suspected to be important since both nitrate and ammonium concentrations from rainfall were as high as 50 μM. The connection between the inner bay and the open ocean through the outer bay and the influence of several small rivers that discharge large amounts of domestic sewage and land run-off during the rainy season, may result in an estuarine ecosystem which is typical of Ambon Bay.

When an outbreak of toxic phytoplankton occurs, the consumption of shellfish and some pelagic fish such as clupeoids, becomes dangerous to humans since these organisms might accumulate toxic compounds from the algae. Therefore, for management purpose, rapid action to disseminate the information about red tide blooms is critical. The major constraint in Indonesia for rapid dissemination of news of a red tide outbreak is basically due to the vast geographical area and the limited awareness of the people of the red tide phenomenon. The red tide in Ambon Bay occurred in a relatively small area. In less than a day, the local people were informed about the outbreak and some precautionary actions were taken to avoid shellfish consumption until further investigations were made.

ACKNOWLEDGMENT

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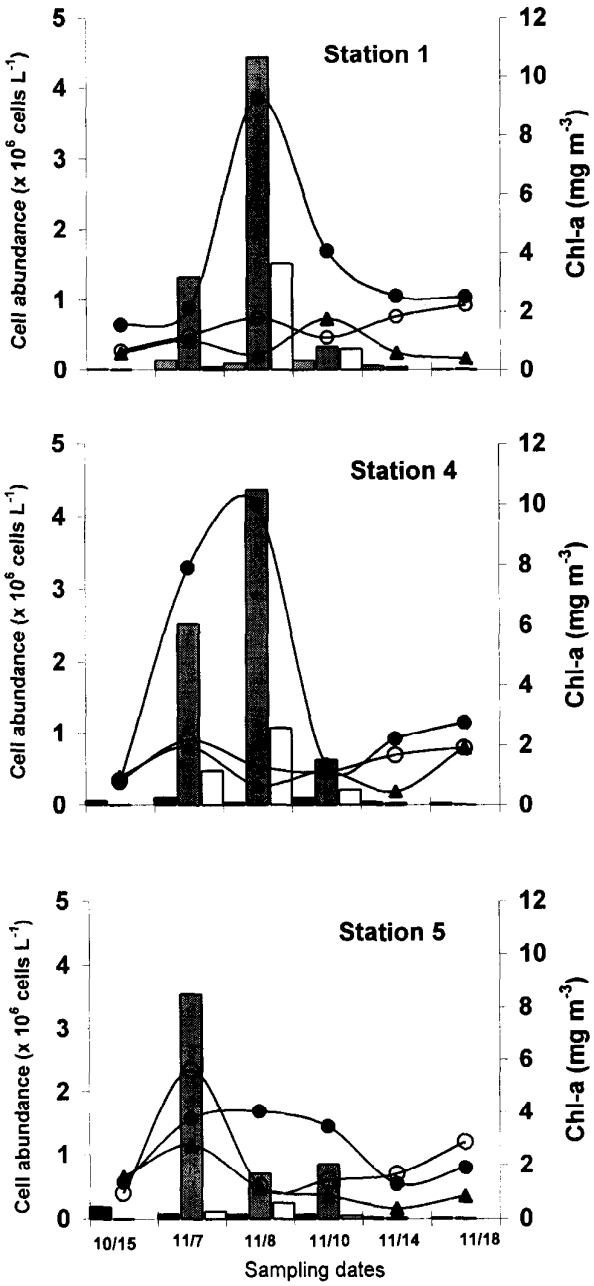

Figure 3. Cell abundance of dominant species (bar graph) during the bloom of *A. affine*. The line graph represents the Chlorophyll-a concentrations at 0 m (—○—), 5 m (—●—), and 20 m (—▲—) at stations 1, 4, and 5.

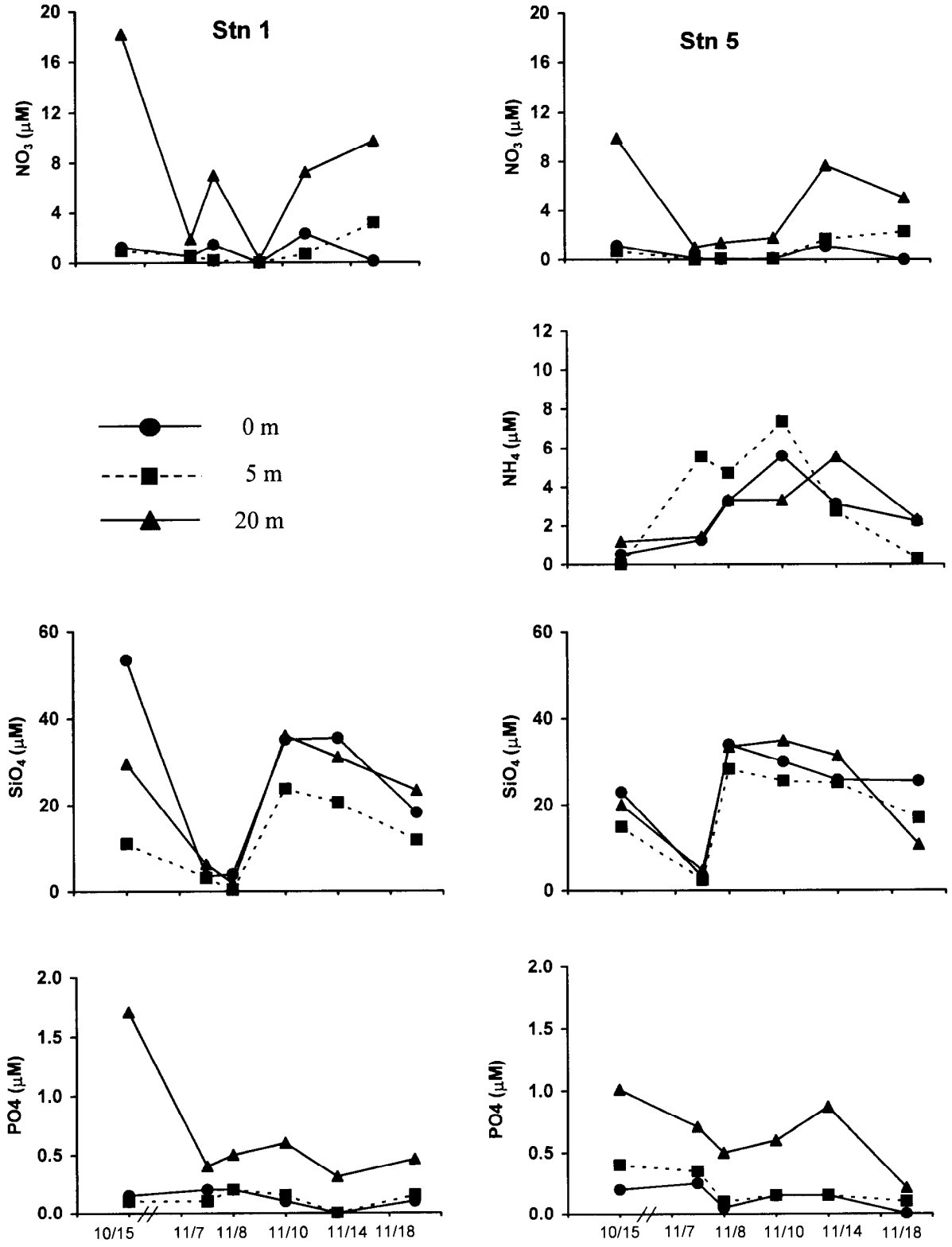

Fig. 4. Nitrate, ammonium, silicic acid and phosphate concentrations at station 1 and station 5 in the Inner Ambon Bay, measured during the bloom of *A. affine* between Oct.15 - Nov.18, 1997. Ammonium concentrations for station 1 were not determined. Results for station 4 were not shown since they had similar values to Station 5.

HARMFUL ALGAL BLOOMS IN INDONESIAN WATERS

Tumpak Sidabutar *, D.P. Praseno * and Y. Fukuyo **

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** ANESC- University of Tokyo. Japan

ABSTRACT

The occurrence of harmful algal blooms in Indonesian waters has been increasing since the beginning of the first red tide programme in this country in 1991. The blooms caused massive fish kills which lead to economic losses in local fisheries, decrease of water quality and even to death of people after eating contaminated shellfish.

It has been found that some red tide species were known to be toxic. Most of them belong to the dinoflagellates, however, only a few have caused bloom phenomena such as *Pyrodinium bahamense* var *compressum, Noctiluca scintillans* and *Alexandrium affine*. The most frequent species which caused red tide phenomena in certain areas were *Trichodesmium, Noctiluca, Chaetoceros*. HAB species that have been recorded in Indonesian waters since the beginning of the programme are as: *Pyrodinium bahamense* var. *compressum, Gymnodinium catenatum, Alexandrium affine, Gonyaulax spinifera, Dinophysis caudata, D. rotundata, Noctiluca scintillans, Prorocentrum lima, Gambierdiscus toxicus, Pseudo-nitszchia pungens, Chaetoceros convulotus, C. concavicorne, Trichodesmium erythraeum* and *T. thiebautii*. The occurrences and distribution of those red tide species in Indonesian waters are discussed.

INTRODUCTION

Recently, one of the environmental problems and management issues that confront the coastal areas in Indonesia are pollution and red tide phenomena. Red tide has been occurring lately in this region in accordance with the tragedy of massive fish kills which caused economic losses in local fisheries. Some areas have experienced harmful algal bloom several times and caused human fatalities, economic losses both in natural fisheries and in aquaculture.

Indonesia for the first time became aware of possible red tide problems in 1991. Red tide was reported mainly in remote places. Following these reports monitoring programs were designed and conducted in these areas. The first report of red tide occurrences which had caused death and illness of local people after eating shellfish was noted in Ambon Bay, where the causative species was *Pyrodinium bahamense* var. *compressum* [1]. This incident had caused serious problems among local people and might be an annual feature of that area. Since then, a monitoring programme has been implemented to observe the reoccurrence of this toxic species and other potential harmful phytoplankton.

It also aimed to study the seasonal variation of phytoplankton population in this bay in connection with environmental disturbances. More importantly, to prevent algal toxin from reaching the human consumers and to minimize damage to living resources as well as economic losses. Owing to the increasing reports of HAB's and the economic impacts that could results from such events, this paper attempts to examine the status of HAB occurrences in Indonesia and their impacts on the natural fisheries and aquaculture since 1991.

METHODS

A monitoring network was established with RDCO-Jakarta acting as the center with several links at locations with trained participants acting as respondents. The laboratory of RDCO in Ambon is functioning as a center for east Indonesia. Presently the monitoring stations cover 12 out of 27 provinces. The Jakarta laboratory covers the western part of Indonesia, while Ambon Laboratory covers the eastern part the country

Plankton samples were collected using small plankton net with mesh size of 20 μm. Water samples were taken using Van Dorn bottle from a depth of approximately 3 meters below the surface. A volume of two liter water samples from each station were then filtered with plankton net. Concentrated plankton were kept in small bottle samples and preserved in Lugol solution, and brough to the laboratory for qualitative and quantitative analysis.

Identification of phytoplankton was conducted under inverted microscope and determined up to species level as possible, especially for toxic and harmful microalgae. Quantitative analyses were done on a 1-ml aliquot part of the sample placed in a Sedgewick-Rafter counting chamber [2]. These counts take into consideration the mouth diameter of the net, sampling depth, and volume of plankton sample.

Monitoring for PSP toxins was conducted for particular areas which had known occurrences of toxic species. The presence of PSP toxin in shellfish and fish is a direct results of *Pyrodinium* red tides and their content in shellfish and fish has been universally used as criterion to determine the severity of red tide. Toxicity determination was conducted if red tide phenomena appeared in the surface water or toxic species occurred in relatively high numbers. The level of paralytic shellfish

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poison (PSP) determined by the standard mouse-bioassay test following the procedures of the Association of Official Analytical Chemists [3]

RESULTS AND DISCUSSIONS

Bloom occurrences in western Indonesia

Incidents of red tide phenomena have been recorded in some areas in Indonesian waters since the beginning of the programme in 1991, which caused losses to fisheries and also impacted on human health. The first HAB impact on fisheries in Indonesian waters occurred in June – September 1991 when mass mortality of cultivated shrimp occurred in the brackish water ponds of the eastern coast of Lampung. The causative phytoplankton was identified as *Trichodesmium erythraeum* [4]. This organism drifted by currents reaching Lampung coastal areas, polluted shrimp ponds on the east coast, and killed shrimp in these ponds.

In December 1993, fish kills occurred in Jakarta Bay caused by high concentrations of ammonia due to *Noctiluca* blooms [5]. It was also reported that the bloom caused mass mortality of cultivated shrimp in brackish water ponds of Kamal, west of Jakarta. The causative organism was identified as *Gymnodinium pulchellum.*

Many other fish kills occurred, however, they went unreported, such as pearl oyster kills at mariculture sites in Molluccas, Lombok, Bali, and Lampung Bay. Fish kill in the Bali Strait and shrimp kill in several brackish water ponds at the northern coast of Java also unreported. The HAB incident in Bangka Strait or Marsegu Island was due to the outbreak of *Pyrodinium bahamense* var. *compressum* with no impact because the island was unoccupied. This mostly occurred in 1996 and 1997. It is unfortunate that there was no research activity conducted during the bloom tragedy.

In January 1998, it was reported that a red tide phenomenon occurred along the coast of West Sumatra. The bloom caused water red-brownish discolourition. Most of the bloom appeared in patchiness and covered most coral reef area along the coast. Many stony coral in that area was observed dead during the occurrence of patchy bloom. The causative species is identified as *Gonyaulax spinifera.* It was observed that around 50 percent of the total phytoplankton was dominated by this species.

Figure 1 showing HAB incidents in Indonesian waters since 1991 until 1997, while the list of red tide organisms that have been recorded in Indonesian waters since 1991 until now are shown in Table 1.

Table 1. List of red tide species found in Indonesian waters.

Species name	Species name
A. affine	*Noctiluca scintillans*
A. cohorticula	*Ostreopsis lenticularis*
A. tamiyavanichi	*Ostreopsis ovata*
Ceratium fusus	*P. emarginatum*
C. tripos	*P. lima*
Dinophysis acuminata	*P. micans*
D. acuta	*P. triestinum*
D. caudata	*Pyrodinium bahamense*
D. miles	var. *compressum*
D. rotundata	*T. erythraeum*
Gambierdiscus toxicus	*T. thiebautii*
Gonyaulax diegensis	*Chaetoceros socialis*
G. polyedra	*C. convulotus*
G. polygramma	*C. concavicorne*
G. spinifera	*Pseudo-nitzschia pungens*
Gymnodinium catenatum	*Thalassiosira mala*
G. pulchellum	*Chattonella antiqua*
	C. subsalsa

HAB occurrences in eastern Indonesia

The first HAB impact on human health was recorded in Kao Bay, Halmahera Island in the eastern region of Indonesia which occurred in 1994 [1]. Actually this was not the first incident, but previous incidents were not associated to HAB. The causative species was identified as *Pyrodinium bahamense* var. *compressum.* This species is similar to that which has caused problems in neighbouring countries, such as Philippines, Sabah-Malaysia, Brunei Darussalam, and Papua New Guinea [6]. Similarly, in 1994 the blooms of *Pyrodinium* caused problems in Ambon Bay, where one child died and several others were treated after consuming contaminated oysters collected from the bay [1]. Since that tragedy, it was realized that the bay had been contaminated by toxic phytoplankton and therefore a red tide monitoring programme was implemented in this bay. Since that tragedy of bloom toxic "red tide" monitoring was conducted in Ambon Bay every two weeks, including the study of monthly phytoplankton fluctuations. It seemed that local climate can play an important role in the variation of phytoplankton population seasonally in this bay. There are two seasons in this region, wet and dry. Figure 2 shows rainfall condition in the eastern region of this country.

Monitoring in Ambon Bay

The abundance of phytoplankton increased in wet or rainy seasons (from June-August) and decreased in dry or hot season (from December-February). The fluctuation of phytoplankton abundance is shown in Figure 3. The abundance of phytoplankton fluctuated following rainfall. The amount of rainfall usually reached a maximum in August every year. An exception

occurred in 1997, when the amount of precipitation changed abnormally due to the El-Nino event in June to May 1997. During this condition the abundance of phytoplankton was lower than normal.

The population of phytoplankton reached a peak in the wet season, mainly at the end of in August. Phytoplankton abundance decreased in the transitional season between wet and dry season. The population grew more slowly in this transition period and was likely correlated with the condition of low nutrient which had been utilized in the previous season. Seasonal patterns of phytoplankton abundance may reflect direct correlation with nutrients in the waters such as dissolved phosphate and nitrate. During rainy season fresh water flowing to the bay through creeks and run-off drained humic substances from surroundung land into the waters. High concentration of nutrients in the rainy season may trigger phytoplankton and lead to red tide phenomena.

Bloom in Ambon Bay

The first case of red tide phenomenon that ever was recorded in Ambon Bay was the outbreak of *Pyrodinium bahamense* var. *compressum*. The bloom of *Pyrodinium* caused water discolouration of the sea surface to a red-brown color. This red tide event had caused to death and sickness of some local people who consumed mussels and oysters collected from the bay during the tragedy [1].

The bloom occurred in the rainy season at the end of July 1994 with number of cells observed around 1.6 x 10^6 per litre. Unfortunately, when the tragedy happened there was no analysis of toxin conducted to examine toxin concentration of PSP in shellfish.

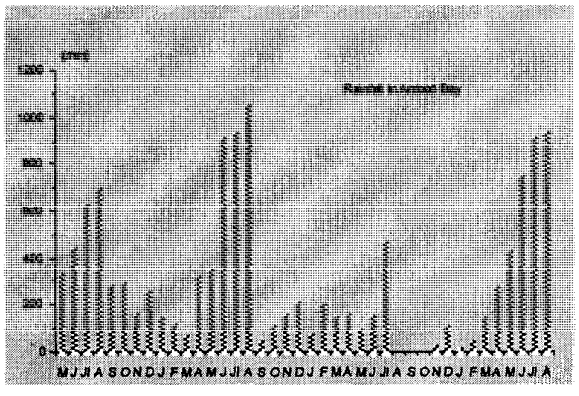

Fig. 2. Rainfall precipitation in eastern part of Indonesia from 1995-1998.

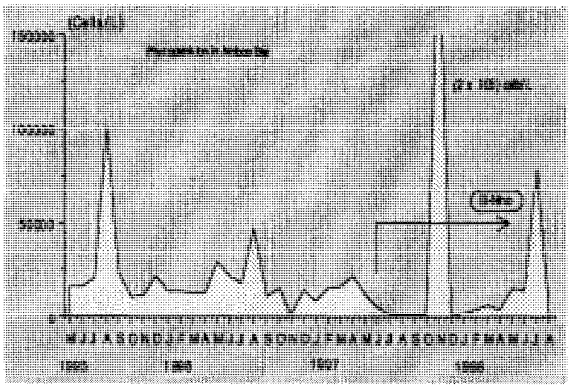

Fig. 3. Phytoplankton population in Ambon Bay since 1995-1998.

A red tide phenomenon due to the bloom of *Trichodesmium erythraeum* occurred in 1995. The bloom appeared in patches during the rainy season in August. The phenomenon caused discolouration of the sea surface to slightly pinkish and spread unevenly around the inner bay. The abundance of *Trichodesmium* was about 3 x 10^4 cells /L, and the total abundance of phytoplankton population was around 1.0 x 10^5 cells/L. There was no harm or damage to the environment.

Another phenomenon was the outbreak of green *Noctiluca scintillans* following the occurrence of *Trichodesmium* in the bay. The bloom of this species scattered around unevenly in the surface of the bay. The bloom occurred mainly in the rainy season at the end of August, and discoloured the sea surface greenish. The abundance of *Noctiluca* dring the bloom was about 1.3 x 10^3 cells/L, and the total abundance of phytoplankton population was around 2.3 x 10^4 cells/L.

The number of *Noctiluca* increased rapidly dependent on the number of *Trichodesmium* in the waters. There seems to be a causal relationships between these two species, in which *Noctiluca* lowered the population of *Trichodesmium* [6]. So far, the bloom has not caused any damage to local fisheries. More recently, the frequent occurrence of *Noctiluca* were considered to decreased the population of bait fishes in this bay. It is likely that the toxicity of *Noctiluca* was due to vacuolar ammonia. Likewise, fisheries in Vietnam experienced blooms of *Noctiluca* which caused fisheries decline [7].

Another phenomenon was due to the outbreak of *Chaetoceros* during the year of 1996. The bloom occurred in the rainy season in August and caused discolouration of the sea surface water to yellowish. The patchy bloom of phytoplankton was composed by the species of diatom up to nearly fifty percent of the total population. The abundance of total phytoplankton population at the highest peak was about 4.7 x 10^4 cells/L, while the abundance of diatom *Chaetoceros sp.* was about 2.0 x 10^4 cells /L. The bloom did not caused

any harm to environtment of the bay or damage to local fisheries.

An unexpected outbreak of the red tide species *Alexandrium affine* occurred in early November 1997. The bloom of this dinoflagellate occurred in the transitional season. So far, it was the first bloom of this species ever known in this water. This red tide species is usually found in low concentration. It was interesting that the bloom of this species occurred during the abnormal condition of climate due to the El-Nino event. The bloom scattered around the bay in patches and caused water discoloration of sea surface to red-brownish colour. The everage density of this red tide species during the bloom was around 2 x 10^6 cells/L. So far, there has been no victim reported or damages to local fisheries. It is known that this causative species of red tide is to non-toxic.

The influence of El-Nino in this region seemed still going on until March 1998. The growth of phytoplankton became normal again since May 1998. The population of phytoplankton tend to grow normally after being stressed in extreme climatic condition due to the El-Nino event. They increased coinciding with the increasing rainfall in this region. It appeared that the number of phytoplankton cells increased and caused a bloom in July 1998, due to the outbreak of *Chaetoceros*. The patches of phytoplankton scattered around the bay unevenly especially in the inner bay. The patchiness caused slight discolouration of sea surface to yellowish colour. The abundance of phytoplankton in July was about 7.8 x 10^4 cells/L and the density of *Chaetoceros* was around 3.8 x 10^4 cells/L. There was no harm during the bloom event in this bay. Table 1 summarises the occurrence of HAB in Ambon Bay since the monitoring.

Bloom during the El-Nino event

The abundance of phytoplankton showed unusual changed from June 1997 until May 1998. Phytoplankton usually reaches its maximum in the rainy season and decreases afterwards in the transitional season. It is interesting to note that during this year the abundance of phytoplankton decreased abnormally in the rainy season. This situation might be due to the influence of the El-Nino event. The impact of El-Nino started in June 1997 showing increased air temperature and there was no rain during June to August.

Table 1. Bloom occurrences in Ambon Bay

Year	Species name	Density (cells/l)	Period
'94	*Pyrodinium bahamense var. compressum*	1.6 x 10^6	rainy
'95	*Noctiluca*	1.3 x 10^3	rainy
	Trichodesmium	3.0 x 10^4	rainy
'96	*Chaetoceros*	3.3 x 10^4	rainy
'97	*Alexandrium affine*	2.0 x 10^6	transition
'98	*Chaetoceros*	4.0 x 10^4	rainy

An unexpected bloom occurred in the bay coinciding with the El-Nino event in the early of November 1997. This red tide was caused by *Alexandrium affine* (Inoe and Fukuyo) Balech. The average abundance of *A. affine* at that time was around 2 x 10^6 cells/liter. This red tide species made long chains up to 30 cells. It was commonly found with chains more than 16 cells [8].

There was no harm to environment or human intoxication. Even though, in less than a day local people were informed about the outbreak to prevent possible intoxication of shellfish consumption. So far, there is no report that this species is toxic [8]. The existence of this dinoflagellate in this water was known for a along time. It is still unknown what is the triggering factors of the outbreak of this species. The El-Nino event influenced hydrological conditions, and caused extreme air temperature with no rain and forest fires.

Several months before the bloom there was haze around the Island due to forest fires from September 1997 to November 1997. It is unclear whether there is any correlation between El Nino-Southern Oscillation (ENSO) and the outbreak of phytoplankton. Maclean (1989) pointed out that ENSO events probably affect different parts of the western Pacific to varying degrees on each occasion [6].

The salinity of surface water showed a marked increase from normal conditions. The range of sea surface salinity in the inner bay usually 30.57 - 33.95 o/oo and in the outer bay ranging from 31.42 - 34.35 o/oo. During this year, the highest salinity ever measured was 34.6 o/oo
in the outer bay. Such high values usually indicates deep water from the Banda Sea. Usually, there are two times of upwelling from Banda Sea which influences the waters of Ambon Bay [9]. Normally, upwelling rise from May to August [10]. This may also lead to enrichment of nutrient needed for the growth of phytoplankton.

Toxic species in Ambon Bay

Dinoflagellate *Pyrodinium* since the first bloom tragedy in July 1994 never again caused red tide phenomena and always was found in low number. The highest density of this toxic species during the monitoring was about 3 x 10^3 cells/L in April 1998. It is still unknown what are the major factors controlling the fluctuation of this species in this bay. Even though, the occurrence of this species is always considered be able accumulate toxin in shellfish and harmful to human if the number of cells is more than 1000 per litre [11].

The other toxic species of phytoplankton in this bay *Gymnodinium catenatum* has appeared frequently in low number since 1996. This species appeared more frequently from May 1996 onwards. The number tended to increase since December 1996 and had a maximum in February 1997. The highest number of this toxic species

ever recorded was in February 1997 with cells density around 2.3 x 10^3 cells/L. During this monitoring there was no discolorisation.

There are three other red tide species *Chaetoceros, Trichodesmium* and *Noctiluca*. All these three species frequently caused blooms in this bay, however none of the blooms caused problems.

Table 2. Red tide organisms in Ambon Bay (1995 – 1998)

No.	Species Name
1.	*Pyrodinium bahamense* var. *compressum*
2.	*Gymnodinium catenatum*
3.	*Alexandrium affine*
4.	*Trichodesmium erythraeum*
5.	*Noctiluca scintillans*
6.	*Dinophysis miles*
7.	*Gonyaulax polyedra*
8.	*Chaetoceros sp.*

PSP in shellfish

During the monitoring, it was observed that cells of *Pyrodinium* were recorded in low numbers, however, this toxic species in April 1997 was around 3 x 10^3 cells/L. In the Philippines-Bataan the number of *Pyrodinium* at 2 x 10^2 cells/L is used as concentration limit for restriction on shellfishery [12].

The other toxic species that has been found in this waters which could produce PSP toxin is *Gymnodinium catenatum*. The highest cells number of this species was recorded in February 1997 with a density around 2.4 x 10^3 cells/L. In the case of cells number of *Gymnodinium catenatum* in Portugal the concentration of 2 x 10^2 cells/L results in implementation of restriction on shellfishery. While some other countries, such as Spain-Andalusia, regulations was initiated at concentration of > 500 cells/L [12].

Mouse assay in May 1997 revealed that the average toxin concentrations in the meat of green mussels was 128 µg/100 gr tissue and in the meat of oysters was about 40 µg /100 gr tissue. The level of toxin accumulation in oyster's meat seemed lower than in mussels.

Toxin concentrations in mussels meat was higher than the critical concentration limit (80 µg/100 gr tissue) which is considered safe for human consumption. Some other countries in the world tend to apply lower level of PSP toxin than this critical concentration limit [13]. During this observation, dissipation of the information to the local people was spread out to prevent people from eating shellfish collected from the bay. So far, no report of human intoxication during the occurrences of those two toxic species in this bay.

CONCLUSSION

Most bloom events in Ambon Bay tend to occur during the rainy season. There might be correlation between bloom events and the availability of nutrient sources in these waters. It appeared that the occurrence of *Pyrodinium* bahamense var. *compressum* and *Gymnodinium catenatum* were responsible for accumulation of toxins in mussels and oysters during May 1997.

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ECOLOGICAL CHARACTERIZATION OF A WIDESPREAD *SCRIPPSIELLA* RED TIDE IN SOUTH CAROLINA ESTUARIES: A NEWLY OBSERVED PHENOMENON

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ABSTRACT

Prior to 1998, the only published record of a harmful algal bloom (HAB) in South Carolina (SC) estuarine or marine coastal waters was a 1988 *Gymnodinium breve* red tide that originated in the Gulf of Mexico and was transported to SC nearshore waters. In spring 1998, a new dinoflagellate species, *Scrippsiella carolinium* (formal description to be presented elsewhere), formed a red tide in Bulls Bay near McClellanville, SC, the first documentation of a red tide localized to SC estuarine waters. In the spring to early summer 1999, the dinoflagellate formed red tides at several sites in SC estuaries, commonly comprising > 99% of phytoplankton biomass, and at times reaching > 10^5 cells ml^{-1}. Results from field monitoring in North Inlet estuary (Georgetown, SC) indicated that bloom initiation followed rain-driven runoff events carrying dissolved organic material (DOM)-rich loads from the surrounding forests. Over the bloom periods, *S. carolinium* abundance varied inversely with DOC, DON, and DOP concentrations, and positively with dissolved inorganic carbon concentrations, suggesting high respiratory activity. Based on these results, we hypothesized that bloom development was related to *S. carolinium*'s ability to use DOM. The recent and recurrent (1998 and 1999) appearance of this widespread (in estuaries over 100 miles apart) and often intense red tide raises important issues regarding the condition of SC estuaries (e.g., is this a natural cyclical or weather-driven event, the result of species introduction, or a signal of changing water quality?).

INTRODUCTION

In contrast to much of the world's marine coastal area, reports of harmful algal blooms (HABs) in South Carolina (SC) estuaries or coastal waters are rare. Prior to 1998, the only published record of a HAB in SC marine waters was a 1988 *Gymnodinium breve* red tide that originated in the Gulf of Mexico and was transported with the Gulf Stream to continental shelf waters off North Carolina and then southward to SC nearshore waters [1]. However, no documentation of a HAB localized to SC estuarine waters existed prior to 1998. It is unknown whether the lack of HAB reports in SC relates to the type of environment characterizing its estuaries (e.g., generally shallow and well-flushed, low-to-moderate nutrient levels) or to the historical lack of research on HAB detection in SC. The issue has important implications because coastal SC is a region marked by one of the U.S.'s highest rates of development, and therefore the "likelihood that these estuaries will develop future problems [related to eutrophication] is inordinately high" [2].

In April 1998, R.E. Ashley, a commercial shellfisherman from McClellanville, SC, observed an unusual rust color in a Bulls Bay tidal creek (Keys Creek). He was concerned because the characteristic appearance of this water was new to his 30+ years of experience in fishing that area. Also, in 1997, major oyster die-offs had occurred from the Bulls Bay region to estuaries as far south as Broad River, Beaufort, SC. On 11 May 1998, J. Monck (SC Department of Natural Resources) sampled "orange/rust"-colored water from Keys Creek, and brought it to the Baruch Marine Laboratory (University of South Carolina), where it was categorized as a dinoflagellate red tide bloom, based on the high abundance (> 10^5 cells ml^{-1}) and nearly monospecific population (> 99% of total phytoplankton biomass) of a dinoflagellate.

That event marked the first report of a "localized" (i.e., apparently originating within the estuary) red tide in a SC estuary. The dinoflagellate was subsequently identified as a new species, *Scrippsiella carolinium* (Fig. 1; formerly to be described in [3]). This study examines the distribution and nutritional ecology of this newly observed SC red tide dinoflagellate.

METHODS

Water samples from estuaries other than North Inlet were collected using acid-clean bottles containing 3% acid-lugols solution or 10% hexamethylenetetramine-buffered formaldehyde [4]. Replicate samples were taken just below the surface (5 cm). Sampling sites were chosen based on the presence of discolored (brown-to-red/orange) water, and samples were taken inside and outside observed patches. This sampling scheme was designed to estimate maximal population abundance near the surface, but not spatial (horizontal or vertical) extent of the bloom.

In North Inlet estuary (NI) near Georgetown, field sampling was conducted either in response to apparent red tide bloom events or as a component of a routine monitoring program at selected sites, including the Clambank Bridge (CBB) site, a target of intensive

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sampling during the present study. Water samples were collected in triplicate 1-L polycarbonate bottles. Temperature was measured by thermometer on site, and salinity measured using a refractometer. From each sample bottle, aliquots

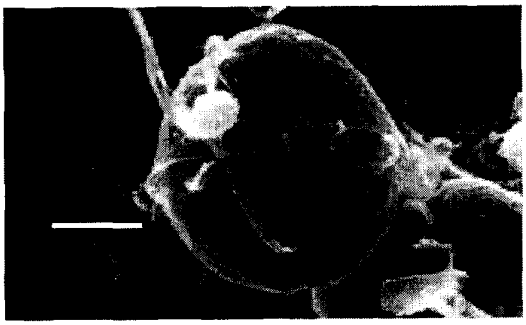

Fig. 1. Scanning electron micrograph of *Scrippsiella carolinium*. Scale bar = 10 μm.

were taken for measurements of phytoplankton community composition and abundance (by microscopic analyses), and analyses of NH_4, NO_3/NO_2, PO_4, dissolved organic carbon (DOC), dissolved organic nitrogen (DON), dissolved organic phosphorus (DOP), dissolved inorganic carbon (DIC), and $Si(OH)_4$.

Epifluorescence microscopy and a hemacytometer (depth 0.5 mm) were used to identify and quantify phytoplankton in samples fixed with 1% glutaraldehyde or 10% hexamethylenetetramine-buffered formaldehyde. Inorganic nutrients were determined by automated colorimetric analyses using Technicon AutoAnalyzers. DOC and DIC were measured using a Shimazu TOC 500. DON and DOP were determined by subtracting total inorganic from total dissolved N or P, measured by the persulfate oxidation technique [5].

RESULTS

After discovery of the red tide in Bulls Bay on 11 May 1998, follow-up water sampling and aerial photographic analysis (from fly-overs) by SCDNR researchers on 12-14 May 1998 revealed that the bloom was noticeable in estuaries from the McClellanville area (as far north as Santee Path Creek, a tidal creek feeding the northern part of Bulls Bay) south to the Stono River (southern part of Charleston); Figure 2. Heavier apparent concentrations of the bloom were observed in tidal creeks of Bulls Bay (e.g., Keys Creek and Santee Path Creek) and the Awendaw area (between McClellanville and Charleston).

Analysis of water samples collected from two Bulls Bay tidal creeks during low tide on 14 May 1998 indicated high abundances (> 10^5 cell ml^{-1}) of *S. carolinium*. Water collected from these creeks was used in biotoxin assays (brevetoxin, ciguatoxin, saxitoxin, domoic acid), which indicated no evidence of toxicity (T. Leighfield, unpub. data). A shellfish bioassay conducted by SCDNR indicated that the growth of juvenile hard clams was substantially reduced in the presence of the dinoflagellate (A.F. Holland, unpub. data).

On 27 May 1998, *S. carolinium* was found in high abundances (> 10^4 cell ml^{-1}) in a blue crab shedding house in Charleston, coincident with a blue crab 30% mortality event (A.F. Holland, unpub. data). In the summer of 1998, *S. carolinium* was detected in samples collected from two fish kill events in the Hilton Head area, but in mixed phytoplankton communities. Also during summer 1998, the dinoflagellate was identified at CBB in samples collected as part of the weekly monitoring program, but in relatively low abundances; 240, 600, and 700 cell ml^{-1} at low tide on 7 July, 13 July, and 12 August, respectively (data not shown).

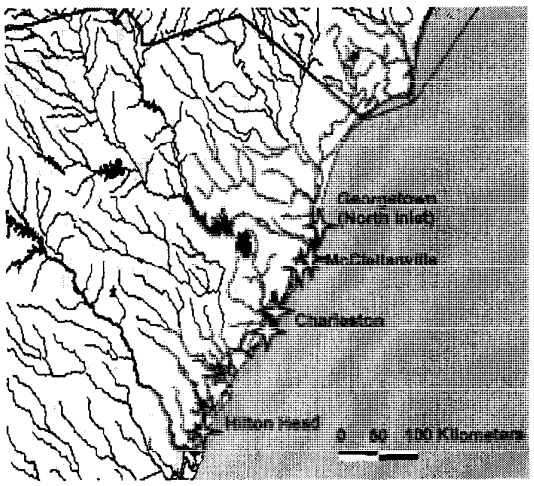

Fig. 2. South Carolina coast, showing estuarine areas (stars) where *Scrippsiella carolinium* blooms were observed in 1998-1999. Map from USGS's National Atlas of the United States of America™

In 1999, the *S. carolinium* bloom was first observed in NI (CBB site) in samples collected during routine monitoring on 14 April (Fig. 3A). That bloom persisted for 13 days. Blooms also occurred in Bulls Bay, but at a later date than in the previous year. *S. carolinium* persisted in both estuaries throughout May, and was detected in NI until mid-June (e.g., Fig. 3A). On 10 May 1999, a commercial fisherman, W. Collins, observed that the water coming out of feeder creeks to Broad Creek, Hilton Head Island, had a deep red-orange color, which he had never seen in 20+ years of fishing in that area. The event lasted for over two weeks, with peak coloration occurring on 25 May (W. Collins, pers. commun.). A sample was collected from the creek on 26 May, but during a time when the bloom was dissipated (i.e., the characteristic color was not apparent). Nevertheless, the water sample contained near-monospecific populations of *S. carolinium* at a mean abundance of 2592 cell ml^{-1}.

The patterns of *S. carolinium* abundance, nutrient concentrations, temperature, and salinity were compared during spring 1999 in water collected from CBB at low tide (Fig. 3). *S. carolinium* bloomed twice during the period, from 14 April to 27 April, and from 11 May to 11 June,

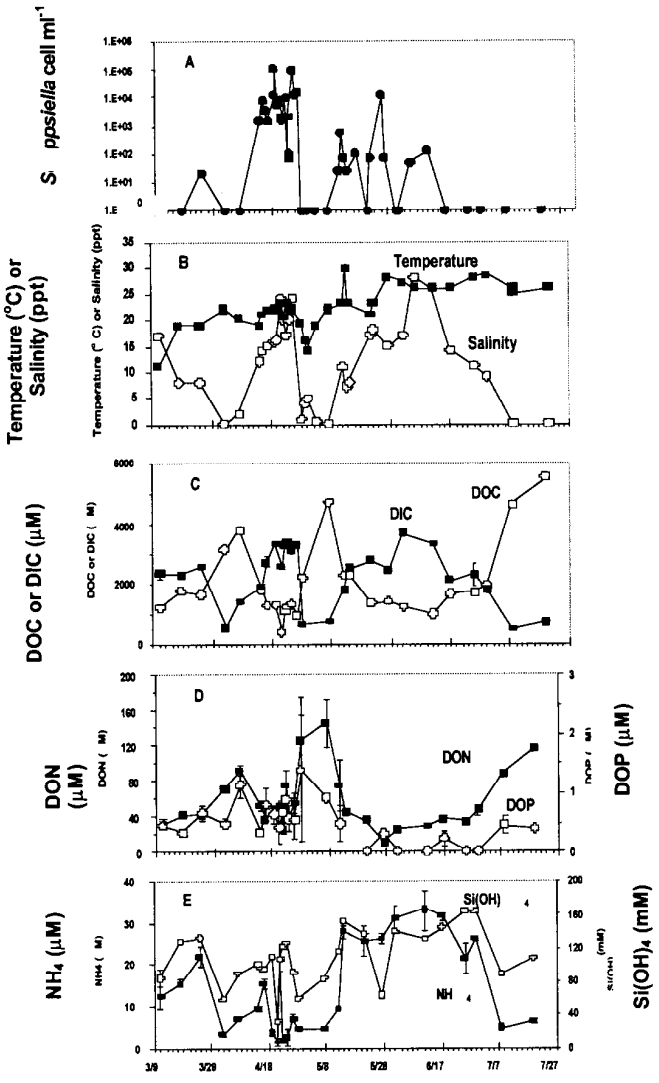

Fig. 3. A. *Scrippsiella carolinium* abundance; B. temperature (■) and salinity (O); C. dissolved organic carbon, DOC (O) and dissolved inorganic carbon, DIC (■); D. dissolved organic nitrogen, DON (■), and dissolved organic phosphorus, DOP (O); E. ammonium, NH_4 (■), and silicate, $Si(OH)_4$ (O) at the Clambank Bridge site from 9 March to 27 July 1999.

although during the latter period the dinoflagellate's abundance was generally much lower and periodically below detection (Fig. 3A). Both bloom periods were preceded by a rain event (salinity 0 ppt; Fig. 3B) that resulted in low DIC and high DOC, DON, and DOP concentrations (Figs. 3C, D). During the bloom periods, DIC increased in the initial stages and remained relatively high until the end of the bloom, when DIC concentrations decreased (Fig. 3C). The opposite trend was observed for DOC, DON, and DOP, which generally were relatively low during the bloom periods and high at other times (Fig. 3C,D). NH_4 and $Si(OH)_4$ levels showed similar trends overall (increased concentrations during the bloom periods) except for the period from 19 April to 24 April, when NH_4 concentrations sharply decreased and were maintained at a relatively low level (Fig. 3E).

DISCUSSION

The *S. carolinium* red tide blooms in SC estuaries in 1998 and 1999 appear to be new phenomena to the region. Although HAB monitoring efforts in SC waters have improved recently, it is unlikely that recognition of the blooms in the last two years, but not previous years, was a function of increased surveillance. At its peak abundance, *S. carolinium* forms distinct red-orange patches (described as "Tabasco Sauce"-colored by one commercial fisherman) that would be recognized as anomalous. In fact, the commercial fishermen who reported the blooms in Bulls Bay (McClellanville) and Broad Creek (Hilton Head) claimed that the observation was new to their experience (20-30 years of fishing those areas). Also, the observation was new to researchers involved in long-term monitoring programs in SC estuaries. These include > 20 years of intensive sampling in NI (i.e., the NSF Long-Term Ecological Research and the ongoing NOAA National Estuarine Research Reserve programs), one of the estuaries where blooms occurred in 1999.

It is premature to offer strong hypotheses explaining the recent proliferation of *S. carolinium* in SC estuaries. One is cognizant of the growing rates of coastal zone development in SC, and the potential influence on estuarine water quality and HAB stimulation. In many of the bloom locations, nutrient loading has increased markedly over the last several years, including the Bulls Bay area, which has been affected by discharges of relatively untreated sewage from a local public school, dumping of crab shucking house waste, and increased use of local waters by recreational fishermen (A.F. Holland, pers. commun.). However, *S. carolinium* also formed pronounced blooms in NI, a protected estuary (National Estuarine Research Reserve site) characterized by a lack of anthropogenic nutrient loading (although influence from atmospheric deposition cannot be discounted). Among other speculative contributors to these anomalous events are natural cyclical weather phenomena (e.g., el Niño/la Niña), long-term climate changes (e.g., sea level rise), or the introduction of the dinoflagellate through ship ballast water.

It is unknown whether the *S. carolinium* blooms were "harmful" per se. Toxin assays suggested that the dinoflagellate is nontoxic. However, *S. carolinium*'s potential to cause adverse ecological effects needs further evaluation. The coincidence between the predicted geographical distribution of the 1998 blooms (as estimated from fly-overs) and the regional extent of 1997 oyster die-offs, the coincidence between *S. carolinium* appearance and high crab mortality (in a blue crab shed), and the demonstrated reduction in juvenile clam growth in shellfish bioassays suggest either a causal relationship between the dinoflagellate's appearance and shellfish mortality or that conditions associated with shellfish problems also favored the growth of *S. carolinium*.

The relationships between *S. carolinium* abundance and nutrient concentrations at the CBB site provide

insight into the dinoflagellate's bloom ecology. The initial periods of bloom formation followed rain events, which resulted in low salinity (fresh) water containing high concentrations of dissolved organic material (DOC, DON, DOP). The CBB site is close to the forested land margin and is characterized by restricted tidal flow. Therefore, water chemistry at this site is markedly influenced by episodic forested runoff events and water column biological processing. The major DOC source to NI is humic material derived from blackwater streams [6], and the association between rain events and high water column DOM is characteristic for this estuary [7].

An association between dinoflagellate blooms and rain-driven runoff of humic-rich water has been repeatedly demonstrated, including a specific association with runoff from forested areas [8-10, and references therein]. The link between DOM loading and dinoflagellate blooms can be explained in several different ways, including potential roles of DOM in enhancing trace metal availability (i.e., chelation) or in supplementing metabolic requirements either through direct uptake of DOM or indirect uptake of DOM catabolic products (e.g., from degradation by bacteria); [8, and references therein].

Based on the relationship between DOM, DIC, and *S. carolinium* abundance, we hypothesize that bloom formation at CBB was related to DOM use by the dinoflagellate. Over the course of the bloom periods, dissolved organic compounds decreased in concentration and were maintained at relatively low levels, while DIC distribution exhibited the opposite pattern. DIC concentrations immediately preceding the blooms were low, possibly a result of freshwater-influenced CO_2 flux to the atmosphere, found to be extremely high under low-salinity (< 10 ppt) conditions in Georgia estuaries [11]. DIC increased as the blooms developed and was maintained at high concentrations until the blooms dissipated. These patterns in DOM and DIC are consistent with a net heterotrophic system, and likely reflect high respiratory rates during the blooms. Pomeroy et al. [12] also found that respiratory rates in a Georgia salt marsh estuary (Duplin River) were exceptionally high during a "*Kryptoperidinium*" bloom.

This study documented the first red tide localized to SC estuaries, and presented some bases for developing hypotheses to explain the ecology of *S. carolinium* bloom formation in one of the affected estuaries. There is a growing recognition that DOM, and particularly DON, loading can be an important stimulatory factor in HAB formation and proliferation [13, 14, and references therein]. Furthermore, in some cases, the mechanism for stimulation can be attributed to direct uptake of DOM by HAB species [15-17]. In NI, *S. carolinium* formed blooms in response to rain event-triggered loading of humic-rich waters from the forested wetland. Evaluating the link between organic loading and *S. carolinium* proliferation may be a key step in understanding its recent widespread occurrence in SC estuaries.

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ON A *LINGULODINIUM POLYEDRUM* BLOOM IN SETÚBAL BAY, PORTUGAL

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ABSTRACT

On 11 September 1996, the HAB monitoring program detected the presence of *Lingulodinium polyedrum* in Setúbal Bay. One week later, a brownish discoloration of seawater was noticed and the species reached concentrations of $7x10^5$ cells.l^{-1}. The bloom lasted 10 days and the accompanying assemblage was dominated by *Prorocentrum micans*. A hydrographic/ phytoplankton cross-shelf section, carried out in the Bay during the bloom period, revealed conditions of stratification, with a vertical gradient of 2.5°C between 10 and 30m depth. *L. polyedrum* was recorded down to 20m, with maximum densities above the thermocline, at 5m depth. One week after bloom detection, the encystment process was already occurring. *L. polyedrum* cysts were observed in the water samples, representing *ca* 0.1% of the species counts. Just after the bloom, and in the following months, sediment samples collected south of Setúbal bay, showed an increase in cysts of *L. polyedrum* with apparently viable cell content.

This bloom event was not associated with shellfish toxicity or any other harmful effects.

INTRODUCTION

Lingulodinium polyedrum (Stein) Dodge is an autotrophic dinoflagellate with a well-known life-cycle, involving a vegetative thecate motile stage, ecdysal stages and a benthic hypnozygote (cyst) [1, 2]. This species has been commonly included in toxic algae lists, although its toxicity has been controversial for a long time. Since the late 1980's this species has been associated with the occurrence of a new group of shellfish neurotoxins, yessotoxins [3, 4].

L. polyedrum is known to be a common species along the Portuguese coast, although never associated with toxicity problems [5, 6]. In 1944, 50km north of Lisbon, this species was for the first time associated with a "red-tide", involving reddish to brown water discoloration and intense night bioluminescence [7]. This was the first and the only published reference of a *L. polyedrum* "red-tide" in Portugal, prior to the here reported event in 1996.

The present report includes data from the Portuguese HAB monitoring programme, an oceanographic cruise carried out in the affected area and data from a long-term dinoflagellate cyst study, started in 1996.

MATERIAL AND METHODS

Samples for the HAB monitoring programme were collected at different sites in Setúbal Bay, by local fishermen (Fig.1). Sampling dates were dependent on shellfish collecting activities in the area, and during the study period sampling was intensified due to observation of water discoloration.

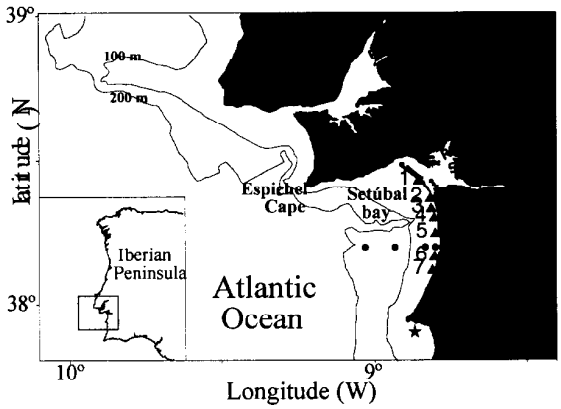

Fig.1. Map of study area showing location of monitoring stations (_), cross-shelf section (•) and cyst station (φ).

At each site, 200ml of surface water samples were collected over natural shellfish beds (water column depth *ca* 30m). Samples were preserved upon collection with acidified formalin to a final concentration of 1.2%. Phytoplankton counts were performed using a Palmer-Maloney chamber, after water concentration by centrifugation (3000rpm for 20min) and the lower limit of detection was of 100 cells.l^{-1}. Results are expressed in cells.l^{-1}.

On 1 October, on board the RV "Capricórnio", a combined CTD/phytoplankton cross-shelf section was carried out in the Bay of Setúbal (Fig.1). CTD data were collected using a SBE19 SEACAT profiler. Water samples were collected with Nansen bottles at 5, 20 and 40m depth. Sampling was performed between 15:00 and 17:00h. Samples were immediately preserved with hexamethylenetetramine buffered formalin to a final concentration of 2.4%. Phytoplankton cells were identified and counted by the Utermöhl method using a Zeiss IM35 inverted microscope with a detection limit of 20 cells.l^{-1}. *L. polyedrum* identification was confirmed with SEM.

For cyst studies, surface sediments (approximately the upper 1-2cm) were collected from a small recreational harbour, built in 1994, inside the main port of Sines (Fig.1). This site is located south of Setúbal bay, and has

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been used, since February 1996 and up to the present, as a long-term study site. Sediment samples were collected using a simple sucking device, and were kept cool and in the dark until treatment. To assure no cyst germination occurred, samples were processed the same day, or the following day. Sample preparation involved sonication in an ultra-sonic bath, wet sieving through 150μm onto a 25μm sieve, and further concentration of this latter fraction using a high density solution of sodium metatungstate (2.016g.cm^{-3}). The lighter organic fraction, floated off by the heavy liquid, was then washed in distilled water, and aliquots were mounted on microscopic slides with glycerine jelly [8]. Samples were examined for both empty cysts and cysts with cell content. Results are expressed as percentage values of the whole cyst assemblage. A total of at least 200 cysts were counted.

Upwelling indices, based on the north-south wind component, were calculated according to [9]. Wind data were obtained from the meteorological weather station located at the cape of Sines (Instituto Nacional de Meteorologia, Boletim Meteorológico Diário).

RESULTS

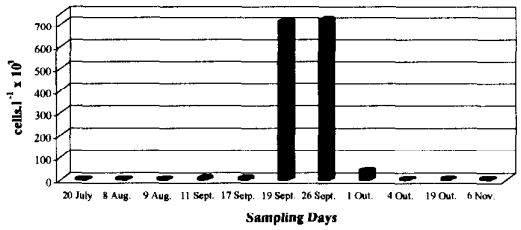

Fig. 2. Maximum concentrations of *L. polyedrum* in the area, at different times.

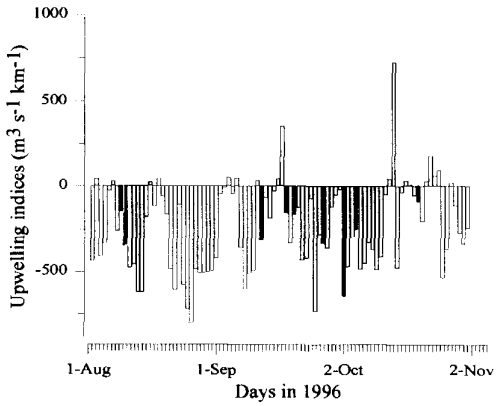

Fig. 3. Upwelling indices at the meteorological weather station of Sines. Negative values indicate upwelling and the black bars the days

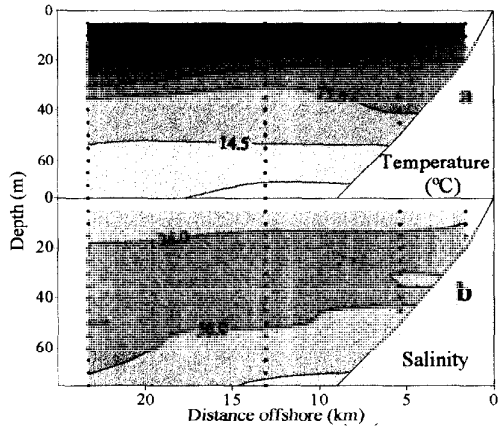

Fig. 4. Vertical distribution of (a) temperature (°C) and (b) salinity at the cross shelf section, 1 October.

Lingulodinium polyedrum was detected for the first time in Setúbal Bay on 11 September, at levels of 4x10^3 cells.l^{-1}. Because the monitoring program in the area involves sampling of different stations at different dates, results shown in Fig. 2 refer to the maximum concentration values observed in the area at each date. On 19 September, the species already reached 710x10^3 cells.l^{-1} and, on the 26th, similar high values were still recorded. Simultaneously, fishermen detected an intense brownish discoloration of seawater. On 1 October, data obtained during the oceanographic cruise at the surface of the innermost station suggested a marked decrease on the concentration of the species. On 4 and 19 October the monitoring program confirmed the bloom decline. In 1996, this was the last detection of *L. polyedrum* in the area.

Wind data at Sines cape revealed that the Portuguese SW coast was under conditions of coastal upwelling during the sampling period (Fig. 3). However, CTD data from the cruise carried out on 1 October, revealed that the water column was well stratified, with an approximate gradient of 2.5°C between 10 and 30m depth, and with no significant vertical gradient in salinity (Figs. 4a-b). Phytoplankton data showed that *L. polyedrum* was present mainly in surface samples and above the thermocline (Fig. 5a). A maximum of 3x10^5 cells.l^{-1} was found *ca.* 5 km from the coast, at 5m depth, where the recorded temperature was 17.5°C and salinity was 36 (Figs. 4a-b). Cysts of *L. polyedrum* were also observed in the water column although concentrations never exceeded 80 cysts.l^{-1}. Cyst distribution showed two maxima, one coinciding with the maximum of the vegetative stage when they represented 0.02% of the *L. polyedrum* population, and the other located near bottom level of the innermost station (Fig. 5b).

The semi-quantitative cyst data on *L. polyedrum*, indicates that this species never reaches high values at Sines harbour. The highest values were recorded between October 1996 and February 1997, with a maximum of 12% recorded in January (Fig.6). It is interesting to note that in October and November 1996, the period immediately following the reported plankton bloom, cysts of *L. polyedrum* showed a marked increase in the cyst assemblage and all the recorded cysts had cell

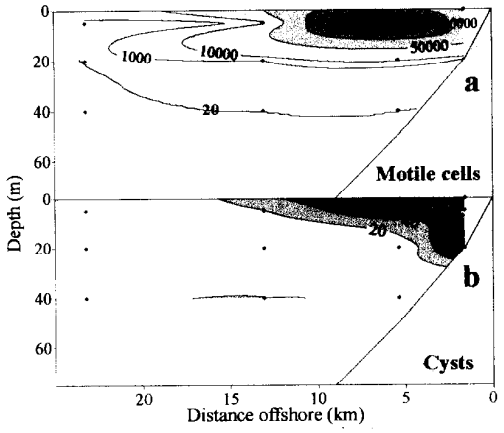

Fig. 5. Vertical destribution of (a) vegetative cells and (b) cysts of *Lingulodinium polyedrum* (cells.l^{-1}) at the cross shelf section, 1 October.

contents. Results also suggest that the annual maximum for this species tends to occur in January.

During the peak of the bloom two main size ranges of *L. polyedrum* cells were observed in the monitoring samples (Fig. 7a). The most abundant cells were the larger ones and measured around 44-45µm in length. The other group of cells measured approximately 33µm and appeared lighter in colour. Ecdysal stages were also observed (Fig. 7b). On September 26, not only these three types of cells were recorded but also fully developed resting cysts (diameter *ca.* 47µm) representing 0.1% of the total recorded cells of *L. polyedrum* (Fig. 7c).

During the bloom period, routine analysis (mouse bioassay and/or HPLC) of ASP, PSP and DSP biotoxins, carried out in the bivalves from the area (*Ensis siliqua* and *Callista chione*), were all negative [10].

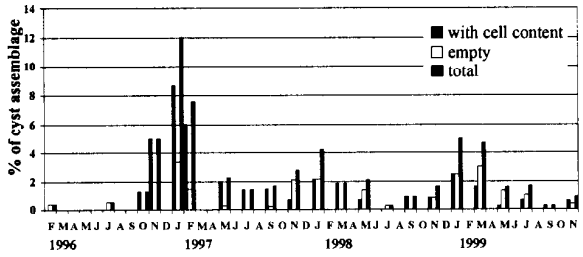

Fig.6. *Lingulodinium polyedrum* cysts at Sines harbour

DISCUSSION

L. polyedrum is a cosmopolitan, euryhaline, warm temperate species, with the northern distribution limit of its cyst linked to the sub-polar/temperate cyst boundary in the northern North Atlantic [2, 11]. The review article on *L. polyedrum* reports different conditions of stratification and nutrient enrichment, in different geographical areas, leading to bloom development of the vegetative stage [2]. These observations support the present day ecological interpretation of the increase in the cyst assemblage of *L. polyedrum* as a nutrient enrichment signal, namely

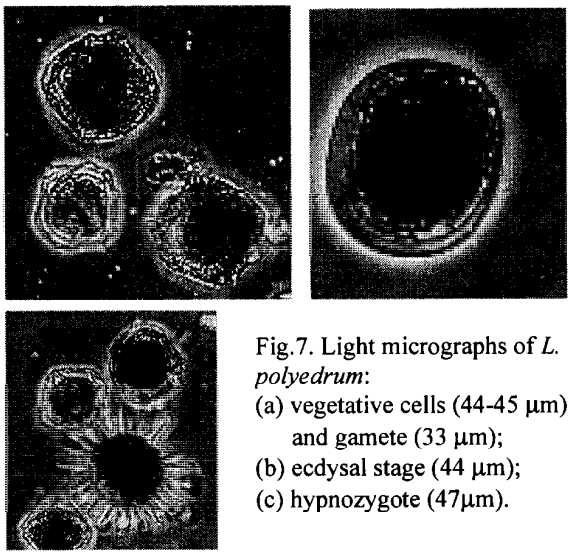

Fig.7. Light micrographs of *L. polyedrum*:
(a) vegetative cells (44-45 µm) and gamete (33 µm);
(b) ecdysal stage (44 µm);
(c) hypnozygote (47µm).

eutrophication or upwelling, rather than a low salinity signal as previously suggested [11].

Here we report a bloom of *L. polyedrum* observed in late summer-early autumn, when the Portuguese coast is characterised by upwelling. On the west coast of Portugal this phenomenon is mainly dependent on the occurrence of northerly winds. During the sampling period, the upwelling indices at cape Sines suggested that this process was occurring along the southwest coast. However, the results of the cross-shelf oceanographic transect indicated that the water column inside Setúbal Bay, where the bloom was recorded, was clearly stratified. This may be explained by several regional features (Fig 1): (i) the bay is limited north by the mountain range Serra da Arrábida, suggesting this formation may act as a barrier to the local influence of northerly winds; (ii) waters upwelled north of cape Espichel are advected southwards, although flowing offshore the sampled area, due to a pronounced coast discontinuity at cape Espichel; (iii) the general topography of the area [12]. This author showed several satellite images confirming the presence of warmer waters inside the Bay bordered by cold upwelled waters.

During the cruise, the species maximum was recorded at the surface during the mid-afternoon. This observation is in agreement with the described vertical diel-migration behaviour of *L. polyedrum* [2, 13]. Previous reports on the appearance of *L. polyedrum* cysts in the water column, as described for other dinoflagellates, show that these tend to occur after an increase in population growth, towards the end of a bloom [1, 14]. On 26 September and during the cruise (1 October), cysts with cell content were already observed in the water column, suggesting *L. polyedrum* had reached its maximum, and the bloom was already declining inside the bay.

Along the cross-shelf section, the maximum of cysts observed near the surface suggests encystment was occurring, while the maximum near-bottom, below the thermocline, may be associated with encystment and sinking that took place in the previous days (Fig. 5b).

During this study and on 26 September, the maximum ratio of cysts to vegetative cells was 1:1000. This ratio is significantly lower than the ratios observed for other closely related species, such as *Gonyaulax grindleyi* (1:2) or *Gonyaulax digitale* (1:500) [15].

Reports from a loch on the West Coast of Scotland give a time-lag of less than one month between the presence of cysts in the water column and the increase in newly formed cysts in the sediment record [14]. Our data also suggests the same pattern. Cysts of *L. polyedrum* in sediments collected during October and November 1996 were only recorded with viable cell content. In January 1997, although empty cysts showed an increase, cysts with cell content reached a maximum.

Since 1996, the monitoring program has not detected blooms of *L. polyedrum*. However, the cyst assemblage shows, for the following years, what may be considered a seasonal pattern, with maximum values occurring in January (Fig. 6). These results suggest that in the south-west coast of Portugal this species may have a seasonal cycle with a general trend similar to what has been described in higher latitudes [2].

During the peak of the bloom, the different size and colour of the swimming cells match the description from the literature for vegetative cells and gametes [16]. These, together with the presence of resting cysts and ecdysal stages suggest that almost all the life cycle stages of *L. polyedrum* were present simultaneously. Sexual reproduction was apparently occurring as evidenced by the presence of gametes and hypnozygotes (Fig. 7). In 1993, it was suggested for the first time that ecdysal stages could be transformed directly into resting cysts [17]. However, the author referred those conclusions could be speculative since the original motile population used in the experiments could have already undergone sexuality. Our field observations confirm that the different life cycle stages can co-occur and sexuality may be coincident with the presence of ecdysal stages. To our knowledge, this is the first field report that clearly identifies the simultaneous occurrence of all these life stages in the water column.

Although nutrients have not been measured, we consider that the influence of upwelled waters, offshore the bay, is a likely source of nutrients for these stratified waters, as observed to occur between upwelling events in the system of southern California [18]. It is therefore interesting to note that cyst assemblages influenced by upwelling off California are dominated by *L. polyedrum* cysts [11]. Our results support the hypothesis that stratification and nutrient enrichment are the main requirements for the development of a *L. polyedrum* bloom [2]. However, the origin of stratification (thermal or haline) and the source of nutrients (*e.g.* anthropogenic, upwelling) seem to be irrelevant.

Although *L. polyedrum* has been associated with yessotoxins production and shellfish toxicity [3, 4], the results of biotoxin routine analysis during the bloom period confirm that no harmful effects are associated with *L. polyedrum* in the Portuguese coast.

From a regional point of view, it is also interesting to note that winds measured at the meteorological station of Sines can not be considered as indicators of stratification/mixture cycles in the Bay of Setúbal, and consequently can not be used as tools to predict blooms in the bay.

ACKNOWLEDGEMENTS

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TOXIC *PROTOCERATIUM RETICULATUM* (PERIDINIALES, DINOPHYTA) IN THE NORTH-WESTERN ADRIATIC SEA (ITALY)

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ABSTRACT

The North-Western Adriatic Sea (Italy) represents the main Italian area producing edible shellfish, mostly blue mussels (*Mytilus galloprovincialis*). Diarrhoetic shellfish poisoning (DSP) due to Adriatic blue mussel ingestion has occurred since 1989 and the toxins have originated from *Dinophysis* spp. During summer 1997 unusual toxicity was detected, by mouse bioassay, in blue mussels from shellfish farms along the coast of Emilia Romagna and high level of yessotoxin and its analogues were detected. Similar toxic events occurred in summers of 1998 and 1999. Phytoplankton analyses revealed the presence of several potentially toxic species including *Protoceratium reticulatum* (Claparède & Lachmann) Buetschli. It was isolated and cultured and very high levels of yessotoxin were detected for the first time in the Adriatic strain: up to 15.736 pg/cell, compared to ~3 pg/cell found in the New Zealand strain.

INTRODUCTION

Different phytoplankton related phenomena such as red tides and toxin production have periodically affected the North-Western Adriatic Sea (Italy) since the 1970s. This causes deep concern because of the importance of this area for tourism and for commercial shellfish harvesting, mostly blue mussels (*Mytilus galloprovincialis*). DSP due to the ingestion of Adriatic Sea blue mussels has occurred since 1989 and the toxins were shown to originate from *Dinophysis* spp. [1,2,3,11]. Severe economic consequences took place due to the closure of shellfish farms for long periods. Since that time toxicological analyses for the detection of DSP, paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP) toxins in the edible shellfishes have been intensified and the water monitored for the presence of harmful algae. The toxicity was mainly caused by okadaic acid, which was detected using NMR [9] and HPLC [6,19]. Dinophysistoxin-1 was also found in Adriatic mussels with ionspray liquid chromatography-mass spectrometry [7]. In June 1995 analyses performed on toxic blue mussels from the Adriatic Sea revealed for the first time the presence of yessotoxin (YTX) together with okadaic acid [4]. In the summer of 1997 unusual toxicity was detected in blue mussels from shellfish farms along the Emilia Romagna coast. Since early July 1997, the mouse bioassays for DSP toxins [17], were positive (survival time < 5 hours), but mice presented neurological symptoms suggesting the presence of YTXs. Since then most mussel breeding areas have been closed for longer periods resulting in greater economic loss. Further analyses on toxic mussels collected from these areas confirmed the presence of YTX and its derivatives homoyessotoxin [8] and 45-hydroxyyessotoxin [5].
The aim of the study presented in this paper was that of discovering the YTX producing organism. Strains of several species were therefore isolated, cultured and analysed and, as reported below, the analyses showed the presence of high toxin concentrations in *Protoceratium reticulatum* cultures.

MATERIALS AND METHODS

Phytoplankton sampling

Water samples were collected weekly or bimonthly, at –0.5 m depth, in nearly 30 natural mussel banks from breeding areas off (6-10 km) Emilia-Romagna coast, Italy, in an area extending for 110 km from Goro to Cattolica. Samplings were performed with Niskin bottles; the water was preserved with 4% formaldehyde and then 25 to 50 mL sample was observed for cell counts in settling chambers at 400x magnification, following the Utermöhl method [16].

Organisms and culture conditions

Protoceratium reticulatum (Claparède & Lachmann) Buetschli, *Gonyaulax fragilis* (Schütt) Kofoid and *Lingulodinium polyedrum* (Stein) Dodge were isolated with the micropipette method [14] from water samples. Cultures were maintained in sterile f/10 *medium* without silicate addition (i.e. a 5 fold dilution of f/2 medium, [10]), salinity 30‰, at 20°C under a 16:8h L:D cycle (about 1600 $\mu W\ cm^{-2}$ from cold white lamps) [12].
For experimental work algal subsamples were inoculated in 500 mL f/10 *medium* without silicate, contained in 1 L Erlenmeyer flasks sealed with cotton plugs. Cell counts were made every other day in settling chambers following the Utermöhl method [16]. Cells were usually collected for toxins analysis in the stationary phase of growth except for *P. reticulatum* which was analysed at different growth phases (day 8, 14, 21 and 23). 500 mL cultures were collected by centrifugation at 10,200 g for 15 min at 4°C in a Beckman J2-HS centrifuge, resuspended in 80% methanol and stored at 5°C until toxin extraction (1 to 2 days). Each experiment was performed in triplicate.

Toxin extraction

Cells were sonicated in 80% methanol for 60 minutes in pulse mode, while cooling in an ice bath. After centrifugation at 3,000 g for 15 min the supernatant was

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evaporated to dryness. The residue (used for fluorimetric HPLC determination of YTXs according to the procedure described elsewhere [18] was dissolved in 0.5 mL of MeOH:H_2O (8:2) and 1.5 mL of 20 mM phosphate buffer (pH 5.8) and loaded onto a Sep-pak C_{18} cartridge column (Waters Chromatography Division U.S.A.).
The column was washed with MeOH:H_2O (2:8) and YTX was eluted with MeOH:H_2O (7:3). The residue obtained after evaporating the second eluate was transferred into an amber vial with 0.5 ml of MeOH and the solvent was evaporated to dryness.

Derivatization and clean-up

The residue was mixed with a fluorogenic reagent 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-ihydroquinoxalin-yl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD, Wako Pure Chemicals). The reaction mixture was kept at room temperature, in the dark, for 2 h and then evaporated. The labelled product was loaded onto a Sep-pak C_{18} cartridge column (Waters S.p.A.) with MeOH:H_2O (3:7). After washing the column with MeOH:H_2O (3:7), the YTX-DMEQ-TAD adduct was eluted with MeOH:H_2O (7:3).

HPLC analyses

The fluorescent adducts were analysed on a Luna C18 (PHENOMENEX 4.6 x 250 mm) with a mobile phase consisting of 40 mM phosphate pH 5.8 buffer and MeOH in a 3:7 ratio.
The YTX standard utilised was in a first time kindly provided by Professor T. Yasumoto and for later experiment purchased from the Institute of Environmental Science & Research Limited (Wellington Science Centre, New Zealand).

Calculations

In our batch cultures the net rate of toxin production was calculated using the equation:
$\ln (T_2/T_1) / (t_2-t_1)$
where T_1 and T_2 are the toxin concentrations at day t_1 and t_2.

RESULTS and DISCUSSION

Phytoplankton observations

Phytoplankton has been monitored for the presence of toxic species year round. In the period during which YTX appeared (from June-August to November of 1997, 1998 and 1999) potentially harmful species such as *Dinophysis* spp., *Lingulodinium polyedrum* and *Protoceratium reticulatum* have been found, but the species present with the highest numbers was the nontoxic *Gonyaulax fragilis* (max cell numbers/L was 1,340,000 in 1997). *Dinophysis* spp. concentrations in that years were similar to those found during DSP appearance (maximum cell number/L was 1,080 in 1997), however, the mouse bioassays had revealed the presence of neurotoxic symptoms, consistent with the presence of YTXs, and no okadaic acid was detected. The other mentioned species were isolated and analysed for YTX content including *G. fragilis* due to its constant presence in high amounts during YTX appearance. As expected, the strain isolated and cultured did not reveal a toxic factor (data not shown). *L. polyedrum* reached a maximum cell number/L of 1,080,000 in 1998; it sustained severe seawater discolourations in the Adriatic Sea from 1976 to 1984 and these were never associated with toxic mussels. However, in 1997 homoyessotoxin was identified in samples collected using a net during a bloom of the same species which occurred in the Adriatic Sea [15]. Yessotoxin analyses performed in a *L. polyedrum* strain isolated during the same bloom and grown in culture in our laboratory gave uncertain results (data not shown).
P. reticulatum was found in the period from June to September of the 3 mentioned years. Although it reached very low cell numbers (max value was 8,900 cells/L on August 1997) it was suspected as the species responsible for the toxicity because a strain from New Zealand had previously been shown to produce YTXs [13]. Toxin analysis of a strain isolated and cultured in our laboratory revealed, for the first time, the presence of YTX in high amounts in *P. reticulatum* from the Adriatic Sea, as below reported.

Cell growth and yessotoxin content of P. reticulatum

Growth of cultured *P. reticulatum* displayed a short lag phase; the exponential growth phase was long (day 3-17)) with a growth rate (μ) of 0.29 ± 0.02 day^{-1} (Fig. 1). A maximum density of 1.3 ± 0.4•10^4 cells/mL was reached in about 23 days.

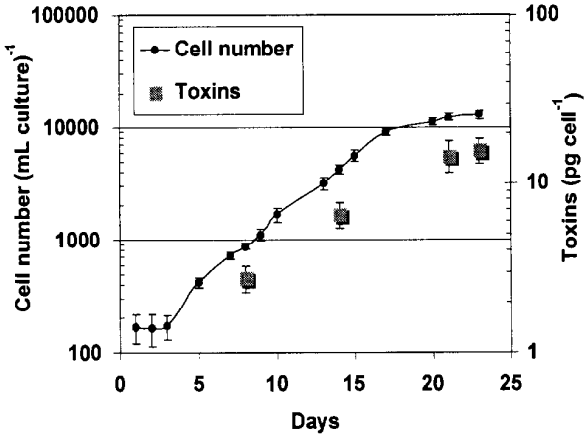

Fig. 1. Cell number and YTX content in *P. reticulatum*.

HPLC analyses of yessotoxins (Fig. 2) was carried out at different growth phases. YTX content was very high ranging from 2.747 pg/cell at day 8 to 15.736 at day 23 (Fig.1); thus, as compared to YTX concentration found in the New Zealand strain (3.0 pg/cell) [13] the Adriatic strain was much more toxic. The increase in toxin

content per cell paralleled the growth pattern (Fig. 1), however the highest production rates were observed during the exponential growth phase (Table 1).

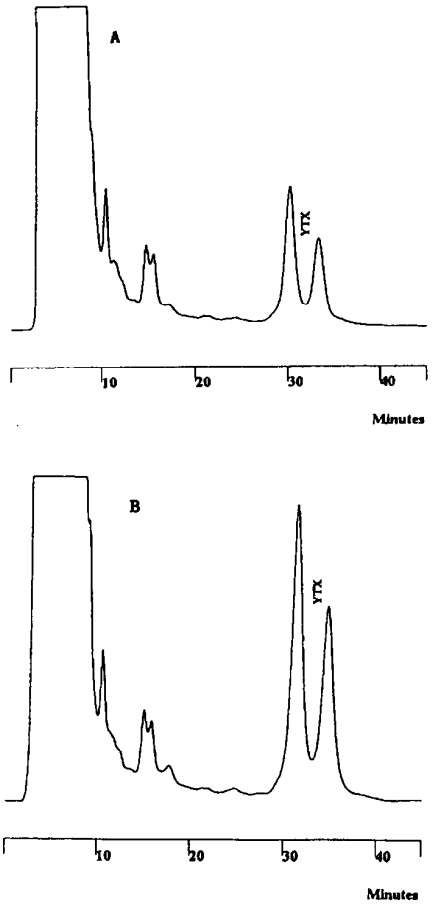

Fig. 2. Fluorometric HPLC chromatograms: A) YTX standard (68 ng); B) YTX in *P. reticulatum* after 23 days of growth (15.736 pg/cell – 10,352 cell injected).

CONCLUSIONS

The above results show, for the first time, the presence of toxic *P. reticulatum* in European waters. The high toxin content per cell underlines the need for a careful monitoring of the phytoplankton present in mussel breeding areas as even a very low number of cells can cause high toxicity. The toxic effects of yessotoxins on humans are still unknown but, as mice are affected by toxin injection, mussels farms were closed during the period of toxicity presence, posing serious economic losses. Research is in progress in order to understand the roles of the environmental conditions, mainly nutrients, salinity and temperature, on YTXs production in that they are highly variable in the studied area of the Adriatic Sea due to the input of various rivers

Table 1. Yessotoxin production rates measured during different growth phases of *P. reticulatum*.

Days	pg/cell/day
8 - 14	0.145
14 - 21	0.115
21 - 23	0.037

ACKNOWLEDGEMENTS

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OCCURRENCE AND SUCCESSION OF POTENTIALLY HARMFUL PHYTOPLANKTON SPECIES IN THE EASTERN HARBOUR OF ALEXANDRIA, EGYPT

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ABSTRACT

Since first observed in 1956 red tides caused by *Alexandrium minutum* Halim remained a recurrent summer phenomenon in the type locality of the species, the eutrophic Eastern Harbour of Alexandria.

The year 1994, saw the last red tide of this dinoflallate, followed by its complete disapperance. Daily observations carried out in the summer of 1999 showed *A. minutum* to have been replaced by a community of other potentially harmful species: *Pseudo-nitzschia pungens, Prorocentrum minimum, P. triestinum, Chattonella* spp. and others. Alternate dominance of this community with diatoms, cyanophytes and rhaphidophytes is modulated by and significantly correlated to wide salinity fluctuations, and stability of the water column interrupted by wind induced turbulence. The amplitude, frequency and duration of the bloom pulses is controlled by grazing pressure of tintinnids, copepods and planktonic larvae. *Chattonella* spp. is a first record for the East Mediterranean.

INTRODUCTION *The problem*

The Eastern Harbour (EH) of Alexandria is a small semiclosed bay about 2.53 km^2, in surface area. It is surrounded by the city along its southern margin and protected from the open sea by a breakwater with two inlets (Fig. 1). Its bottom slopes down to 5 m at the center and 12 m at the inlets. The EH is basically used as a fishing and yachting harbour. It receives a daily volume of domestic waste water amounting to 15 x 10^3 m^3d^{-1}[1], which creates a mixed surface layer, 1-2m in thickness representing a distinct ecosystem. Heavy algal blooms causing discolouration were recorded in the EH since 1956, caused by the proliferation of the dinoflagellate *Alexandrium minutum* Halim [2]. Red tides remained a recurrent but harmless summer phenomenon since [3-6]. Such heavy blooms are triggered by the continuous input of biogenic substances which maintain a high level of nutrients (4.1-7.3 µmole. nitrate $N1^{-1}$ and 2.6-5.8µ mole. PO_4-$P1^{-1}$), accompanied by stable stratification of the water column in summer [6,7].

Occasional fish kills occurred during May 1987[8] at a cell density of $6x10^6$ cell 1^{-1}. They were attributed to localized oxygen depletion and/or gill clogging of fish. During the first week of October 1994 a red tide caused by *A. minutum* broke out and extended out of the EH along 20 km of coast with a peak density of $24x16^6$ cell 1^{-1}[6]. The bloom was accompanied by massive fish kills in the whole area. All fish and invertebrates inside the public aquaria were also killed, in spite of continuous aeration and filtration of the water supplied from the EH. Hundreds of rock crabs migrated out of the water onto the beach[6,9]. Although the toxin was not analysed the dead fish exhibited symtpoms of toxicity with yellowish colouration of the body and gills, others appeared to have lost their equilibrium, swimming on their side or upside down.

The subsequent years however have seen a complete change in the community structure and succession. With this problem in mind, it was decided to follow the dynamic of the summer population at a greater resolution in time than previously.

MATERIAL AND METHODS

Quantitative surface phytoplankton samples were collected daily from 15 May to 5 September 1999 from a station at the center of the EH (Fig. 1). Water temperature was measured and salinity determined according to standard methods [10].

The settling method for fixed phytoplankton counting was followed [11] using an inverted microscope. In addition, zooplankton were counted and sorted into major groups. The data were statistically analyzed using multiple regression, and cluster analysis [12].

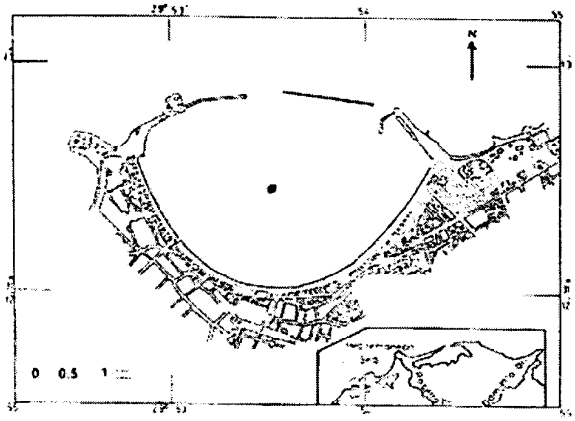

Fig. (1): the Eastern Harbour (EH) and the position of daily station.

RESULTS AND DISCUSSION

The disappearance of *A. minutum* red tides.

Daily observations over 95 days during the summer of 1999 confirmed the total absence of *A. minutum* from the EH population. After 1994 it had already ceased to be the major blooming species, sharing dominance with

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Euglena sp., *Pyramimonas* sp., *Skeletonema costatum* and *Prorocentrum triestinum* [13].

No perceptible alteration in the marine environment in the EH however could be linked to its disappearance. On the other hand, the instability of the bottom sediments appears to have caused the main change in the last decades. Bottom sediments in the EH are subjected to strong and continuous erosional hydrodynamic forces, from the NW waves and currents channelled through the bay inlet. The bathymetric surveys carried out [14] revealed a considerable deepening along the central axis of the harbour. Although the examination of three sediment cores from the E.H. confirmed the presence of *A. minutum* cysts in moderate amounts [15], its seed beds have been scoured at an average rate of 13 cm year^{-1} and about 70% of the eroded material has escaped the harbour. This is further evidence of the link between cyst forming red tide species and their cyst beds in shallow semi-closed basins. It also provides potential red tide management options in similar environments. Dredging surface sediments might help control the recurrent outbreaks of harmful blooms in such environments.

Trend in (Summer 1999). The disappearance of *A. minutum* left an ecological niche now occupied by a group of seven potentially harmful species endemic to the EH. *Prorocentrum minimum, P. triestinum, Scrippsiella trochoidea* and two *Chattonella* spp. are pervasive and bloom forming. *Pseudo-nitschia pungens* and *Skeletonema costatum* are frequent. *Oscillatoria* sp. gave short bloom pulses.

Seventy species were recognized. As observed earlier[16] the summer population is characterized by the dominance of dinoflagellates, both in species richness (49 species) and in numerical abundance. Diatoms follow with 19 species, cyanobacteria with 4 species, Rhaphidophytes with 2 species and Chlorophytes with one species. Cluster analysis (Fig. 2) showed the distribution pattern in time to consist of two twin associations (I and II) briefly interrupted by two minor ones (III and V). Salinity fluctuations (33.27 to 38.9‰) appear to modulate this succession. The two main associations (Table 1) were almost always dominated by *P. minimum* accompanied respectively by *P. triestinum* and *Scrippsiella trochoidea*. They are differentiated by the alternate dominance of a small number of dominant species and by the relative frequency of the less prominent ones: *Skeletonema costatum* and *Pseudo-nitzschia pungens* for association I, *Scrippsiella trochoidea* for association II. *Chattonella* spp. is common to both (Table 1).

It is worth mentioning that *Chattonella spp.* has not been reported before from Egyptian waters nor from any part of the Eastern Mediterranean sea, As a matter of fact no Rhaphidophyte has been reported before from Egyptian waters

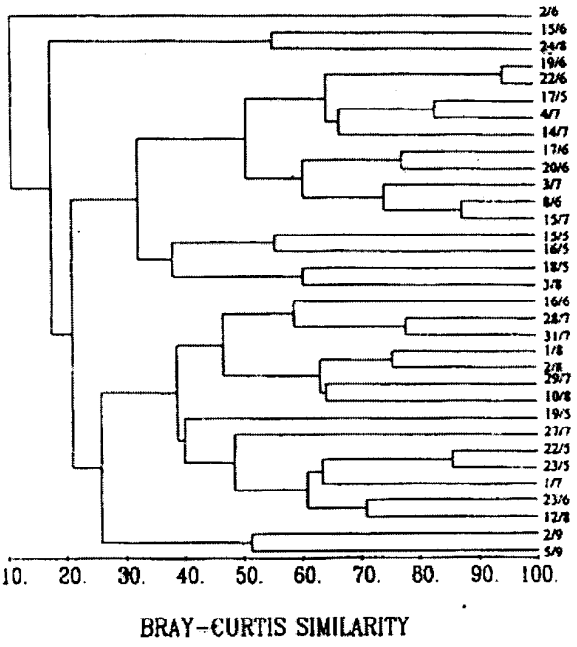

Fig. (2): Dendrogram showing two main and two smaller associations.

Table (1): Associations I and II, their respective diversity and the corresponding blooms.

Association I		Association II	
Species	Average	Species	Average
Prorocentrum minimum	55%	*Prorocentrum minimum*	38%
P. triestinum	15%	*Scrippsiella trochoidea*	27%
Skeletonema costatum	13%	*P. triestinum*	13%
Pseudo-nitzschia	9%	*Chattonella spp*	5%
pungens	8%	*Pseudo-nitzschia*	5%
Chattonella spp		*pungens*	5%
		P. micans	
Blooms: May 15-18, June 22, July 3-4		Blooms: May 19, 22-23, June 23, July 27-31	

Oscillatoria sp. contributed 25% to association III when salinity dropped to 33.2%, followed by *P. minimum*. *Scrippsiella trochoidea* contributed 60% to association IV, again followed by *P. minimum*. Both associations lasted respectively for only two days.

A nutrient replete environment such as the EH would be expected to sustain a quasi-continuous dense bloom. The population, instead, displays blooming phases of a few days interspaced by phases of sparse plankton. Thirty two phytoplankton pulses exceeded 0.5 x 10^6 cell l^{-1}, thirteen of which ranged from 1 x 10^6 to 2.3 x 10^6 cell l^{-1} (Fig. 3).

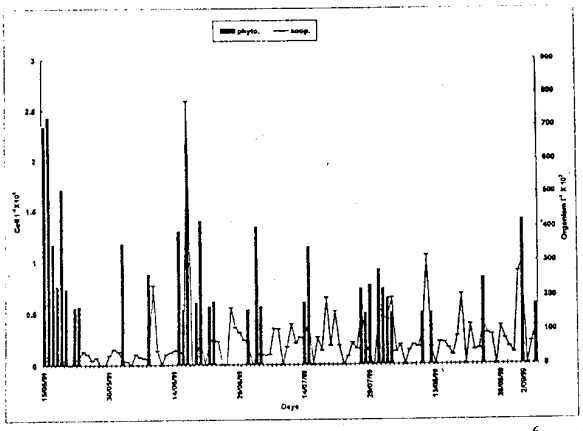

Fig. (3): Phytoplankton blooms exceeding 0.5 x 10^6 cell l^{-1} and zooplankton organism. l^{-1} from 15 May to 5 September 1999.

The population dynamics are governed by wind induced turbulence of the surface layer, on one hand, and the grazing pressure of microzooplankton on the other. Sustained wind speeds exceeding 7 to 12 $m.S^{-1}$ interrupt the blooming phases, as happened in the last week of May 1999 and the second week of June 1999. The negative correlation with the microzooplankton pulses, however, is more significant (0.89 at 95% confidence limit). The frequency, the amplitude and the duration of the blooms are controlled by the grazing pressure. This was most obvious in August, a month comparatively poor in phytoplankton, but with a massive proliferation of planktonic larvae, of tintinnids and ciliates (up to 21 x 10^3 – 32 x 10^3 zooplankton organism per 1).

In conclusion, there processes converged during the summer of 1999 to inhibit the outbreak of harmful blooms in the EH in spite of the presence of several potentially harmful species and of favourable environmental conditions. They were the disturbance of the seed beds, the occasional disruption of the otherwise stable stratification of the water column and heavy grazing pressure. As a result, the phytoplakton population remained below the threshold to cause harmful blooms.

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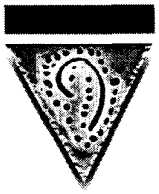
POPULATION DYNAMICS, ECOLOGY AND OCEANOGRAPHY

Harmful
ALGAL
BLOOMS



Ninth Conference
TASMANIA
2000

HARMFUL PHYTOPLANKTON EVENTS CAUSED BY VARIABILITY IN THE IRISH COASTAL CURRENT ALONG THE WEST OF IRELAND

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ABSTRACT

Frequent sampling in summer along the western and northwestern coasts of Ireland showed the rapid onshore development of blooms of potentially harmful phytoplankton species. In both 1998 and 1999, concentrations of *Gyrodinium* cf. *aureolum* rose by four orders of magnitude to over one million cells per litre in Donegal Bay (northwestern Ireland) in less than 10 days. The rapid development of these populations was linked to advection resulting from unfavourable wind-forcing of the Irish Coastal Current (ICC) which runs northwards along the western Irish coast. Current measurements showed that after a particular sequence of changes in wind direction phytoplankton populations could be rapidly advected from areas of slack circulation on the shelf via the ICC into aquaculturally sensitive coastal zones such as Donegal Bay. The model presented is similar to one already demonstrated for the occurrence of toxic events in the bays of southwestern Ireland. Other historical harmful events along the west and northwest coasts relating to substantial losses in both finfish and shellfish culture could also be explained using the model. These include the *G. aureolum* bloom of 1992, the *Prorocentrum balticum* bloom in 1997.

INTRODUCTION

It is now accepted that potentially harmful phytoplankton blooms can arise at coastal sites through physical advection of a community which has developed elsewhere [1,2,3]. Wind-forcing can result in advection of dinoflagellate blooms from the shelf off the south coast of Ireland around the southwest Irish coast and into coastal embayments [4]. This has been demonstrated directly for both *Gyrodinium aureolum* blooms [5], and the advection of a *Dinophysis acuta* community resulting in an accumulation of DSP toxins in farmed mussels [6]. The significance of these events is witnessed by the fact that approximately 80% of the national edible mussel (*Mytilus edulis*) and 60% for the Pacific oyster (*Crassostrea gigas*) production currently come from aquaculture operations along the southwestern Irish coast. A further 25% of national farmed salmon production comes from this region.

More recent development of aquaculture along the northwestern Irish coast has not been without problems. For example, an exceptional bloom (<3 x 10^6 cells l^{-1}) of *G. aureolum* in Donegal Bay in September 1992 was directly linked to mass (80%) mortalities of farmed clams (*Tapes semidecussatta*). A bloom of *Prorocentrum balticum* of over 16 x 10^6 cells l^{-1} was observed in the same region in November 1997 (Irish Dept. of Marine, unpublished records). Both of these events occurred over a time scale smaller than the sampling frequency (<3 days for the *G. aureolum* and < 1 week for *P. balticum*) with the implication that they have arisen via advection.

This paper presents results of physical and biological studies carried out since 1997 in Donegal Bay, NW Ireland and demonstrates the role of wind forcing on the Irish Coastal Current (ICC) in the advection of potentially harmful phytoplankton populations into the region.

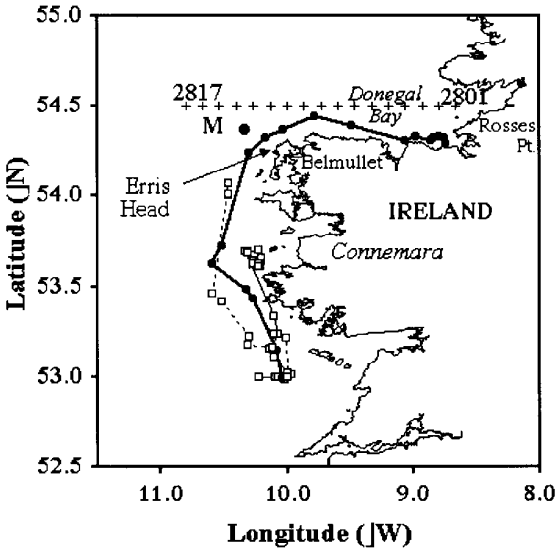

Figure 1: Map of study area. M indicates the position of the current meter mooring PBF-3; the line of crosses refers to the transect of 17 stations sampled on 12-13 August 1999. Included are the tracks of Drogues A (open squares, solid line), B (open squares, dashed line) and C (filled circles). See text for details.

METHODS

The study area is shown in Figure 1. Currents along the west and northwestern shelf off Ireland were measured using both Lagrangian and Eulerian techniques. Vector averaging current meters (Aanderraa Instruments, Bergen) located 45 and 100 m above the seabed were deployed on a mooring on 4 May 1997 at position PBF-3 (54°22'N; 10°20'W) off Erris Head in a water depth of 125 m. On recovery, the data length was 138 days, with a time interval of 20 minutes. In 1999, three Argos satellite-tracked drifters were deployed off the west coast of Ireland at 53°N. The drogues were of a "holey-sock" design, with dimensions 7 m long and 1.5 m in

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diameter, set at 35 m depth in order to be located close to or within the seasonal thermocline where maximum cell densities are known to occur. An average of 2 to 3 fixes per day were obtained for each drifter. Wind data for all years were supplied by the nearest Irish Meteorological Office station located at Belmullet, Co. Mayo (Fig. 1).

In 1998 and 1999, water samples were collected off Rosses Point (Co. Sligo; Fig. 1) at least twice per week from July to September. A 1.3 l NIO water sampling bottle was used to collect discrete water samples from near the surface and at 3 m depth for the determination of phytoplankton and salinity. Phytoplankton samples were enumerated with an inverted microscope after sedimentation using a modified Utermohls technique [7]. *TS* profiles at the location were obtained with a calibrated WTW LF 191 temperature-salinity probe. A transect of 17 stations (Fig. 1) along 54°30'N was sampled on 12-13 August 1999 when a Seabird CTD rosette system linked to an *in situ* fluorometer was used to sample water from the sub-surface chlorophyll maximum.

RESULTS

The mean residual current over the period 5 May to 20 September 1997 was 7.5 cm s^{-1} in direction 003° (T). A more northeastards flow (013°; 13.5 cm s^{-1}) was evident from the record, interspersed with occasional southwestward flow, as occurred between 4-11 May and during the second half of June. These reversals were a direct consequence of wind-forcing from northerly winds (Fig. 2).

G. aureolum counts observed at Rosses Point in 1998 and 1999 are shown in Figure 3. Both data sets show rapid increases of approximately two orders of magnitude within 3 days occurring around 19 August 1998 (Fig. 3a) and 26 July 1999 (Fig. 3b). In both instances cell concentrations reached over 10^6 cells l^{-1}. These increases are faster than known growth rates for this organism [5]. Two other features are apparent in the results. First, the blooms disappeared equally as quickly as they appeared, suggesting physical transport in and out of the sampling location. Secondly, they were both accompanied by an increase in salinity (Fig. 3c,d) indicating physical control of these events. Stick plots for daily wind speeds and direction recorded at Belmullet for the periods covering the 1998 (not shown) and 1999 (Fig. 4) blooms showed that the blooms occurred when winds were from the east.

Phytoplankton counts on samples taken from the surface and from within the sub-surface chlorophyll maximum along the transect of stations at 54° 30'N on the 12 August 1999 did not reveal any measurable *G. aureolum* populations within Donegal Bay until 9°40' W. where a population of 5-10 x 10^4 cells l^{-1} existed (Table 1). These samples were taken 10 days after peak concentrations were observed at Rosses Point, and the results are indicative of the speed of dispersion of the blooms out of Donegal Bay. Furthermore, winds at the time (12-13 August) were westerly, which might favour entrapment of phytoplankton populations within the bay.

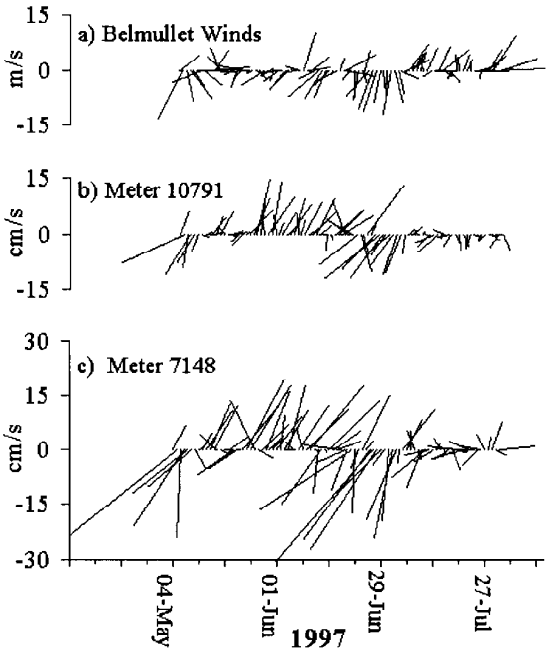

Figure 2: Stick plots of a) winds measured at Belmullet (m s^{-1}) and residual (non-tidal) currents (cm s^{-1}) measured by the current meters b) 10791 located 95 m above the sea bed and c) 7148 located 43 m above the sea bed. Vectors are all scaled to show true direction

The tracks of the drogues deployed at 53° N are shown in Figure 1. All drogues moved north along the shelf. Drogue A halted in its northward track at 53°37'N on 8 August, apparently being caught in a coastal eddy. Drogue B stopped transmitting its location on 7 August. Drogue C, however tracked into Donegal Bay on 23 August, ending up close to the Rosses Point sampling station at the beginning of September. Measured northwards velocities between 53° and 54° north were of 6.8 cm s^{-1} (Drogue A; 28 July to 7 August), 8 cm s^{-1} (Drogue B; 28 July to 7 August) and 22.3 cm s^{-1} (Drogue C; 19 to 22 August). Comparison with wind data shows that these maximal rates occurred when winds were from the northwest.

DISCUSSION

The Irish Coastal Current (ICC) runs along the continental shelf roughly parallel to the western Irish coast [8,9]. Current speeds of the ICC have been estimated to be approximately 7 cm s^{-1} [8], in close agreement to the velocity (7.5 cm s^{-1}) calculated from the extended current meter record (138 days) obtained in the present study. The higher velocity measured from drogue C of 20-25 cm s^{-1} is quite similar to the larger

Table 1. *Gyrodinium* cf. *aureolum* counts at the chlorophyll maximum measured along a transect of stations at 54° 30'N on 12-13 August 1999.

Station	Latitude		Depth	Cells l^{-1}
	(deg)	(min)	(m)	
2801	8	40	22	7600
2802	8	48	30	8900
2803	8	56	24	10100
2804	9	04	20	8900
2805	9	12	28	5000
2806	9	20	16	1200
2807	9	28	23	11400
2808	9	36	23	29000
2809	9	44	23	84000
2810	9	52	25	47000
2811	10	00	25	103000
2812	10	08	30	26000
2813	10	16	25	43000
2814	10	24	0	16500
2815	10	32	30	0
2816	10	40	27	5000
2817	10	48	15	2500

northwards velocities measured at PBF-3 when winds were from the southwest (Fig. 4). It should be noted that the track shown by this drogue is consistent with the hypothesis that phytoplankton blooms can be rapidly imported into Donegal Bay from the continental shelf.

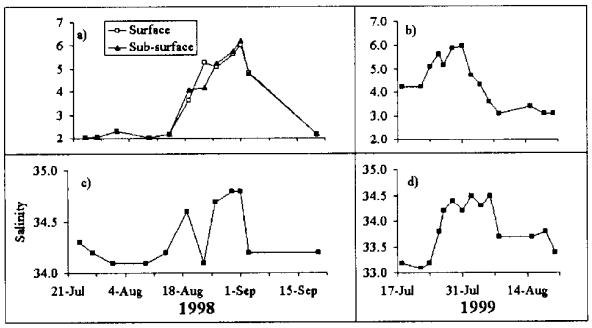

Figure 3: a, b ; *Gyrodinium aureolum* densities (cells l^{-1}) and c, d ; Salinity (PSU) measured at Rosses Point, Co. Sligo during the summers of 1998 and 1999.

Wind-forced water exchange between coastal embayments and outlying water is a well understood phenomenon. In an Irish context, a model has been developed which shows water exchanges to occur as a result of shifts in the axial component of the wind-stress along Bantry Bay, SW Ireland [10]. This process has been directly related to ingress of phytoplankton blooms within the bay [5]. The origin of these blooms were subsequently shown to be a region of slack residual circulation on the adjoining shelf [4].

The results presented here are consistent with a similar advective mechanism occurring in Donegal Bay. Measurements of the local currents demonstrate that phytoplankton can be transported northwards along the western Irish shelf with speeds of up to 20-25 cm s^{-1} and subsequently into Donegal Bay.

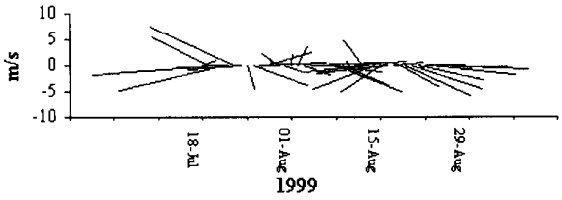

Figure 4: Stick plot of winds (m s^{-1}) measured at Belmullet during July and August, 1999.

Further inferences can be drawn from phytoplankton observations carried out in October 1997, when a bloom of *P. balticum* was observed off west Connemara [11]] Samples containing 10^5-10^6 cells l^{-1} were observed at aquaculture sites, and the presence of this organism was associated with mortalities, starvation, and abnormal behaviour of farmed salmon. Subsequently, the population was noted within Donegal Bay. Again, these observations are consistent with the advective mechanism proposed here, which highlights the importance of wind forcing in transporting potentially harmful phytoplankton populations into aquaculturally sensitive regions.

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THE ADVECTION OF A TOXIC BLOOM OF *GYMNODINIUM CATENATUM* TO THE GALICIAN RÍAS, DETECTED FROM SST SATELLITE IMAGES.

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ABSTRACT

Coastal conditions along the west coast of Galicia at the end of the 1995 upwelling season are presented. Winds, sample data from the monitoring in the Galician Rías and SST imagery from NOAA satellites are combined to study coastal patterns accompanying a toxic algae bloom of *Gymnodinium catenatum* that produced PSP toxicity levels above legal limits in cultured shellfish.

After 20 days of northerly winds, southerly winds were noted, increasing its intensity. CTD data showed a generalised downwelling in the rias, shifting the 17° C isotherm to almost 40 m depth in the outer station of the southernmost ria (Ría de Vigo). *G. catenatum* was found in low concentrations in the inner part of the rias: maximum concentrations were noted in the external part of Ría de Vigo, with more than 30000 cells/L coincident with the strong downwelling. *G. catenatum* was not present in previously collected samples, supporting the advection of the population in the shelf water into the rias.

The temperatures measured in the rias were similar to offshore water temperatures in the previous upwelling period, suggesting that offshore water had moved onto the coast, but SST imagery show a different pattern in the area. Northward moving warm water was found 15-25 km by the coast, in conformity with the measured southerly winds and CTD data. Nevertheless, upwelling features were still present over the shelf break, indicating an equatorward movement of cold water. This equatorward flow blocked the entrance of the oceanic offshore water into the Rias, suggesting that the water that was moved into the Rias with southerly winds was not offshore water, but southern water that was transported northward following the coast from Portuguese latitudes.

INTRODUCTION

Since the first observation of *Gymnodinium catenatum* in the Galician Rías, NW of Spain, in 1976 [1], this dinoflagellate has often been found in the area forming toxic blooms, usually producing PSP toxicity levels above legal limits for cultured shellfish. To ensure protection of public health, a monitoring programme has been in place since 1976, initially overseen by the Spanish Institute of Oceanography (IEO), and from 1992 by the Marine Environment Quality Control Centre (CCCMM). The program evaluates toxicity levels and if above legal limits, production areas are closed and shellfish collection is forbidden.

The aquaculture industry has a great importance in the Galician Rías, with current overall production of mussel approximating 200,000 tonnes, with a first sale value close to 80 million dollars. The sudden closure of production areas causes severe economic losses, not only due to the closure itself, but also due to the mistrust in the consumer on shellfish health, which may lead to a fall of sales after reopening of production areas.

Thus, early detection is desirable, to permit collection of threatened cultured bivalves, and research to this goal has focused on the origin of toxic blooms. Sudden onsets of *G. catenatum* blooms were usually related to upwelling relaxation, at the end of the upwelling season, and several hypotheses about bloom origin have been formulated: advection of an initial population in shelf waters which are moved onto the Rias by downwelling conditions [2]; massive germination of resting cysts of *G. catenatum* from the sediments of the Rias [3]; and selection of this species in the Rias due to strong downwelling conditions, based on the vertical migration capability of *G. catenatum* [4].

In this paper, SST imagery have been combined with CTD data and cells counts from 1995 to support the hypothesis of advection of initial populations of *G. catenatum*. An inshore warm poleward current was detected on 11 September coincident with downwelling measured from CTD casts in Ría de Vigo. A sudden bloom of *G. catenatum* was found at the same date. It is suggested that the population inoculum was advected from coastal Portuguese shelf waters in this inshore current.

MATERIAL AND METHODS

Data from different sources from 8/10/95 to 9/18/95 have been compared. Wind data were obtained from the Instituto Nacional de Meteorología (INM), at Cape Fisterra (42°55'28'' N, 9°17'29'' W), chosen because is not affected by terrain features. The upwelling index based in Bakun [5] has been calculated from the measured winds, as an estimation of upwelled water per km of coast. Northerly winds generate positive values for the upwelling index (upwelling), southerlies generate negative values (downwelling). Upwelling index is shown from 1 August to 18 September (Fig. 1).

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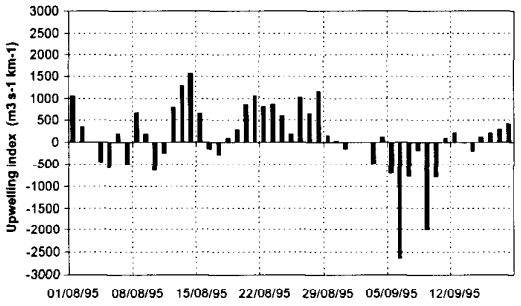

Fig. 1. Upwelling index (m^3 s^{-1} km^{-1}) from 1 August to 18 September 1995.

CTD data and phytoplankton counts were taken from the CCCMM database[6]. The monitoring methodology is described in Pazos and Maneiro[7].

Data from the AVHRR sensor on board NOAA-12 and NOAA-14 satellites have been processed using the "split-window" algorithm [8] in order to obtain the Sea Surface Temperature (SST). SST was obtained for the period from 22 August to 14 September. Due to the continuous cloud coverage that affects the area of study during these dates, no cloud filter has been used. From 28 August, cloud coverage

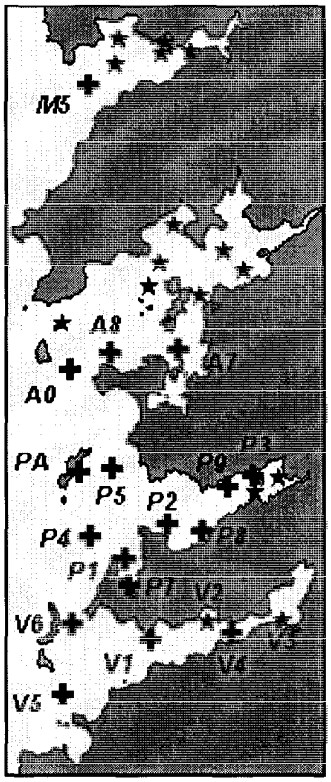

Fig. 2. Stations location in the Rías Baixas. Stations where *G. catenatum* was found in 1995 are represented with crosses, others with stars.

was very abundant in the area, not allowing for the visualization of the zone on 3-6 September.

RESULTS

During the first months of 1995, cells of *G. catenatum* were not found in weekly water and net samples from 33 stations in the Rías Baixas (Fig. 2). Cold and saline water intrusions at depth were detected periodically by CTD measurements following upwelling pulses common in the area.

From 12-15 August, strong winds blew from the north, resulting in upwelling indexes > 1000 m^3 s^{-1} km^{-1}. After 4 days of weak winds, northerly winds were measured from 20-28 August, producing strong and steady upwelling (Fig. 1). On 29 August, vertical stratification was observed in Ría de Vigo, with a strong subsurface thermocline (Fig. 3).

SST imagery provided a mesoscale view (Fig. 4). On 22 August (not shown) coastal temperatures reached 14°C off Cape Fisterra and 16° C off Porto. Oceanic waters, offshore of the upwelling front, were 20-22°C. This situation continued until 28 August, although upwelling increased in the north, and decreased lightly in the south.

After 28 August, winds were variable and weak for a week, with upwelling indexes near zero (Fig. 1). As noted before, cloud coverage was so dense from 3-6 September that no SST data were available. On 5 September (Table 1), *G. catenatum* was detected in very low concentration (<80 cells/L) at two stations (P_3, P_9) located in the middle of Ría de Pontevedra

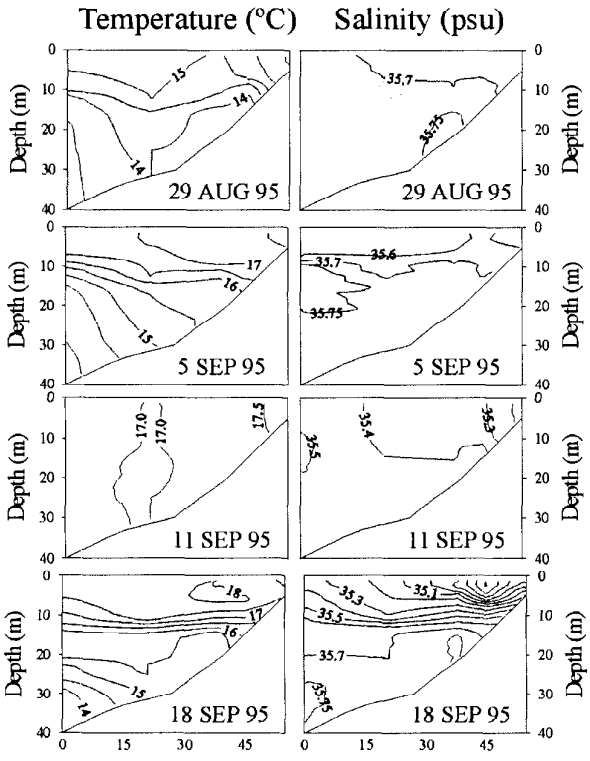

Fig. 3. Temperature and salinity collected from Ría de Vigo.

(Fig. 2). The oceanographic patterns revealed that temperature gradients decreased in intensity and isotherms tended to vertical (Fig. 3). Uniform salinities (between 35.50-35.75 PSU) were seen throughout Ría de Vigo.

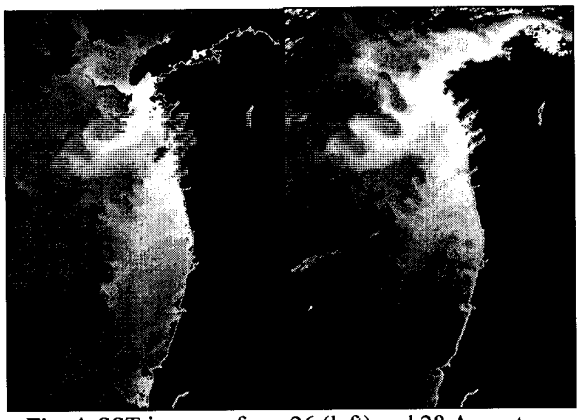

Fig. 4. SST imagery from 26 (left) and 28 August 1995 (right).

Date	Station	*G. catenatum* Concentration
21/08/95	V5	80
21/08/95	P2	20
21/08/95	P4	20
05/09/95	P3	80
05/09/95	P9	20
11/09/95	V1	3760
11/09/95	V4	240
11/09/95	V5	32200
11/09/95	V6	29280
11/09/95	P1	4960
11/09/95	P2	20
11/09/95	P4	2160
11/09/95	P5	1600
11/09/95	P7	120
11/09/95	P8	20
11/09/95	PA	1760
11/09/95	A0	960
11/09/95	A7	20
11/09/95	A8	2840
12/09/95	M5	20

Table 1.- Concentration of *G. catenatum* (cells/L) from 1 March to 12 September 1995. Stations not cited had null counts.

From 5-10 September, strong southerly winds blew, yielding very negative upwelling indexes, below 2000 $m^3 s^{-1} km^{-1}$ (Fig. 1). This strong downwelling homogenized the Ría de Vigo water column (Fig. 3); temperature and salinity were 17±0,5 °C and 35,4 PSU, respectively. On 11 September 32200 cells/L were measured at station V_5, 29280 cells/L at V_6, and values between 240 cells/L and 4900 cells/L were noted in the stations located at the southern and outer part of the other rias, Ría de Pontevedra and Ría de Arousa. Cells were also found in the stations located in the inner part of these rias, and even in the outer station of Ría de Muros, the northernmost ria in the Rías Baixas.

The image from 10 September (Fig. 5-left) shows a cold strip near the coast, and higher temperatures offshore. Finally, the image from 11 September (Fig. 5-right) revealed the coastal structure produced by the reversal of the winds: an inshore northward current of warm water 15-25 km wide following the coast from Porto to the rias, and colder water (1.0-2.0°C) offshore, becoming 75km wide at Porto's latitude, with apparent southward movement. Offshore of this cold water, oceanic water at the same temperature of the coastal current was present.

After this sudden event, downwelling relaxed from 11-18 September, with the upwelling index near zero (Fig. 1). An upwelling pulse began and the vertical gradients of temperature noted earlier were found again in Ría de Vigo (Fig. 3). On 18 September, *G. catenatum* < 300 cells/L was observed in Ría de Vigo and Ría de Pontevedra (with the exception of 2400 at station V_5), with no *G. catenatum* after this date.

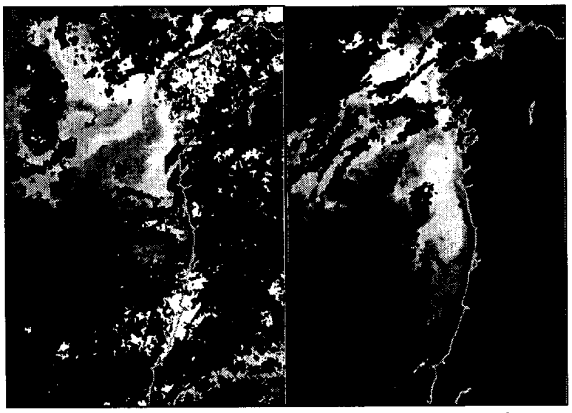

Fig. 5. SST imagery from 10 (left) and 11 September 1995 (right).

DISCUSSION AND CONCLUSION

The sudden onset of a bloom of *G. catenatum* in September 1995 occurred at the end of the upwelling season. Salinity and temperature data were strongly related with the measured winds: southerly winds changed coastal conditions from upwelling to downwelling, and warm water was introduced into the Rías Baixas, breaking down stratification measured in previous weeks. Few cells of *G. catenatum* were detected on 5 September in 2 stations in the middle of Ría de Pontevedra. The presence of high concentrations of cells detected one week later (as high as 32200 cells/L at station V_5) doesn't seem to be related with the cells found on 5 September, because of its spatial distribution and its sudden high concentration. On 11 September the spatial distribution showed an evident gradient from south to north and

from outer stations of the rias inland, indicating advection of this population of *G. catenatum* .

At first examination, winds and CTD data might suggest that water from offshore of the upwelling front was moved into the rias when upwelling ceased, accompanying downwelling. In this case, *G. catenatum* cells would be advected in oceanic water from the upwelling front. However, SST images revealed another pattern, an inshore northward current of warm water following the coast from Porto to the Rías Baixas on 10-11 September (Fig. 5). This water had the same temperature of oceanic water, as can be seen in the images, and cold water with an apparent southward movement separated this coastal water from oceanic water.

We suggest that this inshore poleward current transported an initial population from southern shelf water into the rias. This would explain the sudden onset of the bloom, as well as the CTD data measured in the rias.

ACKNOWLEDGEMENTS

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GYMNODINIUM BREVE (DINOPHYCEAE) IN THE WESTERN GULF OF MEXICO: RESIDENT VERSUS ADVECTED POPULATIONS AS A SEED STOCK FOR BLOOMS

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ABSTRACT

A sampling program in Texas (USA) coastal waters examined near shore waters for *G. breve* and related species. *G. breve* was found inside 15 km in only 6 of 131 samples. Monitoring provided no warning of a *G. breve* fish kill in southern Texas immediately adjacent to a sampling site and suggests that routine monitoring in the Texas coastal zone may provide little or no warning of impending red tides in these waters. *Gymnodinium mikimotoi* was recorded at varying concentrations throughout the year and appears to have a more continuous presence in the near-shore phytoplankton. Since there is no evidence that *G. breve* fish-kills originate <15 km from the coast, we conclude that these events originate offshore and are transported into the area. Support for this hypothesis was found in an transect that noted nutrient-rich water advecting onto the shelf in association with an anticyclonic feature. This feature resembled Loop Current intrusions and could provide both the offshore source waters and the intermittent timing associated with Texas red tide outbreaks.

INTRODUCTION

Gymnodinium breve red tides along the Texas coast (western Gulf of Mexico) are highly disruptive to both fishing and tourism industries. This species has been implicated in fish kills since at least the 1930's [1]. Although the historically sparse population of the Texas coast has limited observations, it appears that until the mid-1980's, major events occurred at approximately 20 year intervals. However, 4 events have occurred since then (1986, 1996, 1997 , & 1999). Fish mortality from beach surveys was estimated at 22.2, 3.0, 21.8, and 0.5 million fish during 1986, 1996, 1997, and 1999, respectively. It is likely that 4-5 times that number remained offshore and were not counted (Texas Parks and Wildlife Department [TPWD] Kills and Spills data). These effects, as well as the impacts on tourism and human health, have focused attention on the origin of *G. breve* red tides along the Texas coast.

Loop Current intrusions are suggested to play a crucial role in the development of *G. breve* red tides along the west Florida shelf [2,3]. The Loop Current does not penetrate to the Texas coast in the western Gulf of Mexico and other hydrographic features must be associated with the hypothesized intrusions. Conversely, *G. breve* may have permanent populations along the near-shore Texas coast that periodically reach red tide concentrations. While this appears unlikely, there are no data to exclude this possibility.

In response to these needs, a simple program was established to monitor *G. breve* populations along the Texas coast. There was no resident inshore *G.breve* population from Nov. 1998 to Jan. 2000. However, a nutrient intrusion along the continental shelf was associated with an anticyclonic feature. This suggests that this type of feature may be important in seeding *G. breve* in a manner analogous to Loop Current intrusions along the Florida coast.

METHODS

Surface samples were collected in 5 areas (<15 km offshore) along the Texas coast (Fig. 1) at approximately 2 week intervals in conjunction with

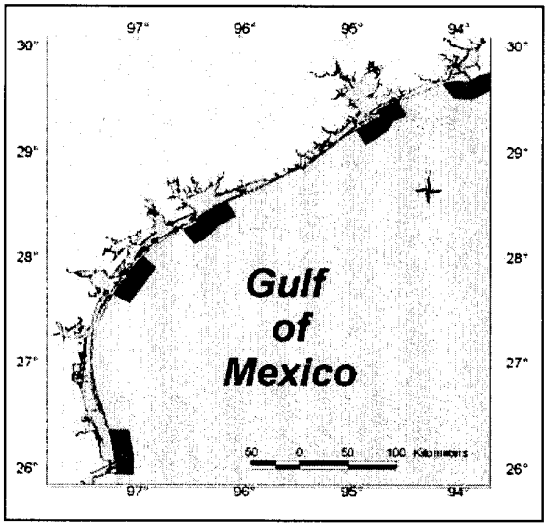

Fig. 1 Sampling areas (in dark boxes) along the Texas coast. From north to south, the stations were off of Sabine Pass, Bolivar Roads Pass, Cavallo Pass, Port Aransas Pass and Brazos Santiago Pass.

the Texas Parks and Wildlife Coastal Fisheries Division's finfish survey. Sites within the sectional grids were randomly determined. Samples were preserved in Lugol's Iodine [4]. Nutrient and chlorophyll samples were filtered (0.45 μM polycarbonate filter and Poretics GF-15 glass fiber

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filter, respectively) and frozen. Temperature, salinity and dissolved oxygen were measured with a YSI meter. Water samples were settled and an equivalent volume of 45 ml examined in a Sedgwick Rafter cell. Nutrients were analyzed on a Lachat Quikchem 8000 and chlorophyll was analyzed fluorometrically in methanol extracts using a non-acidification technique [5]. *G. breve* is positively phototactic, and surface water samples are considered to provide adequate survey data for its presence (Geesey and Tester 1993). In addition, the waters within 15 km of shore are generally well mixed and little persistent stratification is evident.

RESULTS

The two geographic extremes (Sabine Pass in the north and Brazos Santiago Pass in the south) also represent the hydrographic extremes along the Texas coast and will be used to characterize the range of conditions along the coast. Sabine Pass had the coolest winter temperatures and the lowest salinity with the most extreme fluctuation, while Brazos Santiago Pass had the most stable salinity and temperatures (Fig. 2).

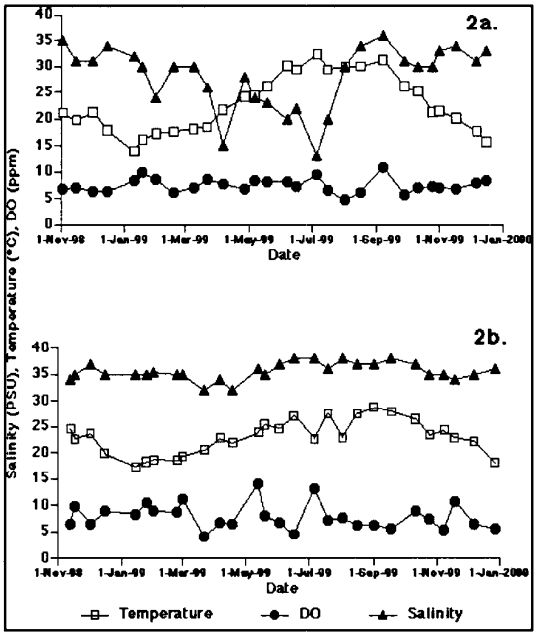

Fig. 2 Temperature and salinity data from two stations along the Texas coast. These stations represent the geographic extremes of the sampling. Fig. 2a. Sabine Pass in the north. Fig. 2b. Brazos Santiago Pass in the south.

The increasing salinity is a reflection of the aridity of the south Texas coast and the general decrease in river flow along the precipitation gradient. Nutrients were highest and most variable at the northern stations and decreased in concentration towards Brazos Santiago pass (Fig. 3). This latter station had values typical of oligotrophic waters over much of the year. Chlorophyll values reflected this same pattern with the highest values in the north (Sabine Pass) and the lowest values near Brazos Santiago (Fig. 4).

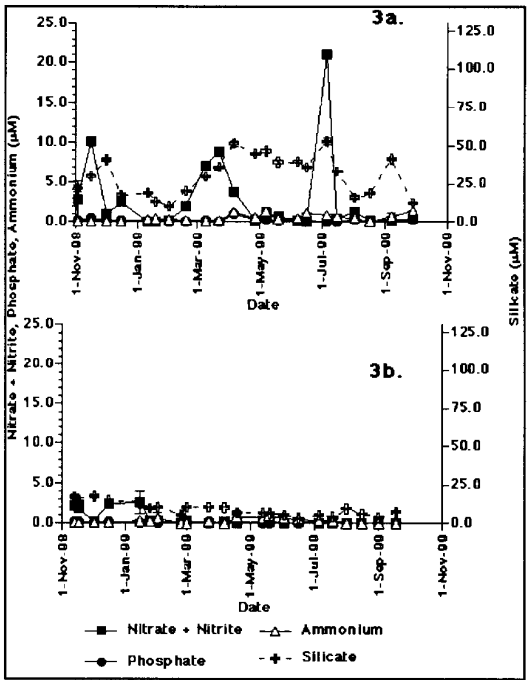

Fig. 3. Nutrient cycles from two stations along the Texas coast. These stations represent the geographic extremes of the sampling. Fig. 3a. Sabine Pass in the north. Fig. 3b. Brazos Santiago Pass in the south.

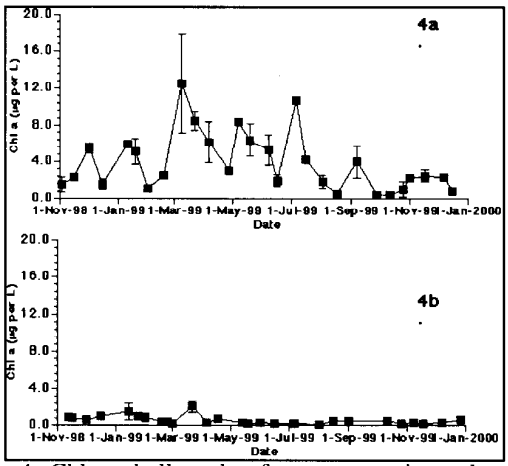

Fig. 4. Chlorophyll cycles from two stations along the Texas coast. These stations represent the geographic extremes of the sampling. Fig. 4a. Sabine Pass in the north. Fig. 4b. Brazos Santiago Pass in the south.

G. breve was noted only 6 times in 131 samples (Fig. 5). It was observed twice off of Cavallo Pass (Fig. 5c), once off of Bolivar Roads Pass (Fig. 5b) and 3 times off Brazos Santiago Pass (Fig. 5e), respectively. The highest *G. breve*

concentration was noted in late October, 1999 after a fish kill near Brownsville.

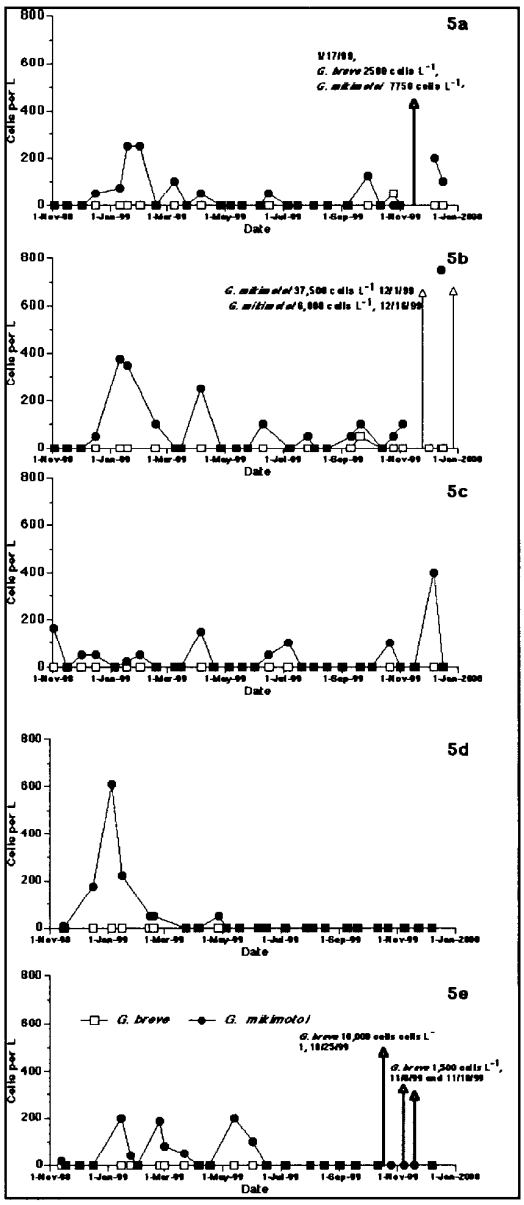

Fig. 5. Cell count data from stations along a north-south direction. Text and numbers on figures indicate data that was offscale. 5a. Sabine Pass, 5b.Bolivar Roads Pass, 5c.Cavallo Pass, 5d. Port Aransas Pass, 5e.Brazos-Santiago Pass.(Brazos Santiago Pass area) in late October, 1999.

The *G. breve* abundance in the fish kill was on the order of 10^6 to 10^7 cells L^{-1}. The event occurred suddenly during a cold front that resulted in 10-15 m s^{-1} winds from the northeast. There was no prior increase in the *G. breve* population noted in the monitoring data, only an abrupt step increase in the concentrations after the fish kill occurred. This fish kill lasted for only a few weeks along the Gulf of Mexico shore, although *G. breve* occurred as intermittent patches for over a month in inland bays and canals. All *G. breve* cells were observed between 18.1-28.4 °C and 30-36 PSU.

In contrast, the closely related species *Gymnodinium mikimotoi* was commonly found (47 of 131 samples), and was present at one or more stations along the coast in all months except August, 1999. In contrast to *G. breve*, *G. mikimotoi* showed evidence of distinct population increases and decreases suggestive of a resident population. This was particularly evident in the November 1998 sampling off of Port Aransas Pass (Fig. 5d). This species was noted down to 15 PSU; however, all occurrences of > 100 cells L^{-1} were noted at >23 PSU, and the bulk of the reports occurred at >30 PSU. No reports of fish kills were associated with *G. mikimotoi* populations.

During a cruise of opportunity in July, 1999 we sampled an anticyclonic feature northeast and offshore of Brazos Santiago Pass (Fig. 6). A discussion of the hydrographic and biological

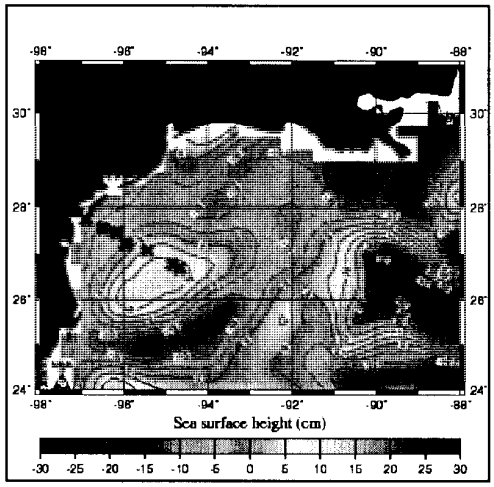

Fig. 6. Radar altimetry data from July 23, 1999 with cruise stations overlaid on it. Note the large anticyclonic feature off the south Texas coast with the cruise track across the northern edge. Data from the Colorado Center for Astrophysical Research website (http://ccar.colorado.edu/research/gom/html/gom.html).

characteristics of this feature is beyond the scope of this paper; however, it is clear from the nutrient profiles (Fig. 7) that substantial transport of nutrient-rich, offshore water onto the shelf was occurring. The intrusion penetrated to within 50 km of the coast and to within 50 m of the surface. Based on light profiles, this would place the upper portion of the nutricline above the 1% light level. The anticyclonic feature dissipated by mid-September, 1999.

DISCUSSION

Previous work has noted low *G. breve* abundance (ca. 100 cell L^{-1}) in the western Gulf of Mexico [6], but no information has been available on seasonal variations in the near-shore area.

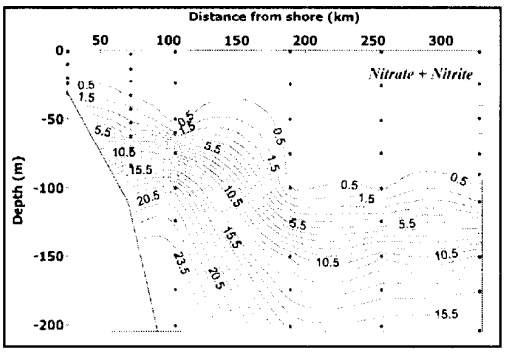

Fig. 7. Nitrate+nitrite contours along the 6 station transect in July 1999 (figure provided by Dr. F. Jochem). The apparent doming at 150 km is an artifact of the software.

In this regard, our report is more notable in what we did not find. *G. breve*, if present, was consistently below our detection ability of approximately 25 cells L^{-1}. There is no evidence of a resident population in Texas coastal waters <15 km offshore at the 25 cells L^{-1} level. While it is possible that persistent populations existed either further offshore or in deeper water, the key point is that the fish killing densities did not arise by gradual increase in local populations. They clearly had their origin further than 15 km offshore.

The two significant pulses, in Oct.-Nov at the geographic extremes of the coast (Sabine Pass and Brazos Santiago Pass), appear to be unrelated. The November increase in the Sabine Pass area was coincident with substantial populations note off the Louisiana coast that may have had their origins farther east (Q. Dortch, per. comm.). It is unlikely that this population initiated the events in extreme south Texas over 500 km away. This fish killing event arose suddenly with no prior warning, and with no cells observed in the adjacent station at Port Aransas Pass.

The Brazos-Santiago fish kill in Oct. 1999 followed a period of strong winds from the northeast that suggest onshore transport. This is similar to the Loop Current intrusion mechanism suggested for the Florida coast, and raise question about the role of mesoscale features off the Texas coast (1, 2). These mesoscale features, both cyclonic and anticyclonic, are long-lived, dominate the western Gulf of Mexico (7; 8) and have direct effects on local productivity and hydrography (Biggs 1992; Biggs et al. 1997). Our observations suggest that nutrient-rich water can be transported onto the shelf in association with anticyclonic features, and reinforces previous observations that these intrusions are typical features of the Texas shelf [11]. While the anticyclone had been replaced by a cyclone by October, 1999, it is possible that introduced populations were stranded on the shelf and advected inshore with the strong northeasterly winds. Salinity fronts have been observed to strand similar intrusions off the Florida Shelf (G. Vargo, pers. comm.) and are noted off the Texas shelf as well. This eddy intrusion mechanism must be confirmed by direct cell counts, but the physical similarities to Loop Current intrusions are striking. These eddy intrusions provides a potential explanation for the origin of *G. breve* red tides off the Texas coast.

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THE HYDROGRAPHIC REGIME, NUTRIENT REQUIREMENTS, AND TRANSPORT OF A *GYMNODINIUM BREVE* DAVIS RED TIDE ON THE WEST FLORIDA SHELF

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ABSTRACT

The 1998-1990 *Gymnodinium breve* bloom that developed on the West Florida Shelf was controlled by hydrographic features, nutrient supply and nearshore currents. Bloom inception occurred approximately 20 km offshore after the breakdown of vertical thermal stratification. The formation of a thermohaline front in coastal waters during January 1999 maintained the bloom near the shore where it was later advected northward by coastal currents. Out-welling of estuarine water from Tampa Bay and Charlotte Harbor with DIN:DIP ratios <1.0 may have supplied sufficient inorganic PO_4 to support growth at 0.2 divisions d^{-1} but the nitrogen requirements were not met by *in situ* concentrations of NO_3. Nutrient availability on the West Florida Shelf was sufficient to support bloom biomass only if organic sources of nitrogen, perhaps from a variety of sources, sustained the population. The combination of hydrographic features and nutrient availability on the West Florida Shelf control the initiation and maintenance of *G. breve* blooms in this region.

INTRODUCTION

The West Florida Shelf (WFS) is a broad (~200 km wide) shallow, oligotrophic shelf with a seasonal pattern of hydrographic features which influence the initiation, growth, and transport of blooms of the toxic, red tide dinoflagellate, *Gymnodinium breve* Davis [1]. Despite the oligotrophic nature of this region, blooms with high biomass persist for months [2].

Well defined seasonal hydrographic changes characterize the shelf environment. Summer thermal stratification with strong vertical gradients is replaced via vertical mixing during the fall transition period when weather fronts penetrate southward [3]. Typical wind patterns throughout the year contribute to periods of coastal up-welling and down-welling [4] which potentially influence the onshore and offshore movements of blooms. Increased rainfall during the summer-fall period may also contribute to the formation of nearshore salinity fronts and enhance nutrient discharge from the coastal estuaries.

G. breve blooms are known to be associated with Loop Current intrusions and thermal fronts that may concentrate and transport bloom populations [5,6]. Therefore, knowledge of the relationship of red tides to hydrographic parameters (e.g. currents, temperature, salinity, frontal regions) is crucial to determining the mechanics of bloom formation, growth, maintenance, and dissipation.

Here we examine several hydrographic features of the West Florida Shelf and their relationship with the inception, growth, transport and persistence of a *G. breve* bloom that started in November, 1998. This bloom persisted for four months and moved from the southern to the northern edge of our study area (Fig. 1) in one month.

METHODS

Hydrographic data and water column samples were collected at 63 locations along three cross-shelf, one diagonal, and two along shore transects (Fig. 1) during monthly quasi-synoptic cruises within an area on the WFS that extends from Tampa Bay to Ft. Myers. This region historically experiences the highest incidence of *G. breve* red tides [1]. Continuous underway measurements of surface temperature, salinity, and chlorophyll fluorescence were made on all cruises. Water column samples were taken with Niskin bottles mounted on a rosette sampler with a Seabird CTD.

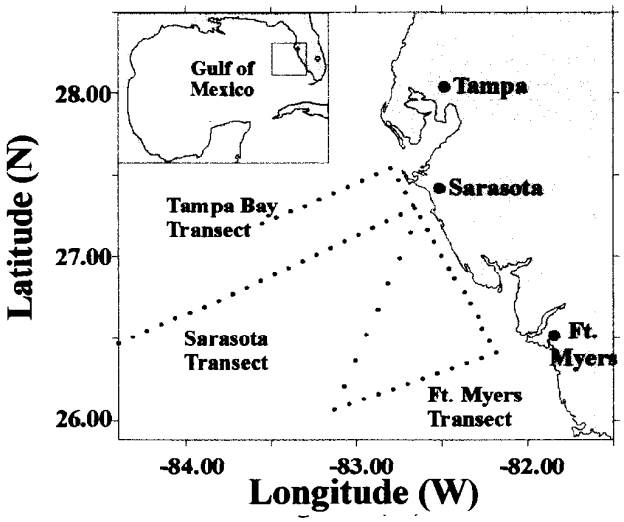

Fig. 1. Location of the ECOHAB: Florida cruise track and stations.

Inorganic nutrients (NO_2, NO_3, PO_4) were determined on frozen, unfiltered water samples taken at discrete depths and analyzed on an Alpkem RFA II segmented-flow nutrient analyzer. Particulate carbon (C) and nitrogen (N) and *G. breve* cell counts were measured as

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described in Heil et al. (this volume). Calculation of the nutrient requirements of the mixed *G. breve* populations were based on measured particulate carbon assuming a growth rate of 0.2 doublings per day [7]. We used the theoretical Redfield ratio to empirically calculate the N and P requirements based on the measured C values since there was no significant difference between our measured C:N:P ratios and the theoretical (T-test, $P<0.05$). Although the bloom consisted of a mixed population, *G. breve* at 10^5 - 10^6 cells • L^{-1} dominated the biomass.

Data from a 10 m bottom mounted ADCP current meter moored along the middle (Sarasota) cross-shelf transect were used to calculate a progressive vector diagram (PVD).

RESULTS

Hydrography and bloom development

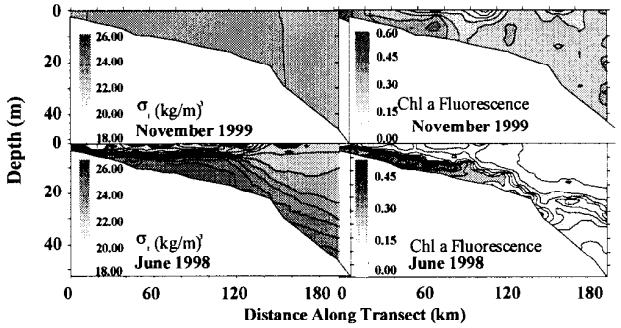

Fig. 2. The vertical distribution of density (sigma-t) and chlorophyll fluorescence along the Sarasota transect during summer (June) and fall (November).

The WFS is characterized by strong summer vertical stratification due to thermal heating. This structure is replaced by a relatively homogeneous water column in the fall due to a progression of atmospheric cold fronts that pass through the region during fall and winter (Fig. 2).

In November, 1998 low populations (10^4 cells • L^{-1}) of *G. breve* were found along the southern cross-shelf transect (Fig. 3) associated with a vertically homogeneous water column (Fig. 2). During the following month *G. breve* populations increased to a maximum of 1.1 x 10^6cells • L^{-1} in December and persisted in the Charlotte Harbor area through January, 1999. By February, 1999 elevated populations were found further north along the Tampa Bay transect while only background concentrations were found along thesouthern transects. No *G. breve* cells were found at any location along the cruise track in March, 1999.

Continuous underway surface temperature and salinity measurements indicated that a temperature and salinity front had developed just west of the 10 m isobath between Tampa Bay and Charlotte Harbor (Fig. 4). Maximum cell numbers in the bloom were found along the 10 m isobath just shoreward of the front.

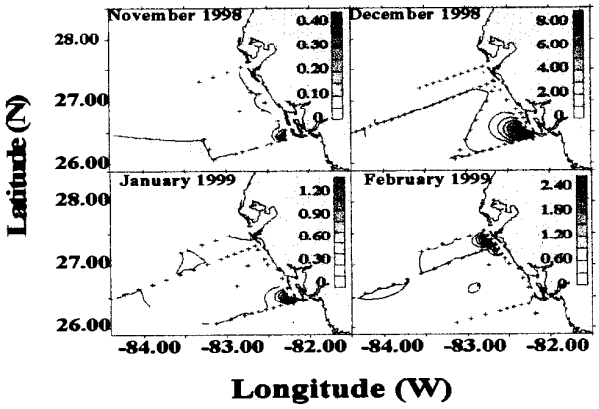

Fig. 3. Location and magnitude of *G. breve* cell concentrations (cells L^{-1} • 10^6) from November, 1998 through February, 1999.

Current meter data indicate a net northward movement of surface and near bottom flows shoreward of the 10 m isobath. Based on a progressive vector diagram (PVD), which displays the cumulative distance traveled by a parcel of water over a set period of time, the general water movement was to the northwest between the January and February cruises (Fig. 5). Based on measured current speeds, the net distance traveled by the bloom was ~ 90 km in the four weeks between cruises.

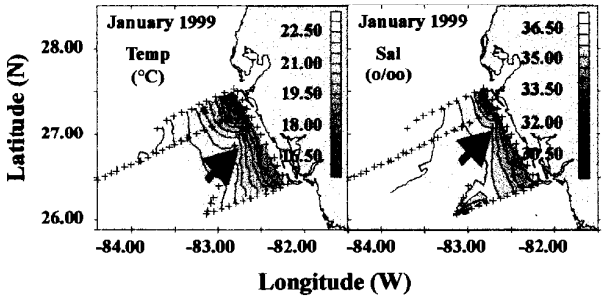

Fig. 4. Continuous underway surface temperature and salinity maps showing location of the thermal-haline fonts seaward of the 10 m isobath (arrows).

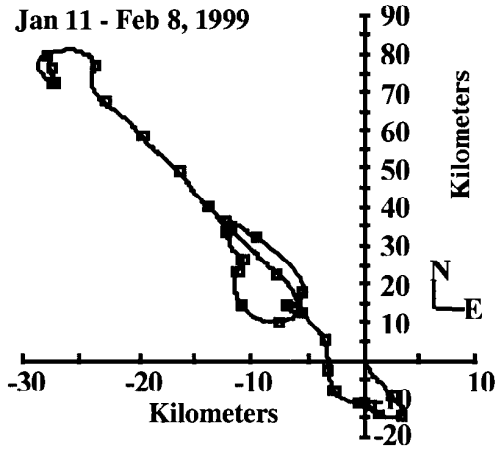

Fig. 5. Net direction and distance traveled by currents along the 10 m isobath from January through February 1999.

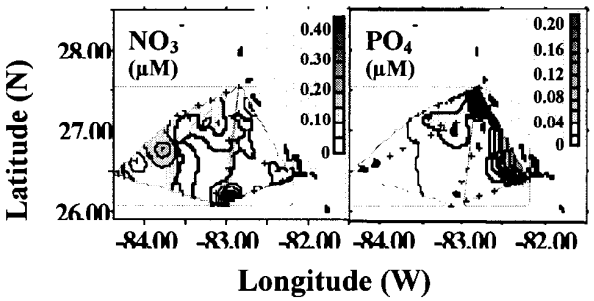

Fig. 6. The surface distribution of NO_3 and PO_4 within the ECOHAB control volume. Note outwelling of PO_4 from Tampa Bay and Charlotte Harbor.

Nutrient requirements and availability

Out-welling of PO_4 typically accompanies fluxes of estuarine water onto the shelf while there is essentially no NO_3 flux (Fig. 6). Typical surface NO_3 and PO_4 concentrations are <0.3 µM and <0.5 µM, respectively.

The PO_4 requirement for all locations ranged from 0.06 – 0.13 µM day^{-1}, while its availability was between 0.15 - 0.31 µM (Table 1). In every instance there was always enough inorganic P available to sustain the mixed bloom populations.

DISCUSSION

Initiation of the 1998-1999 *G. breve* bloom occurred after the water column became vertically homogeneous. Although *G. breve* blooms can occur during any month of the year they occur most frequently in late summer and fall [2]. This is the time of the year when atmospheric weather patterns lead to wind mixing and wind directions that favor upwelling. Both result in a vertically unstratified water column [3,4]. The co-occurrence of significant changes in stratification and our first observations of bloom concentrations of *G. breve* during the '98-'99 bloom suggest that water column dynamics play a major role in bloom initiation. While the mechanisms for transport from mid-shelf to nearshore waters have as yet to be identified, vertical migratory activity with dark dispersal [8] may bring populations into sub-surface, eastward (shore-ward) moving currents that result from offshore, up-welling favorable winds. After transport into near-shore waters, populations may become trapped behind thermohaline fronts.

Winter cooling and out-welling of low salinity water from Tampa Bay and Charlotte Harbor combined with a shoreward intrusion of warm offshore water (Fig. 4) contributed to the formation of the front seaward of the 10 m isobath. Such fronts, when developed in conjunction with upwelling events generated by seasonal wind patterns [4] may act as a seaward barrier to bloom dissipation and effectively trap *G. breve* populations against the coast in a source of estuarine derived water.

Current meter data for surface flows at the 10 m isobath along the Sarasota transect showed a consistent northerly direction to the flow (Fig. 5). Flow was sufficient in duration and rate to transport the bloom from Ft. Myers to Tampa Bay – a distance of approximately 90 km. Therefore, current transport is the most likely explanation for the northward movement of this bloom.

Both Tampa Bay and Charlotte Harbor are phosphate enriched and have inorganic and total N:P ratios <2 [9,10]. Measurements of the inorganic N:P ratio at all stations within the bloom ranged from 0.1 to 1.7; the same range as the near-shore estuarine water. Therefore, it is highly likely that both estuaries contributed PO_4 in support of the bloom, but were not a source of NO_3.

Heil et al.(this volume) found that the proximate C:N and N:P ratios for the bloom were close to Redfield proportions which suggests that the populations were not nutrient limited. Our estimates, based on nitrate availability indicate that population growth would be nitrate limited (Table 1). Thus other sources of nitrogen were apparently available to support growth.

Since *G. breve* can utilize organic sources of nitrogen [11], other potential sources of N for growth and maintenance include: DON from resuspension of near-bottom remineralized diatom populations during the fall over-turn (see Lester et al., this volume), ammonia, urea, and DON derived from N-fixing *Trichoodesmium* blooms that often precede *G. breve* blooms [12], and the remineralization of sea-grasses, macroalgae, and estuarine derived detritus. Based on the $\delta^{15}N$ signature of *G. breve* bloom populations (Table 2) all of the above may be sources of N.

Table 1. Nutrient availability and *G. breve* requirements.

Date	*G. breve**	Chl *a***	Requirement# N	Requirement# P	Available# N	Available# P	%R met by A N	%R met by A P
Nov 98	55	1.39	1.03	0.06	0.01	0.27	1.0	100
Dec 98	1123	5.39	2.08	0.13	0.04	0.31	2.0	100
Jan 99	140	2.06	1.31	0.08	0.04	0.25	3.0	100
Feb 99	422	1.47	1.62	0.10	0.01	0.15	0.6	100

Table 2. $\delta^{15}N$ values of particulate material on the west Florida shelf.

Sample	$\delta^{15}N$ (±SE)
Offshore	
Summer (*Trichodesmium* abundant)	
July 98	3.22 (±0.21)
Aug 98	3.04 (±1.08)
Winter (*Trichodesmium* at blgd. Conc.)	
Nov 98	5.46 (±1.54)
Dec 98	5.78 (±0.13)
G. breve bloom (Dec 98)*	4.88 (±0.15)
Rhizosolenia spp. bloom (Jan 99)	7.09 (±0.89)

**G. breve* concentration > 5 x 10^5 cells L^{-1}

ACKNOWLEDGEMENTS

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EVOLUTION OF A *GYMNODINIUM BREVE* (Gymnodiniales, Dinophyceae) RED TIDE BLOOM ON THE WEST FLORIDA SHELF: RELATIONSHIP WITH ORGANIC NITROGEN AND PHOSPHORUS.

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ABSTRACT

Blooms of *Gymnodinium breve* Davis (Gymnodiniales, Dinophyceae) initiate and are maintained in oligotrophic areas of the West Florida shelf where inorganic nitrogen (N) and phosphorus (P) are at the limits of detection. The role of organic nutrients in *G. breve* bloom dynamics is unknown. We have examined inorganic and organic N and P distributions during a *G. breve* bloom that persisted for four months from November 1998 through February 1999. Highest *G. breve* concentrations coincided with undetectable nitrate concentrations; however, dissolved organic nitrogen (DON) levels at this time ranged from 5 to 15μM Inorganic P and dissolved organic phosphorus (DOP) were both found at or above 0.3μM during highest *G. breve* concentrations, but decreased to below detection limits in January, and remained low in February. Neither DON, nor DOP in surface waters displayed a clear estuarine signal during the bloom period. Mixing of the water column in early fall due to seasonal overturn may resuspend DON derived from near bottom diatom populations at the 30 m isobath, increasing DON concentration in offshore waters. In January, however, DON concentrations were higher inshore than on the outer shelf, coincident with a coastal front which contained the bloom nearshore. Throughout the duration of the bloom, local DON concentrations were not depleted, indicating either that *G. breve* is not using DON in detectable concentrations or that fluxes are offsetting any uptake by the *G. breve* bloom.

INTRODUCTION

Gymnodinium breve Davis (Gymnodiniales, Dinophyceae) blooms on the West Florida shelf (WFS) are hypothesized to initiate in the oligotrophic waters 18-74 km offshore [1, 2]. Blooms of this dinoflagellate may reach concentrations of 10^6 cells L^{-1} within weeks of bloom initiation [3]. Because these high concentrations of *G. breve* occur in waters in which inorganic nutrients are at or below the limits of detection, the question remains: what nutrients are supporting these blooms?

Both inorganic and organic nutrient sources can be utilized by phytoplankton. In culture studies, *G. breve* has been shown to utilize organic N [4, 5, 6], and organic P compounds [7]. Vargo and Shanley [7] also demonstrated production of alkaline phosphatase within a *G. breve* bloom *in situ*, suggesting that DOP sources are available to blooms. The relationship between DON sources and *G. breve* bloom maintenance is unknown.

Possible nearshore sources of organic nutrients available to *G. breve* blooms include estuarine sources (both allochthonously and autochthonously produced), atmospheric deposition, and nutrients potentially derived from resuspended near-bottom phytoplankton populations [8,9]. Potential offshore sources of organic nutrients include the N-fixing cyanobacterium *Trichodesmium* spp. *Trichodesmium* can excrete 40-50% of N_2 fixed as DON [10,11] and significant populations of *Trichodesmium* occur on the WFS year round (C. Heil, unpubl. data). The fate of *Trichodesmium*-derived DON is unknown.

A bloom of *G. breve* persisted in the ECOHAB:Florida study area from November 1998 through February 1999. The bloom occurred off the Fort Myers/Charlotte Harbor area from November 1998 through January 1999, and then moved north to the Tampa Bay/Sarasota region in February 1999 [9]. DON and DOP concentrations on the WFS ranged from 4 to 35μM and 0 to 0.9μM respectively during the bloom. This paper attempts to determine whether DON and DOP were potential nutrient sources for the 1998-1999 *G. breve* bloom by examining the relationship between *G. breve* biomass and inorganic and organic nutrient concentrations, the evidence for nearshore or estuarine sources of DON or DOP, and possible offshore sources of DON.

METHODS

Samples were collected synoptically over a 72h period during monthly cruises. At selected stations, water samples were collected for determination of inorganic (NO_3^-, PO_4^{-2}, and NO_2^-) and organic nutrient concentrations. Inorganic nutrients were determined on unfiltered water samples according to Gordon et al. [12] on an Alpkem RFA II segmented-flow nutrient analyzer. Dissolved organic nutrient concentrations were determined on subsamples filtered through precombusted (2 h, 450°C) Whatman GF/F filters. Total dissolved phosphate (TDP) was determined using the combustion hydrolysis method of Solórzano and Sharp [13]. Inorganic phosphate values were subtracted from TDP values to determine dissolved organic phosphorus (DOP) concentrations. Total dissolved nitrogen (TDN) concentrations were determined using the persulfate oxidation method of Solórzano and Sharp [14] and inorganic nitrogen values were subtracted from TDN values to determine dissolved organic nitrogen (DON) concentrations. *Gymnodinium breve* was counted live

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using a dissecting microscope within two hours of collection.

RESULTS

Relationship between G. breve biomass and inorganic and organic nutrient concentrations

Over the course of the bloom, NO_3^- concentrations were undetectable within the *G. breve* bloom [9]. PO_4^{-2} concentrations remained at or above 0.3μM during the first two months of the bloom; however, they declined after peak *G. breve* biomass was reached in December 1998, in some cases below detectable levels.

DON remained fairly high in the region of the bloom, throughout the duration of the bloom (Fig.1). Highest *G. breve* biomass corresponded with 10-15μM DON (Fig. 2).

DOP demonstrated the same trend as the inorganic phosphate (Figs.1,3), with concentrations remaining above 0.3 μM during the first two months of the bloom, and declining after the peak *G. breve* biomass was reached in December 1998. In January 1999, DOP was below the limits of detection, and though detectable, it remained low in February 1999.

Nearshore and estuarine sources of DON or DOP

There was no significant estuarine signal of either DON or DOP during the *G. breve* bloom (Figs. 2, 3). Although at times DON was higher inshore than offshore, in general there was no clear relationship with either Tampa Bay or Charlotte Harbor as sources. In December 1998, high concentrations of DOP near the mouths of the two estuaries coincided with elevated chlorophyll *a* (Figs. 3, 4). Both of these signals are coincident with phytoplankton blooms: with *Rhizosolenia* spp. and other diatoms off Tampa Bay, and *G. breve* off Charlotte Harbor. This suggests that these high DOP concentrations may be due to *in situ* production, rather than estuarine sources.

A potentially significant source of nearshore DON may be the resuspension of near bottom chlorophyll *a* maxima at the 30 m isobath during seasonal mixing or upwelling events [8,9]. Based on measured particulate N:Chl ratios, this near bottom maximum can be calculated to represent a standing stock of approximately 5μM N. Degradation and remineralization of this stock could potentially yield DON levels similar to those seen in surface waters prior to initiation of the 1998-1999 *G. breve* bloom.

Potential offshore sources of DON and DOP

During the 1998-1999 bloom, offshore DON was high and variable, ranging from 4 to 15μM (Fig 2). Offshore DOP ranged from 0 to 0.6 μM (Fig. 3). The presence at times of high concentrations of both DON and DOP offshore demonstrates that offshore nutrient sources were available for *G. breve* bloom initiation and maintenance.

DISCUSSION

Although DON and DOP were present during some portion of the bloom, there is no direct evidence of utilization of either by *G. breve*. Both DON and DOP exhibited great spatial and temporal variability, and as a result no pattern relative to *G. breve* biomass can be discerned. However, the elevated level of DON (Fig. 1) relative to non-detectable DIN during the course of the bloom suggests that DON may have played a role in the initiation or maintenance of the bloom. Direct use of DON by *G. breve* will have to be demonstrated, however, if it is to be assumed to be the major nutrient supporting *G. breve* blooms.

The depletion of DOP during the month of January (Fig. 3) suggests potential DOP uptake by the planktonic community of the bloom. *G. breve* has been shown to utilize DOP when DIP concentrations are limiting [7]. However, nutrient stochiometry calculations for the bloom suggest that DIP concentrations were not limiting during the course of the bloom. Bacterial hydrolysis of DOP to DIP may be a more likely explanation for the DOP depletion.

Enrichment by estuarine sources has long been a suggested potential foundation of nearshore initiation or maintenance of *G. breve* blooms [15]. No clear nearshore or estuarine source of DON or DOP was detected during the bloom, though both are found in higher concentrations nearshore at various times over the four month period.

It has been observed that *G. breve* blooms are often preceded by, or co-occur with, *Trichodesmium* spp. on the WFS [16]. *Trichodesmium* excretes up to 50% of fixed N as DON [10,11]. The magnitude of this new N input to the WFS is unknown. Although highest concentrations of *Trichodesmium* on the WFS occur from June through August, concentrations as high as 1,500 colonies L^{-1} in surface slicks have been observed in January (C. Heil, unpubl. data).

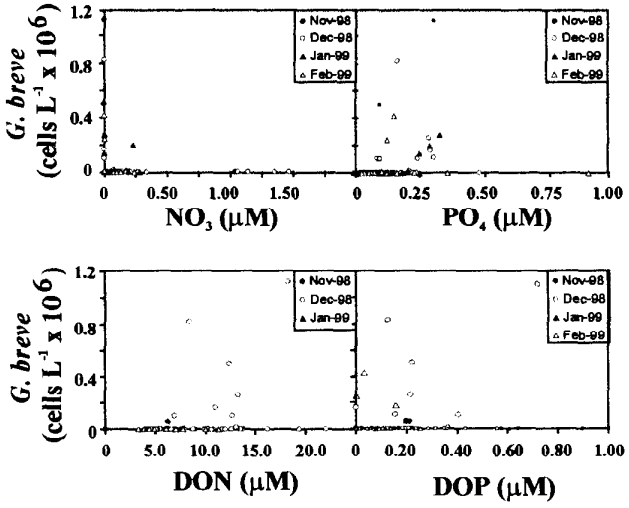

Fig. 1 *G. breve* cell concentrations vs. NO_3, PO_4^{3-}, and DON, and DOP during the months of the 1998-1999 *G. breve* bloom on the West Florida Shelf.

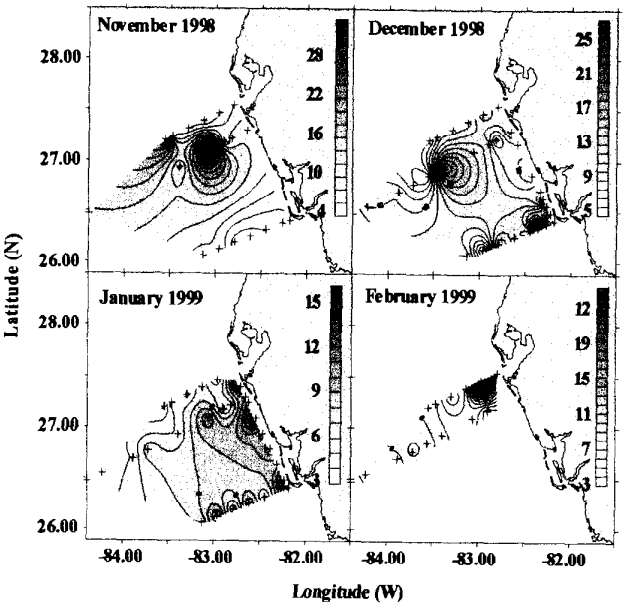

Fig. 2 Surface concentrations of DON ($\mu M\ L^{-1}$) over the four months of the 1998-1999 *G. breve* bloom on the West Florida Shelf.

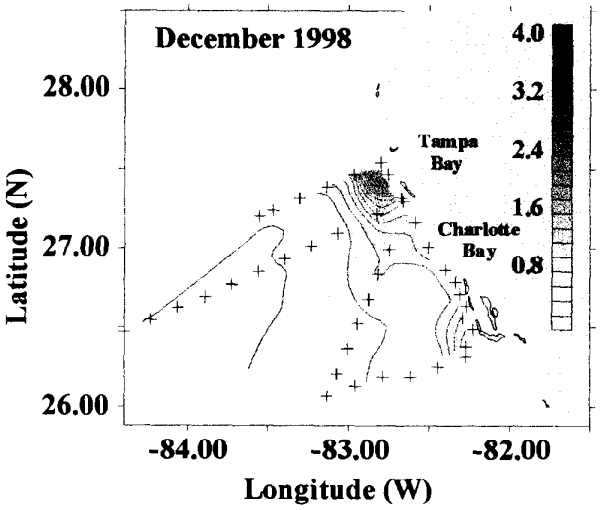

Fig. 4 Surface concentrations of chlorphyll ($\mu g\ L^{-1}$) for December 1998.

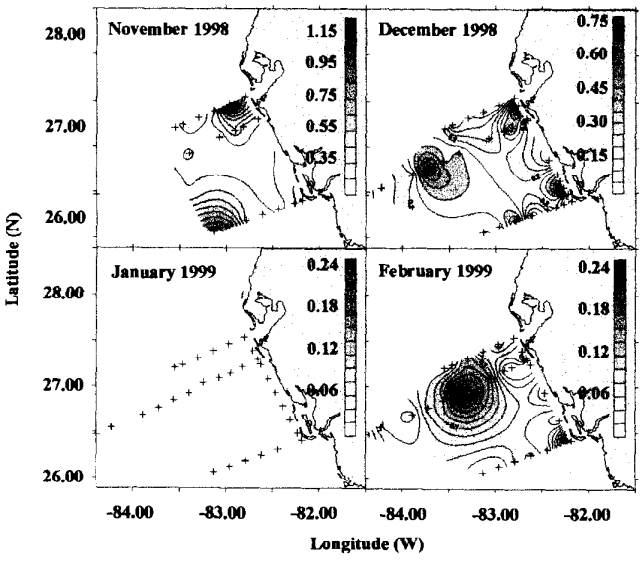

Fig. 3 Surface concentrations of DOP ($\mu M\ L^{-1}$) over the four months of the 1998-1999 *G. breve* bloom on the West Florida Shelf.

Like *G. breve*, *Trichodesmium* has the ability to synthesize alkaline phosphatase, making DOP a potential source of nutrients for the cyanobacterium as well [17].

Further investigation of the potential association of *Trichodesmium* with *G. breve* blooms may provide a clearer understanding of the role DON plays in the initiation and maintenance of these blooms on the WFS.

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NUTRIENT STOICHIOMETRY OF A *GYMNODINIUM BREVE* BLOOM: WHAT LIMITS BLOOMS IN OLIGOTROPHIC ENVIRONMENTS?

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ABSTRACT

Blooms of the Florida red tide dinoflagellate *Gymnodinium breve* Davis (=*Ptychodiscus brevis* Steidinger) occur in oligotrophic West Florida shelf (WFS) waters depauperate in both dissolved inorganic nitrogen (N) and phosphorus (P). The nutrient stoichiometry of a small (maximum concentration 1.1 x 106 *G. breve* L-1), near-shore *G. breve* bloom which persisted off the Sanibel to Tampa region from November 1998 to February 1999 was examined using particulate nutrient (chlorophyll *a* (Chl), carbon (C), nitrogen (N), phosphorus (P)) measurements from both the WFS area and the bloom itself. The *G. breve* bloom was characterized by unique C:Chl, C:N and N:P ratios which differed from surrounding areas (i.e., nearshore surface waters without *G. breve* present, a persistent benthic diatom bloom at the 30 m contour and the deep Chl maximum (DCM) at 50-100 m contour). Chl was only slightly elevated in the bloom (3.6 µg L-1) compared with surrounding waters (0.16 to 1.87 µg L-1 except in areas of a nearshore diatom bloom). N:P ratios from the bloom were generally equivalent to Redfield, suggesting that this bloom was growing at or close to maximum growth rates with sufficient N and P to meet its cellular requirements, except during maximum cell concentrations in December when elevated N:P ratios in the bloom showed a tendency toward P limitation. N:P ratios for other phytoplankton populations (nearshore diatom bloom, 16.8; benthic Chl maximum, 21.8; DCM, 51.1) were consistently equal to or greater than the Redfield Ratio throughout this period. This suggests that phytoplankton populations on the west Florida shelf, including the *G. breve* bloom, were either nutrient replete or had a tendency toward P limitation. Given both the low inorganic N concentrations and low dissolved N:P ratios (0.1-1.7) within this area, an unidentified N source must have been available to both bloom and shelf phytoplankton populations on the west Florida shelf.

INTRODUCTION

Blooms of the Florida red tide dinoflagellate *Gymnodinium breve* are a significant ecological feature of the WFS [1] and have been reported as far north as the US South Atlantic Bight [2,3]. Blooms of *G. breve* can cover areas greater than 3 x 104 km2 and reach up to 60 µg L-1 [4], at times accounting for up to 100% of the annual primary production in WFS waters [5,6].

Blooms of *G. breve* occur in surface waters [7] in areas of the Florida shelf where inorganic N and P concentrations are consistently at or below the limits of analytical detection, hence both N and P potentially limit growth of *G. breve* on the WFS. The nutrient sources which support *G. breve* blooms are unknown. Possible nutrient sources on the 200 km wide WFS include coastal and benthic inputs, atmospheric deposition, input of nutrient rich upwelled water at the shelf break, *in situ* nutrient regeneration from rafting seagrass blades and *Trichodesmium* and diatom blooms. Coastal nutrient inputs do not extend beyond a 1 to 3 km coastal zone, although they can potentially support near-shore blooms [1]. Nutrients within deep water upwelled at the Florida shelf break do not reach surface waters [8] and are of insufficient magnitude to support typical *G. breve* bloom biomass. The magnitude of inputs from atmospheric deposition and *in situ* nutrient regeneration are unknown, but given typical Chl *a* concentrations of <1 µg L-1 and low inorganic nutrient concentrations during non-bloom conditions on the WFS, are probably negligible.

Cells growing at close to their maximum rates take up N and P in a fixed ratio and thus will exhibit an elemental composition close to the Redfield Ratio [9]. Within monospecific blooms, this allows the use of *in situ* particulate N:P ratios to assess the relative availability of these nutrients and infer N or P limitation [10]. This paper analyzes the particulate nutrient measurements from the 1998-1999 *G. breve* bloom to determine the nutrient most likely to have limited the bloom and other phytoplankton populations present during the bloom period on the WFS.

METHODS

Monthly synoptic cruises (53 to 63 stations) were conducted in a prescribed control area on the WFS which historically has experienced the greatest frequency of *G. breve* red tides and includes a region identified as the *G. breve* bloom initiation zone 20-75 km offshore [11,12]. Sampling protocol at alternate stations included determination of Chl *a*, particulate carbon (C), nitrogen (N), and phosphorus (P) at 5 (when depth < 50 m) or at 10 m depth intervals (when depth < 200 m). Water was collected at depth with Nisken bottles and immediately filtered. Duplicate Chl samples were filtered onto Whatman GF/F filters, placed in 10 ml methanol and stored at 4°C in darkness for 3 days. All samples for Chl were analyzed within 1 week of collection using a 10AU Turner Design fluorometer [13]. Duplicate samples for particulate C and N were filtered onto pre-combusted (450°C for 2 hr) Whatman GF/F filters and 3 mls of 10% HCl in filtered (0.7 µm) seawater was added. Filters were lyophilized (24 hr), stored at 4°C in darkness and analyzed with a Carlo-Erba Model 1106 Elemental Analyzer. Particulate P was determined by the method

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Intergovernmental Oceanographic Commission of UNESCO 2001

of Solórzano and Sharp [14]. Concentrations of live *G. breve* cells in surface samples was determined by five replicate counts of 0.2 ml of surface water within 2 hrs of collection.

Several assumptions were made during data analysis. The *G. breve* bloom was assumed to be mono-specific. As some *Rhizosolenia* spp. were observed at the edges of the *G. breve* bloom, only data from stations where *G. breve* concentrations exceeded 5 x 10^3 cells L^{-1} was used in analyses. As no relationship was found between particulate C and Chl in nearshore waters, no correction for the detrital contribution to particulate nutrient values were made; hence all ratios with C are elevated and should be viewed with caution.

RESULTS

The 1998-1999 *G. breve* bloom was localized within a nearshore (5-20 km) region from Sanibel to Tampa, persisting in the Sanibel area from November 1998 through January 1999, then appearing at the mouth of Tampa Bay in February of 1999. Highest *G. breve* concentrations (up to 1.2 x 10^6 cells L^{-1}) were observed in December.

Depth profiles of Chl in three cross-shelf transects within the study area in November 1998 are given in Fig. 1. Dominant phytoplankton on the WFS during this period include the *G. breve* bloom, a near-shore diatom bloom comprised of *Rhizosolenia* spp. at the mouth of Tampa Bay, a near bottom Chl maximum located along the 35 m depth contour comprised of *Rhizosolenia* and *Coscinodiscus* spp. and a deep water Chl maximum at ~60 m depth. The Chl range of each of these features and associated particulate C:Chl, C:N and N:P ratios is given in Table 1. Although highest Chl

Table 1. Characteristics of the dominant biological features of the WFS during the *G. breve* bloom. All values are average (±SE).

	Chl	C:Chl	C:N#	N:P#
Nearshore:				
G. breve Bloom*				
	1.14-3.56	188.8 (77.3)	15.4 (2.5)	39.3 (6.8)
Diatom Bloom				
	0.36-5.03	200.8 (45.6)	10.2 (0.8)	16.8 (3.8)
Near bottom Chl Max**				
	1.22-2.18	127.0 (34.6)	14.8 (1.6)	21.8 (5.5)
DCM***				
	0.21-1.13	306.8 (62.9)	15.4 (3.1)	51.1 (11.3)

#Molar;
*stations where *G. breve* >5 x 10^3 cells L^{-1}
**pelagic diatom bloom present at 35 m depth contour
***50-200 km offshore

concentrations were present in the nearshore diatom bloom, the near bottom diatom bloom, with Chl concentrations up to 2.18 µg L^{-1} and a distribution that extends through the entire sampling area, is a significant feature of the shelf. Average C:Chl ratios ranged from 127.0 (+34.6) for the near bottom diatom population to 306.8 (+62.9) for the DCM. The average C:Chl ratio for the *G. breve* bloom was not significantly ($P<0.001$)

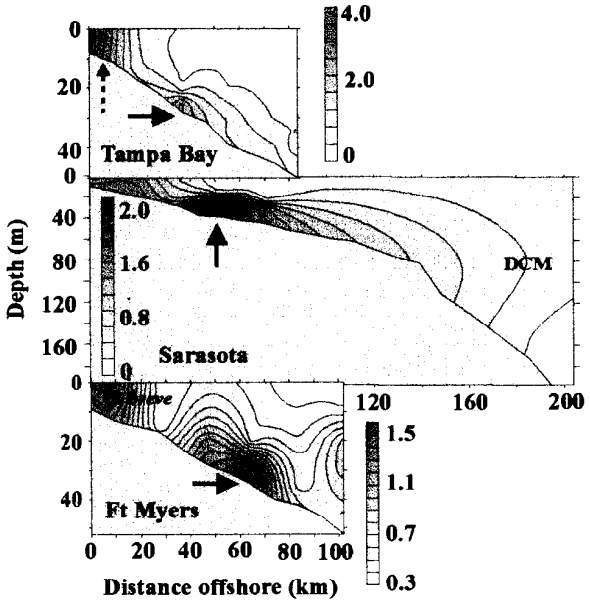

Fig. 1. Depth profiles of Chl (µg L^{-1}) at three cross shelf transects in November 1998 showing the location of the *G. breve* bloom, the near bottom Chl maximum (solid arrows), nearshore diatom bloom (broken arrow) and the deep Chl maximum (DCM).

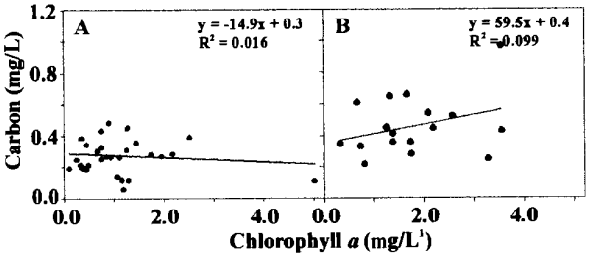

Fig. 2. Relationship between particulate C and Chl at A) nearshore stations with no *G. breve* and B) stations where *G. breve*> 5.0 x 10^6 cells L^{-1} from Nov 1998 to Feb 1999.

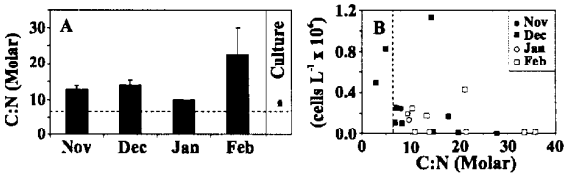

Fig. 3. A) Monthly mean (+SE) C:Chl ratios for the *G. breve* bloom. Culture value is from [21], B) Relationship between C:Chl ratio and *G. breve* concentration.

different than that of the nearshore diatom bloom, although a high degree of variability in values was evident. Average C:N ratios of the *G. breve* and diatom nearshore bloom, 15.4 (+2.5) and 10.2 (+0.08) respectively, were

significantly ($P<0.005$) different, however. Average N:P ratios differed considerably between populations. Both the nearshore diatom bloom and benthic diatom populations had N:P ratios which did not differ significantly ($P<0.05$) from the Redfield Ratio. The *G. breve* bloom and the DCM were both characterized by N:P ratios which were greater than the Redfield Ratio, 39.3 (+6.8) and 51.1 (+11.3) respectively.

The relationship between particulate C and Chl within the nearshore diatom and *G. breve* bloom in given in Fig. 2. No significant relationship was observed for either bloom, thus no corrections for the detrital contribution to particulate nutrient values were made.

Particulate nutrient ratios from the *G. breve* bloom are given in Figs. 3, 4, and 5. Monthly average

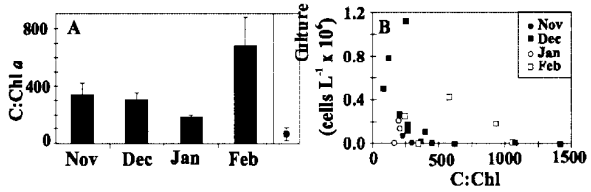

Fig. 4. A) Monthly mean (+SE) molar C:N ratios for the *G. breve* bloom. Culture value is from [15], B) Relationship between C:N ratio and *G. breve* concentration.

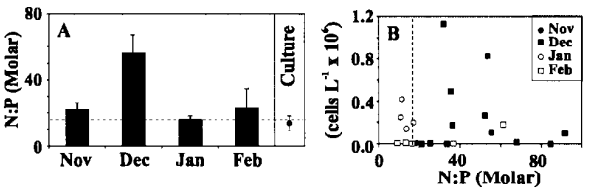

Fig. 5. A) Monthly mean (+SE) molar N:P ratios for the *G. breve* bloom. Culture value is from [15], B) Relationship between N:P ratio and *G. breve* concentration.

C:Chl ratios of the bloom ranged from 203.2 to 357.6 while the bloom was located off Ft. Myers; however, after transport to the mouth of Tampa Bay, C:Chl of the bloom increased to 697.0. At the highest *G. breve* concentrations observed, the C:Chl ratio was 271.3 (Fig. 4). Average monthly C:N of the bloom was also lower in the first 3 months of the bloom when it ranged from 9.8 to 13.8; C:N of the bloom increased to 22.7 after the bloom had moved northward (Fig. 4). Average N:P ratios (Fig. 5) displayed a different pattern, with the highest N:P ratio of 57.1 observed during December of 1998, coincident with the highest concentrations of *G. breve* observed during the bloom. During the other months of the bloom, the N:P ratio was at, or slightly above, Redfield.

DISCUSSION

N:P ratios of *G. breve* populations varied monthly, but were generally at or above 16, implying that *G. breve* were growing at near maximal growth rates during this bloom. N:P ratios of nutrient sufficient *G. breve* in culture range from 9.9-18.7 [15]. Earlier culture and field studies suggest that *G. breve* blooms are not P limited [16,17,18], and Vargo [19] reported that, based on P requirements for growth and *in situ* inorganic and organic P concentrations within a bloom, sufficient P is available to maintain blooms except at extremely high cell concentrations. Highest N:P values (57.1+19.9) were observed in December coincident with the highest observed *G. breve* concentrations (1.1×10^6 cells L^{-1}), suggesting that at the height of the bloom, populations were P limited. Data from Vargo et al. [8] show that, although *G. breve* had sufficient P present to meet calculated cellular P requirements over the entire bloom, at several stations in December only sufficient P was present for <1 day's growth.

N:P ratios of the DCM and the near bottom Chl maximum in WFS waters during this bloom period were greater than Redfield, despite inorganic N concentrations at or near the limits of analytical detection. This suggests that sufficient N is present on the WFS to support growth of these populations. Both the deep and benthic populations are situated such that either could have access to benthic or upwelled NO_3 sources. The only potential N source available to the *G. breve* bloom was organic N [8].

Both C:Chl and C:N ratios within the *G. breve* bloom were elevated compared with culture values. Shanley and Vargo [21] report C:Chl values of 59-124 and C:N values of 7.7-11.5 for nutrient sufficient *G. breve* cultures in artificial media. Heil [15] reported a C:N values of 5.7-10.9 for *G. breve* in seawater based medium. The absence of a significant relationship between particulate C and Chl within both the bloom region and other nearshore stations results in ratio elevation due to nearshore detrital C. The elevated C:N ratios observed *in situ* suggest that either nitrogen was limiting cell growth, the influence of nearshore inputs of detrital C on particulate C measurements were significant or that organic C was utilized by *G. breve*. The N:P ratios of the bloom show that N probably did not limit the biomass present. Bloom initiation in November of 1998 coincided with the advection into the region of a large mass of decaying seagrass (a significant source of DOC via leaching of leaves [22,23]) originating from seagrass die-offs. Organic carbon utilization by *G. breve* has been suggested from toxin synthesis research [24,25] and particulate C:Chl and C:N ratios from the bloom suggest that *G. breve* possibly utilized organic C sources.

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DYNAMICS OF *DINOPHYSIS ACUTA, D. ACUMINATA, D. TRIPOS AND GYMNODINIUM CATENATUM* DURING AN UPWELLING EVENT OFF THE NORTHWEST COAST OF PORTUGAL

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ABSTRACT

The bloom dynamics of *Dinophysis acuta, D.* cf. *acuminata, D. tripos* and *Gymnodinium catenatum* during the development of a weak to moderate upwelling event observed at 41°05'N off the coast of Portugal, is described. All species were mainly distributed in the surface wind driven layer within an equatorward coastal jet. Wind induced mixing, offshore transport and vertical motions were responsible for the distribution and concentration of cells, depending on the species relative position in the water column before the northerly wind event. Generally, wind relaxation was associated with blooming conditions while wind intensification led to dispersion and decrease of cell numbers.

INTRODUCTION

Since 1985, particularly during summer and autumn, blooms of *Gymnodinium catenatum* and species of *Dinophysis*, like *D. acuta* and *D. acuminata* have been associated with recurrent toxic PSP and DSP problems, respectively, along the western Atlantic coast of the Iberian Peninsula [1,2,3,4,5]. After 1996, no *G. catenatum* blooms were recorded off the coast of Portugal.

Between 1985 and 1987, the major outbreaks of *G. catenatum* in the Galician rias took place during autumn, mainly associated with southerly wind events that caused shoreward advection of coastal waters [6]. Cell maxima were observed in the upper 5m in the outer parts of the rias. During summer 1988, weak outbreaks also occurred in partly stratified moderate upwelling conditions. In such cases, surface counts were very low, with the cell maxima observed in the thermocline [6].

In the Galician Rías Bajas, *D. acuminata* proliferate from April to August while *D. acuta* in late summer or early autumn [4]. The early growth of both species has been related to the establishment of thermoclines. On the Portuguese coast, the environmental conditions associated with the southern waters, *e.g.* the lower stratification and higher salinities, >35.9 for *D.* cf. *acuminata* and >36 for *D. acuta*, seem to restrain the bloom development [3]. *D. acuminata* normally blooms to the north of *D.acuta* and is associated with the colder and less saline waters [3].

Proliferation of flagellates has been related to different hydrodynamical conditions within and between water masses. In particular, coastal currents and coastally trapped buoyant plumes have been proposed to play a major role in the development and advection of blooms and their associated toxicity [7,8]. During spring and summer, the Portuguese northern shelf (Fig. 1) tends to be occupied by a lower salinity shallow lens, roughly limited below by the seasonal pycnocline. This lens may result from the combined contributions of water from the NE Atlantic and the Bay of Biscay, modified by local river runoff and interaction with Central Water upwelled near the coast [9]. Upwelling events have been observed to disrupt the structure of this lens near the coast or to push its coastal limit offshore, mainly depending on wind strength and persistence [9,10,11].

In this paper, the cross-shelf and vertical distributions of *D. acuta, D.* cf. *acuminata, D. tripos* and *G. catenatum* in the NW coast of Portugal, during summer 1987, is discussed in relation to the development of an upwelling event and the associated dynamics, as well as with the presence of near-surface stratification.

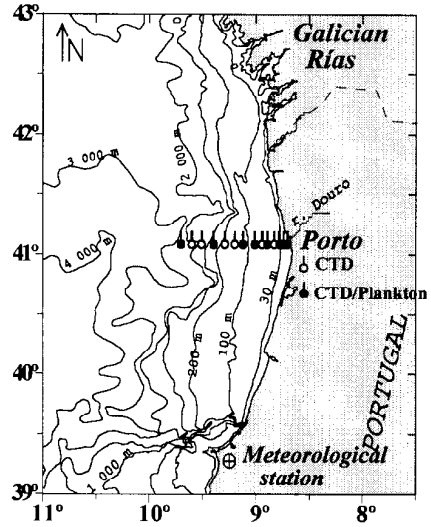

Fig. 1. Location of the repeated transect and position of the automatic weather station.

MATERIAL AND METHODS

From 19 to 27 August 1987, a transect was sampled daily over the western Portuguese shelf and slope off Porto, on board of R/V Almeida Carvalho (Fig. 1). Physical data and phytoplankton samples were collected using a CTD and a rosette sampler or, after the third repetition, *Nansen* bottles equipped with reversing thermometers. Sampling levels were 5, 10, 20, 30, 40, 50 and 75 m depth (or near bottom in shallower areas). Coastal winds were measured at a well exposed area, some 200 km to the south of the section, and were used to estimate an upwelling index [2,12] (Fig.1). The phytoplankton counting procedure was described in [2].

RESULTS AND DISCUSSION

Some patterns appeared during the observations. Regardless of the time evolution, the maximum of each species tended not to be coincident in space. This fact confirms previous studies in Galician waters and mesoscale studies off Portugal: when *D. acuminata* and

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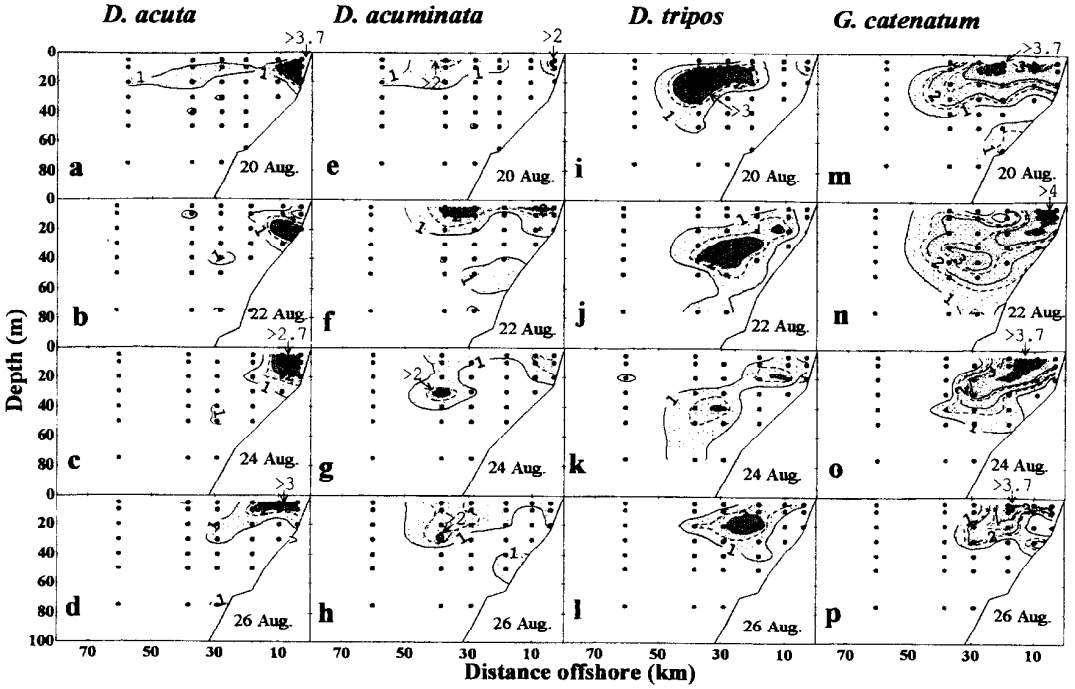

Fig. 2. Vertical distributions of *D.acuta* (a-d), *D. acuminata* (e-h), *D. tripos* (i-l) and *G. catenatum* (m-p) (log_{10} n°.cells l^{-1}+1), respectively on 20 , 22 , 24 and 26 August 1987.

D. acuta coexist, their maxima are not coincident either in space or in time, with *D. acuminata* having affinities with waters of lower salinity [3,4]. *D. acuta* was basically distributed over the inner shelf, with the largest mean values observed during periods of wind relaxation, and water column maxima (> $3x10^3$ cells l^{-1}) found in association with the largest inshore vertical density gradients (Figs. 2a-d, 3a, 4a, 5e-h). *D. tripos* and *D. acuminata* had all concentrations one order of magnitude lower than *D. acuta*. *D. acuminata* probably bloomed either earlier in the year or further north, as observed by [3, 4]. *D. acuminata,* positioned over *D. acuta* or further offshore, appeared to be related to the surface lens of reduced salinities confined below by the isohaline of 35.75 (Figs. 2a-h, 5a-d). *D. tripos* was mainly distributed at mid-shelf and maximum concentrations, at the basis of the pycnocline, decreased after the first wind event (Figs. 2i-l, 4a, 5e-h). The chain forming species, *G. catenatum,* was observed over the whole shelf and the maxima were always at the surface, probably reflecting its higher swimming capacity (Figs. 2m-p). Mean values tended to reflect the cross-shelf water transport associated with wind pulses, clearly visible on sea surface temperature, both as an offshore displacement of the maximum and as a reduction of the number of cells (Figs. 3b, 4b).

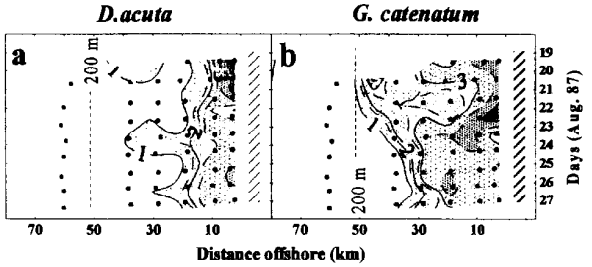

Fig. 3. Space-time distribution of the mean cell count of *D. acuta* (a) and *G. catenatum* (b) in the water column (log_{10} n°.cells l^{-1}+1).

The summer upwelling in 1987 was rather weak, with the favourable wind events having occurred in mid May to mid June and intermittence characterising the remaining months [11,13]. Somehow reflecting this situation, the beginning of the cruise, on 19 August, was preceded by 5 days of upwelling relaxation and eventually downwelling (Fig. 4a). Afterwards, during the sampling period, the wind was weak to moderate, blowing from an upwelling favourable direction and presenting two peaks: a small event that peaked on 19

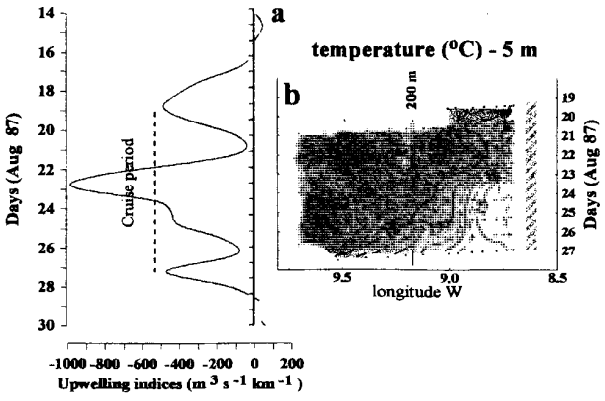

Fig. 4. (a) Upwelling indices at the meteorological station, where the negative values indicate upwelling. (b) Space-time representation of temperature at 5 m depth, where the vertical line indicates the position of the 200m isobath.

Aug. and a stronger and longer lasting one on 22 Aug..

On 19 Aug., the phytoplankton community was dominated by dinoflagellates characteristic of late summer/early autumn, such as *D. acuta*, *Ceratium massiliense*, *Katodinium glaucum*, *G. catenatum*, *Protoperidinium steinii* and, in particular, *Ceratium fusus* ($1.2x10^6$ cells l^{-1}) [4,14]. *D. acuta* and *G. catenatum* represented 5% of the dinoflagellate dominated plankton and reached respectively, $69x10^3$ cells l^{-1} at the inner station and $20.5x10^3$ at the second station (Figs. 3a, 3b). *D. acuminata* and *D. tripos* reached respectively $0.3x10^3$ and $1.4x10^3$ cells l^{-1}, at the second station and at midshelf.

Basically, the upwelling associated cross-shelf circulation consists on an offshore transport in the surface layer and a compensation motion towards the coast at some depth. As in most upwelling systems, the circulation pattern induced by wind favourable events is characterised by longshore shelf currents which are stronger than the cross-shelf flow [15]. The shelf circulation during the cruise and its dependence on wind forcing and stratification was based on direct current measurements along the transect [11]. Matching these results, partly summarised in Figs. 6a-d, on 20 Aug., just after the short northerly wind peak on 19 Aug., the wind driven surface layer was roughly confined below by the isopycnal of 26.8 kg m^{-3} (Figs. 4a, 5e). The offshore extension of the equatorward coastal jet likely coincided with the 35.7 isohaline (Figs. 5a). The poleward undercurrent had its core at 50-60 km from the coast, along and below the coastal limit of the offshore salinity maximum (Fig. 6a). The onshore subsurface motion was most important beneath the offshore salinity maximum and, near the bottom, was likely to bring deeper water to levels above 100 m at mid-shelf (Figs. 5a, 6a).

On that day, 20 Aug., the maximum number of cells of *D. acuta* occurred inshore in the upper 10-20 m, usually in water with salinity above 35.4 and a density anomaly greater than 26.0 kg m^{-3} (Figs. 2a, 5a, 5e). *G. catenatum* showed a similar behaviour but the maximum was found at mid-shelf. This species extended vertically down to the basis of the seasonal pycnocline (Figs. 2m, 5e). *D. acuminata* and *D. tripos* maxima were also present in mid-shelf waters at the surface and 20m, respectively (Figs. 2e, 2i). From the vertical distributions of dinoflagellate species on 20 Aug. we conclude that they were mainly distributed in the wind driven layer within the coastal equatorward jet (Fig. 6a).

Following the wind decline on 19-20 Aug., the surface motion reversed completely. On 22 Aug. the surface water was less saline and less dense, particularly at the innermost 10 km (Figs. 5b, 5f). The surface maximum of *G. catenatum* moved inshore and *D. acuta* moved down in the water column at the innermost station (Figs. 2b, 2n, 6b). However, because a less saline plume occupied the innermost stations, the observed changes in species distributions could also be due to the appearance of a different water body. The pycnocline was displaced downwards which could explain the presence, at mid-shelf, of *D. tripos* and *G. catenatum* in deeper levels (Figs. 2j, 2n, 5b, 6b). Nevertheless, *G. catenatum* was observed at a greater distance from the coast than on 20 Aug., with significant values still being found close to the

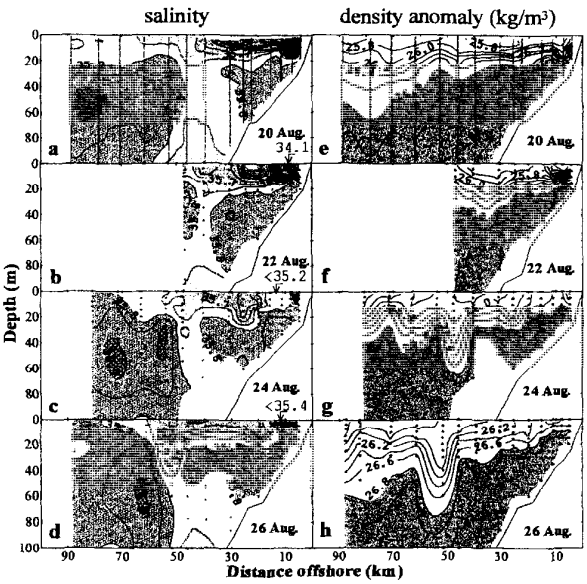

Fig. 5. Vertical distribution of salinity (a-d) and density anomaly (e-h), respectively on 20, 22, 24 and 26 Aug. 1987.

bottom of the pycnocline. In turn, *D. acuminata* concentrations increased in lower salinity waters, at mid-shelf (Figs. 2f, 5b). This increase at the surface can also be related to the species concentration in the convergence area characterised by a deepening of isohalines and isopycnals, although species transport from the north cannot be ruled out.

The wind event that began on 22 Aug gave rise to coastal upwelling and surface mixing, although the water column remained two-layered, which was attributed to the relatively mild wind conditions [11]. The wind driven layer was restricted to the upper 30 m or less, at least over the inner and mid shelf (Figs. 5c, 5g). As a consequence of that wind event, the coastal equatorward jet intensified and underwent offshore displacement, with an inshore counter-current noticeable on 25-26 Aug. (Figs. 6c, 6d).

On 24 Aug., the salinity was above 35.6 everywhere but at the surface in the innermost station (Fig. 5c). Cell numbers of *D. acuta* were similar from near surface to 20 m at the innermost 10-15 km (Fig. 2c). Below, the number of cells was drastically reduced, in contrast with the situation of 22 Aug. Both *D. acuminata* and *D. tripos* concentrations decreased, with the maximum of *D. acuminata* found at deeper depths in association with sinking of less saline waters at mid-shelf (Figs. 2g, 2k, 5c, 6c). At 20km from the coast, the *D. tripos* maximum was disrupted into layers, apparently related to an uplifting of isohalines and isopycnals below 20m depth (Figs. 2k, 5c, 5g). On 23 Aug. (not shown) cell numbers of *G. catenatum* were highest in a band extending from 10 m at 10 km to 30 m at 30 km, roughly following the 26.8 kg m^{-3} isopycnal. On 24 Aug., however, the values were lower, suggesting a greater vertical homogeneity with the exception of the innermost station where no cells were found below 10 m (Fig. 2o).

Wind relaxation occurred again on 25 Aug. and the inshore side of the thermal front approached the coast (Fig. 4b). As a consequence, the average cell number of

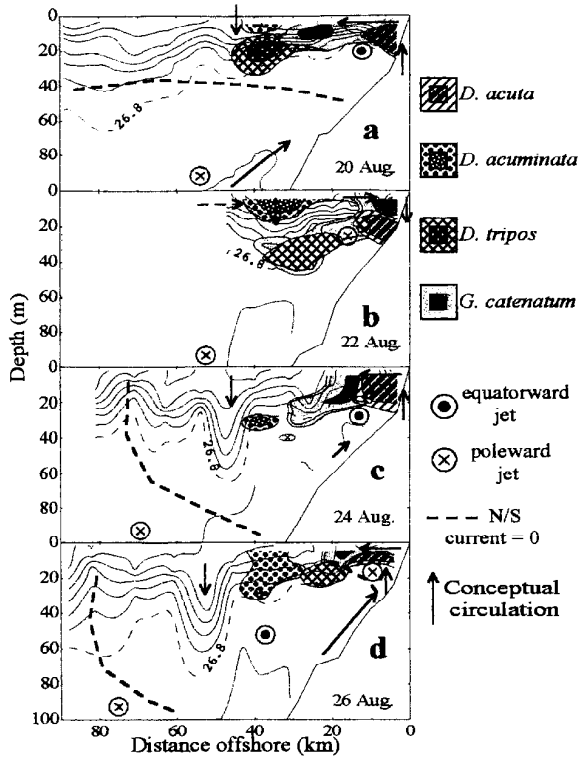

Fig. 6. Space-time dynamics of 4 dinoflagellates in relation to the vertical distribution of density (a-d). Two concentration levels are indicated for each species: the small square inside the larger one, corresponds to the highest concentration. Arrows indicate the conceptual circulation (adapted and interpreted from [11]) and vertical displacement of the pycnocline.

D. acuta seemed to increase nearshore (Figs. 3a, 4a). On 26 Aug. the species was basically confined to the upper 20 m, with maximum values at 5 m in the innermost 10 km (Fig. 2d). In turn, the largest cell counts of *G. catenatum* were obtained in the upper 10 m, at 20 km from the coast (Fig. 2p). The area of distribution as well as the average number of cells of *D. acuminata* increased at mid-shelf, while the outer shelf maximum and limit of distribution of *D. tripos* approached the coast and moved up in the water column (Figs. 2h, 2l).

The abundance of the four dinoflagellates, essentially found in the wind driven layer, reflected the vertical oscillations of its base. This was most evident on the 26th (Fig. 6d). It is interesting to compare the distributions of cells on that day with those on the 20th also after wind relaxation and when surface salinities were similar close to the coast (Figs. 2a, 6a). It is tempting to interpret the differences in cell distributions as due to either totally different stratification conditions, resulting from the different strength of the upwelling events, or different longshore dynamics close to the coast: equatorward jet on 20 Aug., and a poleward counter-current on 26 Aug.. Also, after 23 Aug., lower cell counts in the deeper levels of the innermost stations may be due to replacement of inshore bottom water, in a direct consequence of the upwelling event. Alternatively, one may consider that the offshore displacement of the equatorward jet, as well as of the upwelling source region, may have replaced the water in the coastalmost 10 km, when the inshore counter-current established itself in the last days of the cruise. Where the observations extended to the bottom at mid shelf, they revealed a weak maximum of cell numbers of *G. catenatum* (Figs. 2m,n). The development of upwelling, giving rise to a shoreward compensation current at depth, may have carried this maximum inshore (Fig. 2p).

To conclude, upwelling and the associated surface mixing due to wind appeared to cause offshore transport, dispersion and reduction in the number of cells, which was particularly evident for *G. catenatum* due to its wider distribution area. It is suggested that stronger stratification conditions favour the development of those dinoflagellate species, but it is not clear whether the nearshore dynamics play also a role.

ACKNOWLEDGEMENTS

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POPULATION DYNAMICS AND SPIROLIDE COMPOSITION OF THE TOXIGENIC DINOFLAGELLATE *ALEXANDRIUM OSTENFELDII* IN COASTAL EMBAYMENTS OF NOVA SCOTIA

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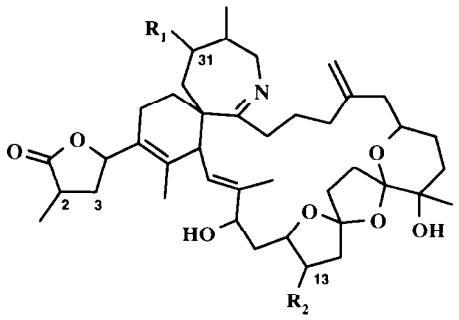
ABSTRACT

Spirolides are pharmacologically active macrocyclic imines discovered in plankton size-fractions and shellfish from the eastern coast of Nova Scotia, Canada. The gonyaulacoid dinoflagellate *Alexandrium ostenfeldii* was recently found to be the cause of spirolide toxicity. Analysis by liquid chromatography-mass spectrometry (LC-MS) showed that spirolide profiles were similar over time and depth within a site, but composition was markedly different among sites. The spatio-temporal distribution of spirolides in the water column is usually confined to late spring (May and June), following the decline in the spring diatom bloom. Highest spirolide concentrations were found in the 26-56 µm plankton size-fractions, during periods when large (>20 µm) thecate dinoflagellates, such as *Alexandrium, Dinophysis. Gonyaulax* and *Scrippsiella*, were dominant. Spirolide concentrations in plankton at Graves Shoal were highly correlated with abundance of cells of *Alexandrium* spp. (r^2=0.93), but attribution to *A . ostenfeldii* was complicated by the co-occurrence of *A. tamarense*, a morphologically similar species that does not produce spirolides.



	R1	*R2*		*MW*
A	**H**	**CH_3**	**$\Delta^{2,3}$**	**691.5**
desMeC	**CH_3**	**H**	**$\Delta^{2,3}$**	**691.5**
B	**H**	**CH_3**		**693.5**
C	**CH_3**	**CH_3**	**$\Delta^{2,3}$**	**705.5**
D	**CH_3**	**CH_3**		**707.5**

Fig. 1. Chemical structure of spirolides. $\Delta^{2,3}$ indicates compounds with a double bond between carbons 2 and 3.

INTRODUCTION

In the early 1990s an unusual "fast acting toxin" was first found in lipophilic extracts of scallop and mussel viscera harvested from certain aquaculture sites along the southeastern coast of Nova Scotia, Canada [1]. The toxicity was associated with a new group of macrocyclic imines called spirolides (Fig. 1) and both pharmacologically active and inactive analogues have been described from shellfish and plankton [2,3]. Since 1994, spirolides have been found in high concentrations in bulk plankton samples, on an annually recurring basis during spring and early summer, at these sites. The seasonal recurrence of this phenomenon, the association of toxicity with the viscera of multiple shellfish species, the coincident abundance of thecate gonyaulacoid dinoflagellates and the apparently restricted geographical distribution, suggested a planktonic origin for these compounds [4]. Yet the exact origin of spirolides remained cryptic until spirolide production was demonstrated in a unialgal isolate of the marine dinoflagellate *Alexandrium ostenfeldii* from Ship Harbour, NS [5] and in micro-extracted samples from natural plankton assemblages [6].

A field program was initiated to establish the dynamics of spirolides and that of the causative organism in Nova Scotian coastal waters. The study reported here is a retrospective analysis of size-fractionated field plankton collected from key sites characterized by high seasonal abundance of spirolides in the water column. The objective was to determine the relationship between plankton composition in particular size-fractions and spirolide composition. This multi-year field study is the first concerted attempt to describe the spatio-temporal distribution of these polyketide-derived metabolites among plankton size-classes.

MATERIALS AND METHODS

In 1997, an aerial survey using an aircraft-mounted passive sensor system (CASI – compact airborne spectrographic imager) was conducted over shellfish aquaculture sites in Mahone Bay, Nova Scotia, Canada. A tethered attenuation coefficient chain sensor (TACCS) (Satlantic, Inc.) for "ocean colour" was deployed on moorages in Mahone Bay, at Indian Point (1997) and Graves Shoal (1998). Physical data were collected at least twice weekly by vertical CTD casts (Applied Microsystems STD-12). In 1999, the focus of the field program was shifted to Ship Harbour for intensive sampling of plankton and physical data, whereas in previous years this site was sampled only during the peak of spirolide abundance.

Bulk plankton samples were harvested by pumping water (>5 x 10^3 L) with a high-volume centrifugal pump (200 L min^{-1}) from near surface (3 m depth) through a 20 µm plankton net, followed by size-fractionation through

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stacked Nitex sieves (21, 26, 44, 56, 76 and 95 µm mesh). Plankton from vertical net tows (20 µm mesh), vertically integrated pipe samples (0 - 7 m) and Niskin bottle casts from discrete depths were preserved in 2% formalin-acetic acid. The species composition was determined by Nomarsky and phase-contrast microscopy.

Plankton concentrated by centrifugation were extracted in 100% methanol (10:1 w/v) by sonication, then filtered through a 0.45 µm spin-cartridge (Ultrafree-MC, Millipore) [5,6]. Spirolides were analyzed by liquid-chromatography coupled with ion-spray mass spectrometry (LC-MS) detection (Perkin-Elmer SCIEX API-III) [7] using appropriate standards of purified spirolides.

RESULTS

The CASI survey (data not shown) of Mahone Bay conducted in the summer of 1977 provided baseline information, such as chlorophyll pigment signature (ratio of 555:443 nm), to be integrated with groundtruthing data on plankton composition and pigment levels. However, there were no distinct optical signatures useful in the species- or group-specific identification of bloom populations. According to both *in vivo* fluorescence and extracted chlorophyll *a* profiles, spirolide occurrence was unrelated to high plankton biomass and elevated chlorophyll levels. Contour plots of the diffuse attenuation coefficient (K_d: 490 nm) from the TACCS sensor (not shown) were compared with chlorophyll data from 1998. Maximum chlorophyll levels remained low (<2 µg L^{-1}) throughout the summer and were associated with a plankton community dominated by diatoms.

Since 1995, at both sites in Mahone Bay and at Ship Harbour, NS, spirolides were consistently associated with the presence and abundance of vegetative cells of the dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech *et* Tangen and GB42 cells. The latter cells are golden-pigmented spherical cells of 42 µm mean diameter that have been identified as athecate forms of gonyaulacoid dinoflagellates, primarily *Alexandrium ostenfeldii* [5].

Spirolide toxicity was first detected in mouse bioassays of shellfish extracts from sites within Mahone Bay and in Ship Harbour, NS [1]. According to the multi-year plankton survey and physical data from the CTD casts, detectable spirolide levels do not typically appear in the water column until the initiation of temperature- and salinity-dependent stratification resulting from vernal warming and increased freshwater run-off in late spring.

In coastal Nova Scotian waters, spirolides often co-occur with paralytic shellfish poisoning (PSP) toxins in the plankton, although blooms of the respective causative organisms *A. ostenfeldii* and *A. tamarense* may be somewhat asynchronous. At Graves Shoal (1997), PSP toxins were present in the water column somewhat earlier (in early May) than spirolides, but the spirolide-producing bloom was more persistent (until the end of June). Over several years, neither group of toxins has re-appeared in substantial abundance in the water column after July.

The plankton data from Graves Shoal (1996) indicate a general trend in spirolide occurrence at this site in subsequent years. The net plankton composition

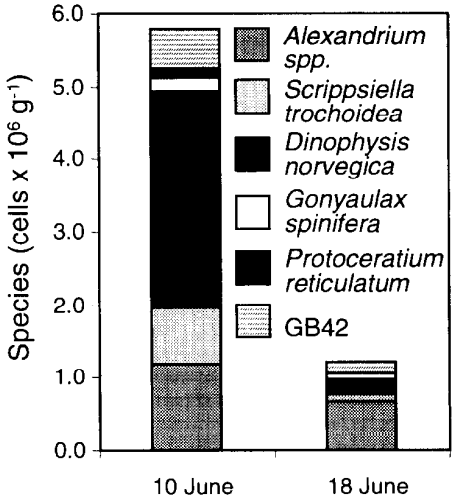

Fig. 2. Dominant dinoflagellates in the 26-44 µm size-fraction of plankton collected from Ship Harbour, NS (1996) during the peak in spirolide abundance.

(>20<150 µm) during the period when spirolides were present was dominated by large thecate dinoflagellates. By the end of June, coinciding with the disappearance of spirolides from the water column and shellfish viscera, diatoms such as *Chaetoceros* spp., had assumed dominance. Spirolides did not recur in the plankton even after a shift back to flagellate dominance in late August. Significantly, *Alexandrium* was not present in vertical net tows after the initial bloom in May and June.

In contrast, in 1996 at Ship Harbour, diatoms continued to dominate the net tow plankton (>80% of total cells) when spirolides occurred in the water column. Nevertheless, an assemblage of large thecate dinoflagellates, similar to that at Graves Shoal, was also present during the peak of spirolide abundance (Fig. 2).

At Graves Shoal (1996), the plankton distributional pattern was reflected in the composition of the bulk samples of the 26-44 µm size-fraction used for spirolide analysis. During the peak in spirolides in early June, the 21-26 µm fraction contained primarily *Scrippsiella trochoidea*, *Protoperidinium brevipes*, *Alexandrium* spp., and smaller "golden balls" (GB cells). At this time, in the 44-56 µm size-fraction, *Alexandrium* spp. (6.8×10^5 cells g^{-1}) co-dominated with mollusc eggs/larvae, along with lesser amounts of *Protoceratium reticulatum*, the tintinnid *Tintinnopsis* sp. and GB42 cells. The larger size-fractions (56-76, 76-95, >95 µm) contained relatively few *Alexandrium* cells (<0.35×10^5 cells g^{-1}) and were typically dominated by mollusc eggs, tintinnids, large dinoflagellates, e.g., *Ceratium* spp., and silicoflagellates.

For Ship Harbour (1996), large thecate dinoflagellates including *Alexandrium* spp., *Dinophysis* spp. and most of the gonyaulacoid species found at Graves Shoal were found in the 26-44 µm size-fractions when spirolides were most abundant in mid-June, although diatoms such as *Chaetoceros* spp were the dominant species (Fig. 2).

The typical spirolide profiles of bulk plankton extracts were very different between the sites in Mahone

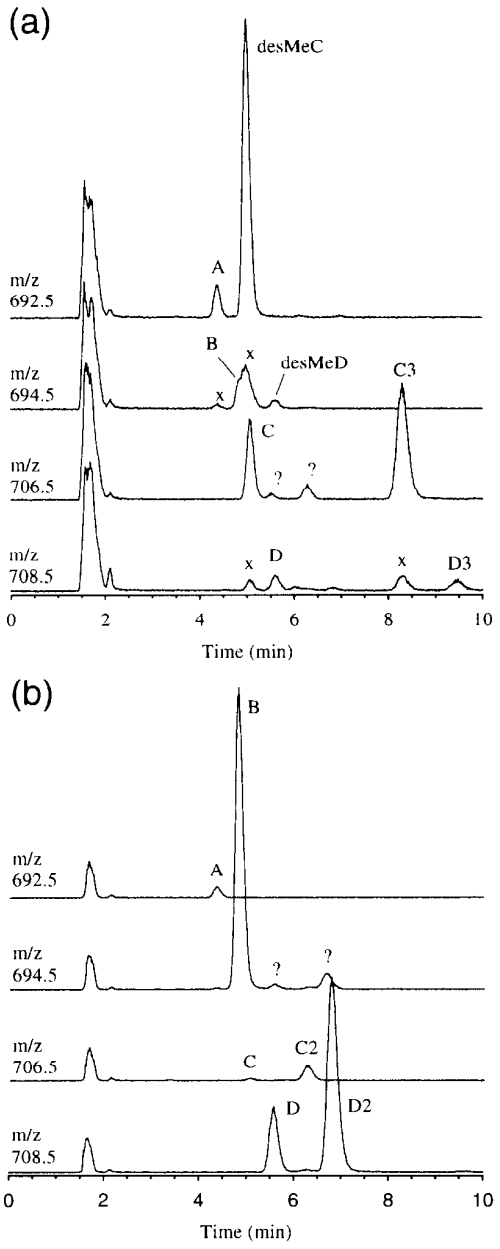

Fig. 3. LC-MS chromatograms of spirolides from plankton size-fractions collected at (a) Ship Harbour (1998) [21-56 µm] and (b) Graves Shoal (1997) [26-44 µm]. x = signals due to ^{13}C-isotope peak interference; ? = unknown spirolide components.

Bay and Ship Harbour, but were relatively consistent over time and water depth within a season. Figure 3 shows a representative spirolide profile determined by LC-MS, from a natural plankton assemblage collected at these respective sites. At Ship Harbour, the profile was particularly rich in des-methyl-C, whereas the primary components in samples from Graves Shoal were spirolides B, D, and D2. Spirolides B2, B3, C2, D2, and D3 are as yet undescribed isomers of C and D,

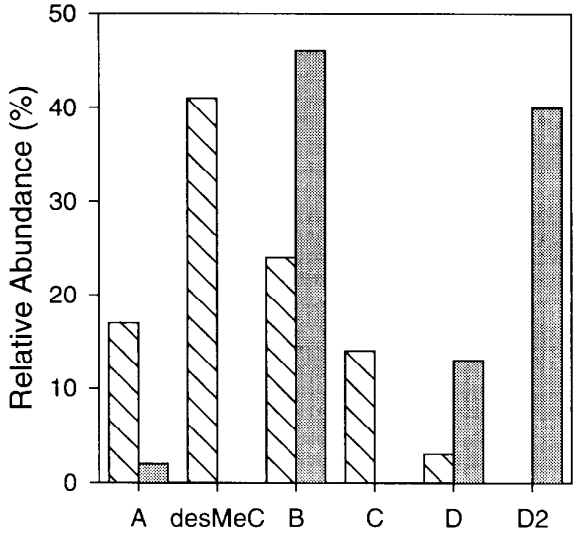

Fig. 4. Mean relative abundance (% of total) of spirolides in bulk plankton size-fractions (26-44 µm) collected at Ship Harbour ▨ and Graves Shoal ▩ in 1996. Components B and C include isomers B2 and B3, and C2 and C3, respectively, which were not identified at the time.

respectively. The bar plot (Fig. 4) shows the seasonally averaged relative composition of spirolides at Ship Harbour and Graves Shoal. The spirolide profile at Graves Shoal has remained essentially constant over several years (since 1995), whereas the profile from Ship Harbour plankton has shown some inter-annual variation.

In coastal Nova Scotia, the distribution of total spirolides in the plankton was highest in the 21-56 µm size-fractions collected from near surface water. The total yield of spirolides (per g wet weight) from the 26-44 µm fraction collected in 1996 was about twice as high at Graves Shoal as at Ship Harbour. Figure 5 shows the time-series concentration of spirolides in the 21-56 µm size-fraction in relation to the abundance of cells of *Alexandrium* spp. from Graves Shoal in 1998. Only trace levels of spirolides were found in the >76 µm fraction.

DISCUSSION

Spirolide toxicity in shellfish is a highly seasonal and repeatable annual phenomenon associated with toxic plankton blooms in coastal waters of Nova Scotia. Field surveys of spirolide distribution in the water column have shown that spirolides originate in the plankton, typically in late spring. The gonyaulacoid dinoflagellate *Alexandrium ostenfeldii* is the primary, perhaps exclusive, source of spirolides in the water column. It has not yet been determined if GB42 cells associated with gonyaulacoid dinoflagellates occur naturally in the plankton in high abundance or if they represent artifacts of plankton sample manipulation. High-volume pumping of sub-surface plankton samples has been shown to cause breakage of long diatom chains and loss of thecae in a fraction of certain armoured dinoflagellates, but complete

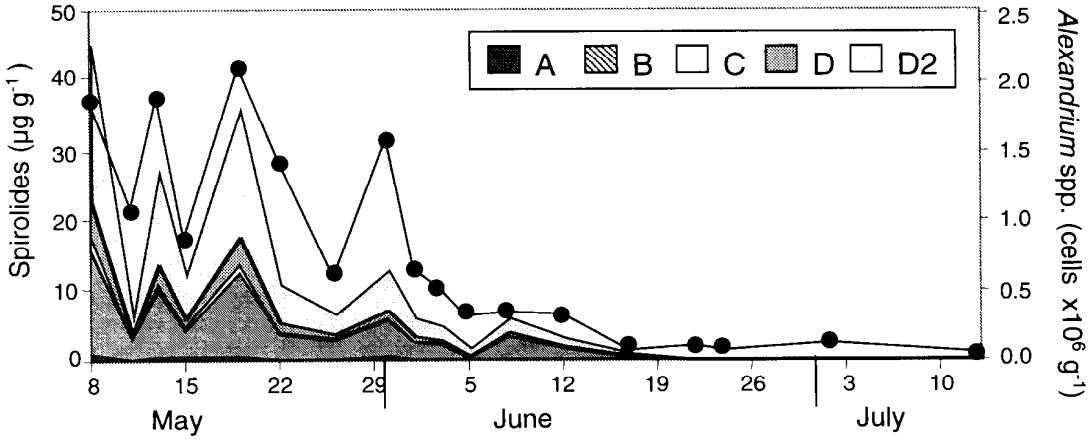

Fig. 5. Spirolide composition and abundance of cells of *Alexandrium* spp. (-●-) in bulk plankton size-fractions (21-56 µm) collected at Graves Shoal (1998). The filled area graph represents changes in cumulative concentrations of various spirolide analogues over time.

cell lysis by mechanical damage does not apparently occur (A. Cembella, unpublished observations). In any case, the consistent cell quota of spirolides in cells harvested from batch cultures and from field populations of *A. ostenfeldii* cells isolated by micropipette from pumped samples [6] indicates that leakage from damaged cells is minimal.

Detection of blooms of the causative organism of spirolide toxicity poses a difficult challenge for airborne remote-sensing and *in situ* optical sensing systems. Blooms of *A. ostenfeldii* in coastal Nova Scotian waters tend to be cryptic sub-surface aggregations at relatively low concentrations (<4 x 10^3 cells L^{-1}) with no obviously distinct pigment signature to discriminate among other gonyaulacoid dinoflagellates in natural plankton assemblages. Maximal spirolide concentrations do not occur within high biomass plankton blooms; i.e. they are not associated with high chlorophyll levels or maxima in plankton biomass-dependent light attenuation.

The appearance of spirolides in the water column coincides with the initiation of stratification and follows the decline of the spring diatom bloom. Physical data (temperature, salinity, σ_t) from the water column indicate that spirolide events are associated with a deepening of the pycnocline and associated stratification of shallow waters in coastal embayments.

Many spirolide derivatives have now been structurally characterized from shellfish and planktonic sources [1,2,3]. Spirolides A, B, C and D, including their respective des-methyl derivatives, are biologically active, whereas E and F, produced by opening the cyclic imine ring, are inactive products found in shellfish [2], but not in significant amounts in the plankton. The spectrum of spirolides produced by geographical populations of *A. ostenfeldii* is complex and distinctive. The fact that natural populations from Ship Harbour exhibit more inter-annual variation in spirolide composition than those from Graves Shoal may indicate a more restricted range of genetic variants at the latter site.

The co-occurrence of spirolides and PSP toxins in the plankton is coincidental not causal. While there is some temporal overlap in the distribution of these toxins, they are derived from different source organisms. The asynchrony in the initiation, peak abundance, and decline of *A. tamarense* and *A. ostenfeldii* blooms may reflect differences in the respective encystment/excystment cycle, as well as differences in *in situ* growth rates. In any case, their morphological similarity in routine plankton analysis poses a significant challenge in assessing the relative toxicity risk posed by these species.

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ON THE LONG-TERM RESPONSE OF HARMFUL ALGAL BLOOMS TO THE EVOLUTION OF EUTROPHICATION OFF THE BULGARIAN BLACK SEA COAST: ARE THE RECENT CHANGES A SIGN OF RECOVERY OF THE ECOSYSTEM-THE UNCERTAINTIES

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ABSTRACT

In the present paper the long-term trends (1954-1998) of phytoplankton blooms are discussed in relation to the evolution of anthropogenic eutrophication (nutrients) and the variability of environmental factors such as temperature, sun spot activity, graizing pressure and large-scale weather patterns.

The results suggest that the recent features of phytoplankton blooms occurence, manifest signatures of ecosystem recovery. PCA analysis, questioned the role of eutrophication as the only controlling factor, providing arguments of a possible global climatic signal of influence too. Thus the 90-ies could be considered an environmental window related to the synergistic effect of anthropogenic eutrophication reduction and natural variability.

INTRODUCTION

Since late 1970s, anthropogenic eutrophication has been identified as a key ecological problem for the Black Sea ecosystem, culminating during the 1980s [1,2,3]. Dramatic alterations of phytoplankton communities and spectacular expansion of phytoplankton blooms have been well documented, the increase of nutrients and the shifts in their ratios reporting as the main forcing factor [4,5,6,7,8]. Besides there is enough evidence that the episodes of algal blooms are controlled by the interaction of many factors: "bottom-up"/"top-down" effects, natural physical and hydrological variance and accompanying climatological changes [9, 10, 11, 12, 13, 14, 15].

A reduction of the Danube river nutrient input [1], a decrease in the frequency of hypoxia [3], increase in fodder zooplankton biomass and a drop off of *M.leidyi* abundance [16] have been reported during the 1990s,, suggesting a relative improvement of the Black Sea ecosystem ecological state, related mainly to a collapsing economy in the riparian Black Sea countries

The objective of the paper is an evaluation of phytoplankton blooms during the 90s (1990-1998) in the context of long-term historical trends (1954-1998) with the aim to highlight the following questions: 1) to what extent can eutrophication be considered the main driving force, within the concept that phytoplankton bloom dynamics rely on the synergistic interactions of favorable physical, chemical and biotic conditions and 2) are the recent changes a sign of recovery of the ecosystem?

MATERIALS AND METHODS

Areas of investigation

The study is based on data from the coastal (3 n.m.) zone off Cape Galata, including Varna Bay, selected as one of the most eutrophicated region along the Bulgarian Black Sea area.

Parameters

The long-term phytoplankton data set was constructed using historical published data, personal published (1981-1996) and unpublished data (1997-1999) analysed in parallel with a long term data array of physical, chemical and biotic parameters (Table 1). The influence of large scale weather patterns is also considered. PCA analysis was applied to score and narrow down the selection of parameters and figure out the underlying mechanisms controlling phytoplankton blooms long-term dynamic.

Table 1. List and abbreviation of parameters

Parameter	Abbr., [data source]
Temperature	T, [NOCD]
Sun spot activity	SSA, [15]
Danube river discharge and associated nutrients-PO_4, NO_3, Si discharge concentrations	DD, DDP, DDN, DDSi, [1]
Nutrients in 3 miles zone (cape Galata)	P-PO_4,N-NO_3, Si-SiO_4, [4]
Dinophyceae bloom abundance	Din, [6, 7, unp. data]
Precipitation	Pr [NOCD]
Total zooplankton biomass	ZB, [16, 17, unp.data]
Noctiluca scintillans abundance	N. sci., [16, 17, unp. data]

RESULTS

Peculiarities of phytoplankton blooms during the 90ies

A characteristic feature of the 1990s is a further increase of blooming species diversity, typical for the period of intensive eutrophication (Table 2). A lot of species have attained bloom densities (39), comprising about 13 % of the total number in the area (305) – exceeding about 1.5 times that of the 1980s, and about 5 times that of the 1960-70s.

The red-tide species list was entered by microalgae typical for the area, but never recorded in a bloom density before-*Dytilum brightwellii (1994), Leptocylindrus minimus (1993, 1994, 1996)), Gymnodinium splendens (1995,1999) Oscilatoria sp. (1995) Monoraphidium convolutus (1992),* or phytoplankters found for the first time off the Bulgarian Black Sea shelf-*Detonula pimula (1995), Oxyphysis oxytoxoides (1994), Apedinella spinifera (1997)), Astasia sp. (1996)* or species not reported for the Black Sea basin-*Alexandrium monilatum, (1991), Gymnodinium uberimum(1994),, Phaeocystis pouchettii, (1989), Rhizosolenia setigera (1998).* Species like *Detonula confervaceae, Chaetoceros curvisetus, Chaetoceros socialis, Cyclotella caspia, Pseudosolenia calcar-avis, Pseudonitzschia seriata* typical for the

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Intergovernmental Oceanographic Commission of UNESCO 2001

1960-70s proliferated more frequently during the 1990s, in comparison to the 1980s.

Table 2. Inventory of phytoplankton blooms at Cape Galata & Varna Bay (A-no of species, B –no of blooms)

Period	1954-1970*		1981-1990		1991-1998	
Taxonomic group	**A**	**B**	**A**	**B**	**A**	**B**
Bacillariophyceae	6	18	14	50	23	57
Dinophyceae	1	4	5	27	8	19
Euglenophyceae	0	0	2	10	2	7
Chrysophyceae	1	2	2	8	2	10
Flagellates			1	1	1	0
Cryptophyceae			0	0	1	3
Cyanophyceae			0	0	2	2
Total	**8**	**24**	**24**	**96**	**39**	**98**
summer blooms		5		35		27
in % from the total		20		38		28
cases of bloom associated mortalities in summer		2		8		2

Although the blooms frequency is maintained an apparent feature of the 1990s is the decrease of both monospecific blooms and their critical levels attained. During 1980-1990, 7 cases when a single microalgae reached concentration about $50x10^6$ cells/l and more (the highest attained by *P. minimum*–481x10 cells/l) were recorded in comparison to only 3 during 1991-1998 (*P. minimum* outbursts during 1997-1998 were of densities within the range $100\text{-}200x10^6$ cells/l). The trend of maximal value reduction is more evident among Dinophyceae species, which average abundance decreased about twice (Fig.1, 2).

Some diatoms like *S. costatum* ($57.2x10^6$ cells/l,-autumn'96), *C. pelagica* ($11.2x10^6$ cells/l–autumn'97), *Ch. socialis* ($35.5x10^6$ cells/l –summer'1993), and the dinoflagellate *P. minimum* continue to generate high numbers but at much lower frequency–Table 2.

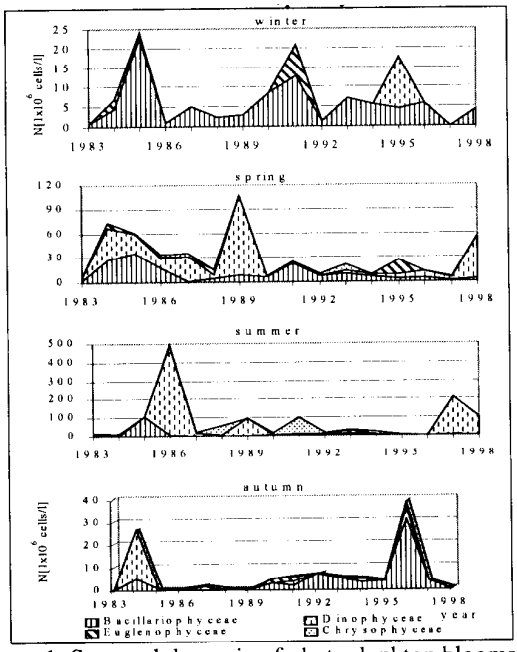

Figure 1. Seasonal dynamic of phytoplankton blooms in Varna Bay.

A peculiarity of the 90ies is the alteration of the seasonal pattern of the bloom episodes (Fig.1).

The ecosystem shifted towards a more harmonized seasonal dynamic-reduced amplitude of seasonal oscillations and year to year variations (spring-summer)-a trend which has been interrupted in 1997. An about two fold increase in autumn ($10.2\text{-}18.8x10^6$ cells/l), and maintained high winter levels are obvious, while on the contrary the maximum average density decreased slightly in spring, and about twice in summer, ($132.2\text{–}76.5x10^6$ cells/l) (Fig. 1), on the account mainly of non-diatoms.

Diatoms sustained high abundance at the level of the 80ies in winter, spring and summer with a substantial increase in autumn (severalfold). In contrast to the 80ies the ratio Bacillariophyceae: Dinophyceae increased in favour of the diatoms, (about 3 times in spring and twice in summer) suggesting a trend of diatom dominance recovery (Fig.1, 2).

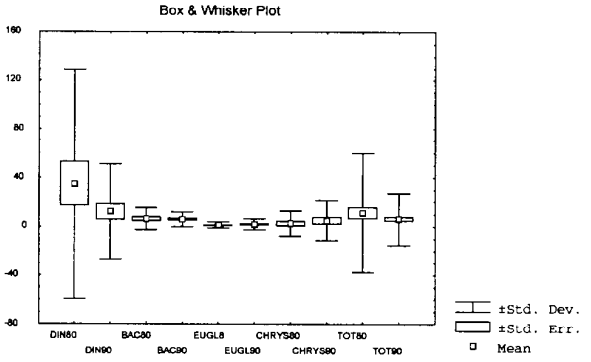

Figure 2. Box & Whisker plot of phytoplankton blooms by taxonomic groups.

When the number of the summer blooms is compared in % of the total blooms the results reveal a dramatic increase during the period of intensive eutrophication (38%) and a decreasing trend during the recent period although still far exceeding the frequency during the reference stage of the ecosystem (Table 2). Consequently the cases of bloom associated fish and zoobenthic mortalities, being almost recurrent during the 80ies (discussed in details in Moncheva *et al.*, [7]) became less frequent (Table 2.). This trend was maintained till 1997, while in summer 1998 and 1999 a spectacular fish mortality associated with the bloom of *Prorocentrum minimum* and co-dominant dinoflagellates was observed.

Environmental parameters and phytoplankton blooms response.

PC Analysis was applied in order to determine and score the most relevant combination of parameters to discriminate by years in order to differentiate between the impact of specific natural variations and the anthropogenic eutrophication (Fig. 3).

The matrix was based on a long term data array of the following variables: T, SSA, DD, DDP, DDN, DDSi, nutrients (PO_4, NO_3, $Si\text{-}SiO_4$) in the investigated area, the latter 5 variables referred to as eutrophication component, ZB and N. sci.(referred to as grazing component) and Dinophyceae bloom abundance (Din) in spring-summer. The period and Dinophyceae blooms dynamic were

selected as the most vulnarable to long-term changes component as suggested by the forgoing analysis. PCA analysis extracts 2 components to which ecological significance could be attached - (PC1) and (PC2) explaining 57.1% of the total variance.

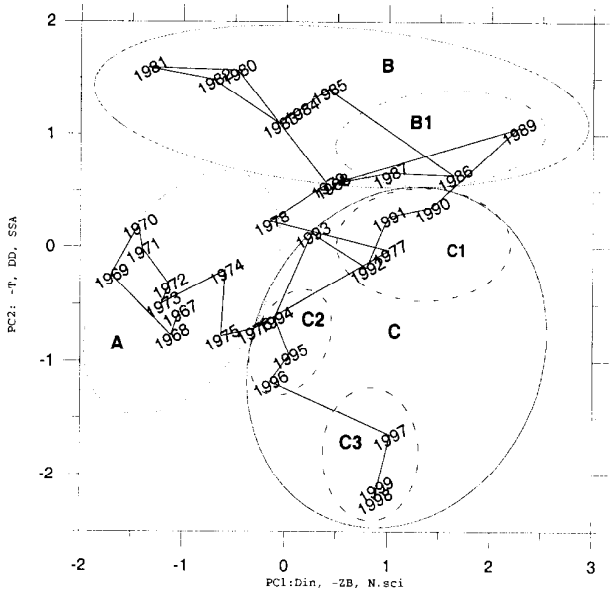

Figure 3. PCA plot of the scores of selected variables.

The score PCA plot displays a clear discrimination between the 1960-70 (cluster A) and 80s (cluster B), the 90s (cluster C) split into three subgroups, in general projected at intermediate coordinates between 70s-80s (Fig. 3). PC1 (a loading of 32.4 % in the total variance) correlates positively with Din and N. sci. and negatively with ZB. PC2 corresponds to a decrease in T, increase in SSA and the eutrophication component (dynamic of variables is presented on Fig.4 and Table 3 for better illustration). The A group (1960-70s), corresponding to the reference period of the ecosystem, is characteristic with low blooms densities and frequency, high grazing and low N. sci., relatively high temperature and low level of eutrophication component-(Table 3). The split of the 1980s (B) into two subgroups - B1 (1980-1985) and B2 (1986-1989) is more apparent along the PC1 axis (blooms/grazing). It is parallel to the different amplitude of Din blooms increase (maximum values recorded during 1986-1989) and the contrasting trend of ZB dynamic (reduction of ZB, more explicit since 1986). Both subgroups are projected at coordinates corresponding to the highest level of eutrophication component (Table 3).

Table 3. Nutrients [μM] in Varna Bay [4] and Romanian Black Sea coastal area [1, 5].

period	DDN	N-NO_3	DDP	P-PO_4	DDSi	Si-SiO_4
1960/70	1.6	4.1	0.34	1.33	36.7	36.54
1971/75		2.6	5.73	0.94	61.2	18.89
1976/80	13.4	7.5	6.38	2.34	30.6	14.4
1983/90	6.9	4.0	6.54	1.41	11.6	6.42
1991/97	5.99	6.56	2.34	0.22	10.1	5.11
1997/98		5.03		0.11		6.3

The 1990-1993 (C1 subgroup) of the recent period marks the transition between the reference (the 70s) and the intensive eutrophication (the 80s) episodes. The association of 1994-1996 (C2 subgroup) to the reference A, reflects more the similarity of the interaction fashion between the selected determinants despite the differences of the real values. A decreasing trend of nutrients and phytoplankton blooms and an increasing trend of ZB was observed but sustained at a higher level (respectively lower for ZB), in comparison to the reference period, (Fig. 4), at higher T and similar SSA.

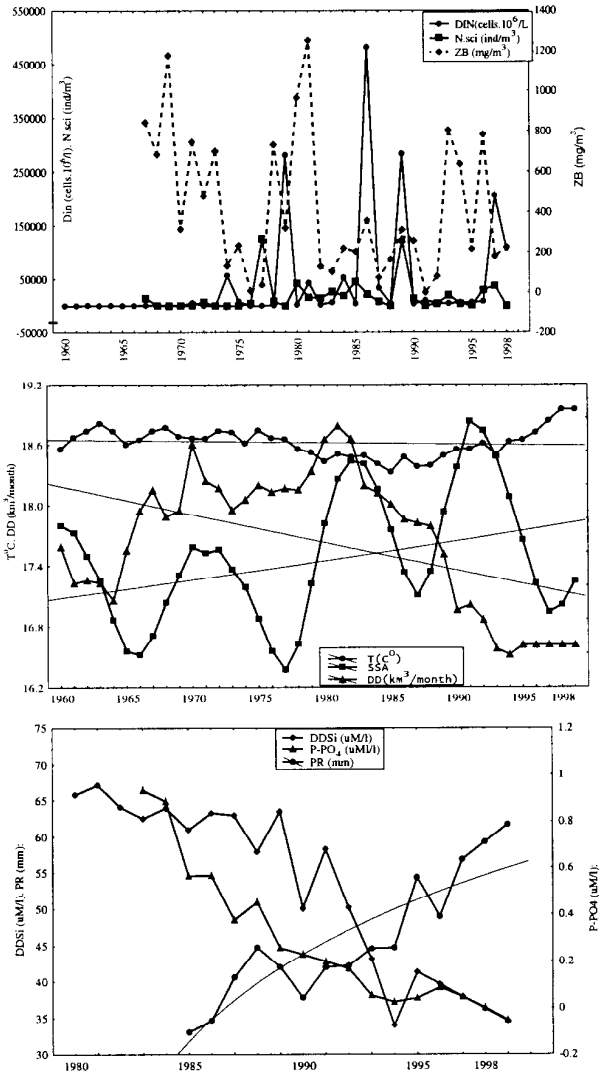

Figure 4. Dynamic of selected biotic and environmental parameters.

The last 3 years (C3 subgroup), are projected at coordinates corresponding to a blooms/grazing mode characteristic for the transition episode (C1) at higher T (the highest) and maintained decreasing trend of eutrophication component. Probably this is a brake down of the general trend of the 1990-1996 suggesting a reversing point of the ecosystem towards the 80ies but at maintained decreasing trend of the nutrients (especially phosphates and silicates)- (Table. 3)

DISCUSSION

The principal questions put forward in this paper are whether the recent changes in phytoplankton bloom dynamic mark a phase of recovery of the coastal Black Sea ecosystem and to what extent the evolution of the eutrophication could be considered the main underlying mechanism.

There exists a widespread belief that the outbreaks of red-tides are becoming more frequent as a consequence of increasing eutrophication in coastal waters [18,19,20]. A lot of examples include the numerical displacement of diatoms with dinoflagelates, chrysophytes, prymnesiophytes and cyanobacteria in geographically diverse regions due to the alterations in nutrient supply ratios from human activities [6,7,21,22,23]. As evident from the results (data reported by Cociasu et al. [1]) since 1990 the DDP content of the DD has decreased steadily from its peak value of 6.5 uM to about 2.0 uM in 1993-1995 with mean concentration d 3.3 uM, in contrast to its consistent increase during 1980-90. Contrary to DDP, the DDSi ranged between 55-70 uM during 1980-1989, and after 1990 declined to 30 uM in 1994 and increased again to 50 uM in 1995. The DDN values between 1998-1995 ranged from 326 uM in 1989 to 176 uM in 1995, indicating a continuous decrease. The nutrients discharged into the basin follow the same trends-the annual input of Si decreased from 500 000 tons in 1980-81 to 175 000 tons in 1994. The annual average of PO4 for 1988-1995 was 18 000 tons. As apparent from the data similar trends of nutrients dynamic is manifested in the investigated area with pronounced decrease in summer [4]. As the nutrient availability sets the biomass carrying capacity, than their reduction could to a certain extent explain the reduced blooms frequency and maximum densities attained during the 90s as compared to the 80s. The nutrients supply ratio hypothesis also hold true, despite the limitations in its application [24], which. could be a factor contributing to the increased dominance of diatoms and the established changes of the phytoplankton blooms seasonal sequence. From the viewpoint of the classical phytoplankton succession in the Black Sea, the summer outbursts are considered a succession perturbation, induced by anthropogenic eutrophication, resulting in a deviation towards selection preferentially of fast growing small microalgae (preferably dinoflagellates and haptophytes – [25]). Initially the shift of species composition in spring-summer to non-diatom opportunistic species (typical for the 80s) and the increase of dinoflagellates bloom prevalence are considered "an alarm bell" of coastal ecosystems ecological degradation, imposed by eutrophication. Thus the reduction of summer incidence and the restoration of the diatoms dominance should mark a stage of improvement. The PCA analysis provide further evidence that the recent period manifest transition patterns and some signatures of positive changes, related to the reduction of the land-based nutrient load into the basin.

The last three years however demonstrate the instability of this trend and questioned the role of anthropogenic eutrophication as a single driving force. As the analysis illustrates both physical (T, SSA) and biotic (zooplankton grazing) interactions count substantially in the discrimination between the different periods of the evolution of the ecosystem. *Mnemiopsis leidyi* dynamic considered a major factor that modified zooplankton oscillations since its successful introduction in the early 80s (Mnemiopsis era, [26]) give further support to this suggestion. During 1986-1990 when the biomass of the Ctenophore reached its maximum in the Black Sea basin [27] concomitant dramatic decrease of meso-zooplankton diversity and biomass was observed [17], coinciding with the maximal level of phytoplankton blooms in summer (especially in 1986, 1989). The fodder zooplankton biomass dropped rapidly to its lowest value in 1992 (80 mg/m^3). The diversity and abundance of Copepods decreased substantially, while *Noctiluca scintillans* became dominant with frequent and massive blooms [16]. In summer 1991-1992 *Mnemiopsis* abundance was the lowest for 1986-1998 (3-16 ind/m^3), starting to increase after 1993 to a maximum of 4 000 ind/m^3 in 1998, despite of the new exotic ctenophore *Beroe ovata* outburst. The percentage share of *N. scintillans* in the total zooplankton biomass decreased on the account of an increase of Copepod species [16].

Thus the predator-prey interactions of *Mnemiopsis*/ zooplankton during the 90ies provide further evidence that *M. leidyi* directly impacts mesozooplankton diversity and biomass and indirectly influences phytoplankton growth by reducing the grazing pressure [28].

In general the peaks of dinoflagellates blooms correlate positively with the temperature maxima (Fig. 4), so the exceptionally high temperature in summer 1998 - 1999 (26-27°C) may contribute to the increased phytoplankton incidence during this period. Mykaelian et al [10] claimed that the climatic quasi-periodic 20 year oscilations of winter air temperature determine the general intensity of Black Sea current system and phytoplankton growth. The sustained concentrations of NO_3 at almost the level of late 80s and the observed increase of diatoms especially in autumn during the 90s, might be attributed to the increased precipitation over the area during the recent period (Fig. 4), as atmospheric input of nitrogen is reported to be partly responsible for the appearance of algal blooms, by either direct input or through the watershed [29, 30, 31].

Saydam [12] has shown that *E.huxleyi* blooms during spring and summer are triggered by the intrusion of desert origin dust over the Black Sea. Nierman *et al.*, [15] examined the long-term dynamic of pelagic species in the open Black Sea during 1980-1995 and other basins in the World Ocean in connection to the global climatic regime and outlined common pattern of zooplankton fluctuations with the Black Sea. It was concluded that the striking changes observed in NAO, SO, ENSO, and ALPI in the second half of the 80s impacted strongly the hydrological and meteorological regime in the northern hemisphere that could have triggered the alterations in phyto- and mezozooplankton of the Black Sea during the 1990s. The oscillation of the global temperature , has its lowest amplitude during the mid 80s and reached its highest summer values during 1998-99. The sun activity (sun spot numbers) was very low during the mid-80s and rose back to high values during the early 90s. The 1990-1995

correspond to five year warm El/Nino episode contrasting to the two year LaNina/El/Nino cycle during the 1996-1999, similar to the 80-ies [www.nnic. noaa.gov/]

CONCLUSIONS

The results suggest that the recent features of phytoplankton blooms: reduced amplitude of seasonal oscillations and year to year variations, the decrease of phytoplankton monospecific blooms and their critical levels attained, especially in summer, the increase in the dominance of diatoms, the shift of the seasonal succession to the Black Sea ecosystem natural annual cycle manifest signatures of recovery.

The demonstrated instability of this trend, the maintained capacity of the ecosystem to sustain critical levels and blooms frequency, the concerted changes of environmental factors such as temperature, grazing, sun spot activity, illustrated by the PCA analysis, questioned the role of eutrophication as the only controlling factor. Similar changes reported for different regions of the World Ocean, suggest a possible global climatic signal of influence too. Thus the 90s could be considered more an environmental window related not to the decrease of anthropogenic eutrophication only.

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PREDICTION OF BLOOMS OF TOXIC DINOFLAGELLATES BY EVALUATING ENVIRONMENTAL FACTORS

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ABSTRACT

Paralytic and diarrhetic shellfish poisonings cause significant damage to coastal bivalve aquaculture in Japan. It is therefore important to predict the occurrence of toxic dinoflagellate blooms, mainly *Alexandrium tamarense* and *Dinophysis fortii*. The environmental factors which were expected to enhance bloom occurrence were examined using a cross correlation method. The annual average density of toxic dinoflagellates observed at a station in Ofunato Bay, Iwate Prefecture, was used as single data points. Environmental factors examined were air temperature, precipitation, sea surface temperature, sunshine hours, wind direction, and wind speed. Moving average values of the environmental factors for 10 days, 20 days, and 30 days were employed as single data points. A correlation diagram between toxic dinoflagellate density and the moving average value of each environmental factor was drawn using a computer software cross correlation method, and the period with the highest correlation between the two values was identified from the diagram. A regression formula using the theoretical density of the toxic dinoflagellates was calculated, derived from multiple regression analysis with toxic dinoflagellate density and each average datum point of the environmental factor. Further, an equation of the cell density from calculated and observed values was derived by the least squares method to evaluate the correlation coefficient. The results showed that the annual average value of *A. tamarense* was highly influenced by the amount of precipitation in March and April. However, no factor was considered to affect that of *Dinophysis fortii* was discovered.

INTRODUCTION

It is important to predict the occurrence and impact of toxic dinoflagellates towards stabilizing fishermen management planned bivalve shipments such as *Patinopecten yessoensis*.

Staffs of fisheries research laboratories in each Japan prefecture are engaged in the prevention of shellfish poisoning damage through projects with the Fisheries Agency, through monitoring of the cell density of toxic dinoflagellates. In addition, marine environmental factors are monitored at each station to define the range of environmental factors coincident with toxic dinoflagellate blooms (Sekiguchi et al. 1989)[1]. Moreover, prediction has been explored using multiple regression analysis and toxic dinoflagellate data and the environmental factor as input terms.

Initially, it is important to understand population dynamics of the object dinoflagellate as a function of the conditions affecting bloom and decline of the species. This will include growth stage and physiological state in order to predict the peak density during blooms of the dinoflagellate. However, currently analyses go no further than calculation of the regression formulas using numbers of a toxic dinoflagellate and multiple regression analysis which employs several physical and chemical environmental factors observed during sampling, followed by evaluation of consistencies between field observations results and the calculated formulas.

In this paper, we employed toxic dinoflagellate densities and reported physical environmental factors in order to develop a more rational prediction method to estimate the density of toxic dinoflagellates. When and what kind of conditions affecting the blooms of toxic dinoflagellate were determined by statistical processing of the correlation between the annual average density of the toxic dinoflagellate and environmental factors, followed by a prediction calculated by using the highly correlated factors with the annual average density. Lastly, the difference between the calculated and observed densities were analyzed for the presence/absence of relationship.

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MATERIALS AND METHODS

Data used were the cell densities of *Alexandrium tamarense* and *Dinophysis fortii* (cells_L^{-1}) observed in twelve layers, the surface and every two meters to the bottom obtained from a station in Ofunato Bay (Iwate Prefecture, unpubl. data), AMeDAS data (Automated Meteorological Data Aquisition System: air temperature, precipitation, sunshine hours, wind direction and wind speed) at the Ofunato Meteorological Station of the Meteorological Agency, and sea surface temperature data at mouth of the bay. Annual average dinoflagellate density was derived by dividing total annual amount by the times samples were collected (Table 1). Moving average value for environmental factors of 10 days, 20 days, and 30 days, respectively, were considered to be an individual datum; the moving average removed short cyclic fluctuations. Environmental factors highly correlated with the annual average values of the toxic dinoflagellate were extracted by using the cross-correlation analysis $\{Rxy(\_) = \lim 1/T\int_0^T x(t)y(t+\_)dt\}$, using the data to clarify highly correlated periods. Where x(t) is the function of the annual average value of toxic dinoflagellate and y(t) is the function of the moving

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Table 1. The annual average density (cells_L^{-1}) derived by dividing total annual density by the number of times sampled when more than one cell density observed.

	1982	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993	1994
A. tamarense	554.2	24.7	28.8	1.0	604	50.0	60.7	408.4	318.0	179.6	9.0	190.8	138.7
D. fortii	---	2.1	15.8	8.7	11.2	8.1	2.8	19.6	7.9	1.8	2.5	11.0	

Table 2. Regression coefficients and correlation coefficients of the equation derived from least squares analysis between cell density of *Alexandrium tamarense* calculated and observed. (see text for details)

Moving Average (days)	Highly correlated environmental factor and the period (days from Jan. 1)-	Coefficient of the equation (Y= aX + b) between cell density calculated and observed	Correlation coefficient
10	precipitation	a 0.414	
---	94_110	b 68.76	0.578
20	precipitation	a 0.353	
---	85_110	b 87.02	0.530
30	precipitation	a 0.447	
---	75_109	b 91.69	0.622

-_these results were derived from the data during 1982 to 1994.

Table 3. Regression coefficients and correlation coefficients of the equation derived from least squares analysis between cell density of *Dinophysis fortii* calculated and observed. (see text for details)

Moving Average (days)	Highly correlated environmental factor and the period (days from Jan. 1)-	Coefficient of the equation (Y= aX + b) between cell density calculated and observed	Correlation coefficient
	air temperature		
10	121_132	a _0.090	_0.037
	precipitation	b 18.17	
	175_190		
20	air temperature	a _0.217	_0.187
	100_141	b 15.09	
30	air temperature	a _0.789	_0.198
	119_151	b 29.54	

-_these results were derived from the data during 1984 to 1994.

average value of each environmental factors. These correlation coefficients were plotted using computer software which shifted days from January 1st on a daily basis up to 180th days (_). Highly correlated environmental factors and their corresponding period were read from this diagram, and a multiple regression formula was calculated based on the average values from 10 years (1982 to 1991) for *A. tamarense* and 8 years (1984 to 1991) for *D. fortii*; average values were selected during the periods that contained the highest correlation. Thereafter, a theoretical value of the toxic dinoflagellates was calculated using the regression formula. Lastly, an equation of cell density was derived from the relationship between calculated and observed densities by least squares method, to compare correlation coefficients.

RESULTS

Alexandrium tamarense

The results of the cross correlation analysis indicated that the annual average value of *A. tamarense* was highly correlated with precipitation in the periods from the 94th _110th days from January 1st in the 10 day moving average, 85th _110th days in the 20 day moving average, and 75th _109th days in the 30 day moving average, respectively. Figure 1 shows the correlation coefficients (Rxy (_)) between the annual average density of *A. tamarense* and the 30 day moving average values for each environmental factor. The period of high correlation (_0.7) is only noted for precipitation during the 75th to 109th day period from January 1st.

A regression formula was obtained from data during 1982 to 1991, the annual average density of *A. tamarense* and the average amounts of precipitation during the period with high correlation (Rxy (_)) against each moving average. The equation (least squares method)

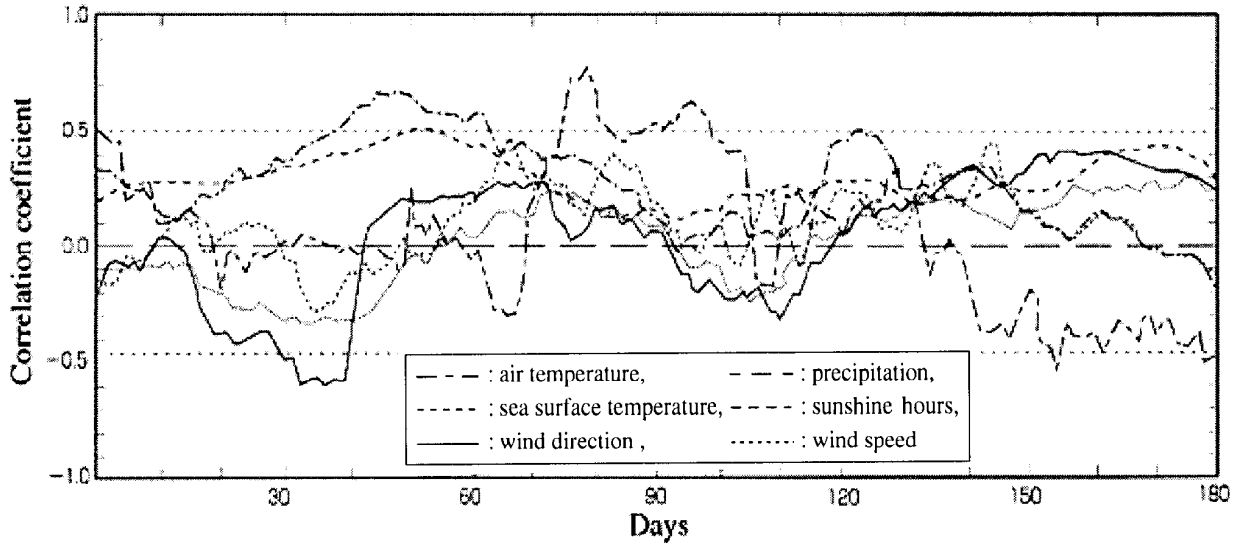

Figure 1. Diagram of the correlation coefficients (Rxy (τ)) between annual average density of *A. tamarense* and the 30 day moving average values of six environmental factors. The period of high correlation (_0.7) is only seen with precipitation during 75th to (79+30)th days from January 1st.

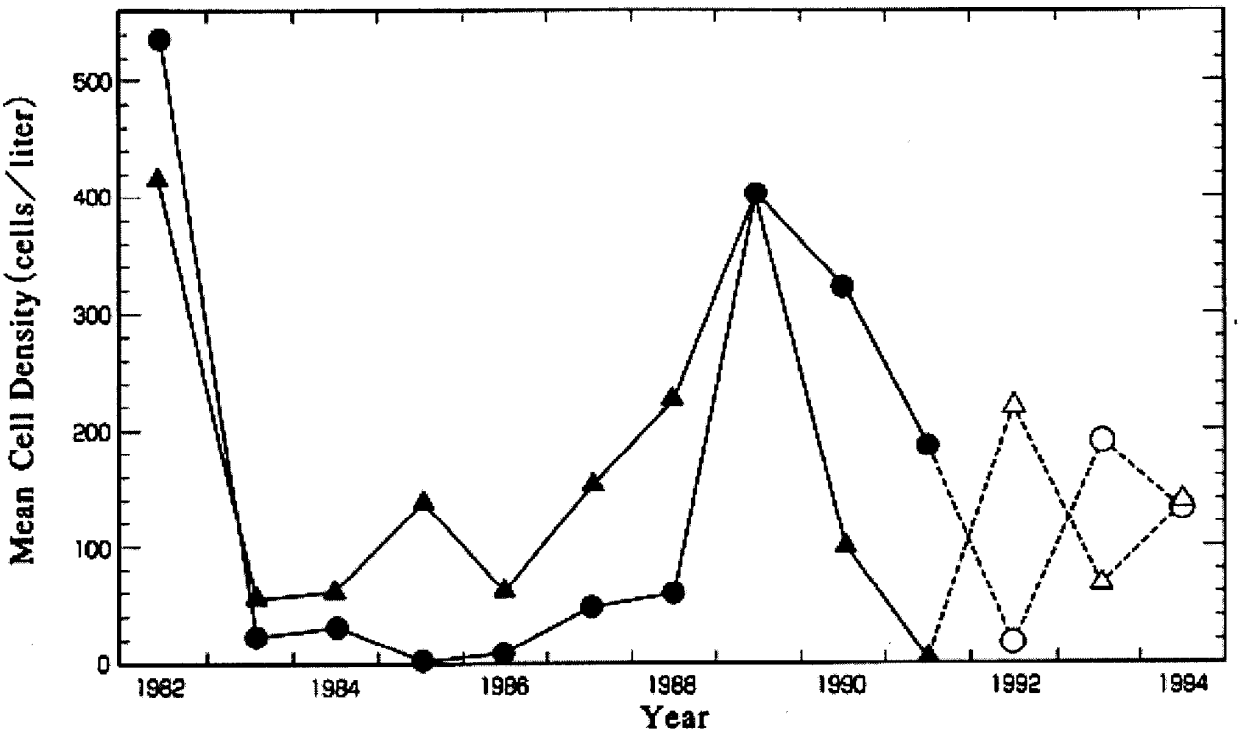

Figure 2. Diagram of cell densities estimated from comparing observed and calculated levels (see text). The cell density observed (●, ○) are the annual average cell densities which were derived by dividing annual total amounts by the number of measurements when more than one cell density was observed. Calculated cell densities (▲, △) were derived by the equation derived in least squares method.

was derived from cell calculated density derived from regression formula and the annual average values that were observed (Table 2). The results indicate the highest correlation coefficient (0.622) was derived for 30 day moving average (Fig. 2).

Dinophysis fortii

The same procedures were also carried out for *D. fortii*. Results of the analysis using cross correlation methods indicated that the annual average density of *D. fortii* was highly correlated with precipitation and

airtemperature. The periods were 121st _132nd days from January 1st of air temperature and 175th _190th days of precipitation in the 10 day moving average, 100th _141st days of air temperature in the 20 day moving average, and 119th _151st days of air temperature in the 30 day moving average, respectively.

Three multiple regression formulas were also estimated from the data during 1984 to 1991, i.e., the annual average density of *D. fortii* and the average amounts of precipitation and air temperature during the four periods which showed high correlations (Rxy (τ)) against each moving average. Also, the equation by the least squares method was derived with the cell density that were calculated with the regression formula and the annual average density that were observed (Table 3), but the correlation coefficients of the equations were very low (Table 3).

DISCUSSION

In this paper, the environmental factors that were highly correlated with the amount of toxic dinoflagellate were selected by the cross correlation method. In the case of *Alexandrium tamarense*, the results indicated that the more the amount of precipitation (snow and rain), the higher the annual average density of *A. tamarense*. It is notable as it indicates that the supply of the nutrients from land and positively affects the multiplication during the middle of March to the middle of April. Hayakawa (1990)[2] clarified the annual changes in the amount of nutrients and chlorophyll from 1979 to 1989 in Ofunato Bay, these data will need to be analyzed to yield correlations of nutrients and cells and to clarify what kind of phenomenon acts as a mediator to cause the growth of *A. tamarense*. In the case of *Dinophysis fortii*, the results also indicated that the increase in the amount of precipitation (snow and rain) and air temperature lead to an increase in the annual average density of *D. fortii*. However, correlation coefficients of the equations were very low, and any significant correlations could not be derived. These results are considered to reflect differences in ecology of the two species.

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ON NUMERICAL ANALYSIS OF HARMFUL ALGAL BLOOMS

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ABSTRACT

Numerical analysis has been shown as an important tool in studying the dynamics of harmful algal blooms, exploring the causes, mechanism and prediction of red tides. In this paper, various multivariate statistical methods and biophysical models are reviewed in examining the roles of biological, chemical, physical factors and biological-physical interactions in red tides. These models have achieved some degrees of success in describing the underlying dynamical processes. However, relatively poor knowledge about physiological responses of bloom species to the physical field has greatly limited the predictive ability of existing models. The improvement of existing models will depend on a better understanding of the physical, physiological, and ecological processes and their interactions.

INTRODUCTION

Harmful algal blooms (HAB), also referred to as "red tides" in the older literature, have been observed frequently and globally [1]. The important aspects for HAB research are how to reveal the causes of their outbreaks, analyze their formative mechanisms and describe the dynamics underlying biological and physical processes. Large amounts of *in situ* data have been accumulated over past decades. However, it is not enough to explore individual environmental factors in laboratory or field qualitative analysis of blooms. Numerical analysis has been used more widely in the last twenty years as an important tool in studying dynamic process of HAB. However, the lack of knowledge in identifying the influence of various physical, chemical and biological factors and their complex interactions on the appropriate temporal and spatial scales has prevented the quantitative assessment of the importance of these factors in causing HAB and the development of predictive models [2]. Until now, statistical models have mainly been used to analyze some limiting factors causing the HAB. Better physical and biological models are still being developed.

MULTIVARIATE STATISTICAL ANALYSIS

Since the 1970's, multivariate statistical methods have been applied in HAB studies in order to decrease the dimensionality of problems. Popular multivariate methods are principal component analysis, regression analysis, interpretative structural modeling, cluster analysis, discriminant analysis and time series analysis.

Principal Component Analysis (PCA)

One of the earliest applications was that of Quchi and Takayama [3], who considered six factors. A three-dimensional "red tide map" was plotted in terms of three principal components in *PCA* and used to predict that *G. mikimotoi* bloom would not occur in 1982 [4]. In another effort, Huang *et al.* [5] used fourteen variables to examine relationships between *S. costatum* and environmental factors in Dapeng Bay of the South China Sea. Results showed that the main factors affecting the amount of *S. costatum* were nitrogen, silicate, and water temperature.

Regression Analysis (RA)

Quchi [6] made a multiple linear regression model of *G. mikimotoi* blooms, from August of 1979 and 1983 in the northern part of Hiroshima Bay. A model of multinomial stepwise regression [7] was applied to analyze the relationship between chlorophyll *a* and environmental factors and results indicated that the soluble iron, COD, and salinity were the most important factors influencing variations of chlorophyll *a*.

Interpretative Structural Modeling (ISM)

ISM modelling was applied to HAB analysis in the middle of the 1980s. Based on literature for *Noctiluca scintillans* blooms in the South China Sea, a simple *ISM* model including 22 basic factors was formulated by Wang and Feng [8]. The relationships suggested for this kind of bloom involved six levels. *ISM* is useful as a "conceptual model" for developing dynamic models of HAB. However, it cannot provide quantitative assessment on the factors responsible for the formation of the HAB.

Cluster Analysis (CA)

One example using *CA* is provided by Qi *et al.* [9] where a dynamic fuzzy cluster was used to analyze *Chattonella marina* blooms from 1991 in the South China Sea. Results indicated that iron ions and wind velocity were decisive factors for the initiation of the blooms. Although *CA* can be used to analyze complicated structures, different cluster criteria can bring about quite distinct results that cause some uncertainty in explaining the dendrogram.

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Discriminant Analysis (DA)

It is a useful tool in predicting the possibility for HAB in an area [10]. For instance, Huang *et al.* [5] constructed *DA* models for *S. costatum* blooms, for an initial period, bloom period, and normal period based on data acquired during 1990 spring blooms in the South China Sea. In order to examine the validity of their resultant discriminant functions, comparisons between predictive results and observed data were made for 1991 spring blooms. The corrective rate for the blooms was only 64 percent. Huang *et al.* [5] found that the accuracy of prediction would be improved with the increasing numbers of data used to construct the DA model.

Time Series Analysis (TSA)

The quantity of an HAB organism fluctuates with time and the variation of biomass forms a series of counts in relation to temporal processes. The key of *TSA* is to set up a stable autoregressive model, such as the autoregressive moving average method. Qi *et al.* [11] applied a random *TSA* to analyze the dynamics of a *N. scintillans* population and found a critical density of the population for both surface and bottom water, what if not reached, no bloom would occur. In fact, however, it is technically difficult to monitor the population density of all causative species in real time.

COUPLED DYNAMIC MODELS OF HAB

In recent years, the dynamics of marine ecosystems have been one of the most studied areas by global oceanic researchers. Usually these dynamics include key physical oceanographic forcing functions that play significant roles in both HAB dynamics and the patterns of toxicity or adverse impacts [12]. Furthermore, interplay or coupling between physical and biological behaviors, such as swimming, vertical migration, or physiological adaptation, holds a key for understanding many HAB phenomena [13]. However, the mechanism governing interactions of physical systems with biological components is ignored in traditional statistical models. In order to understand these interactions, a new program, i.e., the ecology and oceanography of harmful algal blooms (ECOHAB) has been put forward [2]. One of its major aims is to identify, measure, and model the underlying biological and physical processes and their interactions.

Since Kierstead and Slobodkin [14] proposed the first model to examine the opposing effects of growth and diffusion in the formation of HAB, many models have been developed to explore the dynamics of bloom species. These models can be divided into various types in accordance with different classification. For instance, according to expiated complexity, there are aggregated biological models [15], multispecies biological models [16], and biological models coupled with simple physics [17] or detailed physics [18].

Modeling HAB ecosystems demands abundant knowledge of chemical and biological interactions operating within the physical environment and must be formulated by a multidisciplinary team. Over the past four decades, studies of HAB in laboratory, field, and theoretical work have greatly contributed to the theory of how biological and physical processes and their interactions affect the dynamics of HAB. The European Regional Seas Ecosystem Model (*ERSEM*), proposed in the early 1990s, is hitherto one of the most successful ecosystem models and has been used in HAB simulation.

The ecosystem described in *ERSEM* is considered to be a series of interacting physical, chemical, and biological processes, which together exhibit a coherent system behavior [12]. Almost all the biota, important elements, and physical processes are included in a bulk model. It is very promising for predicting long-time effects of anthropogenic inputs of nutrients on marine systems, but fails to reproduce a scenario when a bloom is collapsing [18].

A relatively simple model was proposed by Xia *et al.* [19] to describe the dynamics of *N. scintillans* blooms. The model used two-dimensional physical dynamics coupled with the biological dynamics.

$$\frac{\partial D}{\partial t}+u\frac{\partial D}{\partial x}+v\frac{\partial D}{\partial y}=A_h\frac{\partial^2 D}{\partial x^2}+A_h\frac{\partial^2 D}{\partial y^2}+S_D$$

where u and v are the seawater velocities, A_h is the diffusion coefficient, D represents the nutrient concentration E, and the biomass N in seawater, respectively. S_D is a biological term.

$$S_E = H\left[C_1(E_0-E)-a\frac{E}{E_m+E}N\right]E,$$

$$S_N = H\left[-C_2+b\frac{E}{E_m+E}N\right]N$$

where H is the water depth, C_1, C_2, a, and b are the rates of nutrient consumption, algal death, maximum photosynthesis, and nutrient weight conversion, respectively. E_0 is the initial nutrient concentration and E_m is the nutrient half saturation constant.

Although the model is fairly simple, the results show a good agreement between numerical simulation and observation. It can be considered as a basis for developing more complex models.

DISCUSSION

Nearly all dynamic models stem from a similar framework of physical and biological equations. Differences between models are parameterization and initialization of processes.

Variable selection

Numerous studies have shown that every causative alga responsible for HAB grow and reproduce at special optimum conditions. All possible factors affecting the

formation of HAB can be monitored, but not all factors are equally significant. Therefore, it should be possible to obtain an acceptable result by restricting parameters by some amount. Every area has characteristics of temperature, salinity, current, tide, topography, and meteorology. The selection of parameters can be site-specific through the analysis of historical HAB events. For example, in the study of necessary conditions for *Chattonella antiqua* red tide outbreaks, the model was expressed as a set of equations for PO_4^{3-}, NO_3^-, NH_4^+, P cell quota, N cell quota, temperature, and salinity [20].

Data assimilation

Models of HAB are very sensitive to initial and boundary conditions. Slightly different initial conditions can lead to widely different results. The accuracy of results is also affected by inaccurate and inconsistent data from different meteorological, hydrological, chemical, and biological measurements. Moreover, HAB generally lasts for less than a week and presents irregular spatial patterns as a function of turbulence, internal waves, and sharp fronts [21]. Insufficient *in situ* sampling performed temporally and spatially prevents models from providing adequate insights into HAB dynamics.

An assimilation technique has been developed to obviate the effects of inaccurate initial conditions and the inescapable chaotic divergence of model and reality [22]. Some models that incorporate data obtained by remote sensing techniques have been proposed [23]. Data assimilation can greatly improve the agreement between model simulation and observation.

Dimensional selection

Models of HAB can be classified as one, two, and three-dimensional. Most flagellates swim to surface waters during the day and sink at night, so a one-dimensional model often depicts the vertical migration of flagellates. A two-dimensional model usually describes the effects of wind forcing and vertical migration upon the formation of the HAB. A three-dimensional model might include diel vertical migration, spatially variable steady wind, and tides. Three-dimensional simulations have already been proposed [24]. Models become more complex as the model dimension increases. Suitable dimensions should be chosen in accordance with species of algae, the features of current, such as tide, upwelling, and the prevalence of wind.

Model coupling

An ecosystem with HAB can be considered to be a series of interacting complex physical, chemical, and biological processes, which together exhibit a coherently systematic behavior. Thus, a HAB model underlying bloom dynamics usually consists of biological, chemical, and physical submodels. Detailed descriptions of biological submodels can be found for a population growth model of algae [25] and multispecies models [26]. Lee and Arega [27] proposed chemical dynamics of nitrogen, phosphate, and silicate. Hydrodynamic models have been well documented to determine watermass movements such as river inflows [28], wind-forced upwelling [29], and advection [30].

It is difficult to integrate biological submodels with physical submodels due to numerous variables varying in time and space. Comparative studies between model simulations and experiments show that relatively simple reliable modules or compartments may have better results in some cases because errors caused by inserting certain unreliable submodels may spoil the larger model simulations [31]. Nonetheless, fine interdisciplinary models with interaction among different compartments reveal a great deal and make predictions of HAB possible.

CONCLUSIONS

The ultimate goal of HAB models is prediction. However, many parameters and linkages, such as poor knowledge of competition of multispecies and life-historic strategies of causative species, limit predictive ability. Before models of HAB act as a useful tool like weather forecasts, the following efforts will be required in the future:

(1) To improve algal fundamental physiological and ecological knowledge;

(2) To investigate and explore dynamic processes underlying HAB formation both in laboratory and in field, especially biological and physiological responses to physical forcing;

(3) To apply newly developed techniques such as remote sensing and DCMU techniques in HAB observation so as to gain detailed sets of spatial and temporal data; and

(4) To develop better parameterization and data assimilation in order to improve predictive power of models.

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TAXONOMY :
ADVANCED IDENTIFICATION METHODS

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THE USE OF SEDIMENT SLURRY CULTURE TO SEARCH FOR ORGANISMS PRODUCING RESTING STAGES

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ABSTRACT

Sediment slurries were used successfully in experiments aiming to survey the northern Swedish west coast for dinoflagellate cysts as well as in a pilot study to investigate the presence of phytoplankton resting stages in ballast water and sediment.

Untreated aliquots of sediment or concentrated (by filtration) ballast water were cultured using filtered seawater to allow germination of resting stages. The resulting mixed cultures were studied for a period of two weeks and documented directly from the flasks using an inverted microscope. This simple and unselective germination of resting stages proved a very useful complement to a "traditional" dinoflagellate cyst survey or a survey for resting stages in ballast sediment or water. Many organisms appeared in the cultures that were not found as resting stages, probably due to their resting stages being few, small and inconspicuous or of unknown appearance. Cultures of sediments from the Swedish west coast contained copepods, rotifers, ciliates (at least 46 types), dinoflagellates (at least 47 species), diatoms (at least 128 species), cyanobacteria, haptophytes, cryptophytes, euglenophytes, prasinophytes, chlorophytes and amoebae. Cultures of ballast water and sediment contained mainly cosmopolitan diatom species.

INTRODUCTION

Culturing of untreated sediment samples is a simple method, which has been employed in a number of previous studies. In the beginning of the twentieth century Huber and Nipkow [1] studied the limnic dinoflagellate *Ceratium hirundinella* after germinating it from cysts in sediment samples. They also refer to earlier experiments with germination of *Ceratium* from cysts in lake sediments. In 1979 Dale [2] mentioned "raw cultures" as a good method to determine the organisms present in marine sediments. A number of authors have used "most probable number" techniques with sediments to determine the presence of species otherwise undetectable by visual means (Harris *et al.* [3] and references therein). The technique has also been used to determine the viability of resting stages in stored sediment samples (Lewis *et al.* [4]).

The method for incubation and examination of sediment slurries described here was used as a valuable complement to a dinoflagellate cyst survey and a pilot ballast water research project in Sweden.

MATERIALS AND METHODS

Sediment samples

Sampling: Surface sediment samples were collected by scientists from NIVA (Norwegian Institute for Water Research) along the western Swedish coast in September 1995. The top centimetre of sediments was sub-sampled from boxcores and stored in plastic jars in the dark at 4°C until analysis (about four months). Ten of the samples were used to germinate sediment slurries as part of a cyst survey (Persson *et al.* [5]).

Culturing and microscopy: Each sediment sample was thoroughly mixed to a uniform consistency. Dry weight (DW) was determined in triplicate using samples dried to constant weight at 105°C. For culturing 0.7 - 1 g sediment was added to each of five 60-ml Nunclon®-flasks containing 58 ml filtered deep-sea water (Whatman GF/F glass fibre filter, nominal pore size 0.7 µm, adjusted to a salinity of 26 ‰ with distilled water). No extra nutrients were added. The flasks were shaken and cultured (standing in an upright position) at 16°C, 80 $\mu E\ m^{-2}\ s^{-1}$, 12:12 h light:dark. For microscopy the flask was gently positioned (to maintain settled sediment) in the holder of an inverted microscope (Zeiss Axiovert 135, LM and fluorescence microscopy), and examined daily (except weekends) for two weeks. Organisms were video-filmed (Panasonic F15 S-VHS), photographed (Olympus OM4), drawn and thorough notes were made. After two weeks culture samples were preserved with glutaraldehyde to a final concentration of 1%. Dinoflagellate cysts were counted quantitatively from microscope slides. The data from the cyst counts are presented in [5] together with cyst data from ten more sites. For identification of germinated dinoflagellates 3 - 4 g sediment was weighed and added to a 60 ml Nunclon® flask containing filtered deep-sea water. Sonication and sieving was performed as for cyst counting [5], but with filtered deep-sea water instead of distilled water. Incubation was as for untreated sediments until a dense culture of mixed dinoflagellates was present (about 3 days). This culture was used for identification of living dinoflagellates and for photomicrography. A very useful method was to concentrate approximately 15 ml of this mixed culture to approximately 0.5 ml by gentle filtration and then add a drop to a slide coated with an agar solution. In this way the dinoflagellates swam very slowly or not at all and could be viewed and photographed in high resolution. The agar also delayed the destruction of cells at high-resolution microscopy. For calcofluor preparations (slides) about 15 ml of dinoflagellate culture was mixed

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with a few drops of calcofluor [6] and filtered down on a Nuclepore polycarbonate filter (nominal pore size 0.2 µm) on top of a supporting Millipore cellulose acetate filter (pore size 0.8 µm). The polycarbonate filter was laid on a slide coated with immersion free oil and a drop of oil was put on the filter and a coverglass on top. The slide was turned upside down on a Kleenex paper and gently pressed for the oil to spread evenly. The identification of dinoflagellates was obtained from the study of the tabulation and arrangement of the thecal plates. Scanning electron microscopy (SEM) was performed as described in [5]. Diatoms were mounted on microscope slides in Naphrax after cleaning in HNO_3 and distilled water. Species-specific autofluorescence [7] was used to see spiny cysts covered with detritus and cysts and diatoms in copepod pellets.

Ballast water/sediment

Sampling: Three ships in Göteborg were sampled. Water samples were taken with a hand pump or a bucket from a manhole and concentrated by filtration through a 10 µm net. Parts of the samples were pre-served (with glutaraldehyde and Lugol's iodine solution). Additionally sediment samples were taken by hand from the bottom of empty ballast tanks in one of the vessels.

Culturing and microscopy: The samples were examined directly to see which organisms were alive at the time of sampling (this was later also established from the preserved samples). Incubation experiments: 5 ml of the concentrated water sample was added to 60 ml Nunclon®-flasks containing 55 ml filtered deep sea water and enriched with f nutrients [8] at f/10. Aliquots of the sediment samples were cultured in the same way as the concentrated water samples. Additionally, parts of the sediment samples were sonicated for 7 minutes and sieved (fraction 100-25 µm used) to concentrate any occurring dinoflagellate cysts. Parts of these fractions were also incubated. There were at least five replicates from each tank cultured at a final salinity of 26 ‰ and five at 15 ‰ (diluted with Milli Q-water). The flasks were incubated at 16°C and 150 µE $m^{-2}s^{-1}$ in 12:12 h light:dark cycle. The microorganisms were observed directly in the flasks as above for two weeks and then after a month. Living cells were documented on videotapes and photos. Some specimens were also examined by SEM.

RESULTS AND DISCUSSION

Sediment samples

A large number of well known restingstage producing organisms appeared in the cultures as well as organisms for which no resting-stages are known and organisms known or assumed to be able to survive heterotrophically for long times in the sediment (Table 1).

Table 1. Number of species found in ten sediment samples from the Swedish west coast.

Taxon (number of species)	Number of species found in initial samples	Number of species found in slurry cultures
Dinoflagellates:		
Cochlodinium (2)	0	2+
Gymnodinium (4)	1	4+
Gyrodinium (2)	0	2+
Nematodinium (1)	0	1
Polykrikos (2)	2	1
Alexandrium (3+)	3+	3+
Fragilidium (1+)	+	1+
Gonyaulax (5)	5	5
Lingulodinium (1)	1	1
Protoceratium (1)	1	1
Pyrophacus (1)	0	1
Glenodinium (2)	0	2
cf. *Helgolandinium* (1)	0	1
Pentapharsodinium (1)	1	1
Scrippsiella (2+)	1+	2+
Diplopelta (2)	2	1+
Diplopsalis (2)	2	1+
Oblea (1)	0	1
Heterocapsa (1)	1	1
cf. *Chaconina* (1)	0	1
Protoperidinium (14+)	12+	7+
Ciliates (46+)	0	46+
Cyanobacteria (5)	0	5
Flagellated protists except dinophyceae:		
Euglenophyta (3+)	0	3+
Kinetoplastida (3+)	2+	3+
Bicosoecales (2)	+	2
Pedinellales (3)	0	3
Haptophyta (1)	0	1
Cryptophyta (3+)	0	3+
Chlorophyta (1+)	0	1+
Prasinophyceae (5)	0	5
Choanomastigotes (1+)	0	1+
Incertae sedis taxa (5)	0	5
Amoebae (2+)	0	2+
Diatoms:		
Centric (32+)	3+	32+
Pennate (96+)	4+	96+
Multicellular organisms:		
Copepods (1+)	1	1+
Rotifers (2)	0	2
Other (5)	4+	1

+ indicates presence of additional unidentified species

The dinoflagellates were given most attention in order to compare with the cyst survey that was made in combination with the germination experiments [5]. Many planktonic diatoms are known to produce spores whereas the benthic pennate forms are assumed to survive heterotrophically. Ciliates are known to produce cysts, but descriptions of cysts are rare. Some flagellates are known to produce resting cells and others live heterotrophically. As for dinoflagellates some species have well-known cysts whereas others have not. A detailed discussion and the complete species list of organisms will be reported elsewhere.

The large advantage with the use of sediment slurries is the easy handling: just add aliquots of untreated sediment into the desired medium in flasks that fit directly onto an inverted microscope, place in culturing chamber and check regularly. It is inexpensive, does not take much extra time and gives much information. Of course, all resting stages and resting organisms present in the sediment will not germinate, but I found many more than was expected from looking at the sediment initially. If the conditions after germination are good for growth only a few resting stages are needed to make a dense culture in a few weeks. The species composition of the cultures changes over time due to species-specific differences in such characteristics as excystment, growth and survival. Predation and competition are probably also important factors that affect the species composition. To exemplify the advantages of a combination of methods (cyst counting and slurry culture) we can use the dinoflagellates: some species are easy to identify as cysts but not as freeliving, whereas some species have cysts without species-specific characters but an easily identified free-living stage. In that way some will be detected as cysts and some as vegetative stages.

For many of the harmful resting stage producing organisms details are known of suitable media, temperature, light etc. for successful culturing as well as details of the encystment, excystment and the length of resting periods. For these organisms the use of sediment slurries in the future may be a valuable complement to or substitute cyst counting if used in combination with modern recognition methods that are being developed for recognition of toxic species in plankton samples. Thus, besides increasing the detection of organisms the use of slurry cultures can increase the speed and economy in surveys.

Ballast water/sediment

Most of the few species that were alive at the sampling occasion soon disappeared, but after a week there was a dense culture of planktonic diatoms, some ciliates, some small naked dinoflagellates as well as bacteria and small heterotrophic flagellates (Table 2).

Table 2. Number of species found in ballast samples from three vessels.

Taxon (number of species)	Number of species found in initial samples	Number of species found in slurry cultures
Dinoflagellates (3+)	0	3+
Ciliates (4+)	4	3
Bacteria (2+)	2+	2+
Chlorophytes (2)	0	2
Flagellated protists except dinophyceae:		
Euglenophyta (1+)	0	1+
Kinetoplastida (2+)	+	2+
Cryptophyta (1)	1	0
Incertae sedis taxa (2+)	1	2+
Diatoms		
Centric (15+)	7+	12+
Pennate (8+)	6+	3+
Multicellular organisms (3+)	1+	3+

+ indicates presence of additional unidentified species

No dinoflagellate cysts were observed in any of the samples, which was probably due to the cysts of the small naked dinoflagellates appearing in the cultures being small and inconspicuous.

Most species found are common in Swedish waters and have a global distribution. Although few living cells were seen initially, the incubations showed that there were large numbers of living organisms present as resting cells or living heterotrophically in ballast water and sediments. When ballast water and sediment are discharged, these "hidden" resting cells might germinate and grow. We had no reason to believe that these cultured organisms could not have been established in our coastal waters. Thus, new clones of cosmopolitan species most probably have been introduced to Swedish waters by ballast and the risk of introduction of harmful plankton species should not be neglected.

Thus incubation of concentrated samples gave much more information than just examining the samples (fresh or preserved). With sediment slurries it is possible to use water of different possible recipients/ different media/ salinities/ temperatures etc. to evaluate the risks for establishment of unwanted species. The disadvantage with the methods currently used for resting stages (microscopic examination and individual picking with a micromanipulator) is that resting stages of unknown appearance or resting stages that are rare will be missed.

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DINOFLAGELLATE CATEGORISATION BY ARTIFICIAL NEURAL NETWORK (DICANN)

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ABSTRACT

DiCANN is an advanced pattern recognition tool for laboratory use that is being developed to automatically categorise specimens of marine dinoflagellate species involved in HAB episodes. A prototype of the system demonstrated accurate categorisation of 23 species of dinoflagellates from microscope images. The project partners are also developing calibration techniques and standards for this new class of marine observation method. DiCANN uses artificial neural networks, an internet distributed database and advanced image analysis techniques to perform natural object categorisation.

INTRODUCTION

Taxonomic categorisation of marine dinoflagellates is normally a time-consuming task for expert ecologists. Yet both marine ecology and fisheries health and safety monitoring depend on the taxonomic identification of specimens from marine water samples by manual inspection.

Routine analysis of large volumes of seawater is therefore not possible, ensuring that sampling regimes are always sparse and have a high cost associated with them. This coupled with a dwindling interest in marine taxonomy emphasises the need for automatic analysis machines to assist taxonomists.

There is a need for automation instruments, which should provide categorisation of species to family, genera, or species level. They need to operate without supervision, but do not need to have a comprehensive range of categories within which they operate. This is because they are not replacing the taxonomist, merely taking the more tedious taxonomic tasks away from the human.

Automation of categorisation is not a new goal. Historically feature-based methods have prevailed, using digital image processing methods to measure various morphological parameters of specimens [1]. For example Lassus *et al.* [2] successfully used discriminant analysis of morphometric criteria extracted with a digital pattern-recognition system to differentiate species of *Dinophysis* (*Dinophysis fortii* and *D. acuta*).

However, the selection of salient features is species specific and is also a time consuming process to develop. Digital image analysis performance is also reduced by the presence of noise, detritus and clutter, which is typical of pictures taken direct off-microscope.

For an automatic categoriser to become a laboratory instrument it needs to be robust and easy to configure as well as being accurate in its specimen labelling. Recent development of trainable artificial neural network pattern categorisers [3,4] has simplified the development of such a tool for operation with field collected specimens, showing both intra- and inter-specific morphological variance.

This paper outlines the development of a new laboratory based machine designed for this purpose, called DiCANN.

DICANN OVERVIEW

DiCANN applies the coarse coded channel method for image analysis [5] (see Fig. 1). Specimen images are processed at low resolution through several complimentary channels. The resulting 142 bit descriptor is fed into an automatic categoriser for training and testing.

An early prototype has been trained on 100 specimens per species drawn from an image database of over 5,000 field collected Dinoflagellates. Fig. 2 shows the best mean performance on test data drawn from the same database. The DiCANN processing is invariant to specimen rotation and translation in the field of view. It is also partially invariant to scale, allowing up to 10% variation in specimen size. DiCANN recognises 3D objects from different view points through training on a range of views, which are then interpolated through the training process [6]. DiCANN may not succeed at recognising an object from an unusual view angle if it is easily confused with another object. In this manner DiCANN is no different to a human taxonomist.

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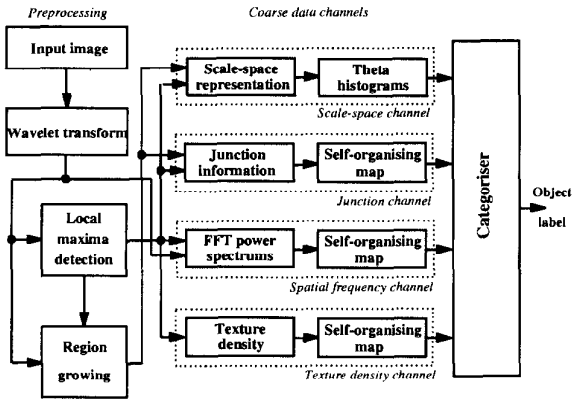

Fig. 1 Diagram of DiCANN system

The DiCANN prototype had four categorisation systems, two artificial neural networks - Radial basis Function (RBF) and Back Propagation of error perceptron neural Network (BPN) and two multidimensional statistical classifiers – Quadratic Discriminant Analysis (QDA) and k-Nearest Neighbour (k-NN). The results of each were compared to assist in future development of the system and to benchmark against existing statistical methods [3]

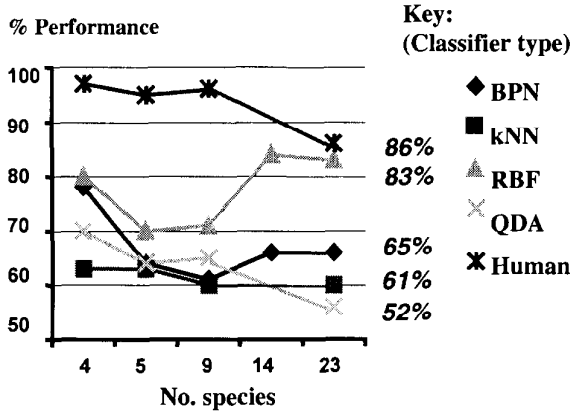

Fig. 2 Best mean accuracy of categoriser Legend: BPN is Multi layer Perception with back propagation of error, k-NN is k-Nearest Neighbour, RBF is Radial Basis Function, QDA is Quadratic Discriminant Analysis, Human comprised a panel of six expert taxonomists

Automatic classifier performances are similar in behaviour over the range of species, with the RBF neural network leading with an accuracy of 83% best performance over 23 species on unseen data. This compares favourably with the expert panel of taxonomists who, over a subset of the same data, returned a best performance of only 86%. This poor human performance reflects the difficulty of the task. The other neural network system (BPN) follows with a 65% accuracy. The multivariate statistical categorisers (QDA and k-NN) lag with 61% and 52% respectively.

Table 1 shows the detailed performance across the four classifiers by species for data bmix2, one of the randomised data sets drawn from the data archive. It may be seen that the performance of all classifiers varies with particular species. It appears that this behaviour is correlated to population size and to population morphological variance, as shown for a sub set of these data in McCall *et al.* [7]. Large intra-species data pools with low morphological variances within the pools give rise to high accuracies of identification.

Species	1	2	3	4	5	6	7	8	9	10	11	12	13
RBF	81	58	73	82	62	94	68	76	75	85	81	79	90
BPN	79	56	64	77	53	81	49	60	75	85	67	55	27
QDA	87	52	59	75	53	57	42	84	89	36	67	30	45
k-NN	72	50	73	68	40	84	45	43	7	75	88	33	50

Spp.	14	15	16	17	18	19	20	21	22	23	mean
RBF	89	86	100	100	100	85	100	68	89	100	**83**
BPN	67	86	67	67	100	63	0	40	82	0	**65**
QDA	79	0	0	100	67	68	100	0	64	0	**61**
k-NN	70	29	67	67	100	45	22	0	67	0	**52**

Table 1. Classification performance (%) for edited 23 species data (rounded to integer) for data set *bmix2*

Key: Species 1:*Dinophysis acuta*, 2:*D. acuminata*, 3:*D. rotundata*, 4:*D. sacculus*, 5:*Ceratium longipes*, 6:*C. arcticum*, 7:*C. horridum*, 8:*C. tripos*, 9:*C. azoricum*, 10:*C. furca*, 11:*C. fusus*, 12:*C. lineatum*, 13:*C. macroceros*, 14:*C. pentagonum*, 15:*Prorocentrum lima*, 16:*P. triestinum*, 17:*P. micans*, 18:*Dinophysis tripos*, 19:*D. caudata*, 20:*D. punctata*, 21:*D. dens*, 22:*D. norvegica*, 23:*Peridinium spp.*

CURRENT SYSTEM

The current system employs the Radial Basis Function categoriser as it consistently proved more accurate at labelling specimens than the other categorisers.

The pilot version of the system was running on a Unix machine, and employed off-the-shelf classifiers for categorisation. The coarse analysis channels were coded in different programming languages. Version 1.13 DiCANN incorporates its own categoriser and has been re-coded as a single C++ package, which can be used on any PC. Therefore it is easier to use in routine analysis at marine laboratories. A graphical interface enables the user to select specimens in the field of view to be used to train or test DiCANN (see Fig. 4). The diagram in Fig. 4 shows the specimen chooser window in the background, with the categoriser results in the foreground window. The categoriser reports *D. fortii* as the label for the specimen shown with a confidence ranking well above chance for four of the available six coarse channels. A

future version of DiCANN will combine these into a single probability score for the species label.

On a Pentium 450 MHz computer experiments show that it only took 40 seconds to segment an object and to add it to the training set. Once the software is trained the categorisation of the specimen only takes 5 seconds. Therefore to construct a new training set with 120 images of 6 specimen, the user will need approximately 3 hours. Once trained the system can process new images every 5 seconds.

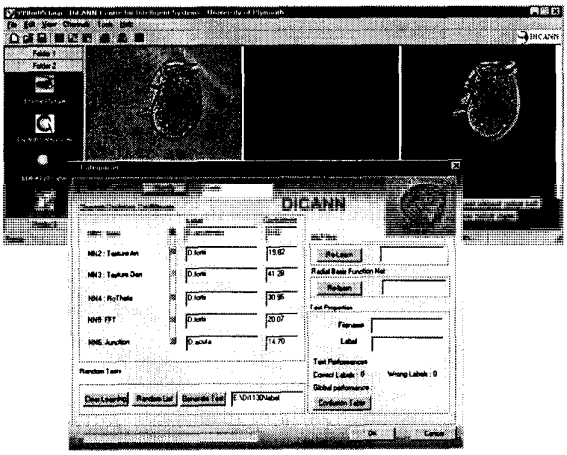

Fig.3 Screen shot of DiCANN v1.13 system

Its performance on unseen data will be monitored. DiCANN is expected to perform as well as the earlier prototype version reported above. Early results suggest that on average DiCANN can achieve about 80% to 90% correct labelling across a selection of HAB species of Dinoflagellate.

SPECIMEN TREATMENT

It has been shown in an earlier study [8] that a minimum of 40 specimens of each species are required as training data. Unused data from the data pool is then used to test categoriser performance on independent material. New material can then be used to extend the performance studies on the trained categoriser.

The assumption is that the archive of field collected specimens is a uniformly distributed sub-set of the natural population. Therefore random selection of data for training and testing is held to be representative of the natural populations and the categorisation results obtained from the automatic classifiers is also representative of their behaviours on fresh specimens collected from the field. The training and test protocols are normally repeated many times to gain a mean performance across many random data pools.

Specimen images are taken using calibrated microscopes and 1:1 aspect ratio video or digital cameras. Images are stored in an Internet database for access by all project partners. DiCANN is being trained on many hundreds of examples. Its performance on unseen specimens will be monitored.

Tests are in progress on the efficacy of DiCANN in assisting the assay of cell cycle morphological changes in *Dinophysis* species and on how effective DiCANN can be as an assistant to HAB monitoring personnel. Some example images being used in the study are shown in Fig.4. The DiCANN system is resilient to detritus in low concentrations, but high densities of detritus can cause the image segmentation algorithms to fail. All the images shown in Fig.4 were correctly processed by DiCANN.

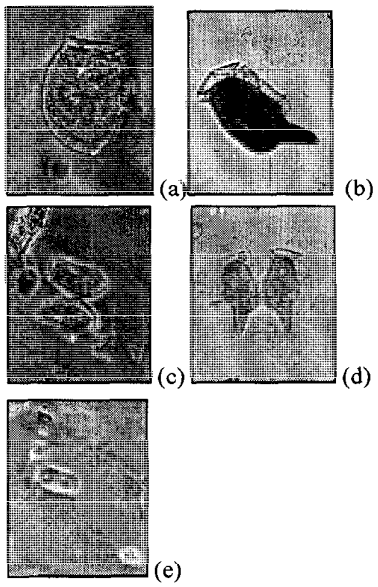

Fig. 4: Example specimen images

(a) *Dinophysis acuminata*, (b) *D. caudata*, (c) *D. fortii*, (d) *D.caudata*, (e) *D. sacculus*[i]

DISTRIBUTED IMAGE DATABASE

A Distributed Image Database (DIB) on the Internet is a key part of DiCANN system (please refer to http://www.dist.unige.it/DICANN). The DIB provides the DiCANN members facilities and a query language on the Internet network to insert, modify and retrieve information about the specimens' images. These facilities can be accessed through a web interface available at the server site. The Internet User can query the DIB and directly retrieve the images and the related information from the web pages generated by the DIB that the server.

This on-line access of images and validation data provides a mechanism for DiCANN to train itself. The computer program can be scheduled to request and process images from the database, overnight and ready the next morning for use on a new labelling task in the laboratory. A future release of DiCANN will possess this facility.

THE PARTNERS

The University of Plymouth is leading the software development work. DiCANN testing is being performed in Centro Oceanográfico de Vigo and Laboratorio di Biologia Marina. Both marine laboratories are collecting, labelling and validating Dinoflagellate specimens for this purpose. DiCANN performances are being assessed on *Dinophysis* cell cycle morphologies, *Alexandrium* plate organisation and on categorisation of multiple specimen images of Dinoflagellates. The Università di Genova has developed the distributed database for the consortium partners.

THE FUTURE

Currently trials are being carried out on recognition of *Alexandrium* species from ultraviolet fluorescence light microscopy images (see Fig. 5 below). Recognition of species of this genus is problematic as surface pore and plate features are often the only discriminant. It is hoped that DiCANN can return performances better than chance, indicating that further work may enable a future version of DiCANN to automate their categorisation, perhaps in combination with fluorescent tagging.

The task of biological material sorting and categorisation is complex and requires expert judgements to be made routinely and repetitively. Fatigue and other well known psychological effects cause people to degrade their performance with time.

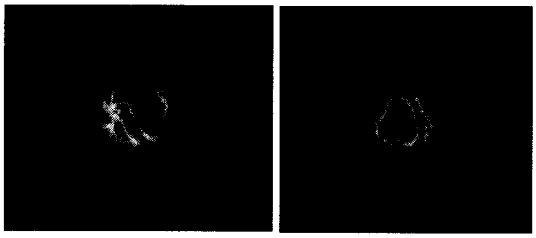

Fig. 5: Example *Alexandrium tamarense* images[ii]

To overcome this degradation the repetitive tasks need to be automated. Progress in automatic categorisation of biological material over the last decade suggests that this is possible and that machines such as DiCANN will be commonplace in the near future. Before they can succeed in helping taxonomists, however, their behavioural characteristics need quality assurance. This can only come about through a wide spread acceptance that automation in specimen labelling is possible. This will allow laboratories to explore the available systems, to understand their shortcomings and to provide feedback to systems developers so that they may be improved. The wholesale rejection of automation is counter productive to ecological science as a whole and should be resisted.

One day tools similar to DiCANN will be a feature of every marine laboratory. They will be categorisation assistants to the expert taxonomists of the future, reducing the burden of routine analysis.

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[i] Sources of the example specimen images shown in Fig. 4. A) - *D. acuminata*: 630x, Ría de Vigo (Galicia, NW Spain), B) - *D. caudata*: 400x, Ría de Vigo (Galicia, NW Spain), C) - *D. fortii*: 320x, Gulf of Trieste (Italy), D) - *D. caudata*: 320x, Gulf of Trieste (Italy), E) - D. sacculus: 320x, Gulf of Trieste (Italy)

[ii] Sources of the example specimen images shown in Fig. 5. *Alexandrium tamarense* (Marina Cabrini, LBM Trieste, IT. from laboratory culture)

HPLC PIGMENT COMPOSITION OF PHYTOPLANKTON POPULATIONS DURING THE DEVELOPMENT OF *PSEUDO-NITZSCHIA* SPP. BLOOMS

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ABSTRACT

Pseudo-nitzschia blooms from the Ría de Pontevedra (NW Spain) were studied by light microscopy and HPLC pigment analysis during years 1998 and 1999. Two main *Pseudo-nitzschia* blooms were registered: the first one in summer (July 1998) had up to 800.000 cells L^{-1} and the second in winter (December 1999) had up to 68.000 cells L^{-1}. During the first bloom amnesic shellfish poisoning (ASP) was not detected and the dominant species was *P. fraudulenta*. During the second bloom ASP toxicity was detected, and the dominant species was *P. australis*. Pigment analyses from both blooms showed Chl c_2 and Chl c_3 as major components of the Chl *c* family, with Chl c_1 a minor component.

Although Chl c_3 is usually associated with members of Prymnesiophyceae, Pelagophyceae and Dinophyceae, it has also been detected in *Pseudo-nitzschia* species as *P. fraudulenta*, *P. delicatissima*, *P. pungens* and *P. pseudodelicatissima*. However, chl c_3 is not present in *P. multiseries* and *P. australis*, both able to synthesise domoic acid, the causative agent of ASP. The parallel increase of Chl c_3 levels and *Pseudo-nitzschia* cell numbers (throughout the development of a quasi mono-specific blooms of *Pseudo-nitzschia* spp) can be used as preliminary information while domoic acid analysis and species identification by EM are performed.

INTRODUCTION

Several species of the genus *Pseudo-nitzschia* such as *P. multiseries* and *P. australis* have been associated with ASP toxicity [1, 2]. In Galician coastal waters populations of *Pseudo-nitzschia* spp. have been detected since 1994 as the causative agent of ASP toxic events, affecting many shellfish areas in the Galician Rías [3]. Due to the economic importance of aquaculture, a monitoring programme of HAB species was set up in Galician waters.

Secure taxonomic identification of *Pseudo-nitzschia* species requires TEM, a time consuming technique. The chemotaxonomic approach using HPLC analysis of taxon-specific pigments allows to interpret composition of phytoplankton populations, but several important markers are shared by different algal classes.

In spite of it, traditional HPLC methods have ignored the value of Chl *c* pigments as taxonomic markers, focussing mainly to carotenoids.

In a previous work studying Chl *c* distribution in 30 strains of 7 *Pseudo-nitzschia* species [4] we found three pigment types: type I, Chl c_1 and Chl c_2 (*P. multiseries*, *P. australis*), type II, Chl c_1, Chl c_2 and Chl c_3 (*P. delicatissima*, *P. pseudodelicatissima*, *P. pungens*, *P. fraudulenta*), type III, Chl c_2 and Chl c_3 (*P. cuspidata*).

Therefore, *P. australis* and *P. multiseries* most relevant species associated with ASP toxicity constituted the single Chl c_3-lacking type I. We used this information to study Chl *c* patterns during *Pseudo-nitzschia* blooms from the Ría de Pontevedra and Chl c_3 as a marker pigment to differentiate between potentially toxic and non-toxic *Pseudo-nitzschia* blooms.

METHODS

Seawater samples were collected weekly from a station in the Ría de Pontevedra throughout 1998 and 1999. Sampling was based on depth integrated samples from 0-15m in order to obtain representative integrated profiles.

Pigments were extracted from 1.5 L seawater, concentrated and size-fractionated by sequential filtration through a 47 mm diameter Whatman GF/D filter (nominal pore size 2.7 μm) and a Whatman GF/F filter (nominal pore size 0.7 μm). Pigments were extracted with 95% methanol, filtered and immediately injected into a Waters Alliance HPLC equipment, including a Waters 2690 separation module and a Waters 996 diode-array detector, interfaced with a Waters 474 scanning fluorescence detector by means of a Sat/In analog interface.

HPLC pigment separation was performed using a monomeric C_8 column (Symmetry) and pyridine containing mobile-phase [5]. Chlorophylls and carotenoids were detected by diode-array spectroscopy (350-750 nm). Chlorophylls were also detected by fluorescence (Ex: 440 nm, Em: 650 nm).

Aliquots of each integrated water sample (0-15m) were preserved with Lugol's solution, phytoplankton were allowed to settle for at least 12 h followed by observations with a Nikon Diaphot TMD inverted

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Intergovernmental Oceanographic Commission of UNESCO 2001

microscope. The chamber was examined at 100x to enumerate and identify larger and less frequent microplankters, then 200x and 400x were used for identifying and counting smaller organisms. The identification of *Pseudo-nitzschia* species from net samples was made by light microscopy on cleaned samples following the method outlined in [6].

RESULTS

A comparison of *Pseudo-nitzschia* cell numbers and total diatoms in the sampled station over 20 months (1998-99) are shown in Figs. 1A and B, respectively.

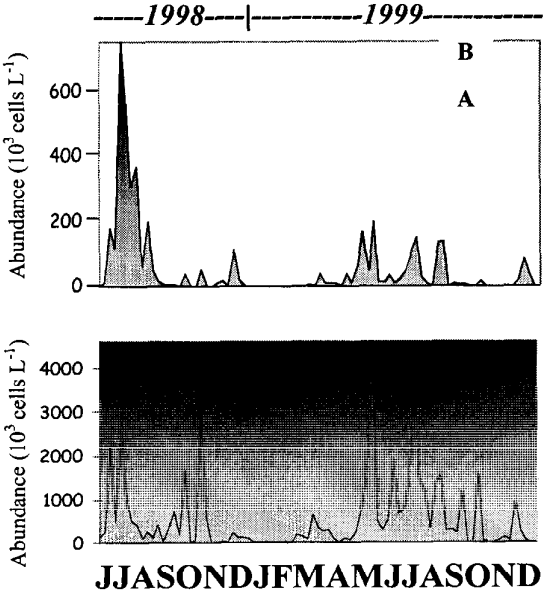

Fig. 1. Abundance (10^3 cells L^{-1}) of A) *Pseudo-nitzschia* spp. and B) total diatoms sampled over 20 months (1998-99) at station P2.

During June-July 1998, a bloom of *Pseudo-nitzschia* spp. was observed mainly dominated by the non-toxic *P. fraudulenta* (confirmed by TEM). Up to 800.000 cells mL^{-1} were present which was around 90% of the total diatoms.

During December 1999, a toxic *Pseudo-nitzschia australis* bloom was detected (68.000 cells mL^{-1}) which was only 30% of the total diatom abundance. HPLC pigment chromatograms corresponding to these two *Pseudo-nitzschia* blooms are shown in Figs. 2A and B.

During the summer 1998 bloom (Fig 2A) dominant accesory chlorophylls were chl c_2 (0.657 µg L^{-1}, 69 % of the total chl *c*) and chl c_3 (0.188 µg L^{-1}, 20 %), with lower levels of chl c_1 (0.110 µg L^{-1}, 11 %). A chl *c*-like compound eluted close to the chl c_3 peak and was identified as chl *c*-like pigment detected previously in *Pseudo-nitzschia* species [4].

Fucoxanthin (Fuco) constituted the major carotenoid (1.24 µg L^{-1}) and very low concentrations of fucoxanthin acyloxy derivatives were detected showing minor contributions by groups other than diatoms. The summer bloom of *Pseudo-nitzschia* was dominated by *P. fraudulenta*, confirmed by light microscopy and TEM.

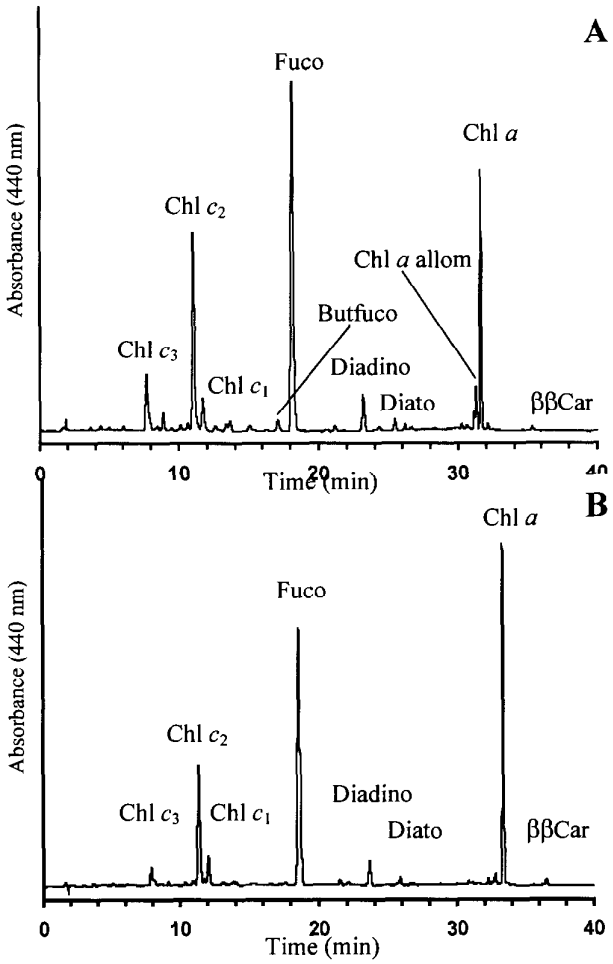

Fig. 2. HPLC Chromatograms obtained from phytoplankton samples during A) *Pseudo-nitzschia fraudulenta* bloom in July 1998 and B) *Pseudo-nitzschia australis* bloom in December 1999.

The winter 1999 bloom (Fig. 2B) was similar in its pigment composition showing dominance of chl c_2 (72% of total chl *c*) with lower contributions of chl c_3 (10%) and chl c_1 (18%). The expected pigment composition from *P. australis* was not reflected in the field sample due to the larger abundance of other diatoms such as *Chaetoceros socialis* and *Chaetoceros didymus*. Pigment analysis of cultures obtained from *Pseudo-nitzschia* isolated from this bloom (Fig. 3) revealed a chl *c* pattern

corresponding to that previously described for *P. multiseries* and *P. australis* [4] (chl c_3 absent).

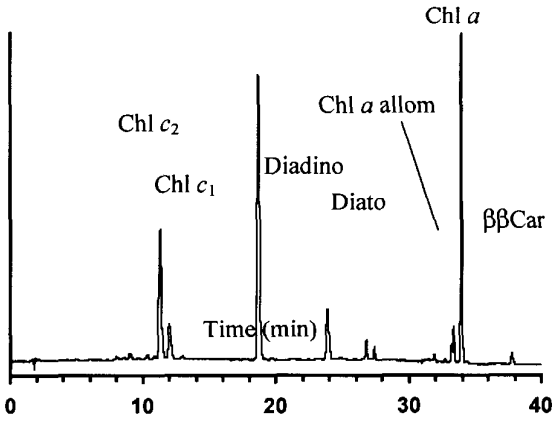

Fig. 3. Chromatogram obtained from a culture of *Pseudo-nitzschia australis* isolated in December 1999. Note the absence of chl c_3.

DISCUSSION

The most common diatoms found in samples from the Galician Rías include different species of *Chaetoceros*, *Skeletonema costatum*, *Leptocylindrus danicus*, etc. They have the classical pattern of diatom pigments of Chl c_1, c_2 and Fuco as dominant components [7,8].

However, Chl c_2 and Fuco are also present in other algal classes present in field samples. Examples of these are Cryptophyceae, which posses Chl c_2 but can be easily identified by the carotenoid alloxanthin, some members of the class Prymnesiophyceae (Chl c_3 and 19'-Hexanoyloxyfucoxanthin), Pelagophyceae (Chl c_3 and 19'-Butanoyloxyfucoxanthin), etc.

As we described before, *Pseudo-nitzschia* species have shown three pigment types as based on Chl *c* pigments [4]. Chl c_3 and a Chl *c*-like compound eluting close to this chlorophyll have been detected (in addition to the normal pigments found in diatoms) in non-toxic species, as *P. fraudulenta* and *P. delicatissima*, most commonly found in samples from the Rías. By other hand, the toxic species causing ASP events in our coast, *P. australis*, is interestingly lacking Chl c_3. In that sense, detection of Chl c_3 and Fuco during bloom episodes of *Pseudo-nitzschia* without significant levels of the fucoxanthin derivatives, can suggest that toxic *Pseudo-nitzschia* is absent while confirmation is obtained by domoic acid analysis and species identification by TEM are performed.

CONCLUSIONS

Absence or low levels of Chl c_3 together with quasi-monospecific *Pseudo-nitzschia* spp. blooms indicates that dominant species are either *P. multiseries* or *P. australis*.

Thus, HPLC analysis of Chl *c* pigments in samples dominated by *Pseudo-nitzschia* spp. can provide preliminary and fast information in harmful algae monitoring programmes about *Pseudo-nitzschia* blooms due to *P. multiseries* and/or *P. australis* while domoic acid analysis and TEM techniques are performed.

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IN SITU PROFILES OF PHYTOPLANKTON: ALGAL COMPOSITION AND BIOMASS DETERMINED FLUOROMETRICALLY

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ABSTRACT

A new methodology enabling rapid evaluation of the phytoplankton community structure and distribution with high spatial and temporal resolution in situ is introduced. This is based on the concept that four 'spectral groups' of phytoplankton (green, blue, brown, mixed) are each characterised by specific photosynthetic pigment compositions and, consequently, by specific excitation spectra of chlorophyll-fluorescence. We have developed a submersible fluorometer which measures the emission intensity for excitation in five characteristic wavelength ranges employing pulsed light-emitting diodes (LED's). These five spectral excitation ranges can be used to differentiate the four spectral groups of microalgae in situ within a few seconds. In examples of depth profiles of phytoplankton populations in a eutrophic lake we also show a significant correlation of deconvoluted fluorescence profiles to corresponding biovolumes.

INTRODUCTION

In the last two decades toxic phytoplankton blooms have increased in coastal areas [1,2,3]. Commonly human activity and raise in human population is given as main reason for this. With growing problems for health and costs caused by Harmful algal blooms (HAB's) there is a need for a better understanding of the origins and evolution of HAB's. This requires methods for an quantitative and qualitative analyses of phytoplankton concentrations in natural waters.

As far back as 1890, Haeckel [4], already considered phytoplankton counting a task which could not be accomplished without 'ruin of mind and body'. The determination of algal biomass has always been problematic and laborious. The challenge associated with recording phytoplankton community structure is, not least, due to a lack of methods. Current methods, although quite accurate, involve rather time consuming enumeration to species or major taxonomic groups using counting chamber methods [5,6,7]. However, the greatest problem is that these methods are usually retrospective and not suited to instant assays in situ. Often, aspects such as patchiness, vertical algal migration and nanoplankton, are not detected because of enumeration problems [8, 9].

Another important method for the determination of phytoplankton abundance is the chlorophyll measurement by High Pressure Liquid Chromatography HPLC. Via marker carotinoids this method enables the determination of taxons [10,11,12, 13]. This method is also retrospective.

Here we present examples selected from numerous others derived from different waterbodies, including seawater, of the vertical distribution of algae in a eutrophic lake as determined with a new submersible instrument in comparison with standard microscopical biomass determinations and prove the feasibility of monitoring algal assemblage composition and biovolume in situ using a sensor. Five spectral excitation ranges are used to differentiate groups of microalgae in situ within a few seconds. We show that this is also significantly correlated with algal biovolume.

Fluorescence emission measured around 685 nm is widely accepted as a measure of chlorophyll contents of algae in aquatic systems. Depth profiling of chlorophyll fluorescence has been carried out since the early seventies [14]. Since then attempts have been made to distinguish different groups of phytoplankton using their fluorescence properties in vivo [15,16,17]. Kolbowski and Schreiber [18] used light-emitting diodes and were able to distinguish fluorescence kinetics caused by different groups of algae. Gilbert et al. modelled the oxygen evolution from in vivo fluorescence from different algal taxa [19]. Cowles et al. [20] show vertical profiles of phycoerythrin containing algae and Desiderio et al. [21] of fluorescence emission spectra without relation to the biomass or diverse algal groups.

The concept is based on the fact that fluorescence is emitted mainly by the chlorophyll *a* of the Photosystem II (PS II) antenna system, which consists of the evolutionarily conserved chlorophyll *a* core antenna and species-dependent peripheral antennae.

The chlorophyll fluorescence intensity $F(\lambda_{ML})$ can be described by

$$F(\lambda_{ML}) = N_{PSII}\, c_{instr}\, I_{ML}\, \{A_{peri}(\lambda_{ML}) + A_{core}(\lambda_{ML})\}\, \Phi^{F} \quad (1)$$

where: N_{PSII} is the number of PS II in the sample volume; c_{instr} is a fluorometer dependent constant; I_{ML} is the intensity of the measuring light, λ_{ML} is the

wavelength of the measuring light and Φ^F is the fluorescence yield, A_{peri}: absorbance of the peripheral antenna, A_{core}: absorbance of the core antenna.

This association (1) results in spectral differences in the fluorescence excitation spectra. We distinguish four spectral algal groups with different spectral signatures, 1) green: chlorophyll *a/b*, 2) blue: phycobilisomes rich in phycocyanin, 3) brown: chlorophyll *a/c* and green light absorbing xanthophylls and 4) mixed: chlorophyll *a/c* and phycoerythrin.

METHODS

We measured fluorescence excitation spectra for several species of the four spectral groups using five distinct excitation wavelengths [22]. The mean excitation probabilities per chlorophyll *a* concentration (in calibrated instrument dependent units) were calculated. These were used as 'norm-spectra' in a deconvolution approach. Thus, it was possible to quantitatively determine the algal population distribution (i.e., given in terms of chlorophyll *a* units per spectral algal group) within the sample volume of a submersible instrument.

Optics and electronics

The optics and electronics [22] are mounted in a stainless-steel housing (l = 45 cm, ∅ = 14 cm) with robust windows suitable for water-depths up to 100 m. Algal chlorophyll *a* and accessory pigments are excited with light from five LED's (emission wavelength of the LED's: 450 nm, 525 nm, 570 nm, 590 nm and 610 nm). The LED's are switched alternately (with 5 kHz) by a microcontroller. Data might be stored in the probe or transferred via RS 485 intersection to a PC. The PS II-fluorescence emission peak (685 nm) is detected rectangularly using an optimised optical band-pass-filter combination and a red sensitive miniature photomultiplier. A cover prevents the incidence of direct sunlight on the detector, which might cause perturbation of the measurement. High sensitivity and dynamic range enables measurement of fluorescence excitation spectra at extremely low chlorophyll concentrations. During measurement the probe is in water. The spectra are recorded automatically with an integration-time of a second. Measurement of the pressure enables the calculation of the submersion-depths. An iterative gaussian fit weighted with the standard deviations of the norm spectra facilitates the estimation of the distribution of the spectral groups.

Normspectra

Normspectra (see Table 1) were obtained from pure cultures: Green spectral group: *Chlorella vulgaris*, Blue spectral group: *Microcystis aerigunosa*, Brown spectral group: *Cyclotella sp.*, Mixed spectral group: *Cryptomonas sp.*

Growth of cultures and sampling

Cultures were grown for 14 days in white light (light intensity 20 $\mu E\ m^{-2}s^{-1}$) in WC medium [23] at a temperature of 20 °C. Samples were diluted with WC medium for fluorescence measurement within the linear range of the fluorometer. Spectra of pure algal cultures in the exponential growth phase were normalized to the chlorophyll content obtained from an HPLC analyses (see below).

Spectral algal group	Excitation wavelength				
	450 nm	525 nm	570 nm	590 nm	610 nm
Green	1.16	0.38	0.20	0.29	0.40
Blue	0.32	0.28	0.76	1.33	1.93
Brown	1.22	0.73	0.28	0.26	0.30
Mixed	1.15	0.73	1.04	0.95	0.76

Table 1: Normspectra of spectral groups of algae according to the species (Green spectral group: *Chlorella vulgaris*, Blue spectral group: *Microcystis aerigunosa*, Brown spectral group: *Cyclotella* sp., Mixed spectral group: *Cryptomonas* sp. These normspectra were used in the fitting procedure. Values are given in relative fluorescence intensity per $\mu g\ L^{-1}$ Chlorophyll-a

Determination of chlorophyll concentrations

Concentrations of chlorophyll were determined according to [24] using HPLC. The liquid chromatographic method used was a shortened version of the Mantoura & Llewellyn [25] method. It was a binary gradient method where solvent as 'A' consisted of a 80:10:10 methanol:water:ammonium acetate solution and solvent 'B' was a 90:10 methanol:acetone mixture. A third solvent 'C' was a methanol: propanol mix in a volume ratio of 10:7.7. This allowed a better separation of the carotenes. All solvents were degassed nanograde HPLC solvents. The flow rate used was 1 mL.min-1, the column was a reversed phase 5C18, 25 cm long column packed with Nucleosil.

Determination of Biovolume

Biovolume concentrations were calculated from phytoplankton cell counts of water samples taken at the same time in ten 1 m depth intervals. Samples were taken with a Ruttner water sampler and preserved with Lugol's solution. Total cell numbers were counted in 1 ml sedimentation chambers and average specific biovolumes were calculated with a geometrical formula using linear measurements of dimensions of 50 cells of each species.

RESULTS

Two depth profiles of a five point fluorescence excitation spectrum were recorded with the submersible probe in a eutropic lake, the Plußsee in Northern Germany, in August 1998 in a two hour interval. One example of the deconvoluted depth profile is presented in Fig. 1A.

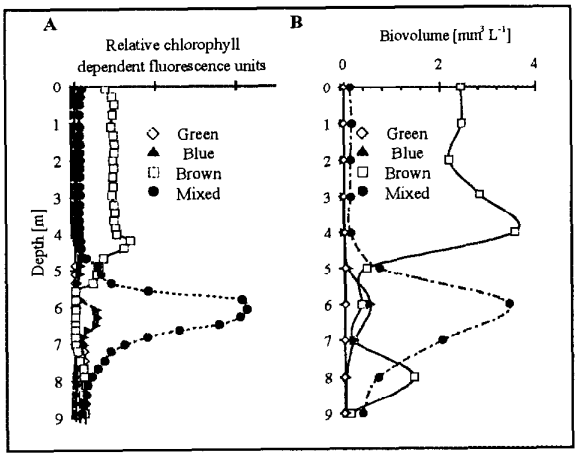

Fig. 1. (A) Depth-profiles recorded with the submersible probe and deconvoluted to chlorophyll-dependent fluorescence units in August 1998, 8:00 *am* at the eutrophic, stratified lake Plußsee of four spectral algal groups (green, blue, brown and mixed) and (B) the corresponding calculated phytoplankton biovolume concentrations for species of the four spectral groups: green (*Closterium acutum*), blue (*Limnothrix rosea*), brown (*Ceratium furcoides, C. hirundinella, Mallomonas caudata*), mixed (Cryptophyta).

This profile is representative of the typical summer phytoplankton community in this lake. Both fluorescence profiles were compared to the corresponding biovolume concentrations (Fig. 1B).

The algal taxonomic groups found by microcopic examination of different depth-samples were cyanobacteria, dinoflagellates, chrysophytes, cryptophytes and chlorophytes. The measured fluorescence intensity for each spectral group was significantly correlated to the biovolume of associated algae found at different depths for these groups. In August (Fig. 1) the blue spectral group (y) was represented by cyanobacteria (x) where $r = 0.96$, $y = 5.57x$. The brown spectral group was due to dinoflagellates (y) and chrysophytes (x) $r = 0.94$, $y = 3.42$ x. The mixed spectral group (y) was attributed to cryptophytes (x), $r = 0.94$, $y = 11.5$ x. The green spectral group (chlorophytes) was in the range of the limit of detection (below 0.077 mm^3 $liter^{-1}$ biovolume in this case) for both methods.

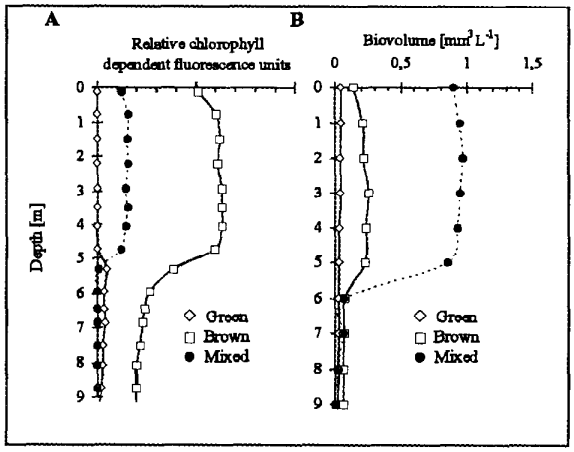

Fig. 2. (A) Depth-profiles recorded with the submersible probe and deconvoluted to chlorophyll-dependent fluorescence units in March 1999, 10:00 *am* at the eutrophic lake Plußsee of four spectral algal groups (green, blue, brown and mixed) and (B) the corresponding calculated phytoplankton biovolume concentrations for species of three spectral groups: green (*Closterium acutum*), brown (*Asterionella formosa, Mallomonas akrokomos, Stephanodiscus hantzschii, Stephanodiscus parvus*), mixed (Cryptophyta).

Another profile (Fig. 2A) was recorded at lake Plußsee in March 1999 with the submersible instrument. The phytoplankton community consisted of species typical for spring in this lake. The profile was again compared to biovolume concentrations (Fig. 2B) calculated from phytoplankton cell counts of water samples taken simultaneously in ten 1 m depth intervals. The algal groups found at the different depths in this profile were diatoms, chrysophytes, cryptophytes and chlorophytes. The fluorescence intensities found for each spectral group were also significantly correlated to the biovolume of associated algae found at different depths for these groups.

The ratio of fluorescence intensity to biovolume in spring (Fig. 2) was different from the summer results (Fig. 1). The brown spectral group (y) was correlated to the biovolume of diatoms and chrysophytes (x) as: $r = 0.97$, $y = 1.48$ x. The mixed spectral group (y) was attributed to cryptophytes (x), $r = 0.94$, $y = 27.25$ x. The green spectral group (chlorophytes) were in the range of the limit of detection for both methods (below 0.045 mm^3 $liter^{-1}$ biovolume in this case).

DISCUSSION

The successful correlation of biovolume determination and the in situ data from the submersible probe clearly demonstrates that correct and rapid

assessment of the phytoplankton community structure in situ with unsurpassed spatial and temporal resolution now is really possible. However, as is to be expected, the ratio of fluorescence to biovolume changes over time and with different algal species, the correlation needs to be calibrated for the specific algal association being examined.

The short measurement duration for a depth profile (50 m-profile in two minutes) is an enormous step forward and generally should suffice in most aquatic investigations. The accuracy of the algal group differentiation is probably limited by the species dependent variability within an individual algal group and by the influence of environmental factors on the fluorescence yield. This would be of considerable use in many oceanographic and limnological applications. Our new method represents an new approach to qualitative and quantitative assessment of phytoplankton, in situ, with high spatial and temporal resolution, enabling a rapid evaluation of the community structure and its distribution. We, therefore, believe that the submersible probe will become an important new tool in aquatic ecology, for monitoring harmful algal blooms and for supervision of aquatic resources. Further developments and measurement refinements will permit a more detailed classification of algal groups in future.

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APPLICATION AND FLOW CYTOMETRIC DETECTION OF ANTIBODY AND rRNA PROBES TO *GYMNODINIUM MIKIMOTOI* (DINOPHYCEAE) AND *PSEUDO-NITZSCHIA MULTISERIES* (BACILLARIOPHYCEAE)

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ABSTRACT

Cells of *Gymnodinium mikimotoi* and *Pseudo-nitzschia multiseries*, harvested during exponential and stationary growth, were probed with species-specific monoclonal antibodies (MAb's) against cell surface antigens, and universal or *P. multiseries*-specific rRNA-targeted DNA probes, to measure the variability of label intensities with flow cytometry. *G. mikimotoi* cells labelled with either the direct MAb or the rRNA probe showed no significant changes in FITC intensity under nutrient stress. Likewise, the APC intensity of indirectly MAb-probed *P. multiseries* cells was stable. However, the FITC intensity of rRNA labelled *P. multiseries* declined significantly when cells were growth-limited, which has consequences for the applicability of flow cytometric counting of *P. multiseries* in field samples.

INTRODUCTION

The study of harmful algal bloom dynamics would benefit from rapid species-specific identification and enumeration techniques. For identification purposes, antibodies against cell surface antigens and DNA probes against intracellular ribosomal RNA, both coupled to fluorescent reporter molecules, have shown great promise [1, 2, 3]. Cell identification in combination with flow cytometry not only enables rapid enumeration, but also the possibility of simultaneous measurements of cell physiology [4]. However, in contrast to microscopy, flow cytometry yields limited morphological information. Therefore, a prerequisite for species-specific flow cytometric counting is that probed cells have acquired an increase in fluorescence, significantly above the autofluorescence of non-probed cells of (near)-equal morphology. Secondly, the probe fluorescence should be independent of cell physiology. In *Alexandrium fundyense* and *G. mikimotoi*, the antibody label intensity is hardly affected by the physiological state of the cells [3, 5]. On the other hand, rRNA-probed *A. fundyense* cells show a reduction of intensity when their growth is nutrient-limited [3].

To study the effect of nutrient limitation on the label intensity of *G. mikimotoi* and *P. multiseries* both exponentially growing and growth-limited cells were probed with monoclonal antibodies and rRNA probes. Label intensities were measured using flow cytometry.

METHODS

cultures and sample fixation

G. mikimotoi strain Ts175Ga1 and *P. multiseries* strain CNL-1 (courtesy dr. S.S. Bates), were grown in PEP-Si culture medium [6] made from North Sea water (33.5 psu). All nutrients were added 5 times the normal strength, with the exception of nitrate, because N was to become the limiting nutrient. The final N-concentration was 112 µM (N:P = 3.5). Si was added to the *P. multiseries* culture only (N:Si = 0.5). N-limitation was checked by (i) analysing N and P concentrations, and (ii) spiking 50 mL culture in stationary growth with 100 µM nitrate and measuring cell growth response. Nutrient-replete control cultures of both species were diluted regularly with normal strength medium. All cultures were grown at 19.5 W m^{-2} in a 12:12 L:D cycle at 15°C.

Samples for measuring cell concentrations were fixed to 0.1% w/v formaldehyde (FA) and counted in a Sedgewick-Rafter chamber. Cells for monoclonal antibody labelling were fixed to 1% w/v FA and stored in a refrigerator (4°C). Samples for rRNA-probe labelling (50 mL) were centrifuged 15 minutes at 1000 rpm (140 g_{av}). After 40 mL of supernatant was discarded, the cells were resuspended, fixed with 40 mL of a saline ethanol fixative (22 mL 96% ethanol + 5 mL dH_2O + 3 mL 25xSET, modified from ref. 2) and stored refrigerated. Storage times of up to 8 months in this EtOH fixative did not significantly affect rRNA label intensity of either species.

monoclonal antibody (MAb) labelling

Cells of both species were concentrated by centrifugation. All centrifugation steps were performed at 15°C, 140 g_{av}, for 15 minutes. The pelleted cells were resuspended and blocked in 1 mL PBST/BSA for 15 minutes [7].

G. mikimotoi cells were labelled directly with 10 µL FITC-conjugated monoclonal antibody α-GA_8 [8] (dilution 1:100). *P. multiseries* cells were first labelled with 40 µL monoclonal antibody Ppm6 (dilution = 1:25) [7], then with goat-anti-mouse IgM-biotin (Sigma, secondary antibody, dilution = 1:250), and finally with streptavidin-APC (Molecular Probes, dilution = 1:1000). All dilutions were made in PBST/BSA. During the first label step, *P. multiseries* red (FRR) autofluorescence was bleached with 200 W m^{-2} white

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light from two Schott KL1500 lamps, each equipped with two Philips-6423 bulbs. After each incubation step (45 minutes at room temperature) the cells were washed with 8 mL PBST, centrifuged, and resuspended in 1 mL PBST [7]. In the negative controls the primary antibody was omitted. Separate tests with both *G. mikimotoi* and *P. multiseries* showed that FBG and FRR intensities were independent of cell concentration (results not shown). Labelled cells were stored refrigerated until analyses; storage for up to 1 week did not significantly affect the label intensities of MAb-labelled cells of either species.

rRNA probe labelling

Cells of both species were centrifuged 15 minutes at g_{av} = 140 at room temperature and the pellet was resuspended in 2.5 mL hybridisation buffer, centrifuged again and resuspended in 390 µL hybridisation buffer. Then 10 µL probe (= 2 µg) was added, and the samples were incubated at 45°C for 2 hours. *G. mikimotoi* was labelled with a universal positive (uni+). *P. multiseries* was labelled with the uni(+) and a species-specific probe (muD1). Negative controls were a universal negative (uni-) probe and, a tube with none of the probes added. All probes were coupled to fluorescein. Probe sequences and buffer compositions were as described previously [9]. The label reaction was stopped by adding 1 ml 5xSET buffer and centrifugation. Excess probe was removed by adding 1 mL pre-warmed (45°C) 5xSET to the pellet, 2 minute incubation (45°C), and centrifugation. The supernatant was removed and the pellet was resuspended in 2 mL 5xSET.

For both species, there was no effect of the cell concentration on FBG intensity (results not shown). Probed cells were stored refrigerated until analyses. Storage for up to 3 weeks did not significantly affect the label intensity of rRNA-probed cells of either species.

microscopy

Microscopic evaluation of fluorescence intensity of labelled cells was as described previously [7], except that an Olympus AX70 microscope was used. Filter settings: FITC: λ_{ex} 460-490 nm, λ_{em} 515-550 nm; APC: λ_{ex} 595-645 nm, λ_{em} >670 nm.

flow cytometry

Measurements were performed with the EurOPA flow cytometer [4]. Fluorescein label intensity was measured as FBG (λ_{ex} 488 nm, λ_{em} 500-550 nm). APC label intensity was measured as FRR (λ_{ex} 633, λ_{em} >670 nm). The triggering variable was Perpendicular Light Scatter (PLS: *G. mikimotoi*) or Forward LS (FLS: *P. multiseries*). See ref. 4 for abbreviations. All samples were stored refrigerated until analysis, which usually took place within 24 hours.

To compare labelled and unlabelled cells, histograms were made of raw flow cytometer data. Further data analysis was performed in DATADESK (Odesta, USA); cells were selected in plots using autofluorescent chlorophyll (FGR or FRR) and size (PLS or TOF), with the exception of MAb-labelled *P. multiseries*, which were selected in FGR histograms or FGR*FRR plots (APC-labelled cells only). The means and standard deviations of 1000-5000 cells (depending on sample concentration) were calculated. 2 µm FITC-coated carboxy-YG spheres (Polysciences, Warrington USA) were used for optics alignment and FBG calibration. FRR was calibrated with chlorophyll beads (FCSC, San Juan USA).

Ratio's of rRNA to MAb labelling of *G. mikimotoi* were calculated from Datadesk mean values. *P. multiseries* values were corrected for day-to-day instrument variability with calibration sphere data, then the mean values of the negative controls were subtracted.

The effect of the experimental differences on label intensity was calculated with ANOVA's. 95% confidence intervals of mean fluorescence intensities were calculated as: c.i. = $t_{0.95}(df)$. stdev / $\sqrt{(n)}$, in which n = number of measurements, stdev = the standard deviation from the mean and $t_{0.95}$ = the value of t from a t-distribution at degrees of freedom (df) = n - 1 and α = 0.95.

RESULTS AND DISCUSSION

G. mikimotoi and *P. multiseries* grew exponentially until the 45th and 15th day respectively (Figs. 1a, 3a). *P. multiseries* had a higher growth rate than *G. mikimotoi*, and after day 35, the culture entered the death phase (Fig. 3a). The *G. mikimotoi* culture did not enter death phase (Figure 1a).

A subsample of the *G. mikimotoi* culture from day 36 was spiked with nitrate. In the following days this subsample reached an 80% increase in cell concentration relative to the experimental culture, evidence that this culture was N-limited in stationary phase. Nitrate spiking of a *P. multiseries* subsample from day 24 (when DIN was still 46 µM), did not lead to enhanced growth. This means that another nutrient was growth limiting, or that the cells were not viable anymore.

G. mikimotoi

The direct labelling of *G. mikimotoi* cells with the FITC-conjugated MAb resulted in an even and bright green fluorescence, which made the cells easily distinguishable from unlabelled cells, both microscopically (not shown) and flow cytometrically (Figure 2a), as shown previously [5, 8].

Labelling of *G. mikimotoi* cells with the uni(+) rRNA probe led to bright green speckled labelling inside the cells (not shown). These rRNA-probed cells were smaller than the monoclonal labelled cells, due to cell shrinkage in the saline ethanol fixative relative to cells fixed in 1% FA. Uni(+) labelled *G. mikimotoi* cells had a three times higher FBG intensity than cells labelled with the uni(-) probe although there was considerable overlap in the intensity histograms (Fig. 2b). Despite this overlap, rRNA probed *G. mikimotoi* cells could be separated in Data Desk dot plots. The uni(-) control (mean FBG 1746 ± 45) did not have a significantly different FBG (1758 ± 49) than the no probe control.

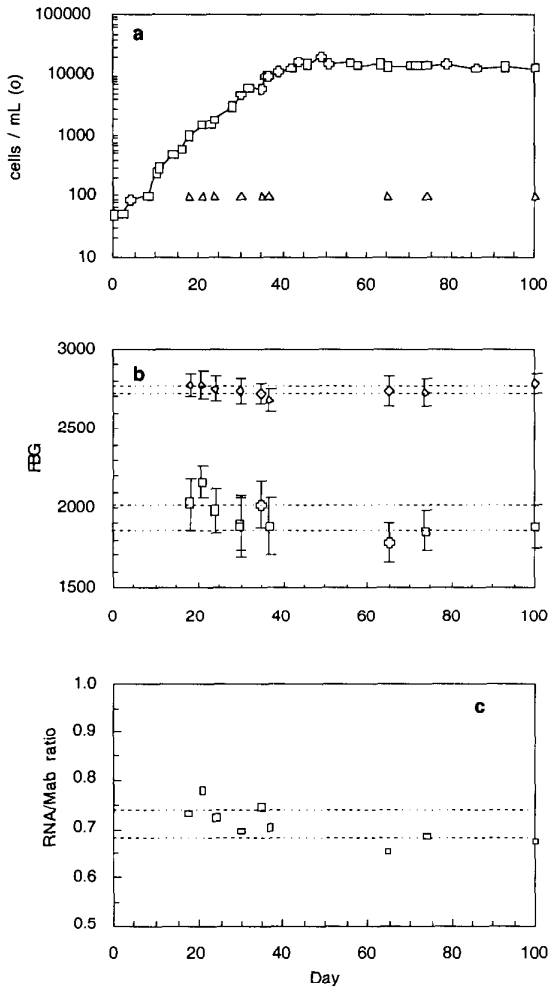

Figure 1. *G. mikimotoi* a. growth in batch culture (Δ = sample for label experiment), b. Green FITC fluorescence (FGB) of MAb (◊) and rRNA (o) labelled cells. Horizontal lines are 95% confidence intervals around the mean FGB intensities of nutrient-replete control cultures), c. rRNA to MAb label intensity ratio.

G. mikimotoi cells labelled with the monoclonal antibody had a significantly higher fluorescence (FBG 2742 ± 25) than the RNA-labelled cells (FBG 1940 ± 79), see Figure 1b. FBG values of both labels slightly decreased during exponential phase. Monoclonal-labelled cells showed a slight increase in FBG during stationary growth phase, while the rRNA-labelled *G. mikimotoi* cells then reached their lowest values. For both labels, the differences in FBG between the two growth phases, were not significant. However, the ratio of RNA:MAb label in stationary phase was slightly (10%), but significantly ($P < 0.05$) lower than in exponential phase (Fig. 1c).

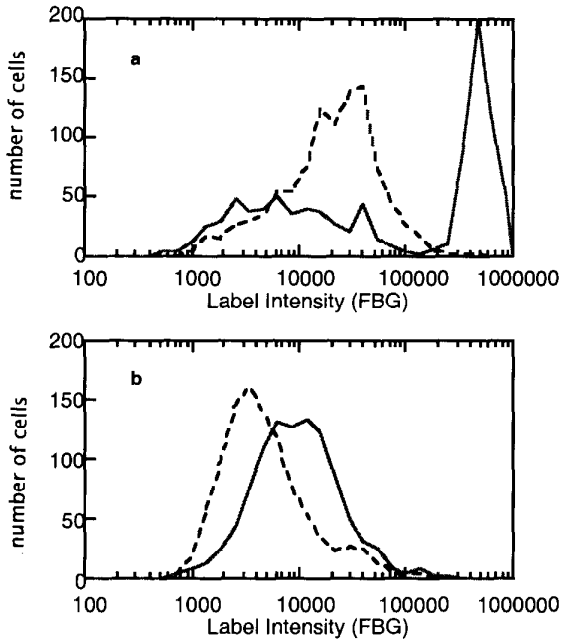

Figure 2. *G. mikimotoi* label intensities of a. MAb, and b. rRNA (uni+) probed cells. Solid line = labelled cells; dashed line = unlabelled control.

P. multiseries

The monoclonal labelling of *P. multiseries* with APC led to an FRR intensity that was much higher than that of

the negative controls; these non-labelled cells had an FRR intensity less than 1% (Fig. 4a). With the microscope, APC-labelled cells were glowing dark red, while the controls did not visibly fluoresce (not shown). However, the long wavelength (> 670nm) of the emitted light by APC-labelled cells, does not make this fluorochrome amenable to routine field sample labelling and microscopic counting.

With both the uni(+) as with the species-specific rRNA probe, exponentially growing *P. multiseries* was labelled homogeneously through the cell [cf. 9]. Positively labelled cells could be separated from negative controls in Data Desk dot plots. In histograms (Figure 4b), the peak separation was not complete, although better than for *G. mikimotoi* (Fig. 2b). The uni (-) control (mean FBG 1309 ± 171) did not have a significantly different green fluorescence than the no probe control (FBG 1465 ± 81).

The shift in FRR of MAb-labelled *P. multiseries* cells after day 24 was due to an unexplained decrease in calibration sphere intensity; cell properties such as TOF did not change. *P. multiseries* cells in exponential phase, stationary phase and in the control culture that had been labelled with the uni(+) probe had a slightly higher (+ 75) FBG intensity than cells from the same sample labelled with the species-specific probe ($P < 0.005$). For both the uni(+) and the species-specific probe, the FBG differences between the three growth phases (Figure 3b), were significant ($P < 0.005$). The rRNA to MAb ratio became practically zero during

stationary growth phase (Fig. 3c), due to the low FBG intensities of the rRNA-probed cells (Fig. 3b). Eventually, *P. multiseries* cells in

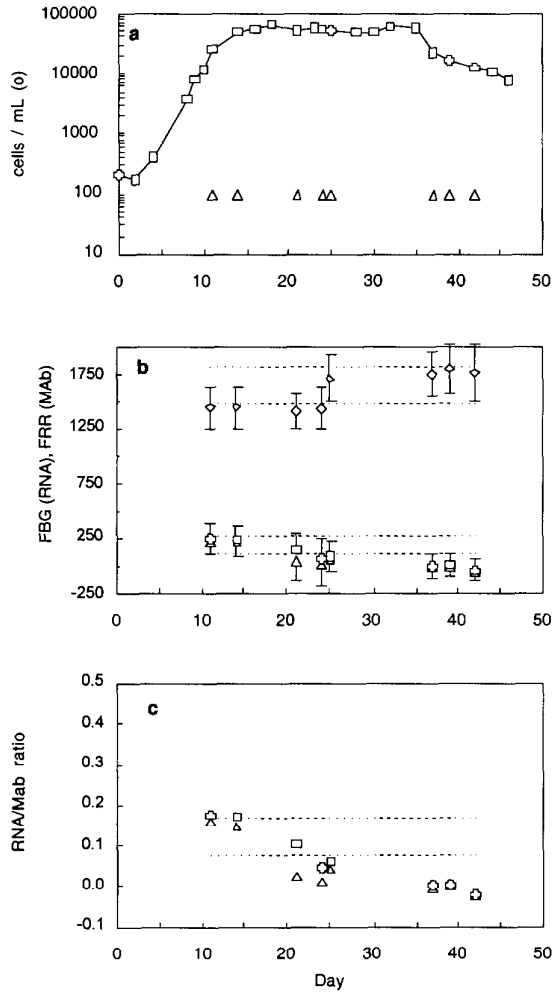

Figure 3. *P. multiseries*. a. growth in batch culture (Δ = sample for label experiment), b. Red APC fluorescence (FRR) of MAb (◊) and Green FITC fluorescence (FGB) rRNA universal + (o) and species-specific (Δ) labelled cells; unlabelled controls subtracted. Horizontal lines are 95% confidence intervals around the mean of nutrient-replete control cultures), c. rRNA to MAb label intensity ratio.

death phase had a FBG intensity not different from the uni(-) probe, which was confirmed microscopically.

The strong decline in rRNA-probe, FBG intensity after exponential growth (Fig. 3b) means that growth-limited *P. multiseries* may remain undetected in both flow cytometry and microscopy. If such cells are not viable anymore, this would have little ecological consequences. Likewise, the counting of MAb-labelled dead cells, would lead to an overestimation of the size of the viable population.

It is concluded that: (i) the monoclonal antibodies tested in this study are not sensitive to the nutritional status of cells, (ii) the effect of growth limitation on rRNA label intensity differs between *G. mikimotoi* and *P. multiseries*, (iii) rRNA label intensities should be enhanced to facilitate separation from non-labelled,

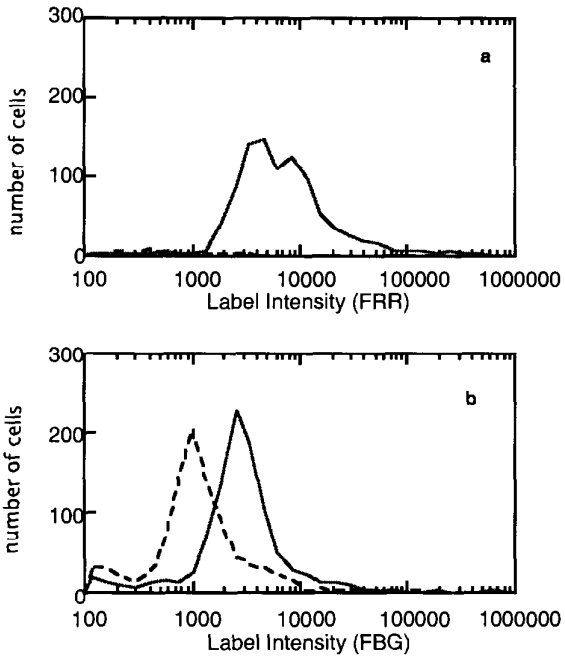

Figure 4. *P. multiseries* label intensities of a. MAb, and b. rRNA (uni+) probed cells. Solid line = labelled cells; dashed line = unlabelled control. The mean FRR intensity of the unlabelled control cells in (a) is <100.

green autofluorescent controls, e.g. by fluorescence enhancement techniques or by choosing a non-green fluorochrome, (iv) a combination of MAb and rRNA labelling of *P. multiseries* may provide a means to detect in situ nutrient stress or cell viability.

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PARALYTIC SHELLFISH TOXINS IN *GYMNODINIUM CATENATUM* STRAINS FROM SIX COUNTRIES

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ABSTRACT

The dinoflagellate *Gymnodinium catenatum*, which may have been introduced to Tasmania via ships ballast, has more recently been reported from several sites on mainland Australia, possibly derived from Tasmanian populations. To examine this hypothesis we compared paralytic shellfish toxin (PST) profiles of a range of Australian (27 from 6 sites), Japanese (3), Portuguese (1), Spanish (5), Uruguayan (4) and Hong Kong (1) (China) strains. Two novel toxin-like compounds were detected in isolates from Australia, Spain, Portugal, Uruguay and Hong Kong. These toxins, designated here as C5 and C6, were similar to PSTs in chromatographic behaviour and fluorescence properties and their demonstrated activity in the saxiphilin binding assay which has a highly specific affinity for PST compounds. Hong Kong and Uruguayan strains exhibited similar profiles to Portuguese and Spanish strains but differed in their low proportion of GTX5,6. Australian strains contained a broad combination of PSTs (C1-4, GTX1-5, dcGTX2,3, dcSTX, STX, C5 and C6, and deoxy-PSTs) as previously reported. PST profiles of Port Lincoln (South Australia) strains were similar to those from south eastern Tasmania, supporting the idea of *G. catenatum* dispersal from Tasmania to mainland Australia. A number of Australian strains exhibited aberrant PST profiles (traces of C5,6 and/or dcGTX2,3 only) which was correlated with their isolation as the products of single cyst germination. Understanding the characteristic toxin fingerprints of *G. catenatum* is continuing to provide valuable insights of dispersal and population relationships for this species.

INTRODUCTION

The distribution of the toxic dinoflagellate *Gymnodinium catenatum* appears to have increased substantially during the past 20 years and it has now been identified from the coastal waters of all continents [1]. Toxic blooms of *G. catenatum* first appeared in Tasmanian waters during the mid-1980s, however benthic cyst records indicate that populations of this species were probably not present prior to 1973 [2,3]. This suggested that bulk carrier shipping, which commenced in the early 1970s, may be responsible for the introduction of *G. catenatum* to Tasmanian ports via ballast water discharges [4]. Disturbingly, *G. catenatum* has now been reported from mainland Australia and these newly discovered populations may have been derived from Tasmanian populations [5].

Successful sexual crossing experiments confirmed compatibility between Australian and Japanese strains [6] but additional studies of four populations indicate that Spanish, Portuguese and Japanese strains are more similar to each other than either is to Australian strains [7]. This has also been supported by RAPD-PCR studies [5,8]. Australian strains were genetically diverse but still formed a distinct cluster from the other populations examined, however the number of Australian and international populations sampled was low and the possible source of Australian strains remains unclear [5].

G. catenatum contains up to 17 saxitoxin analogues including the N-sulfocarbamoyl-11-hydroxysulfate toxins, (C1-4) the gonyautoxins (GTX1-6), decarbamoylgonyautoxins (dcGTX2,3), decarbamoyl saxitoxin (dcSTX), neosaxitoxin (NEO) and the deoxydecarbamoyl PSTs (doGTX2,3, doSTX) [9,10]. Despite potential transformation of PSTs via epimerisation, chemical hydrolysis and enzymic activity, modification of toxin profiles can be minimised by culturing under consistent conditions and harvesting while the cells are growing in the late logarithmic phase [6,11]. The PSTs are therefore practical and functional biomarkers to differentiate *G. catenatum* populations. Consistent differences in PST composition between populations from Tasmania, Japan and Spain are known [10]. In this study we compare PST profiles of a wider range of Australian (27 from 6 sites), Japanese (3), Portuguese (1), Spanish (5), Uruguayan (4) and Chinese (Hong Kong) strains and relate this to possible dispersal via national and international shipping.

MATERIALS AND METHODS

Cultures

Strains of *G. catenatum* were isolated as single chains or as products of individual (single) resting cysts or germinations of several cysts together (mixed), from water and sediment samples collected at a number of localities in Tasmania, Port Lincoln (South Australia), Cowan Creek (New South Wales), Deepwater Bay (Hong Kong) and Mar del Plata (Uruguay). Strains from other regions were kindly provided by Dr I. Bravo (Spain), Dr S. Franca (Portugal) and Professors S. Yoshimatsu and Y. Oshima (Japan). Stock cultures were maintained as previously described [12] at 20°C and 100 µmol photons PAR m^{-2} sec^{-1} with a 12:12 light:dark cycle.

Toxin extraction and analyses

Cultures were grown in 125 ml flasks containing 100 ml of GSe medium [12] to late-logarithmic phase and harvested by gentle filtration onto 47 mm glass-fibre

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filters (Whatman GFC). The filters were transferred to 10 ml centrifuge tubes containing 2 ml of 0.05 N acetic acid and sonicated for 20 seconds (Braun Labsonic, small probe 80 watts) on ice three times. Extracts were stored frozen at –20°C until analysis. High performance liquid chromatography (HPLC) analyses of the three classes of PSTs (C-toxins, gonyautoxins and saxitoxins) were performed using published methods [10] with modifications [13] (Fig.1). Toxins were identified by comparison of retention times and fluorescence emission maxima with standards, the disappearance of peaks by eliminating post-column oxidation and spiking experiments [14]. The same extracts were examined for binding activity to saxiphilin, a soluble circulatory protein isolated from vertebrates and arthropods, which specifically binds PST compounds [15]. Toxin standards were kindly donated by Professor Y. Oshima and Dr. H. Onodera of Tohoku University, Japan.

Data analysis

PST content of strains examined were expressed as molar percentages the mean PST composition calculated across locations and PST profile types (Table 1). This dataset was converted to a relative abundance matrix, with epimer pairs pooled, using the following scoring criteria: not detected = 0; <5% = 1; 5% - 40 = 2; > 40% = 3 (representing the four distinct sets in the distribution of relative concentrations). This matrix was converted to pairwise euclidean distances between groups and a dendrogram constructed using the unweighted pair group mean average (UPGMA) algorithm using Statistica, release 5.1 (1997, StatSoft inc. USA) (Fig. 2).

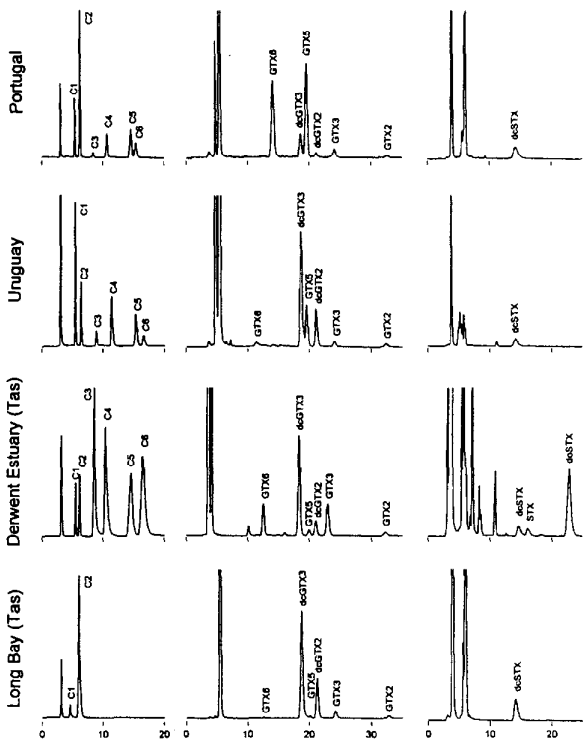

Fig. 1. Chromatograms of extracts from *G. catenatum* (not to scale).

RESULTS

Plankton derived cultures from the Derwent Estuary (DERW) and Triabunna (TR-P) in Tasmania produced profiles dominated by the C-toxins (C1-C4) and also lower proportions of GTX2,3, GTX5, GTX6, dcGTX2,3, dcSTX, STX and doSTX. No standards were available for deoxy-PSTs but identification was made by comparison with published chromatograms [10] and by fluorescence properties (Fig. 1, Table 1). Very similar toxin profiles were observed for strains from Port Lincoln (PTLN) in South Australia. An additional pair of peaks with identical spectral properties to PSTs were observed, initially in Australian strains, during the C-toxin HPLC runs (Fig. 1). After isolation from other known PSTs these compounds exhibited activity in the saxiphilin binding assay which has an exclusive specificity for PST compounds [15], indicating these compounds may be undescribed PSTs (to be published elsewhere). We designated these compounds C5 and C6. Spanish and Portuguese strains also produced the C5,6 peaks but displayed higher proportions of GTX5 and GTX6 than Australian strains, and did not contain deoxy-PSTs. Uruguayan and Hong Kong strains contained C1,2, C3,4, C5,6, dcGTX2,3 and low proportions of GTX2,3 and GTX5,6, but no detectable STX or doSTX that were conspicuous in typical Australian strains (Table 1, Fig. 1).

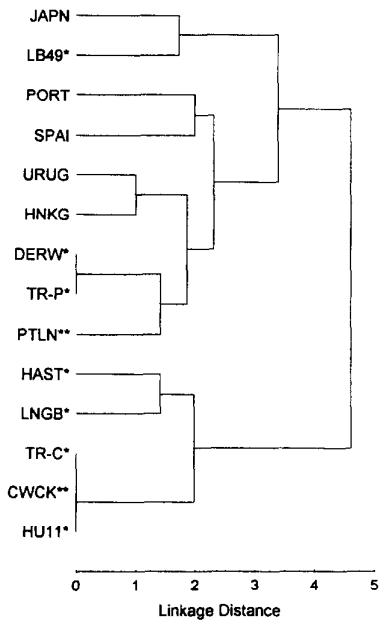

Fig 2. Similarity dendrogram for *G. catenatum* cultures based upon toxin profiles. Each location is depicted by a code (see Table 1). *designates Tasmanian and **mainland Australian strains.

Table 1. Mean toxin profiles of *G. catenatum* from 6 countries. Strains were isolated from plankton (P), as mixed cyst incubations (M) or as single cysts (S) and cultured under identical conditions. No standards were available for doSTX, C5 and C6 so presence (+) or absence (-) only is reported. *designates Tasmanian and **mainland Australian strains.

Strain source	N	isol-ation	C1,2	C3,4	GTX2,3	GTX5	GTX6	dcGTX 2,3	dcSTX	STX	C5,6	doSTX
Japan (JAPN)	5	P	83.5	-	0.4	7.5	3.1	4.9	0.6	-	-	-
Portugal (PORT)	3	P	31.8	10.4	1.8	26.9	15.1	8.1	5.5	0.6	+	-
Spain (SPAI)	11	P	22.7	15.3	2.0	29.2	22.8	4.5	3.5	-	+	-
Uruguay (URUG)	4	M	36.1	43.0	0.3	5.0	2.4	10.9	2.4	-	+	-
Hong Kong (HNKG)	1	M	40.0	35.5	0.3	4.0	1.1	16.4	2.5	-	+	-
Australian strains												
Derwent Estuary*	6	P	21.3	66.3	0.4	1.8	0.3	8.7	1.0	0.2	+	+
Pt. Lincoln (PTLN)**	6	M,P	14.8	67.7	0.6	2.5	0.4	7.2	6.9	-	+	-
Triabunna (TR-P)*	3	P	14.8	70.8	0.3	1.1	0.5	10.2	2.4	0.03	+	-
Triabunna (TR-C)*	2	C	-	-	-	-	-	100.0	-	-	+	-
Hastings Bay (HAST)*	4	C	-	-	-	-	-	77.4	20.1	2.5	+	-
Long Bay (LNGB)*	24	C	-	-	0.7	-	-	95.7	2.9	0.8	+	-
Long Bay (LB49)*	2	C	77.0	-	2.1	2.6	1.4	16.2	0.6	-	-	-
Cowan Creek (CWCK)**	6	C	-	-	-	-	-	100.0	-	-	+	-
Huon Estuary (HU11)*	1	C	-	-	-	-	-	100.0	-	-	+	-

A number of Australian strains derived from the products of wild resting cysts germinated in the laboratory exhibited atypical PST profiles for *G. catenatum*. Strains from Cowan Creek (New South Wales) exhibited only low concentrations of the PSTs usually found in *G. catenatum* and many of these were below the detection limit (approximately 0.1 ng). One strain from Long Bay, Tasmania (LB49) was most similar to Japanese strains (Table 1), due to the absence of doSTX, C5,6 and the N1-hydroxy analogues C3,4.

When the data were used to construct a similarity dendrogram, several major clusters were evident (Fig. 2). Strains from Japan and a single strain from Tasmania (LB49) clustered together; Portuguese and Spanish strains clustered together. Strains from Uruguay and Hong Kong clustered between the European and Australian strains. Toxic strains from mainland Australia (PTLN) exhibited similar toxin profiles to Tasmanian isolates (see Table 1). The Australian strains that contained mostly dcGTX2,3 and/or C5,6 clustered together. These strains were all isolated from sediment core cysts.

DISCUSSION

Earlier research on *G. catenatum* toxin profiles demonstrated consistency within populations but variation between populations [9,10]. The essential differences were that: (1) Australian strains were unique in their ability to produce deoxy-PSTs; (2) Australian and Spanish strains produced C1-4 but Japanese strains produced C1,2 and not C3,4, and (3) Spanish strains produced higher amounts of GTX5 and GTX6. [10]. This study confirms these results with additional strains and shows that Portuguese strains are, not surprisingly, similar to Spanish strains in producing a full suite of C1-C4 and relatively higher proportions of GTX5 and GTX6. The absence of doSTX and the presence of C3,4 distinguished Hong Kong and Uruguayan strains from Australian and Japanese strains respectively; the low mol% of GTX5,6 distinguishes them from Spain/Portugal. The profile is perhaps most similar to that reported for Korean strains [16] which were dominated by C1-3 and dcGTX2,3 (although it is not clear whether doSTX and the novel C5,6 compounds were present). Recent analysis of other Uruguayan strains revealed similar profiles to the results in this study however they contained additional toxins corresponding to neoSTX and GTX1,4 [17]. Strains from Port Lincoln, South Australia exhibited a toxin profile typical of Tasmania strains, supporting earlier genetic data which suggested that mainland Australian populations may have derived from Tasmanian populations, possibly via a domestic shipping vector [5]. Recent dated core studies indicate that this mainland population appeared at a similar time to Tasmanian populations in the early 1970s [18].

Previous studies have shown that *G. catenatum* strains from Australia, Japan, Spain and Portugal are genetically heterogeneous but can be distinguished as distinct clusters [5]. This study also shows discrete groups based on toxin composition and provides a useful visual illustration of the relationships between the different toxin profiles. Interestingly, despite the geographic proximity of the Hong Kong, and particularly the Korean strains, to Japanese populations, they can be clearly differentiated by the presence of C3,4 (this study, [16]). This biochemical divergence is similar to the limited (partial) rDNA variation noted between Japanese and Korean/Chinese *A. tamarense* [19] which suggests that Japanese dinoflagellate populations are, or have been, biologically isolated from those on mainland China/Asian continent. The similarity of Uruguayan strains to those from Hong Kong is not surprising considering *G. catenatum* has been documented in Uruguayan ballast water delivered to Hong Kong ports [20].

The additional peaks (C5,6) in the C-toxin HPLC profile are intriguing. Similar peaks (designated as non-toxic fluorescent compounds) may have been present in chromatograms published by Oshima et al. [10]. The peaks in our chromatograms show similarities to PSTs in fluorescence properties, disappearance in the absence of post-column oxidation, and activity in the saxiphilin

assay, which indicates a close structural relationship to PSTs. At the very least, C5,6 peaks can function as useful biochemical markers for *G. catenatum* populations as Australian, Spanish, Portuguese, Uruguayan and Hong Kong strains all exhibited these peaks consistently. Further research is being undertaken to confirm our preliminary results and determine the structure and toxicity of these compounds.

The aberrant toxin profiles of a number of Australian strains, only producing combinations of dcGTX2,3 C5,6, dcSTX and STX is unusual. Total toxin yield from these cultures was generally low and most could be considered at least functionally non-toxic. For example, mouse bioassays with concentrated aqueous extracts of strain GCCC36 (Cowan Creek, Australia) elicited PSP symptoms in mice indicating a low level of PST toxicity (0.6μg STXeq/100 ml culture), that may be due to the presence of dcGTX2,3 and C5,6 in the extract. The aberrant strains reported here were isolated by 3 different workers, at several sample locations, on different dates ranging from 1987 through to 1998. Furthermore, the low yield and toxin profiles of these single cyst isolates appear stable despite more than 10 years in laboratory culture in some cases.

Careful examination of culture isolation histories of aberrant strains show that they are all products of single cyst germinations rather than plankton cell isolates, as was a previous report of a non-toxic *G. catenatum* strain (GCHU10, see Oshima et al. [10]). This influence of isolation history (i.e. plankton vs single cysts) on toxin production is difficult to explain. One possibility is that benthic cyst beds may be a separate "low toxicity" population, however, this seems highly unlikely, especially across several Australian locations. It is more likely that PST synthesis is affected in some way by the combination of events involved in germination of resting cysts as a source of strains. These could include genetic events linked with meiosis along with isolation history. PST content of *Alexandrium* spp. cysts and *G. catenatum* cysts is similar to, or higher than, plankton cells, but β-epimers dominate indicating that PST synthesis is reduced or halts in the early stages of cyst formation [21,22]. It therefore seems more likely that PST synthesis may not re-establish when cysts are germinated in a laboratory environment due to some unknown biological, chemical or physical factor. Future experimental work is planned to examine the underlying causes of the correlation of isolation and life history with PST profiles and low toxicity in *G. catenatum*.

The use of PST profiles as biogeographical markers in *G. catenatum* continues to provide valuable insights into population relationships of this species, but the potential effect of culture history shows that care should be exercised when interpreting such data. Further studies are required to elucidate the effect of life history and laboratory cyst germination on PST synthesis and production. The available evidence suggests that Australian *G. catenatum* are distinct from the known populations examined here, but domestic shipping may have played a role in the dispersal of *G. catenatum* from Tasmania to the mainland Australian ports, or vice-versa.

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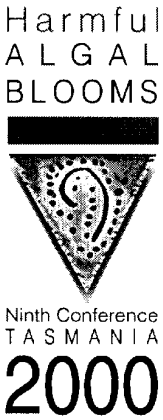
MOLECULAR AND IMMUNOLOGICAL IDENTIFICATION METHODS



TOXIC AUSTRALIAN *ALEXANDRIUM* DINOFLAGELLATES: INTRODUCED OR INDIGENOUS?

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ABSTRACT

The genetic relationships of *Alexandrium catenella* (5 strains), *A. tamarense* (2) and *A. minutum* (5), representative of Australian and New Zealand populations of these dinoflagellates, were examined using a variety of methods. Analysis of the D1 and D2 regions of the large-subunit (LSU) ribosomal RNA gene proved an adequate technique for the characterisation of *A. catenella*, but not *A. minutum*, as some strains produced double amplification products. However, the use of the 5.8S gene and flanking internal transcribed spacer (rDNA-ITS) regions proved suitable for the discrimination of all strains studied. Sequence analysis of fragments of the rDNA-ITS and LSU rDNA indicate that Australian and New Zealand *A. catenella* is genetically homogeneous and that two genotypes of *A. minutum* exist in Australian waters. Paralytic shellfish toxin profiles also discriminated the different species/ genotypes. It is hypothesised that all Australian and New Zealand populations of *A. catenella* have been introduced from temperate eastern Asia (Japan) in recent times. In contrast, Australian and New Zealand *A. minutum* may represent indigenous populations.

INTRODUCTION

Regional populations of *Alexandrium* species in the '*tamarense* complex' differ genetically according to their geographic origin [1], rather than morphotaxonomic distinctions. As such, populations of the .'*tamarense* complex' have been classified into rDNA-sequence groups or 'ribotypes'. However, the geographical variation of rDNA sequences among other *Alexandrium* species, such as *A. minutum*, has not yet been examined in detail.

A. catenella, *A. tamarense* and *A. minutum* are widespread in southern Australia, and rDNA sequences of selected populations from Victoria, South Australia and Tasmania have been characterised [2]. A northern Tasmanian population of *A. tamarense* possesses a unique ribotype, while Victorian populations of *A. catenella* share the same ribotype as Japanese strains [1].

Mussel toxicity on the New South Wales south coast suggests the presence of Paralytic Shellfish Toxins (PST) in southern Australia as early as 1935 [3]. Wood [4] described a chain forming dinoflagellate (*Gonyaulax conjuncta*) in N.S.W. waters and it has been suggested this record is actually the first report of *A. catenella* in Australia [5]. The presence of this species along the NSW coast has now been verified [6]. The near identical LSU-rDNA sequences of Victorian and Japanese strains of *A. catenella* has prompted the hypothesis that this species has been recently introduced to Australia via human activities [7]. This hypothesis is, however, based only on sequences obtained from Victorian strains, and does not adequately reflect the Australian distribution of *A. catenella.*

Both the LSU rDNA and the rDNA-ITS can discriminate between regional populations of Alexandrium [1, 8]. Here we use sequences from both to examine the relationships between Australian populations of *Alexandrium tamarense*, *A. catenella* and *A. minutum* and their overseas counterparts, and determine the likelihood of introduction(s) via ballast water. By examining samples spanning the known Australian distribution of *A. catenella,* we aim to more accurately reflect the population genetics of this organism.

MATERIALS AND METHODS

Clonal, non-axenic cultures of three *Alexandrium* species (Table 1) were maintained in a seawater based GSe medium of 28 g kg^{-1} salinity [9]. Stock cultures were maintained in 30 ml of growth medium at a constant temperature of 17° C or 20° C, under a 12:12 light:dark photoperiod and approximately 100 µmol m^{-2} s^{-1} irradiance.

DNA extraction , PCR and sequencing

Vegetative cells were harvested from 10 ml of mid- to late-logarithmic phase cultures, and DNA was extracted by gentle lysis and purified using the methods described by Bolch *et al.* [10].

The rDNA-ITS and the D1-D2 region of the LSU-rDNA were amplified in 100 µl volumes, using the primers "ITS A" [11], and "ITS B" [12], and "D1R" and "D2C" [13] respectively.

PCR products were electrophoresed and examined for amplification artefacts, then purified and sequenced [14]. Automated base calls were checked by visual inspection of the electropherograms of both the forward and reverse sequence. Ambiguous calls and conflicts were resolved by alignment and comparison of both strands using the program Sequence Navigator, version 1.0.1 (Perkin-Elmer). Consensus sequences were aligned using the clustal alignment option of Sequence Navigator and the default settings for gap inclusion and extension, and compared with several published sequences . Sequence alignments were refined visually and phylogenetic analysis was carried out using PAUP* (Version 4.0b2a) computer program [15]. Phylogenetic analyses were carried out under the assumptions that all sequence positions were unordered characters with equal weight. In all analyses, single- and

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Table 1. *Alexandrium* species used in this study, their source locality, strain number, isolator and year of isolation.

Species	Locality	Strain code	Isolator	Year of isolation
A. catenella	Sydney Harbour, NSW (1), Australia	ACSH01	S. Norwood	1993
A. catenella	Cowan Ck., NSW (2), Australia	ACC501	J. Valentine	1996
A. catenella	Newcastle, NSW (3), Australia	ACNC50	M. de Salas	1998
A. catenella	Tauranga, New Zealand	ACCWD44	L. MacKenzie	1997
A. catenella	Kashima, Japan (Ballast water)	ACCS-316	C. Bolch	1989
A. catenella	Triabunna, TAS, Australia	ACTRA02	C. Bolch	1997
A. tamarense	Triabunna, TAS, Australia	ATTRA03	C. Bolch	1997 (now lost)
A. tamarense	Bell Bay, TAS, Australia	ATBB01	C. Bolch	1987
A. minutum	Bunbury, WA, Australia	AMBU03	C. Bolch	1996
A. minutum	Adelaide, SA, Australia	AMAD06	S. Blackburn	1987
A. minutum	Newcastle, NSW, Australia	AMNC04	C. Bolch	1997
A. minutum	Croisilles Harbour, New Zealand	AMCWD13	L. MacKenzie	1994

multiple-base insertions and deletions were treated as a single evolutionary event, and scored as either present or absent relative to the outgroup *A. (Gessnerium) margalefi.*

Toxin extraction and analysis

Approximately 75 ml of each culture were harvested during mid- to late- logarithmic phase. Cultures were gently filtered through glass fibre filters, placed in 3 ml of 0.05N acetic acid and sonicated (Braun Labsonic; small probe, 80W) on ice, three times for 20 seconds. Extracts were frozen at -20° C until analysed. Two milliliters of each culture were fixed with Lugol's iodine solution and triplicate cell counts of were carried out using a 1.0 ml Sedgewick-Rafter chamber. PST analyses of saxitoxin derivatives, gonyautoxins and C-toxins were conducted according to the method outlined by Negri & Llewellyn [16].

RESULTS

Sequencing of the D1-D2 region of the LSU rDNA was not possible in *A. minutum* strains from Newcastle, NSW, due to the presence of a smaller sized pseudogene. Attempts to isolate a single band and sequence failed due to the production of two overlapping sequences.

Both genetic and toxicological analysis separated Australian and New Zealand populations of *A. minutum* and the '*tamarense* complex' into consistent groups.

All Australian *A. catenella* strains exhibited a similar toxin profile, in which GTX1, GTX4 and C-toxins dominated (Fig. 1). Toxic Tasmanian *A. tamarense* has a very different profile, dominated by GTX 2 and 3, with minor amounts of saxitoxin. Unfortunately this strain was lost before seqencing was possible. All *A. catenella* populations belong exclusively to Scholin's [2] Japanese-Temperate Asian ribotype, and are clearly distinct from the Tasmanian ribotype of (non-toxic) *A. tamarense*.

Populations of Australian and New Zealand *Alexandrium minutum* form two main groups, supported by molecular and toxicological data (Figures 1 & 3).

1. An eastern "trans-Tasman" group, comprised of NSW (Australia) and New Zealand subgroups, in which saxitoxin is the dominant toxin, and smaller amounts of neo-saxitoxin and gonyautoxins may be present. Sequences from these populations are distinct from other *A. minutum* populations, but differ little from each other.
2. A western Australian, group, which has genetic affinities to European *A. minutum* (*lusitanicum*), and a toxin profile dominated by GTX 1 & 4, with minor amounts of GTX 2 & 3.

DISCUSSION

The possibility that regional populations of dinoflagellates responsible for recent toxic events may be the result of human-mediated introductions instead of directly attributable to a changing environment has often been reviewed [17].

In Australia, there is circumstantial evidence that some toxic dinoflagellate species have been introduced, both on a regional and local scale. McMinn *et al.* [18] have shown that *Gymnodinium catenatum*, which has fossilisable cysts, was not present in appreciable density in Tasmania or South Australia until the early 1970s, a time when woodchip exports from Tasmania to Japan and eastern Asia began.

Similar evidence is mounting for *Alexandrium* species. Long term sampling records from the international shipping ports of Triabunna, Tasmania, and Port Adelaide, South Australia, also indicate that introduction of toxic dinoflagellates, from domestic and/or international sources, may have occurred during the 1980s and 1990s. *Alexandrium* blooms in the Port River, S.A., consisted exclusively of *A. minutum* during the late 1980s [19], and *A. catenella* cysts were not detected during sediment

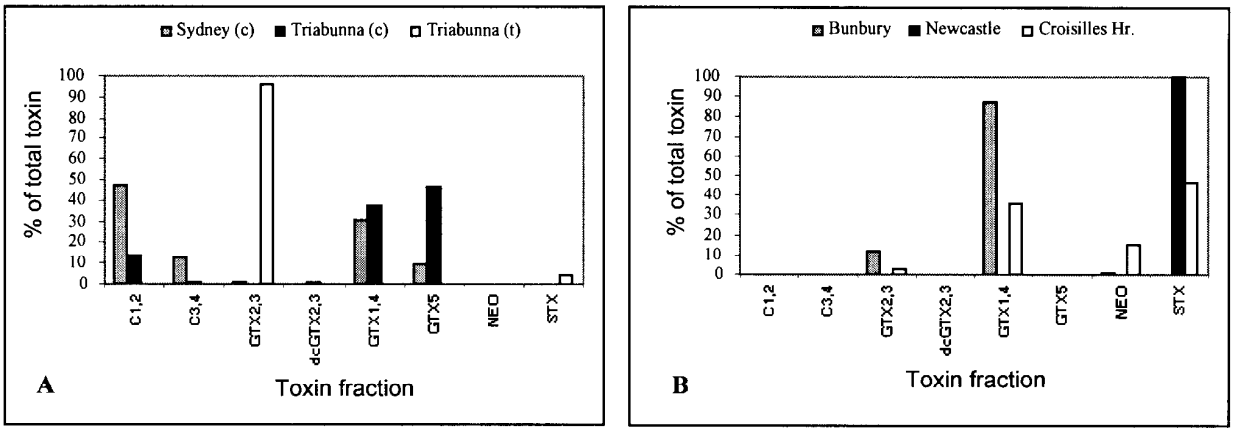

Figure 1: Comparison between toxin composition of **A:** three "*A. tamarense* complex" strains from eastern Australia (c = *A. catenella*, t = *A. tamarense*), and **B:** three *A. minutum* strains from Australia and New Zealand.

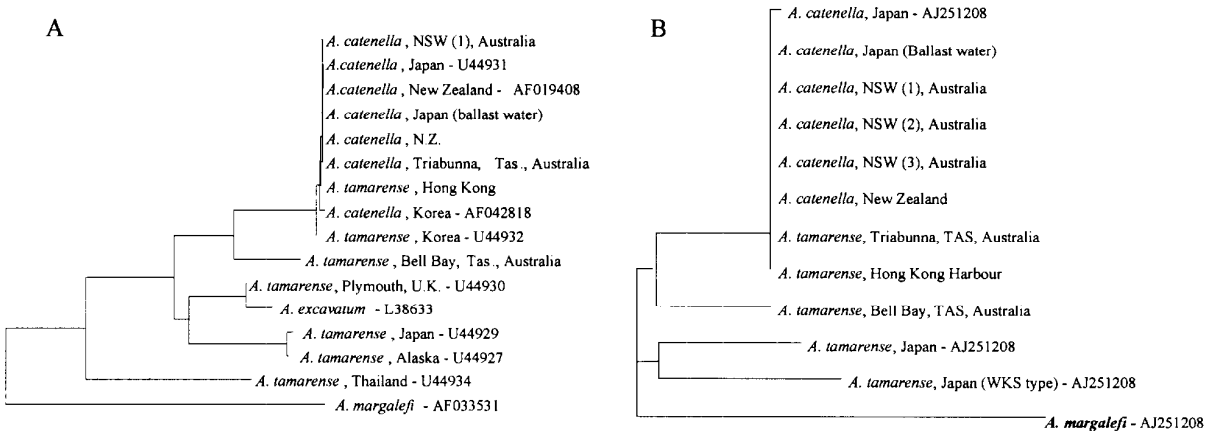

Figure 2: Phylogenetic analysis of strains of the *A. tamarense* complex, **A**: Analysis of LSU rDNA sequences. **B**: Analysis of rDNA-ITS sequences. Strain sequences obtained from GeneBank [20] contain accession number.

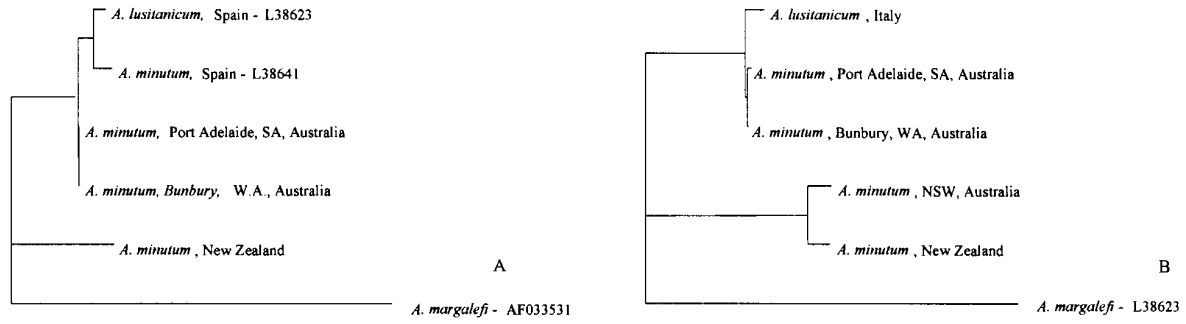

Figure 3: Phylogenetic analysis of strains of *A. minutum*, **A**: Analysis of LSU rDNA sequences. **B**: Analysis of rDNA-ITS sequences. Strain sequences obtained from GeneBank [20] contain accession number.

surveys at this time [21]. However, sediment and plankton sampling during the late 1990s show that *A. minutum* has been replaced by *A. catenella*. Similarly, in Tasmania, surveys in the port of Triabunna in the 1980s [22] found no evidence of *Alexandrium* cysts, yet sampling during 1997 has demonstrated the presence of *A. catenella* and *A. tamarense* (Table 1).

Earlier PST records indicate that PST-producing species may have been present on the N.S.W. coast since the mid 1930s [3]. *A. tamarense* from Bell Bay, Tasmania, seems to be of a unique, non-toxic ribotype [1, 7, 23], so it is probable that *A. catenella* was responsible for the earlier report of PST. Whether it was introduced prior to the 1930s or is indigenous is still unclear. If Australian *A. catenella* were indigenous, a continuity of ribotype along the most probable natural dispersal route, through southeast Asia, would be expected. However, Korean and Chinese ribotypes (as far south as Hong Kong) differ slightly from

those of Japan and Australia, which appear identical [this study and 1]. This suggests a discontinuity through the temperate and tropical Asian region, supporting a recent introduction of Australian *A. catenella* and possibly the Triabunna *A. tamarense* population. Additionally, the unique ribotype of tropical Asian *A. tamarense* (Fig. 2A) supports this discontinuity [7, 8].

PST toxin analyses of Triabunna *A. tamarense* indicate a toxin profile dominated by the potent carbamate (GTX2,3, STX) toxins, rather than C-toxins, consistent with toxin profiles of northern Japanese strains [24], or those of northeast and/or northwest American strains [25].

Australian populations of *A. minutum* were found to exhibit two discrete ribotypes, which could also be clearly distinguished by PST-toxin analyses. The southern/western type is genetically and toxicologically very similar to European (Mediterranean) strains of this organism [26]. The other ribotype is found on eastern Australian coasts, and is very similar to strains isolated from New Zealand [27]. This latter ribotype may represent an indigenous population of *A. minutum* in NSW and New Zealand waters, while WA and SA populations may have resulted from one or more introductions from Europe. Until a more comprehensive set of known *A. minutum* (e.g. French, temperate and tropical Asian) populations have been examined, the likely source of *A. minutum* cannot be determined, nor can the possibility of endemism of Australian *A. minutum* be discounted.

The emerging variety of disparate ribotypes of *A. tamarense* complex and *A. minutum* populations in Australian waters is at odds with the genetic homogeneity found in most other regions of the world. As more strains are examined it is clear that heterogeneity can exist in areas of "ribotype overlap" (UK and northern Europe [28], northern Japan [1]). The genetic homogeneity of Australian *A. catenella* and their ribotype identity with Japanese populations, compared with the unique Tasmanian ribotype (ATBB01), points towards a recent introduction of this organism from Japan to Australian waters. Nevertheless, it cannot be discounted that the unique Tasmanian ribotype has been introduced to the port of Bell Bay, Tasmania, from another as yet unsampled location.

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BLOOMS OF *ALEXANDRIUM TAYLORI* (DINOPHYCEAE) IN THE MEDITERRANEAN: A PRELIMINARY MOLECULAR ANALYSIS OF DIFFERENT ISOLATES

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ABSTRACT

High-biomass blooms of *Alexandrium taylori* Balech are reported from new areas of the Mediterranean Sea. In 1995-1999, a number of sites in Spain and Italy were affected by summer blooms of *A. taylori* with associated events of yellowish to green-brown discoloration of the water. Clonal cultures of *A. taylori* established from Italian and Spanish seawater samples were used for the sequence analysis of the 5.8S rDNA gene and ITS regions in order to initially study the genetic variability of different geographical populations of *A. taylori* in the Mediterranean Sea. From the sequence analysis, the Italian and Spanish strains of *A. taylori* proved to be closely related to each other, but distinct from species with similar morphological features, such as *A. margalefi* Balech and others, such as *A. minutum* Halim. Thus, species boundaries and groupings previously established by morphological criteria coincided with the molecular data, although with some exceptions. An oligonucleotide probe specific to the *Alexandrium* genus was designed for the conserved 5.8S rDNA gene, whereas the highly variable ITS1-ITS2 regions of *A. taylori* can be used to target the species level.

INTRODUCTION

High-biomass harmful algal blooms (HB-HABs) are massive, nearly-monospecific outbreaks that, although often non-toxic, induce adverse effects on the marine ecosystem and loss of the recreational value of coastal regions, with considerable economic setbacks to the local tourist industry [1]. Whilst some recurrent HB-HAB species are well-documented (e.g. the colonial haptophyte *Phaeocystis* sp. in Northern European waters), few studies have been conducted on other taxa, such as *A. taylori* Balech, a dinoflagellate recently identified as responsible for summer events of yellowish to green-brown discoloration of the water along the Mediterranean coast of Spain and Italy [2-4]. Studies by these authors have shown the morphological variability of *A. taylori*, the high diel production of temporary cysts, and the existence of a sexual resting stage, as a part of its life cycle. Up to now, the identification of this dinoflagellate and its discrimination from species with close morphological similarities (e.g. *A. margalefi* Balech) have relied largely on time-consuming microscope observations, in some cases limiting the monitoring of *A. taylori* to a few coastal areas.

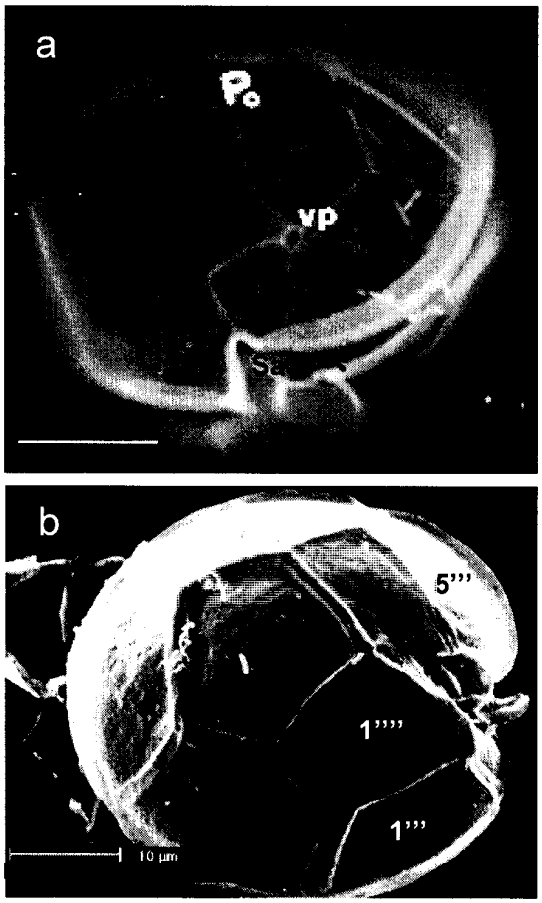

Fig. 1. Micrographs of *A. taylori* specimens from Vulcano (Tyrrhenian Sea, Sicily). (a) LM, epifluorescence. Epithecal view showing the complete disconnection of the 1' apical plate from the Po apical pore plate (*Gessnerium* type). Location of the ventral pore (vp) slightly variable in this species. (b) SEM. Hypotheca with posterior sulcal plate (Sp) sloped to the right. Scale bars = 10µm

In order to study the genetic variability of different geographical populations of *A. taylori* and develop further molecular markers for a rapid diagnosis of HAB species, we examined the ITS-5.8S rDNA gene sequences of *A. taylori*, as well as other congeneric species from the Mediterranean areas. Additional information on blooms of *A. taylori* in new Mediterranean localities is also provided.

Harmful Algal Blooms 2000
Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J. (eds)
Intergovernmental Oceanographic Commission of UNESCO 2001

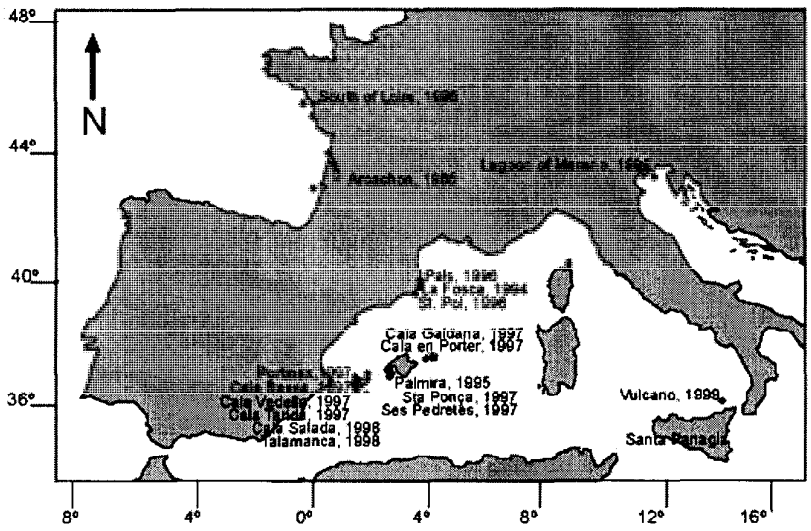

Fig. 2. Distribution and year of first report of *A. taylori* blooms in the Mediterranean Sea and the European Atlantic coast.

MATERIALS AND METHODS

Sample collection and microscopy

The *A. taylori* assemblages were collected in 1995-1999 from tourist localities in the Balearic Basin (Mediterranean coast of Spain, Mallorca, Menorca, Eivissa) and the Tyrrhenian Sea (Vulcano, Aeolian Islands) during the summer blooms and events of water discoloration (July-September). Subsamples fixed with Lugol's solution or formaldehyde (1% final concentration) were settled in Utermöhl chambers and counted with an inverted microscope for a quantitative estimate of the bloom density. The specific identification was obtained by staining the cells with Calcofluor White M2R [5] and observing them under Axioplan microscope equipped with epifluorescence (Zeiss UV filter set 487902). Alternatively, *A. taylori* specimens were treated for scanning electron microscopy [4]. In some cases, the species identity was confirmed by comparing the morphology with an Ionian clone already available in our lab (AT-4 strain, isolated from S. Panagia, Syracuse - Sicily), a Spanish clone (AV-8 strain, La Fosca - Catalan coast), and a Tyrrhenian clone of *A. margalefi* (AM-1 strain, from Green Pool – Sicily).

Nutrients and biomass

Additional seawater samples were taken at Vulcano, Sicily during *A. taylori* blooms for the analysis of chemical and biological parameters, including nitrate, nitrite, ammonia, phosphate, total phosphorus, silicate, and chlorophyll *a* [6].

Molecular analysis

The *Alexandrium* strains used for the molecular analyses were: *A. taylori* AT-4 and *A. margalefi* AM-1 (M.G. Giacobbe & X. Yang), *A. taylori* AV-8 (E. Garcés), and A. *minutum* LAC27 from the Northern Adriatic Sea (G. Honsell) kindly provided by E. Graneli. The cultures were maintained in f/2 medium [7]. Light was provided by cool-white fluorescent tubes at a photon flux of 100 μmol · m^{-2} · s^{-1} on a 14:10 h LD cycle.

Total nucleic acids were isolated from each culture and the ITS regions containing the 5.8S rDNA were amplified as reported previously [8] using primers ITSA and ITSB. Double-stranded PCR products were sequenced directly using an ABI PRISM 310 Genetic Analyzer (Perkin Elmer, Applied Biosystems, Foster City, CA) and the dye terminator method was used according to the manufacturer's instructions (ABI PRISM Big Dye Terminator Cycle Sequencing Ready reaction Kit, Perkin Elmer Corp., Foster City, CA).

DNA sequences were read directly into a computer and the termini of 5.8S rDNA coding regions and the starting positions of the ITS regions determined by comparison with published 5.8S rDNA and ITS sequences [9]. Multiple sequence alignments were performed using Sequence Navigator (Perkin Elmer, Applied Biosystems). The *A. taylori* AT-4 sequence was submitted to BLAST database (Basic Local Alignment Search Tool) at the NCBI (National Center for Biotechnology Information) archive to search similarity with other nucleotide sequences. A distance matrix was generated using the DNADIST of PHYLIP with Kimura's two parameter method [10], and a distance tree was constructed using NEIGHBOR program (Neighbor-Joining method) of the PHYLIP package (PHYLIP version 3.52 c; [11]). Bootstrapped data sets (100 replicates) were generated using the SEQBOOT program, and these were analysed using the DNADIST and NEIGHBOR programs with the multiple analyses option. In the analysis, *Saccharomyces cerevisiae* was used as an outgroup.

RESULTS AND DISCUSSION

Main morphological features

The microscopical analysis of plankton samples collected both in the Balearic and Aeolian area showed assemblages of *A. taylori* (Fig. 2). The thecal morphology of this species is characterised by the complete disconnection of the Po and 1' plates, typical

of the subgenus *Gessnerium*. To date, this dinoflagellate has probably been under-reported since it may easily be confused with other *Alexandrium* spp. present in the Mediterranean area, e.g. *A. margalefi* (Tyrrhenian Sea), although this last organism is considered as clearly differentiated within the subgenus *Gessnerium* [12]. Both species have a similar shape of the 1' plate and location of the ventral pore. However, a more detailed analysis shows some fine differences in the general cell shape, 6" and Sa plates, as well as 1'-2' relationships (compare with illustrations of *A. margalefi* [12, 13]).

Table 1. Isolates of *Alexandrium* species used in this study and nucleotide sequence length of the ITS-5.8S rDNA regions.

Species & Strains	Isolation locale	PSP Toxicity	Length 5.8S	ITS1	ITS2	Total	GenBank Accession No.	Authors[1]
A. taylori AT-4	Ionian Sea, Italy	No	160	168	168	496	AJ251653	This study
A. taylori AV-8	Catalan Coast, Spain	No	160	168	168	496	AJ251654	This study
A. margalefi AM-1	Tyrrhenian Sea, Italy	No	161	155	165	481	AJ251208	This study
A. minutum LAC27	Adriatic Sea, Italy	Yes	160	184	196	540	AJ005050	Penna & Magnani 1999
A. insuetum S1	Shoudoshima, Japan	No	160	188	177	525	AB006996	Adachi et al. 1996
A. affine H1	Harima-Nada, Japan	No	160	173	192	525	AB006995	Adachi et al. 1996
A. tamarense FK-788	Funka-Bay, Japan	Yes	160	165	194	519	AB006993	Adachi et al. 1996
A. tamarense WKS-1	Kushimoto, Japan	No	160	167	190	517	AB006991	Adachi et al. 1996
A. tamarense CU-15	Thailand	No	160	170	188	518	AB006992	Adachi et al. 1996
A. catenella MI7	Harima-Nada, Japan	Yes	160	166	192	518	AB006990	Adachi et al. 1996
A. pseudogonyaulax H1	Harima-Nada, Japan	No	161	184	173	518	AB006997	Adachi et al. 1996

[1]Individuals who sequenced the 5.8S-ITS regions of the strains.

High-biomass blooms

A. taylori blooms took place in a number of Spanish localities and new Italian areas (Fig. 1), giving a yellowish to green-brown discoloration of the surface waters.

Maximum cell densities were detected at La Fosca, St. Pol, Paguera (S.ta Ponça area), and Vulcano (Baia di Ponente). This last site, was subject to a first event of discoloration in early August 1999, followed by another late summer outburst. The environmental/meteorological scenario was comparable with that recently reported from La Fosca, Spain [14]. In contrast, a high amount of dissolved inorganic nitrogen (DIN) - mainly due to ammonium (99%) – was found in late summer at the Aeolian location, being probably connected with the increased anthropic pressure and discharge of sewage. In terms of biomass, *A. taylori* blooms represented exceptionally significant proportions, with maximum abundance on 2 August (12 x 10^6 cells l^{-1}; 135 µg of Chl *a*) under balanced nutrient ratio (N:P = 15). Thus, a relevant role of nutrients in the bloom development and maintenance cannot presently be ruled out at the Italian site, requiring further studies extended over all the summer period.

Molecular analyses

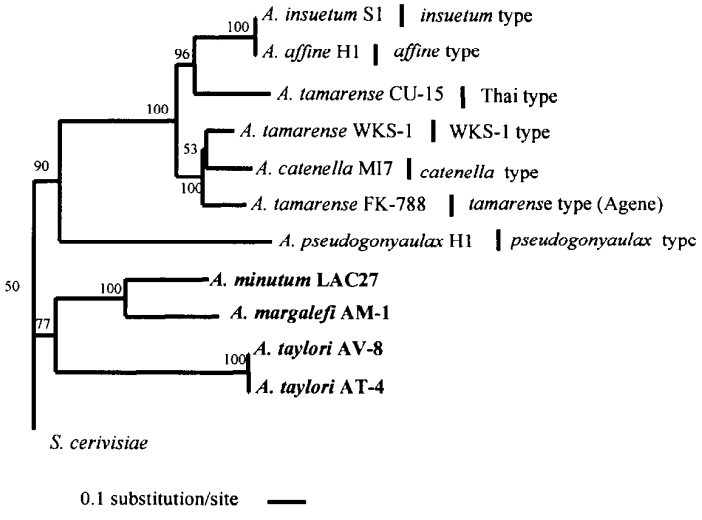

Fig. 3. Molecular phylogenetic tree inferred from the 5.8S rDNA and ITS in *Alexandrium* species using the neighbor joining method (Kimura 1980). A distance of 0.1 is indicated by the scale. The numbers at nodes indicate the bootstrap value (100 replicates) (≥ 50%). *Saccharomyces cerevisiae* was used as an outgroup.

Table 2. Distance values between the 5.8S rDNA and ITS sequences of 11 representative isolates of *Alexandrium*.

	1	2	3	4	5	6	7	8	9	10	11
1. *A.taylori* AT-4	0.0000										
2. *A.taylori* AV-8	0.0000	0.0000									
3. *A.affine* H1	1.2965	1.2965	0.0000								
4. *A.insuetum* S1	1.2965	1.2965	0.0000	0.0000							
5. *A.tamarense* FK-788	1.3460	1.3460	0.3982	0.3982	0.0000						
6. *A.tamarense* WKS-1	1.2097	1.2097	0.3460	0.3460	0.2068	0.0000					
7. *A.tamarense* CU-15	1.2957	1.2957	0.3630	0.3630	0.4438	0.3937	0.0000				
8. *A.catenella* MI7	1.2676	1.2676	0.4279	0.4279	0.2355	0.1961	0.4765	0.0000			
9. *A.pseudogonyaulax* H1	1.5903	1.5903	1.1124	1.1124	0.9949	1.0615	1.1402	1.0679	0.0000		
10. *A.margalefi* AM-1	0.9558	0.9558	1.1317	1.1317	1.1754	1.0615	1.1262	1.1517	1.1196	0.0000	
11. *A.minutum* LAC27	0.9142	0.9142	1.1255	1.1255	1.0486	0.9467	1.1489	0.9381	1.1717	0.4718	0.0000

The ITS-5.8S rDNA regions of the four *Alexandrium* Mediterranean isolates available in our laboratory (*A. taylori* AT-4 and AV-8, *A. margalefi* AM-1, *A. minutum* LAC27) were sequenced and compared with the ITS-5.8S rDNA regions of other *Alexandrium* species obtained from GenBank (Table 1). The rDNA regions vary in fragment length only at the species level. The 5.8S rDNA conserved region length is identical in all the species, except *A. margalefi* AM-1, which is one base longer. There was no sequence variation between the Italian and Spanish *A. taylori* strains, but a high level of variability in the ITS regions was found between the species of *Alexandrium*.

The distance tree (Fig. 3) derived from a neighbor-joining analysis of the ITS-5.8S rDNA revealed a clear divergence of the *A. taylori*-clade from the *A. tamarense/catenella* group. The maximum distances were found with *A. pseudogonyaulax* H1 and *A. tamarense* FK-788 (Table 2). Further, *A. margalefi* AM-1 and *A. insuetum* S1 (subgenus *Gessnerium*) clustered with *A. minutum* LAC27 and *A. affine* H1 (subgenus *Alexandrium*), respectively. Thus, previous groupings of species based on morphological criteria appeared in some cases incongruous with the molecular data reported here. Since this study was limited to the comparison of the ITS-5.8S rDNA sequences of a few *Alexandrium* Mediterranean species with *A. tamarense/catenella* Asian isolates, more comparative data on the ITS-5.8S and 18S rDNA sequences are needed to better define the phylogenetic position of *A. taylori*.

The molecular information presented here provides data for the design of unique target sequence for *Alexandrium* probe design: the conserved 5.8S rDNA gene has been used to obtain an oligonucleotide probe (ITSAa) targeting the genus level [8]. The highly variable ITS1-ITS2 regions may be useful for the designation of a species-specific probes which could speed the routine monitoring of *A. taylori* in the Mediterranean.

Additional studies are in progress to define genetic divergences and species boundaries, based on the evidence that further *Alexandrium* key-species are affecting new coastal areas of the Mediterranean.

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GENETIC ANALYSIS OF THREE SPECIES OF *DINOPHYSIS* CAUSING DIARRHETIC SHELLFISH OUTBREAKS IN GALICIA (NW SPAIN).

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ABSTRACT

The development of a protocol for the preparation of DNA from single cells clean enough to be used in enzymatic treatment such as PCR, allowed us to amplify the rDNA-ITS and 5.8S rRNA gene of three *Dinophysis* species from the Galician Rías (NW Spain), *D. acuminata, D. sacculus* and *D. tripos*, that are associated with Diarrhetic Shellfish Poisoning episodes. Sequence analysis of this region showed 99% sequence identity between *D. acuminata* and *D. sacculus*, suggesting that they might be morphotypes of the same species. However further studies need to be performed to completely ascertain this matter.

INTRODUCTION

Species of the genus *Dinophysis* are the main organisms responsible for the presence of Diarrhetic Shellfish Poisoning (DSP) toxins in European bivalves, causing prolongued closures of bivalve marketing and considerable economic losses to shellfish industries. *Dinophysis acuminata* Claparède & Lachmann is the main agent of DSP episodes in Southern Galicia [1, 2] but its toxin content has proved to be extremely variable, and values ranging from traces to 40 pg okadaic acid (OA) · $cell^{-1}$ have been reported [3, 4, 5]. *D. sacculus* Stein is the most abundant *Dinophysis* species in Northern Galicia (north of Cape Finisterre) and in the Mediterranean Sea, but toxic episodes associated with this species are very mild if any. Preliminary results from Catalonia specimens showed very low levels of toxin per cell (1-5 pg OA· $cell^{-1}$) [6]. *Dinophysis tripos* Gourret is a minor component of the *Dinophysis* assemblage in Southern Galicia and Portugal [7], and its contribution to total toxicity in this area has not been established, however Japanese specimens showed high toxin content (36 pg OA · $cell^{-1}$) [8].

D. acuminata and *D. sacculus* are distinguished morphologically by the shape of their large hypothecal plates, a character which varies considerably in both species [9]. Where both morphospecies co-occur, intermediate forms appears which are difficult to ascribe to one species or the other. Identification and species counting are therefore difficult for monitoring centres that must survey the presence of potentially toxic phytoplankton species to comply with European Union directives [10]. In some cases, intraspecific variations in morphology and toxin content might be attributed to biological (potential sexual cycle, cell cycle, feeding behaviour) and environmental factors [11, 12, 13], but it is possible that variations may be caused by genetically differentiated strains or regional varieties of the same species. To date, establishing long term cultures of *Dinophysis* has proved impossible and, combined with the usually low concentrations of field populations (< 200 cell · l^{-1}) this has limited studies of life cycle, toxicology and genetic variability of *Dinophysis* species, to those based on single cell isolations by capillary micromanipulation. The development of advanced molecular techniques capable of processing very small samples is essential to improve our knowledge of *Dinophysis* species. This paper presents results on the genetic analysis of natural populations of *Dinophysis acuminata, D. sacculus* and *D. tripos* from Galician coastal waters.

MATERIALS AND METHODS

Dinophysis cells were collected using vertical phytoplankton net-hauls (10 or 20-µm mesh size) in the Rias Bajas (NW, Spain) during different Diarrhoeic Shellfish Poisoning (DSP) outbreaks and seasons between 1994 and 1999. Net hauls were filtered through a 100-150 µm sieve to eliminate larger organisms; aliquots of water samples were placed in sedimentation chambers, and *Dinophysis* cells were identified under inverted microscope with 25x and 100x magnification.

Cells were isolated with a capillary pipette and transferred to 0.22 µm (Millipore) filtered sea water. Finally the cells were placed in buffer A (_ volume of 1x PBS; _ volume of 0.5M EDTA pH 9.5) and mixed with 1.6 % low melting agarose (InCert Agarose, FMC BioProducts) at 37°C. The cell-agarose mixture was then deposited as a drop on parafilm and kept at 4°C until solidification. The agarose beads were incubated at 26°C in solution B (0.4 M D-Mannitol (Sigma) pH 5.8; 1% Cellulase (Calbiochem); 1% Hemicellulase (Sigma)) for 3 hours and then with ESP solution (0.5M EDTA pH 9.5; 1% Lauroyl-Sarcosine (Sigma); 1mg/ml Proteinase K (Sigma)) for 48 hours at 50°C (15). Agarose embedded DNA was stored in ESP at 4°C until use. Before using these samples for PCR an exhaustive wash with TE buffer (10mM Tris-HCl, 1mM EDTA pH 8.0) was performed.

PCR amplification and analysis of products

The 5.8S rDNA and flanking internal transcribed spacers (ITS1 and ITS2 regions) were amplified using ITSA and ITSB primers [14]. The primers were synthesised by Isogen Bioscience BV. Amplifications were performed in a Perkin Elmer Gene Amp PCR System 2400. Reaction mixtures contained in 100 µl:

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agarose embedded genomic DNA, 250 µM each of dATP, dCTP, dGTP, dTTP, 100 pmol of each primer, 2.5 mM $MgCl_2$, 1x reaction buffer Perkin Elmer and 2.5 U of Taq Polymerase (Perkin Elmer). Thermal cycling was as follows: 94°C for 5 min; 30 cycles of 1 min at 94° C, 1 min at 40°C and 1 min at 72°C; and an additional elongation step of 7 min at 72°C.

The PCR products were analysed by electrophoresis on 1% agarose gels. The band containing the 5.8S rRNA gene and the ITS1 and ITS2 was purified from the agarose gel using a QIAquick Gel Extraction Kit (50) (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Sequencing of purified DNA was performed at the Department of Molecular Biology, Universidad Autonoma of Madrid with primers ITSA and ITSB. Nucleotide sequence alignment and comparisons were carried out by using the BLAST and PILEUP programs (GCG software package).

RESULTS AND DISCUSSION

To date little is known of the biochemistry, genetics and ecology of species of *Dinophysis* because they are notoriously difficult to culture and remain largely recalcitrant to most routine culture protocols. For molecular biology applications, the preparation of clean DNA is required, however the existing protocols for this purpose [14] need to start with a high number of cells. This is particularly difficult to achieve for *Dinophysis* species since cells have to be individually collected by micromanipulation from seawater samples. We have developed a method to prepare, from samples as little as one cell, DNA clean enough to be used for PCR amplification [15]. The method used to prepare DNA uses combined physicochemical and enzymatic procedures on cells embedded in agarose plugs or beads. The use of this method for DNA preparation has allowed us to amplify by PCR and sequence the ITS1-5.8S rRNA gene-ITS2 region of three *Dinophysis* species: *D. acuminata*, *D. sacculus* and *D. tripos* (Fig 1). For *Dinophysis* species no sequence of this region is available. Since the primers ITSA and ITSB have been used successfully for different species of dinoflagellates we tried them on *Dinophysis* DNA. Only morphotypes unequivocally corresponding to these three species were used (Fig.2).

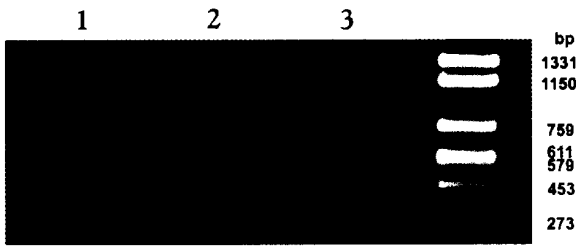

Fig.1. PCR products obtained from 1) *D. acuminata*, 2) *D. sacculus* and 3) *D. tripos*, with the primers for the ITS1-5.8S rRNA-ITS2 genomic region.

All the samples were successfully amplified and a band of similar size around 650 bp was obtained. This band from the three species was purified from the agarose gel and sequenced. The sequence shows that they correspond to the expected locus.

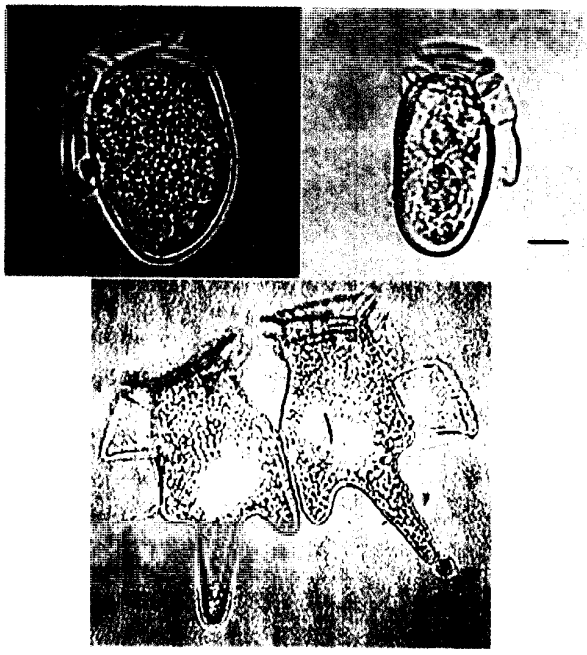

Fig.2. Light microscopy observations of a) *D. acuminata*; b) *D. sacculus*; c) *D. tripos*. Scale bars = 10 µm.

Analysis of the sequences (Fig 3) indicates a very high sequence similarity between *D. acuminata* and *D. sacculus* (99%), while sequences from *D. tripos* were more divergent (approx. 88%). The highest sequence similarity is found in the 5.8S rRNA sequence. The ITS regions of the three species are also quite similar. It is usual that related species of many organisms show higher similarities in their respectives ITS2 regions than in the ITS1 regions, however this is not the case for the *Dinophysis* species studied here. The 5.8S rDNA sequence is similar to that of other dinoflagellates species, showing highest similarity with *Prorocentrum micans* (84.9%), *Gyrodinium impudicum* (84.3%) and *Gymnodinium sanguineum* (83.6%).

The ITS sequences have frequently been used for phylogenetic studies in many other species. Nevertheless, the results presented here suggest either that the ITS sequences are not useful for the genetic characterization of some *Dinophysis* species or that in the case of *D. acuminata* and *D.sacculus* we are dealing with different morphotypes or regional varieties of a species with high morphological plasticity.

Edvardsen et al. [16] also found that ITS1 sequences in *D. acuminata* and *D. norvegica* isolates from Norwegian waters were very similar, with maximum differences of less than 10 bp. More molecular studies need to be done with more species of *Dinophysis* from different regions to analyze the genetic variability within the species of this genus.

```
                1                          ↓ITS1                                    60
D. acuminata    GAACCTGCGG AAGGATCATT --CGCACGCA TCCAATATCC ATAACTTGAA ATTT-CTTGT
D. sacculus     .......... .......... --........ .......... .......... ....-.....
D. tripos       C..G..T..- ...CG-..A. AAG..T.--T .-..G.CC.. .......... ....T..-..

               61                                                              120
D. acuminata    GTGAGCTTCT GGGTGAGGTT GAACAAAGTG TTGCCTTCAT GTGGAAGCTC GAGGGTAGAT
D. sacculus     .......... .......... .......... .......... .......... ..........
D. tripos       .......... .......... .......... .......-.. .......... ..........

               121                                                             180
D. acuminata    GAACTGAAGC AGTGTGGTCT TGCTGTTTCG TGGGCGCTAC CGTCTGCTTG GCTCACACTG
D. sacculus     .......... .......... .......... .......... .......... ..........
D. tripos       .......... ....CT.C.. .....C.... .......... .......... ...A...T..

               181                                                  ↓5.8S rRNA 240
D. acuminata    CCTTGCGGTT GAACCTTTAT TT--GTTTGT ATGTGCATGC TGTATGTATC ACAATTTTCA
D. sacculus     .......... .......... ..--...... .......... .......... ..........
D. tripos       ......T... .GG....... G.AA...... G.......AT ...C..CTC. ..........

               241                                                             300
D. acuminata    GCGATGGATG CCTCGGCTCG AACAACGATG AAGGGCGCAC CGAAGTGTGA TAATCTTTGT
D. sacculus     .......... .......... .......... .........G .......... ..........
D. tripos       .......... .......... .......... .......... ....C..... ..........

               301                                                             360
D. acuminata    GAATTGCAGA ATTCCGTGAA TCAATAGAGC TTTGAACGTG TCTTGCGCTT TCGGGCTATA
D. sacculus     .......... .......... .......... .......... .......... ..........
D. tripos       .......... .......... .......... .......... .T........ ..........

               361                          ↓ITS2                              420
D. acuminata    CCTGAAAGCA TGCCTGCGTT GGTGTCTGTA TGGCCTCATT CAGTCAGCAA CACTAAACCT
D. sacculus     .......... .......... .......... .......... .......... ..........
D. tripos       .......... .TTT...... .......... ...T...... .......... ..........

               421                                                             480
D. acuminata    CATTGGTAGT GTGATGTCTG TGTGTCGGTG TGTGC-AAGG TTCACCCTTG TTACACAGAA
D. sacculus     .......... .......... .......... .....-.... .......... ..........
D. tripos       T..G...... ...C..AT.. ......AA.. A..T.A.... G......... .....AC...

               481                                                             540
D. acuminata    -TGCATGTTT ATTATCGTTG CCCAACAGCT TGCTGTGCCT CTGTATAGTG GTTAACCTTC
D. sacculus     -.......... .......... -.......... .......... .......... ..........
D. tripos       A......C.. T..G...... -.......... ........-- ....G..A.. ......T...

               541                              572
D. acuminata    TGTTGGCACG AAATCCAAAG CACATGCCCC -A
D. sacculus     .......... ......-... .......... C.
D. tripos       .......... ..C...-... ......T.A. -.
```

Fig.3. Alignment of ITS1, ITS2 and the 5.8S rRNA coding region for *D. acuminata, D. sacculus and D. tripos.* Identities in % are: *D. acuminata:D. sacculus*: 99; *D. acuminata: D. tripos*: 87; *D. sacculus: D. tripos*: 88.

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MOLECULAR CHARACTERIZATION AND CLASSIFICATION OF THE CIGUATERA DINOFLAGELLATE *GAMBIERDISCUS.*

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ABSTRACT

The benthic dinoflagellate *Gambierdiscus* is regarded as the primary causative agent of Ciguatera Fish Poisoning, a disease prevalent in the Pacific regions, the Carribean and the Indian Ocean. Six distinct species within this genus, most of them being toxic, have been described so far on the basis of morphological criteria. The genetic characterization of various strains of *G. toxicus* has also been addressed in recent studies. In the present work, we investigated the usefulness of the rRNA genes for the molecular characterization and classification of *Gambierdiscus* isolates distributed in 5 of the 6 species described to date.

Sequences of the 5.8S+ITS rDNA and the LSU rDNA D8-D10 regions of 11 Polynesian isolates were compared for phylogenetic analysis. Both regions proved to be useful biogeographical markers, as a grouping of these isolates according to their geographic origin (northern versus southern islands) was globally observed.

To investigate the potential interest of the LSU rDNA D8-D10 regions in *Gambierdiscus* systematics, sequences of 8 isolates from distinct geographic origins, distributed among 5 of the 6 morphospecies described to date, were also compared. Four molecular types corresponding to *G. australes*, *G. polynesiensis*, *G. yasumotoi* and *G. toxicus/G. pacificus* lineages could be distinguished. These results suggest that, within this genus, genetic data are fairly consistent with morphological classification by SEM microscopy. They also indicate that distinguishing species of *Gambierdiscus* can combine both molecular and traditional morphological criteria.

INTRODUCTION

Ciguatera Fish Poisoning (CFP) is a serious problem affecting public health and fishing industries in tropical regions. Its primary causative agent is a benthic dinoflagellate, *Gambierdiscus toxicus* Adachi et Fukuyo, first discovered in the Gambiers Islands in the late 70s [1]. Species recognition in thecate dinoflagellates is primarily based on their plate morphology e.g. the shape and size of cells, apical pore (Po) and posterior intercalary (1p) plates which provide useful conservative characters [4]. For instance, light microscopy was used to describe *G. toxicus* [1] whereas SEM was used to identify *G. belizeanus* Faust and *G. yasumotoi* Holmes [9,13]. However, morphology-based species identification remains difficult because phenotypic convergence or plasticity of cells cannot be completely ruled out, especially when laboratory-acclimated clones are compared to cells collected in the field. For this reason, recent studies tend to combine both morphological and molecular (DNA) analyses. Using this approach, three additional toxic species of *Gambierdiscus* were recently identified from French Polynesian coral reef habitats, *G. australes* Faust et Chinain, *G. pacificus* Chinain et Faust, and *G. polynesiensis* Chinain et Faust [7].

DNA analyses have proven to be a very useful tool in *Gambierdiscus* systematic and phylogenetic studies. Previous studies based on *G. toxicus* SSU- and LSU rRNA genes have revealed its specific phylogenetic position as compared to other dinoflagellate species, as well as the existence of a great genetic diversity among clones of this species [3,6,18]. However, a number of molecular studies of various groups of algae often show inconsistencies between morphology classification and molecular data in several dinoflagellate species [8].

To contribute to the debate, we examined the relationships between morphotaxonomy and molecular classification in the genus *Gambierdiscus*, by means of the sequence comparison of the 5.8S+ITS and D8-D10 LSU rRNA genes of various isolates. The potential interest of these 2 rDNA regions as useful biogeographical markers was first investigated among 11 Polynesian clones of *Gambierdiscus* distributed among the morphospecies *G. toxicus*, *G. australes*, *G. pacificus*, *G. polynesiensis*. The phylogenetic tree inferred from the D8-D10 LSU rDNA sequence comparison of 8 clones distributed among 5 different morphospecies of *Gambierdiscus*, including *G. yasumotoi*, was also examined, to assess the taxonomic interest of this region at the species level.

MATERIAL AND METHODS

Origin of isolates

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Fifteen clones from various ciguatera-endemic areas were isolated from *Jania* sp. and *Amphiroa* sp. macroalgal substrates attached to coral rubbles. They were maintained in laboratory conditions for >3 years. The following *Gambierdiscus* species were examined: *G. toxicus* isolates: GTT2, GTT-91; PAP1 (Tahiti); TUR (New Caledonia); REN (La Réunion) // *G. pacificus* isolates: GTT5; HIT25 (Tahiti); HO-91; HO5 (Hao); MUR (Mururoa) // *G. australes* isolates: RAV-92 (Raivavae); GAMB4 (Mangareva) // *G. polynesiensis* isolates: RG-92 (Rangiroa); TB-92 (Tubuai) // *G. yasumotoi* isolate: GTY (Singapore). For a detailed map of the Polynesian sampling locations, please refer to [5]. Morphospecies assignments for each *Gambierdiscus* isolate were based on either SEM descriptions [7], or epifluorescence observations of plate patterns using Calcofluor White M2R staining method [11] combined with PCR assays using species-specific primers sets.

PCR amplifications

Protocols used for total DNA extraction and purification followed published methods [2]. The ITS regions containing the 5.8S rDNA were amplified using ITSA and ITSB primers, complementary to the 3' end of the SSU rDNA and the 5' end of the LSU rDNA, respectively [2]. The so-called D8, D9 and D10 hypervariable domains of the LSU rDNA were also amplified using FD8 and RB primers [7], because of their potential interest in phylogenetic and taxonomic analyses of closely related species [15]. PCR reactions typically contained a 50 µL mixture: 2U Taq Polymerase (Promega), 1X reaction buffer (Promega), 10-100 ng total genomic DNA, 5 mM of each dATP, dCTP, dGTP and dTTP (Pharmacia), 1.5 mM $MgCl_2$ and 100 pmol of each primer. Amplifications were conducted as follows: 4 min denaturing at 94°C followed by 25 cycles of 30s denaturing at 94°C, 1 min annealing at 55°C, 2 min elongation at 72°C, and completed with a final elongation step of 10 min at 72°C.

DNA sequencing

PCR products were cloned into pAmp1 vector using the CloneAmp^R pAmp1 kit (GibcoBRL). Plasmids were propagated in *Escherichia coli* strain XL1Blue. Plasmid preparations were obtained using PlasmidPURE^{TM} DNA Miniprep kit (Sigma). For each *Gambierdiscus* isolate, 5 bacterial clones containing plasmids with a single insert were selected and sequenced to resolve heterogeneities at intra-clone level. Both strands of the inserts were sequenced using BigDye™ Terminator Cycle Sequencing ready reaction kit (PE Applied Biosystems) and primers M13 and rM13 [7] of the pAmp1 vector.

Phylogenetic analyses

Sequence data were aligned using Clustal V [12] and phylogenetic analyses were performed using the distance method. The aligned data set was converted to a distance matrix using the Kimura two-parameter correction of the DNADIST computer program of PHYLIP 3.5c package [10,14]. A phylogenetic tree was constructed from the distance matrix using the neighbor-joining algorithm [17] of PHYLIP. The sequence from the dinoflagellate *Prorocentrum micans* was used as outgroup to root the trees. As an indication of confidence in the branching order, a bootstrap analysis (1000 replications) was completed. The phylogenetic trees shown are majority-rule (50%) consensus trees.

RESULTS

PCR amplifications of the 5.8S + ITS rDNA and LSU rDNA D8-D10 regions of the *Gambierdiscus* isolates examined in this study resulted in fragments of ≈ 450 bp and 950 bp, respectively. Sequence data confirmed the ribosomal nature of these fragments.

Biogeographic patterns.

The phylogenetic trees inferred from the multiple sequence aligment of the 5.8S + ITS and D8-D10 LSU rDNA of 11 *Gambierdiscus* isolates distributed among the morphospecies *G. toxicus*, *G. australes*, *G. pacificus* and *G. polynesiensis* are shown in Fig. 1A & 1B, respectively. In both trees, isolates tended to cluster into 2 main groups representative of Northern Islands (Tahiti and Rangiroa) and Southern Islands (Tubuai & Raivavae in the Australes Archipelago, and Mururoa & Mangareva in the Gambiers Archipelago), with the exception of TB-92 and RG-92 *G. polynesiensis* isolates which fell variously into the North (Fig. 1A) or the South group (Fig. 1B).

Morphological versus genetic variations.

Sequence data revealed a small degree of heterogeneity between D8-D10 LSU rDNA clones for a given *Gambierdiscus* isolate: % identity varied between [96%-100%] for *G. yasumotoi*, *G. australes*, *G. pacificus* and TUR and REN *G. toxicus* isolates, and between [92%-100%] for *G. polynesiensis* and GTT-91 *G. toxicus* isolates (data not shown). The clustering patterns observed in Fig. 2 indicate that *G. yasumotoi* significantly differed from the other four morphospecies. Grouping of *G. polynesiensis* isolates (RG-92, TB-92) within the same clade was strongly supported by bootstrap values (100%) while this species joined *G. australes* (RAV-92) with moderate suppor (77%) (Fig. 2). Grouping of *G. toxicus* isolates (GTT-91, REN and

TUR) with *G. pacificus* (HO-91) was also supported by high booststrap values (100%).

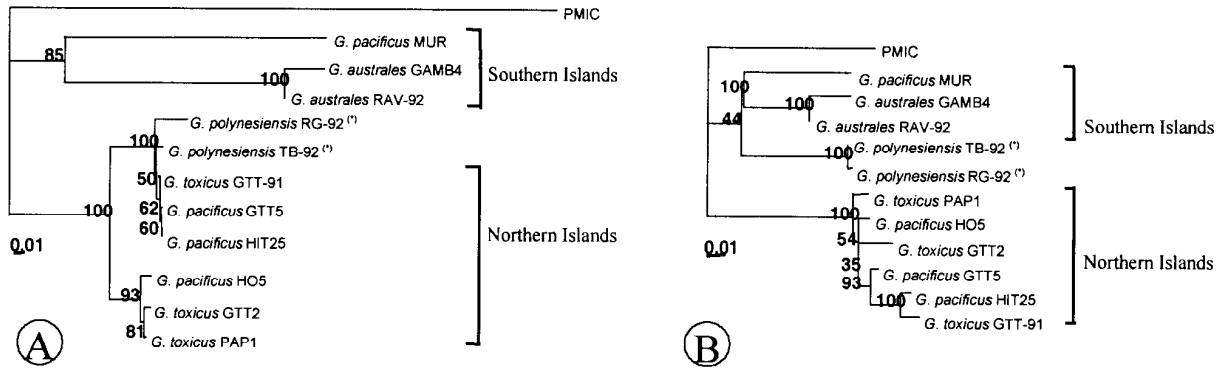

Fig. 1. Phylogenetic analysis of relationships among 11 *Gambierdiscus* isolates from French Polynesia, performed on A) 5.8S + ITS rDNA and B) D8-D10 LSU rDNA aligned sequences using a distance method. Outgroup species was *Prorocentrum micans* (PMIC). Bootstrap values (1000 replications) are given at the internal nodes. The trees were constructed using a neighbor-joining analysis of a Kimura distance matrix. Bar: 1% divergence. (*) TB-92 and RG-92 originate from Tubuai (southern island) and Rangiroa (northern island), respectively.

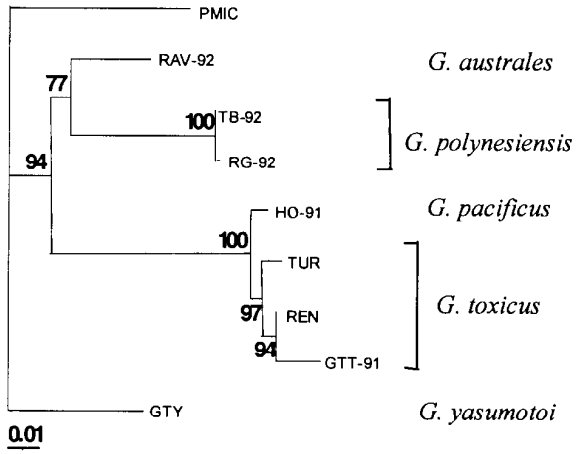

Fig. 2. Phylogenetic analysis of relationships between 8 *Gambierdiscus* isolates distributed among 5 of the 6 morphospecies described to date, and performed on D8-D10 LSUrDNA aligned sequences using a distance method. Outgroup species was *Prorocentrum micans* (PMIC). Bootstrap values (1000 replications) are given at the internal nodes. The tree was constructed using a neighbor-joining analysis of a Kimura two-parameter distance matrix. Bar: 1% divergence.

DISCUSSION

So far, 6 distinct morphospecies are described within the genus *Gambierdiscus*: *G. toxicus*, *G. belizeanus*, *G. yasumotoi*, *G. australes*, *G. pacificus* and *G. polynesiensis* [1,7,9,13]. *G. belizeanus* and *G. yasumotoi* look morphologically quite distinct from the 4 other morphotypes, with respect to cell shape and thecal plates areolation [9,13] whereas *G. toxicus*, *G. australes*, *G. pacificus* and *G. polynesiensis* appear morphologically more similar (for more details about morphological differences, see [7]). Previous studies also provided evidence that biochemical and genetic heterogeneities exist within *G. toxicus* populations [3,5,6]. These observations are consistent with the idea that marine phytoplankton have remained conservative in form while accumulating genetic variability within them [20]. This explains why, for some groups of dinoflagellates, it might be appropriate to complement morphological features with molecular data, when referring to a particular morphospecies [2,7]. In this study, we applied molecular biological methods to the characterization and classification of several *Gambierdiscus* isolates distributed among 5 of the 6 morphospecies described to date, i.e. *G. toxicus*, *G. yasumotoi*, *G. australes*, *G. pacificus* and *G. polynesiensis*.

The potential role of 5.8S + ITS and D8-D10 LSU rDNA as biogeographic markers was first assessed among 11 Polynesian isolates of *Gambierdiscus*. Globally, the sequence comparison of these 2 rDNA regions resulted in the grouping of these isolates on the basis of geographic origin, suggesting that these two groups of markers may be useful for fine-scale population analyses of *Gambierdiscus*. Our findings parallel those of Adachi et al. [2] and Scholin et al. [19] conducted on the 5.8S + ITS and D1-D2 LSU rDNA regions, respectively, of *Alexandrium* isolates. This

biogeographical pattern of rDNA variation, however, did not apply to *G. polynesiensis* isolates which seemed to cluster more on the basis of morphology than geographic origin. However, the possibility that this clustering pattern may just reflect the existence of a species cline from North to South, with *G. polynesiensis* being an intermediate morphotype between *G. toxicus/G. pacificus* lineage in the north and *G. australes* in the south is unlikely, as (i) *G. pacificus* MUR isolate was found to cluster with *G. australes* clones (Fig. 1A & 1B) and (ii) *G. polynesiensis* appear morphologically more similar to *G. toxicus* as compared to *G. australes* and *G. pacificus*, especially with respect to plate patterns (e.g. 1p plate) [7].

The taxonomic interest at the species level of *Gambierdiscus* D8-D10 LSU rDNA regions was also demonstrated, as comparison of their sequences among 8 *Gambierdiscus* clones distributed in 5 of the 6 morphotypes described to date, proved useful in discriminating the species *G. yasumotoi*, *G. polynesiensis*, *G. australes* and *G. toxicus* from one another. The clustering pattern of *G. yasumotoi* (GTY) which appeared clearly different genetically from the 4 other morphotypes is fairly consistent with morphological data [13]. Further investigations on *G. belizeanus* specimens would be necessary to confirm such a result. The D8-D10 LSU rDNA sequences, however, showed some limits in discriminating *G. pacificus* from *G. toxicus*, as our results tend to indicate that isolates HO-91, TUR, REN and GTT-91 should be regarded as one unique molecular type despite their classification in 2 distinct morphogroups [7]. In conclusion, the five morphospecies examined in this study could be classified into four distinct molecular groups, corresponding to *G. polynesiensis*, *G. australes*, *G. yasumotoi* and *G. pacificus/G. toxicus* lineages.

This discrepancy in morphological and molecular classifications points out the issue of the species concept currently debated in dinoflagellates. Truly, for species (or genera) in which apparently identical morphological features mask genetic diversification, molecular data can provide useful, additional taxonomic and genetic markers at the species and/or population levels. In term, this should allow the development of a "molecular species concept". However, it is also clear that molecular data should not be seen as the answer to species problems, as they appear to be confronted with many of the same difficulties as morphological data, i.e. some appear to be informative whereas others can be misleading [16], as illustrated by the results in *G. toxicus* and *G. pacificus* phylogeny. In conclusion, our data indicate that distinguishing species of *Gambierdiscus* should combine both molecular and traditional morphological criteria.

ACKNOWLEDGEMENTS

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A MOLECULAR ANALYSIS OF CYANOBACTERIAL BLOOM EVENTS IN ONE WATER BODY

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ABSTRACT

Bloom-forming toxigenic cyanobacteria occur worldwide and some species, such as *Microcystis aeruginosa* and *Anabaena circinalis*, are a particular problem in Australia. This study focuses on a molecular analysis of cyanobacterial blooms at one site in the New England region of Australia. Genetic variation was determined by examination of the intergenic spacer region (IGS) of the β- and α-phycocyanin subunits. We show that it is possible to routinely obtain template for DNA amplification by PCR and differentiate the strains in bloom samples. Differentiation of PCR products was by restriction endonuclease digestion of the IGS (Restriction Fragment Length Polymorphisms [RFLPs]) and/or DNA sequencing. Genotypically different strains of the one morphotype have been identified in samples from a succession of bloom events at one site.

INTRODUCTION

Taxonomists have traditionally used morphology as the basis for identification of cyanobacterial species. However, in many species, some taxonomic characters can be quite variable. For example, in an examination of serial collections of *Anabaena circinalis* taken from recurrent blooms in dams in New South Wales, Australia, over several years, variations in morphology were noted [1], such as trichome coiling, shape of akinete and relative position of heterocysts. A further problem is that the identification of Australian taxa has been based on taxonomic texts from Europe and North America. The assumption of taxon identity from 'comparative iconography' may not be valid for all Australian taxa [2].

Recent advances towards a molecular taxonomy for the bloom-forming cyanobacteria have shown that DNA sequencing can provide strain-specific information and form the basis for a phylogenetic description of organisms [3, 4, 5].

Phycocyanin is one of the accessory pigment proteins in the photosynthetic apparatus of cyanobacteria, rhodophytes (red algae) and cryptophytes, and it is highly conserved. The phycocyanin operon consists of five open reading frames (ORFs), separated by noncoding intergenic spacers (IGS) which may be highly variable. Studies have shown that the IGS sequence between the β- and α-subunit ORFs, which encode for phycocyanins *b* and *a* respectively, can be used to differentiate cyanobacterial species and strains [4, 6, 7]. The β-α-phycocyanin IGS shows variation in the strains of known toxigenic cyanobacterial species throughout the world [4, 6, 7] although one study has shown that genetic variation of strains within one region may be limited [7]. However, it should be noted that these studies employed only strains cultured under axenic conditions. We decided to employ PCR to amplify the IGS from environmental samples, thus providing an analysis of all bloom strains/species and circumventing the need to obtain isolates and ensuing problems of culturability.

The study area was the New England tablelands region of Australia, situated about mid-way between Sydney and Brisbane and 120 km inland from the eastern seaboard. Drinking water for the city of Armidale (population 26,000) is supplied from Malpas Dam, located at an elevation of 1170m above sea level. The average annual rainfall is 890mm. Average summer temperatures range from 10^0 to 23^0C, with a winter range of -1^0 to 10^0C. The catchment area lies in grazing land and contains basalt rock with a relatively high phosphorous content, about three times the world average [8]. Levels of soluble reactive phosphorous in Malpas Dam exceed the value considered conducive to cyanobacterial growth [9]. Because of the relatively low rainfall and low turnover of water, cyanobacterial blooms frequently occur and are occasionally toxic. Both hepatotoxicity from *M. aeruginosa*-dominated blooms and neurotoxicity from *A. circinalis*-dominated blooms have been detected by mouse bioassay [10].

MATERIAL AND METHODS

Sampling and amplification of DNA

Water samples were collected at dams in the New England region as part of the regular monitoring procedure. A 1L sub-sample was used for microscopic and molecular analysis. Small fractions were examined microscopically and the species composition and features, such as presence of akinetes, were noted. DNA from bloom samples was extracted by a modification of a technique originally developed for plant material [11]. Oligonucleotide primers were used to amplify, as previously described [4], an approximately 700 bp segment of DNA spanning the intergenic spacer (PC-IGS). The sequences of the primers are:

PCβF (forward) 5'-GGCTGCTTGTTTACGCGACA-3'
PCαR (reverse) 5'-CCAGTACCACCAGCAACTAA-3'

RFLP analysis

The PCR products were purified (QIAquick PCR Purification Kit, Qiagen) and digested separately with the enzymes *Alu* I, *Hha* I, *Sau* 3AI, *Scr*FI and *Hae* III (New England Biolabs). RFLP patterns were analyzed by gel

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electrophoresis in 1.8% Metaphor agarose (FMC BioProducts) in TBE. As a reference, RFLP analysis also included PCR-amplified products from cultures of *A. circinalis* UNSW 1 & UNSW 2 and *M. aeruginosa* PCC 7806.

Sequence analysis of PC-IGS PCR products

The PCR products were cloned, using a pGem®-T Easy Vector System (Promega Corp.). The plasmids with inserts were isolated and purified using Wizard® Minipreps (Promega Corp.). The plasmid DNA was used for automated sequencing with dye-deoxy terminator chemistry at Sydney University Prince Alfred Macromolecular Analysis Centre.

Sequences were compared with 'Bestfit', which uses the local homology algorithm [12]. 'Pileup' was used to create multiple sequence alignments, using a simplification of the progressive alignment method [13]. The subsequent multiple sequence files were used for analysis with 'Eneighbour', which uses the neighbour-joining method [14]. IGS nucleotide sequences were compared to entries deposited in the databases GenBank, EMBL, DDBJ and PDB using 'BLASTN' [15].

RESULTS AND DISCUSSION

RFLP patterns

RFLP patterns from digestions with the enzymes *Alu* I, *Sau* 3AI, *Scr*FI and *Hae* III did not differentiate the Malpas bloom samples. However, four different RFLP patterns were produced with restriction enzyme *Hha* 1, indicating differing strain composition between all four blooms (Fig. 1). Interestingly, all four samples appeared by microscopy to contain only one morphotype, *Anabaena circinalis*.

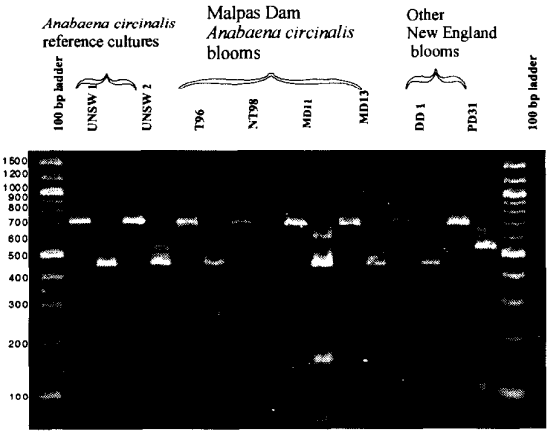

Fig. 1. Restriction digests of the PC-IGS of bloom samples with *Hha* 1. For each sample, the undigested PCR product precedes the digested product.

In some cases, we could deduce from the size of the PCR products (prior to restriction enzyme digestion) the presence of more than one type of PCR amplification product and thus the presence of more than one strain type. We based our deduction on the observation that amplification from reference cultures of *M. aeruginosa* PCC 7806 and *A. circinalis* UNSW 1 (data not shown), produce products of approximately 690 and 720 bps long respectively. It has been shown that some cyanobacterial species possess more than one phycocyanin operon, with both length and sequence differences between the duplicated operons [6]. This could produce two different length PCR products and different sequences in the IGS region within the one strain. However, there have been no reports of duplicated operons in the common Australian bloom-forming cyanobacteria and no indication in this study that the species examined contained more than one phycocyanin operon.

In the case of sample T96 (1996 bloom) two PCR products of approximately 690 and 720 bps could be resolved, indicating the presence of both *Microcystis* and *Anabaena*. Sequence analysis of six clones produced from a library of the PCR products confirmed that this was indeed the case: comparison of the IGS regions showed that the genotype designated as Type 2 clusters with database entries of strains of *Microcystis* (see Fig. 3 & Fig. 2). In contrast, analysis of the sequences derived from the genotype designated as Type 1 (Fig. 2), clustered with entries belonging to the genus *Nodularia* and also showed strong similarity with an entry for *Anabaena*, PCC7120. Both *Anabaena* and *Nodularia* belong to the same order, *Nostocales*. However, the length of the IGS of PCC7120 is 91 base pairs [16] compared with a length of 99 bps for the sample T96 clones. Whilst these conclusions appear reasonable, it should be noted that the current taxonomic coverage of the PC-IGS sequences in the DNA sequence databases is quite restricted and places limitations on molecular identification based on sequence similarity.

A single PCR product of approximately 720 bps was observed from sample NT98 (1998 bloom) suggesting the presence of *Anabaena* only. Restriction digestion produced an inconclusive banding pattern, too faint to discern well, and further analysis is required.

Only a single PCR product of approximately 720 bps was observed from sample MD11 (1999 bloom). However, the cumulative molecular weights of the bands from the restriction digest are much more than 720 bps (approximately 1540 bps) suggesting the presence of at least 2 PCR products. Moreover, the banding pattern is different from that produced from sample T96, thus indicating sample MD11 has a different strain composition.

A single PCR product of approximately 720 bps was observed from sample MD13 (1999 bloom, separate from MD11). As the cumulative molecular weight of the bands from the restriction digest is approximately 720 bps, the presence of only one PCR product is suggested. The banding pattern is very similar to that produced with

sample MD11, indicating (though not conclusively) that the same strain may be present in both MD11 and MD13 bloom samples.

As a comparison, we performed similar analyses on two bloom samples which occurred in two other water bodies, in the same area of Malpas Dam but with different geology and catchments. The sample from Dumaresq Dam, DD1 (1998 bloom), was microscopically a monospecific bloom of *Anabaena circinalis*. Amplification by PCR produced a single band of approximately 750 bps. The cumulative molecular weight of the bands from the restriction digest is approximately 720 bps suggesting the presence of only one PCR product. The difference between the cumulative molecular weight and the PCR band can be explained by, for example, the presence of co-migrating fragments. Alternatively, very small fragments may have been generated and can easily be missed.

The sample from Puddledock Dam, PD31 (1999 bloom), was, by microscopic examination, predominantly *Aphanizomenon*, a species frequently mis-identified as *Anabaena*. Amplification by PCR produced an apparently single product of approximately 720 bps. The cumulative molecular weight of the bands from the restriction digest was approximately the same as the PCR product indicating the sample is genotypically monospecific. The digest pattern of PCR products was clearly different from that of the samples which contained predominantly *Anabaena*.

Sequence comparisons

To investigate the efficacy of the RFLP analysis, we constructed a plasmid library of the PCR product from sample T96 and sequenced a number of the clones. Sequence analysis included both part of the β- and α-subunits encoding phycocyanin genes and the IGS region. Multiple sequence alignments of the IGS region of the cloned fragments (Fig. 2) indicated that at least two genotypes were present, designated as Type 1 and Type 2.

Interestingly, although there was a strong sequence similarity between the IGS regions of the Type 1 clones from the T96 bloom sample (*Anabaena* by microscopic examination) with both the sequences from the *Nodularia* and *Anabaena* (PCC7120), there were significant differences in the overall sizes of these regions. The size of the region from the sample T96 clones was 99 bps, whereas those from the *Nodularia* strains and *Anabaena* PCC7120 were 79 and 91 bps respectively.

Alignment of the IGS of the two Type 2 sequences showed six regions of difference (in total, 11 nucleotide differences) which cannot be accounted for by sequencing and/or proof-reading error by the *Taq* polymerase. This indicates the presence of two related strains of *Microcystis*.

Identity of clones

Comparison of the IGS sequence from one of the Type 2 clones against nucleotide sequences in the databases showed very strong identity (not less than 98%) with all *Microcystis*. Comparison of one of the type 1 clones showed strong similarity to those entries from the Order *Nostocales*, Family *Nostocaceae*, of which *Anabaena* is a member.

CONCLUSIONS

The reported work assesses the utility of PCR amplification of DNA fragments of environmental cyanobacterial bloom samples and analysis by RFLP and DNA sequencing.

```
                1                                                  50
T96-clone 1 IGS  AAATTACAAA TTAACT.CCC GTCTAAATTG AGCTTTTTTC ATAACCTGCA
T96-clone 2 IGS  AAATTACAAA TTAGCT.CCC GTCTAAATTG AGCTTTTTTC ATAACCTGCA
T96-clone 3 IGS  AAATTACAAA TTAGCTCCCC GTCTAAAATG AGCTTTTTTC ATAACCTGCA   Type 1
T96-clone 4 IGS  AAAATACAAA ATAACT.CCC GTCTAAAATG AGCTTTTTTC ATAAACTGCA
T96-clone 5 IGS  .......... .......... .......... ...AACCTGG GGCTAGTCTT
T96-clone 6 IGS  .......... .......... .......... ...TCCCTGG GGCTAGTCTT   Type 2

                51                                                 100
T96-clone 1 IGS  TGGCGTAGCC ATACCAAAC. AAGCAAGAAA CAATCTAGGA GATTTTCACC A
T96-clone 2 IGS  TGGCGTAGCC ATACCAAAC. AAGCAAGAAA CAATCTAGGA GATTTTCACC A
T96-clone 3 IGS  TGGCGTAGCC ATACAAAAC. AAGCAAGAAA CAATCTAGGA GATTTCACCA     Type 1
T96-clone 4 IGS  TGGCGGAGCC ATACAAAACA AAGCCAGAAA CAATCTAGGA GATTTTTGCC C
T96-clone 5 IGS  AATTAAACCC GTAGGAAACT TATTGCAA.. GATATTGGGA GATACCAAAA CA
T96-clone 6 IGS  AATTAAA.CC GTAGGAAACT TATTGCAAAG ATTATTGGGA GATACCAAAC C   Type 2
```

Fig. 2. Multiple sequence alignment of forward sequences of the IGS region of 6 clones of sample T96
Note the different lengths of the IGS region: Type I - 99 bps, Type 2 - 66 bps.

We have found that bloom samples can be successfully analyzed at three levels of detail. In some cases, subjecting the PCR products to gel electrophoresis enabled the resolution of fragments of differing sizes, indicating the presence of more than one genotype, although microscopic analysis indicated that the sample was monospecific. RFLP analysis also indicated differences in strain composition of samples of the one morphotype from the one site, taken at different times, and also showed which samples were likely to contain more than one type of PCR product. Sequence analysis of amplified products from one sample, which was apparently monospecific *A. circinalis*, indicated the presence of two different genera-*Anabaena* and *Microcystis*. Since both *Anabaena* and *Microcystis* blooms occur at this site, we have shown that these methods are able to detect minor components in bloom samples. Sequence analysis also revealed two different strains of the minor component, *Microcystis*.

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THE USE OF MOLECULAR TECHNIQUES TO CHARACTERISE TOXIC CYANOBACTERIA

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ABSTRACT

This study describes a number of approaches to identify and characterise toxic cyanobacteria of the genus *Anabaena* and the species *Cylindrospermopsis raciborskii*. We have used DNA sequence of a DNA dependent RNA polymerase gene (*rpoC1*) to place cyanobacterial isolates in a phylogenetic tree. We also demonstrate how derived *rpoC1* sequence can be used to design species specific PCR assays applicable to environmental samples. A series of short tandemly repeated repetitive (STRR) sequences common to a range of cyanobacteria were also targeted in PCR reactions and resulted in repeatable strain specific profiles more discriminatory than *rpoC1* sequencing alone.

INTRODUCTION

Cyanobacteria are known to occur naturally in fresh and marine waters worldwide, and have gained attention because of their potential toxicity and associated effects on human and animal health. Cyanobacteria produce toxins that have hepatotoxic, neurotoxic, cytotoxic and allergenic effects [1, 2]. *Cylindrospermopsis raciborskii* and *Anabaena circinalis* are of particular importance in Australian fresh waters. *C. raciborskii* is well known for producing the potent alkaloid hepatotoxin cylindrospermopsin and has been implicated in several significant poisoning events [3-6], whilst *A. circinalis* is the only *Anabaena* species in Australia known to produce the saxitoxin-group neurotoxins (paralytic shellfish poisons).

Baker and Humpage [7] recently identified up to seven coiled morphotypes of *Anabaena* co-existing in the Murray-Darling Basin in Australia, with 41% of samples containing *A. circinalis* and 55% of these shown to produce neurotoxins. In such instances, differentiation of *A. circinalis* from other coiled *Anabaena* species with microscopic methods is difficult and time consuming. The microscopic identification of *C. raciborskii* is also difficult, with two distinct morphotypes, coiled and straight, being observed for this species [8]. In view of the potential toxicity of these cyanobacteria, it is important that they are correctly identified when monitoring environmental samples. A combination of genotypic and microscopic identification techniques is of potential value.

Here we summarise the use of *rpoC1* gene sequence data to discriminate isolates of *C. raciborskii* and members of the genus *Anabaena*. We also illustrate how this sequence information can be used to design species specific PCR tests, using *Anabaena circinalis* as an example. Short tandemly repeated repetitive (STRR) sequences are also used to successfully discriminate straight trichome forms of *C. raciborskii* from coiled forms.

METHODS

rpoC1 PCR and sequencing

Reference and environmental strains were obtained from the algal culture collection of the Australian Water Quality Centre. Culture conditions, DNA extraction methods and the procedures used for *rpoC1* analysis are as previously described [8]. The cyanobacterium-specific primers used for PCR were:
rpoC1-1 5'-GAGCTCYAWNACCATCCAYTCNGG
rpoC1-T 5'-GGTACCNAAYGGNSARRTNGTTGG
The PCR products were sequenced directly with the Big Dye Terminator Cycle Sequencing Kit. The nucleotide sequences were aligned using the program ClustalX [9] and phylogenetic analysis done with the MEGA program [10] as previously described [8].

STRR analysis

A series of STRR primers were used to obtain banding profiles for strains of *C. raciborskii* [8]. The primer sequences are:
STRR1F 5'-CCCCARTCCCCART
STRR1R 5'-GGGGAYTGGGGAYT
STRR3F 5'-CAACAGTCAACAGT
STRR3R 5'-ACTGTTGACTGTTG
For phylogenetic analysis of the STRR PCRs, individual bands were recorded as present or absent for each isolate, converted to binary data, and a similarity tree drawn with PAUP* [11].

A. circinalis specific PCR

The primer sequences used for PCR were Ana2, Ana4 and Ana-ICF:
5'-GATAGCATCCTCAATTTCTAGCCATTGG-3',
5'-CTCTGAAGCCAGAAATGGACGGC-3'
5'-TAGCCATTGGCATATCCAAGAGAATAGC-3'
respectively. An internal control fragment (ICF) was produced for spiking into all PCR reactions, this yielded a product of 276 bp whilst the diagnostic product was 377 bp (Fig. 2). The ICF was constructed by performing firstly a PCR reaction with *A. circinalis* genomic DNA, Ana4 and Ana-ICF. Ana-ICF recognises sequence internal to that recognised by Ana2 and Ana4, however Ana-ICF also contains a 10 bp sequence at its 5' end (underlined above) which is complimentary to 10 bases at the 3' end of Ana2 (underlined). The PCR process incorporates this 10 bp in the final product and when this is used in another

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PCR with primers Ana2 and Ana4, the complete sequence of Ana2 becomes incorporated into the new product. It is this product which is used as the ICF as it recognises Ana2 and Ana4, but yields a smaller sized product than the diagnostic product resulting from amplification of *A. circinalis* DNA. Conditions for the *A. circinalis* PCR were essentially the same as previously reported for *C. raciborskii* [8] except between 1-10 ng of genomic DNA and 2 pg of internal control fragment (ICF) were used per reaction. The thermal-cycling conditions were: 94°C for 10 min, 1 cycle; 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, 35 cycles; 72°C for 15 min, 1 cycle; 4°C, hold.

RESULTS AND DISCUSSION

A 609 bp fragment generated by PCR from 19 geographically diverse *C. raciborskii* isolates representing both coiled and straight trichomes was sequenced and found to be 99.8 -100% homologous. This data taken with data collected on 16S rRNA gene sequence indicates that both straight and coiled trichome forms of this cyanobacterium are the same species and that the definition of a coiled form as *C. philippinensis* is not supported [12]. A 612 bp fragment was amplified from 12 isolates of *Anabaena circinalis* and 1 isolate of *Aphanizomenon* collected from bloom samples around Australia. In addition *rpoC1* sequence was derived from 3 isolates morphologically identified as *A. solitaria*, 2 as *A. spiroides* f. *spiroides*, 2 as *Anabaena* sp., 2 as *A. flos-aquae*, 2 as *A. perturbata* f. *tumida*, 1 as *A. aphanizomenoides*, and 1 as *Aphanizomenon gracile*. An alignment of the sequences obtained showed that in 4 out of 12 *A. circinalis* strains examined, identical nucleotide changes were observed at 18 of 540 positions. This data suggests that genotypic groups of *A. circinalis* exist (termed type I and type II). The type I group could be further split, as 3 isolates had an identical nucleotide change at position 57, which in fact coincided with an identical change seen in the group II isolates. The available *rpoC1* sequence data for other organisms is insufficient to indicate whether the level of variation observed between strains of *A. circinalis* is indicative of discrete species.

A phylogenetic analysis of *C. raciborskii* partial *rpoC1* gene sequence and a range of cyanobacteria has been previously presented [8]. Fig. 1 introduces the additional information on *Anabaena* isolates reported here and includes *C. raciborskii*. A cluster consisting of the heterocystous cyanobacteria was evident in the earlier analysis [8] and this grouping is still present, extending from *A. circinalis* ANA175A to *Fischerella* PCC7414 in Fig. 1.

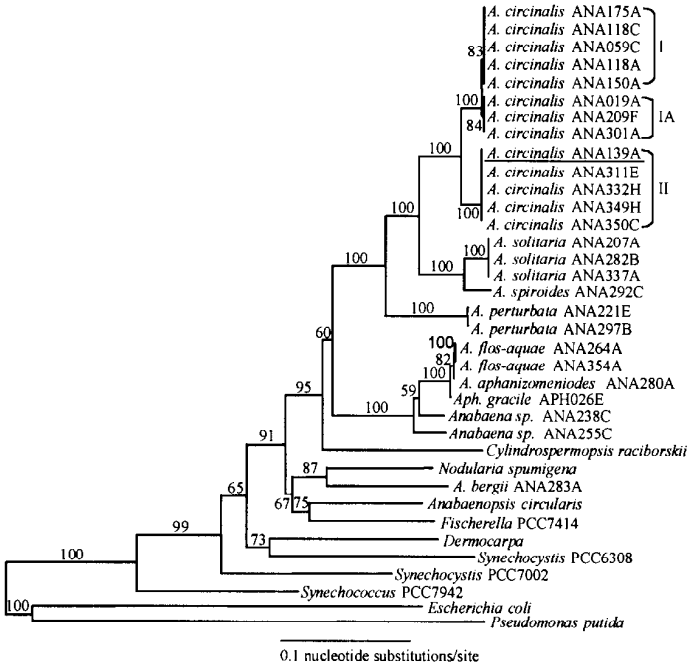

Fig. 1. Phylogenetic tree for the cyanobacteria based on analysis of aligned *rpoC1* nucleotide sequences. The *A. spiroides* ANA139A isolate re-classified as *A. circinalis* ANA139A is underlined. Bootstrap valued derived from 500 replicates of the sequence data are shown.

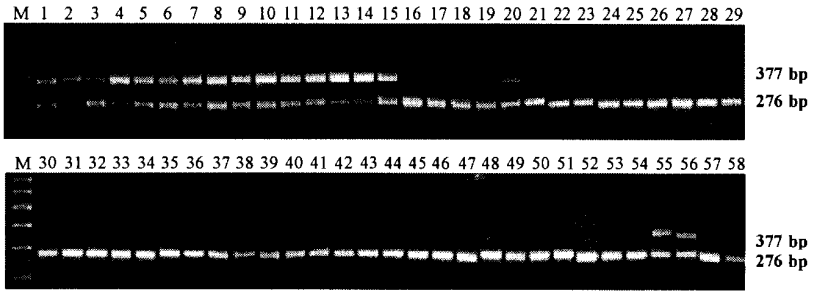

Fig. 2 *A. circinalis* specific PCR assay. The diagnostic product (377bp) and the ICF (276bp) are indicated. Lanes 1-15 are *A. circinalis* isolates, lanes 16-19, *A. flos-aquae*; lanes 20-22, *A. spiroides* f. *spiroides*; lanes 23-25, *A. spiroides* f. *minima;* lanes 26-31, *Anabaena* sp.; lanes 32-37, *A. aphanizomeniodes*; lanes 38-44, *A. perturbata* f. *tumida*; lanes 45-49 *A. solitaria*; lanes 50-52 , *Aphanizomenon issatschenkoi*; lanes 53-54, *Aph. gracile*, lane 55, environmental sample containing *A. circinalis* (Geelong, Victoria); lane 56, environmental sample containing *A. circinalis* (Gawler, South Australia); lane 57, environmental sample containing *C. raciborskii* (Broken Hill, New South Wales), lane 58, ICF only and lane M, molecular weight markers.

Interestingly *A. circinalis* ANA139A (underlined in Fig. 1) was originally identified as *A. spiroides* by morphological criteria but *rpoC1* sequencing indicated it was a type II *A. circinalis*. Only *A. circinalis* is known to be toxic in Australia, thus illustrating the usefulness of *rpoC1* typing for *Anabaena*. Morphological identification alone could lead to the erroneous assessment of a bloom as non-toxic.

The sequence alignments with other species and genera of cyanobacteria was used to design species specific PCR tests for both *C. raciborskii* [8] and *A. circinalis*. It is common practice for PCR reactions to contain some type of internal control fragment (ICF). This reveals whether failure to amplify a diagnostic PCR product is due to genuine absence of the target sequence or because the PCR failed due to sample inhibition. We applied a technique successfully used previously [8] to design an *A. circinalis* specific PCR which contained an ICF. Fig. 2 shows an example of gel electrophoresis of the diagnostic PCR to specifically identify *A. circinalis* in a range of cultured *Anabaena* isolates and environmental samples collected from locations around Australia. The test is demonstrated to be species specific and capable of identifying *A. circinalis* when applied directly to environmental samples.

Combinations of cyanobacterium specific STRR primers were used to characterise *C. raciborskii* isolates [8]. A DNA fingerprint pattern could be established for each isolate following gel electrophoresis of PCR products. Primers STRR1R and STRR3R yielded similar patterns for all strains (data not shown), whereas STRR1F and STRR3F showed variation between strains (Fig. 3). Differences were even observed among strains isolated from the same population, eg *C. raciborskii* CYP003A (lane 1) and CYP003K (lane 2). A similarity tree was constructed (Fig. 4) to represent the STRR data. There was no evidence to support the clustering of toxic and non-toxic isolates of *C. raciborskii*. The coiled isolates (geographically separated) clearly grouped together.

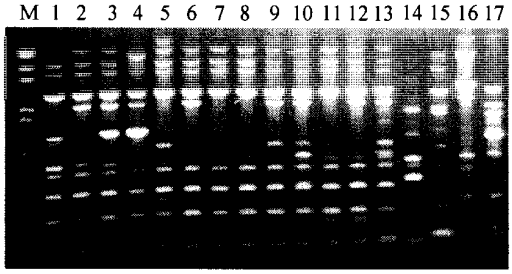

Fig. 3. STRR profile analysis of 13 isolates of *C. raciborskii* (lanes 1-13) and other representative genera (lanes 14-15, *A. circinalis*; lane 16, *M. aeruginosa*; lane 17, *N. spumigena*) obtained by PCR amplification with primers STRR1F and STRR3F.

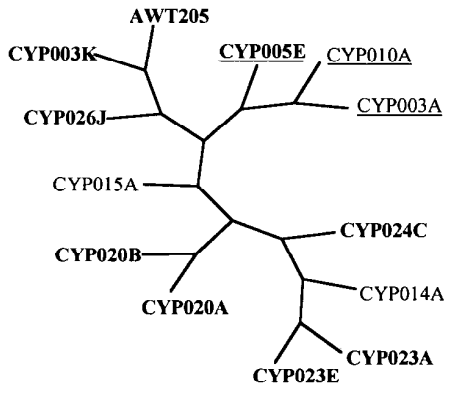

Fig. 4. Branching pattern of *C. raciborskii* STRR profiles from total character differences calculated with the Neighbour-Joining method. Toxic isolates are in bold type. Coiled isolates are underlined.

This study demonstrates that a combination of molecular typing approaches are required to characterise different groups of toxic cyanobacteria. Whereas *rpoC1* analysis did not discriminate isolates of *C. raciborskii,* when this analysis was applied to *A. circinalis* it was discriminatory at the strain level. Because this determinant is considered an essential "house-keeping" gene within the cell, and is useful therefore as an evolutionary clock, this may reflect a more distant origin of this organism. However, *C. raciborskii* isolates could be discriminated by STRR analysis which targets a range of genetic loci on the cyanobacterial chromosome. We are currently investigating the unique regions amplified within coiled isolates of *C. raciborskii* to evaluate their possible role in this phenotype.

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THE NON-TRANSCRIBED SPACER OF THE rRNA LOCUS AS A TARGET FOR SPECIFIC DETECTION OF THE DINOFLAGELLATE *PFIESTERIA PISCICIDA* AND PROTISTAN PARASITES (*PERKINSUS* SPP.)

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ABSTRACT

We have partially characterized the non-transcribed spacer (NTS) of the rRNA gene cluster from clonally cultured dinoflagellate *Pfiesteria piscicida*. With a similar approach to that applied for *Perkinsus* parasites, we targeted the intergenic spacer and the small subunit (SSU) region for the development of a species-specific PCR-based assay for this toxic dinoflagellate. *P. piscicida*-specific primers were designed (one forward primer in the NTS and a reverse primer in the SSU) for a PCR assay yielding a 429 bp amplicon. The specificity of the assay was assessed by testing clonal cultures of *P. piscicida*, three presumptive *P. piscicida* isolates (CCMP 1830, 1831 and 1834), *Gyrodinium galatheanum*, *Prorocentrum minimum*, and 21 unidentified CCMP isolates. CCMP 1828, 1829, 1830, 1831, and 1834 tested positive for *P. piscicida*. CCMP 1828 and 1829 appeared to be mixed cultures, verified by SEM and sequence of the amplicons. To validate the application of the PCR-based assay to environmental samples, 69 water samples from Chesapeake Bay tributaries were tested resulting in 19 samples positive for *P. piscicida*. Amplicon sequences were identical to that of *P. piscicida*.

INTRODUCTION

Massive fish mortalities, in some cases associated with health problems in watermen working along the lower Eastern Shore of the Chesapeake Bay, were reported during the late summer of 1997 [1]. Since then, similar episodes, although of lesser scale, have occurred in Chesapeake Bay tributaries. Like those reported earlier in North Carolina [2], these outbreaks have been attributed to toxic blooms of *P. piscicida*.

P. piscicida is a heterotrophic dinoflagellate with a multiphasic life cycle that includes flagellate, amoeboid, and cyst stages [3]. This complex life cycle comprises putative toxic and non-toxic stages. Other *Pfiesteria*-like dinoflagellate species have been isolated in the similar episodes, although of lesser scale, occurred in Chesapeake Bay region. The routine method for the identification of these dinoflagellate species has been by plate tabulation, a technique that relies on scanning electron microscopy (SEM) to define the number, shape, and position of the plates of the theca. Although plate tabulation constitutes a reliable method for the classification and identification of armored dinoflagellate species, the need for urgent identification of species that may result in toxic blooms requires complementing the above mentioned morphological approach with a rapid, practical, sensitive and specific assay. These assays should be based on a methodology that provide accuracy, sensitivity, speed and high throughput, which can be implemented in most research laboratories and environmental agencies.

P. marinus is a protozoan parasite that affects the eastern oyster (*Crassostrea virginica*) along the Gulf and Atlantic coasts of United States. Recently, based on ultrastructural features and phylogenetic studies of a new species, *Parvilucifera infectans*, and a critical review of the pertinent literature, Norén, Moestrup, and Rehnstam-Holm (1999) created the new phylum Perkinsozoa that includes *Perkinsus* species, and bridges the Dinoflagellata and Apicomplexa. [4]. The lack of specificity of the traditional methods of detection of this parasite and related species, underscored the need for the development of specific and reliable diagnostic tests [5].

Ribosomal RNA genes (rRNA) have been the target sequences of choice for development of specific PCR assays. The rRNA gene cluster is constituted by the 5S, 5.8S, small subunit (SSU), and large subunit (LSU) encoding regions. Intergenic regions such as the internal transcribed spacer (ITS), and a non-transcribed spacer (NTS) can be found between the above mentioned genes (Fig. 1). The rRNA units are usually organized as tandem repeats, in variable number of copies. These copies are nearly identical within an organism [6]. High copy number and broad database on rRNA sequences have made these regions suitable targets for the development of diagnostic tests. In addition, rRNA sequences are used to infer phylogenetic/evolutionary relationships among organisms. Nucleotide sequences of rRNA genes have been used to distinguish between species and strains of bacteria, fungi, and protozoa [5; 7-13].

Because the NTS is not transcribed [14], it was hypothesized that this intergenic spacer could potentially accumulate a large number of base substitutions without phenotypic consequences for the organism [5]. Therefore, NTS sequences should exhibit a considerable degree of inter-specific variation, and to a lesser degree, intra-specific variation. Under this hypothesis, we have developed PCR-based assays for several *Perkinsus* spp. that target the NTS (Fig. 1).

We report herein a PCR-based diagnostic assay for *P. piscicida* based on the NTS and SSU sequences and, based on our preliminary studies on *Perkinsus* species. We discuss the use of this region of the rRNA of *P. piscicida* as target for detection assays. We present preliminary data on the specificity of the test and its potential uses for specific detection of *P. piscicida* in environmental samples (water and sediment) and as a tool for laboratory experiments.

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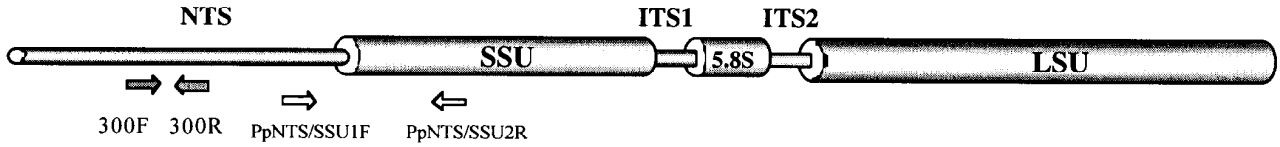

Fig. 1. General organization of rRNA locus. *P. marinus* specific primers (300F/300R [5; 17]). *P. piscicida* specific primers (PpNTS/SSU1F/PpNTS/SSU2R).

METHODS

Sampling

Several sites on the western half of the Delmarva peninsula in Maryland were sampled at locations where runoff eventually reaches the Chesapeake Bay. The sites included the Kings Creek and St. Peter's Creek (Manokin River tributaries), Jenkin's Creek, (a Tangier Sound tributary), and the Pocomoke, Nanticoke, Chicamacomico, and Middle Rivers.

Cultures

P. piscicida cultures were maintained in enriched f/20 medium under alternating periods of 14 h of light and 10 h of dark (white-fluorescent; 150 $\mu E\ m^{-2}s^{-1}$) at 23 °C, [15] using *Rhodomonas* sp. (CCMP 768) as prey. *P. marinus* cultures were propagated under conditions previously described [16]. *G. galatheanum* DNA was kindly provided by Dr. D. Stoecker, UMCES Horn Point Laboratory. A total of 25 unidentified CCMP isolates (1828, 1829, 1832, 1833, 1835, 1836, 1838, 1839, 1840, 1841, 1842, 1843, 1844, 1845, 1872, 1873, 1874, 1875, 1876, 1877, 1878, 1879, 1880, 1881, and 1880) were used to test the specificity of the *P. piscicida* PCR-based assay.

DNA extraction

DNA from *in vitro* propagated dinoflagellates and *P. marinus* was extracted using the QIAamp tissue kit (QIAGEN, Valencia, California) following the manufacturer's instructions. DNA was eluted with 50 µl sterile water and concentrations were determined by optical density at 260 nm. Environmental water (50 ml) and cultured samples (1-2 ml) were centrifuged (2000 xg, 10 min), the pellet resuspended in 100 µl of TE, frozen on dry ice and thawed at room temperature, and then boiled for 10 min. Samples were clarified by centrifugation and the supernatant used for PCR. One gram of sediment was resuspended in 10 ml of and 1 ml of the suspension was extracted as above. Aliquots of 10 µl of the DNA sediment extraction were used for detection test of *P. piscicida.*

PCR amplification and sequencing

PCR primers derived from *P. piscicida* rRNA gene cluster (PpNTS/SSU1F: 5'- TTC GGC GAT TTC GTG CTT CG -3' and PpNTS/SSU2R: 5'- TTC TCC GTT ACC CGT CAT TGC C -3') were used for the detection of the dinoflagellate. These primers are localized in the NTS (PpNTS/SSU1F) and in the SSU (PpNTS/SSU2R) of the *P. piscicida* rRNA gene cluster (Fig. 1). Positive controls consisted of PCR reaction mixtures with either *P. piscicida* DNA 2-10 ng or the cloned *P. piscicida* NTS-SSU region. In negative controls, template DNA was substituted by autoclaved Milli-Q-filtered water. Controls for the integrity of the DNA PCR templates were "universal" actin primers (kindly provided by Dr. G.W.Warr, Med. Univ. of South Carolina). PCR products were resolved by electrophoresis in a 1.2% agarose gel (w/v) in the presence of ethidium bromide (10 ng/ml final concentration) in Tris/acetate/EDTA buffer. Bands of interest were recovered form agarose gels with QIAquick Gel Extraction Kit (QIAGEN) for direct sequencing.

Tissue samples DNA from oysters *C. virginica* and clams *Ruditapes decussatus* was extracted as above and tested using *P. marinus* and *P. atlanticus* specific PCR assays [5; 17; 18].

RESULTS

The PCR-based assay developed for *P. piscicida* yielded a 429 bp amplicon (Fig. 2A). DNA from *Rhodomonas* sp., *G. galatheanum, P. minimum*, and 25 unidentified CCMP isolates was not amplified. CCMP 1828, 1829, 1830, 1831, and 1834 tested positive for *P. piscicida.* CCMP 1828 and 1829 resulted to be mixed cultures verifying by SEM and sequence of the amplicons. Performance of the PCR-based assay for *P. piscicida* on environmental water samples was satisfactory (19 samples positive out of 69 samples from the Chesapeake Bay region) in that all amplicons yielded identical sequences. Detection of *P. piscicida* in sediments (Fig. 2B) required dilution of the sample in order to overcome effects of environmental inhibitors on the PCR amplification. Sequence of the amplicons of samples positive for *P. piscicida* showed no variability in the NTS.

The specificity of the PCR assay based on the NTS regions of *Perkinsus* spp. is shown on Fig 2. DNA from oyster tissue samples infected with *P. marinus* was not amplified with *P. atlanticus* PCR-based assay (Fig. 2C and 2D). Likewise, DNA samples from clams infected with *P. atlanticus* were positive with the *P. atlanticus* PCR-based assay, but not with the *P. marinus* specific assay (Fig. 2C and 2D).

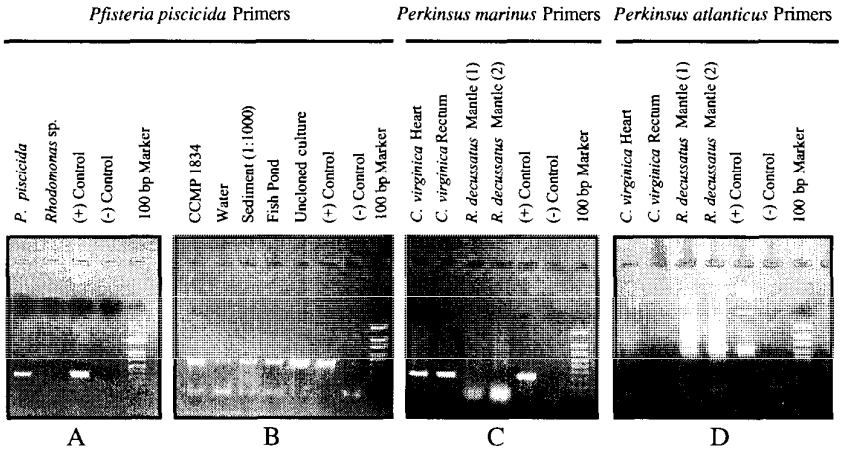

Fig. 2. Agarose gel electrophoresis of amplified products of the PCR-based diagnostic assay based on the NTS region of the rRNA genes. **A.** Specificity of *P. piscicida* PCR assay: only *P. piscicida* DNA and no *Rhodomonas* DNA was amplified using the PCR assay. **B.** Performance of *P. piscicida* PCR assay: *P. piscicida* DNA was detected in samples from several sources including dinoflagellate clonal culture, environmental water, environmental sediment, fish pond water, and dinoflagellate uncloned culture. **C. and D.** Specificity of *Perkinsus* spp. PCR assays: *C. virginica* and *R. decussatus* environmental samples infected respectively with *P. marinus* and *P. atlanticus* were specifically amplified with *P. marinus* PCR assay (C) or *P. atlanticus* PCR (D). The two upper bands in the (+) control (D) correspond to excess of plasmid.

DISCUSSION

Discrimination of species and strains requires a detailed analysis of phenotypic characteristics and the correlation of relevant biological traits with genetic markers that evolve at a rate that accurately reflects divergence within the genus. "Signature sequences", such as those from ribosomal RNA genes, are frequently identifiable as characteristic of a particular taxonomic group of organisms [19]. Some positions or stretches in rRNA sequences, however, change often enough to result in stable differences among closely related strains within the same species [11; 12]. Although the NTS is not transcribed, it has been suggested that it contains a putative RNA polymerase A promoter [20; 21] and participates on the propagation of extrachromosomal copies of the rDNA unit [22; 23]. Among *Saccharomyces* species a high degree of identity in the NTS region has been reported [24]. Using *P. marinus* as the model organism we hypothesized that because the NTS sequences are not transcribed, this region could be subject to frequent base changes without detrimental consequences for the parasite [5]. Therefore, it would constitute a suitable target for the detection of differences between individuals, strains, and higher taxonomic categories [5]. As predicted, the *P. marinus* PCR diagnostic assay based on the NTS region is species-specific and very sensitive; none of the other *Perkinsus* spp. tested were amplified [5; 17]. Non-transcribed intergenic spacers have also been used to develop specific probes for the identification of yeast (Henriques *et al.* [25]). Analysis of the NTS of *P. marinus* from the Atlantic and Gulf Coasts of USA revealed 2 distinct sequence types, designated as types I and II, which exhibited distinct geographic distributions [26]. Types I and II can be distinguished either by enzymatic restriction or by a type-specific PCR assay. We found no variability in any other regions of the *P. marinus* rRNA locus from either NTS type (Table 1). To date, all monoclonal cultures established in our laboratory of various *P. marinus* isolates have resulted of either type I or type II NTS, and all ITS sequences from these strains have been identical to the published *P. marinus* ITS sequences [26]. Because the ploidy of *Perkinsus* spp. is unknown, further investigation is required in order to elucidate if these results may be due to allelic variation. Table 1 summarizes the inter- and intraspecific variability of the different regions of the rRNA locus for two *Perkinsus* species. Among all genes and intergenic spacers in the rRNA locus, the NTS shows the highest variability between *P. marinus* and *P. atlanticus*. Within *P. marinus*, where all rRNA transcribed regions are 100% identical, the NTS exhibits a 2% difference that enables the identification of two strains, and their discrimination by PCR or restriction analysis.

Table 1. Percentages of identity of several *Perkinsus* rRNA regions. *P.m.*(I/II) *P.marinus* types I and II; *P.a. P. atlanticus*; Max. Id. Maximum Identity; Min. Id Minimum Identity.

	SSU	ITS1	5.8S	ITS2	NTS
P.m (I/II)	100	100	100	100	98.0
P.m./P.a.	98.0	76.6	100	92.3	62.2
Max. Id.	99.7	100	100	100	64.6
Min. Id.	97.1	71.6	92.5	92.3	34.5

Similarly, the PCR NTS-based detection assay for *P. piscicida* proved specific, since neither the other dinoflagellate species tested nor *Rhodomonas*, the algal prey used for culturing *P. piscicida*, were amplified. The PCR-based assay performed well with samples from diverse sources, which included clonal and mixed dinoflagellate cultures maintained in our laboratory, and water and sediments from Chesapeake Bay sites and ponds from a fish farm where fish deaths have been associated with the presence of toxic dinoflagellates. Sequences of all amplicons obtained from the above-

mentioned samples were identical to that obtained from clonal *P. piscicida*.

Application of the *P. piscicida*-specific PCR detection assay to the limited number of cultures and environmental samples tested so far, has failed to reveal variability in the relatively short stretch of NTS used as amplification target. A comprehensive study that includes the complete NTS sequence, however, will be required to rigorously assess the presence of intra-specific NTS sequence types, as observed in *P. marinus*. Studies currently underway in our laboratory are aimed at investigating the degree of NTS variability among the several regional *P. piscicida* isolates, and its relationship(s) with putative phenotypical traits.

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ELECTROCHEMICAL DETECTION OF DNA OR RNA FROM HARMFUL ALGAL BLOOM SPECIES

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ABSTRACT

Newly developed electrochemical methods make it possible to detect low concentrations of DNA or RNA from harmful algal species. This paper presents the principles of electrochemical detection and describes our initial attempts to design a convenient assay for detecting *Pfiesteria piscicida* and a related *Cryptoperidiniopsoid sp.* The electrochemical detection method being developed is a modified sandwich hybridization technique. In order to develop the assay, species-specific gene sequences that can serve as hybridization targets must be identified. Suitable target sequences have been identified from the ribosomal 18S regions of *P. piscicida* and a related *Cryptoperidiniopsoid sp.* The electrode detection system is sensitive, portable, and inexpensive to manufacture.

INTRODUCTION

Electrochemical techniques are currently being developed for routine detection of pathogenic microorganisms [1,2] and disease related genes [3] in medical laboratories. A goal of our current research is to adapt this technology to develop a quick, reliable, economical, and quantitative method of detecting specific Harmful Algal Bloom (HAB) species in natural phytoplankton assemblages. This paper describes the electrochemical detection method, presents some preliminary data for detection of PCR products using this method, and briefly discusses the potential strengths and weaknesses of electrochemical assays and other molecular methods.

Present electrochemical assays represent modified sandwich hybridization assays where a unique DNA or RNA from a target organism is quantified electrochemically, rather than using fluorometric, chemiluminescent, or radioactive detection methods [4-9]. The first step in developing these assays involves identification of unique DNA sequence from the HAB species of interest. The candidate gene chosen for this study was the small subunit (SSU) ribosomal or "18S" gene. The complete SSU gene from *Pfiesteria piscicida* and three closely related *Pfiesteria*-like organisms (PLOs) were sequenced [10]. Sufficient variation was found in at least three regions of the 18S genes of these species to begin testing various electrochemical detection methods.

METHODS

The "PCR Detect Method" developed by AndCare, Research Triangle Park, NC USA, involves amplification of a unique target sequence from RNA or DNA extracted from a culture, sediment, or phytoplankton sample [11,12]. In the case of RNA, a reverse transcriptase reaction is performed prior to the PCR reaction to convert the RNA sequence of interest into complementary single-stranded DNA (cDNA) suitable for amplification. The PCR amplification is carried out using a modified forward primer, which has a biotin molecule attached to the 5' end, and a modified reverse primer possessing a 5' phosphate group (Fig. 1). A portion of the PCR amplification product is then incubated for several minutes with λ exonuclease. λ exonuclease recognizes and completely degrades the DNA stand containing the 5' phosphate group. What remains is a pool of single-stranded DNA molecules, which has a biotin group at the 5' end.

The single-stranded DNA is then incubated with a reporter probe, a short piece of synthesized single-stranded DNA 15 to 23 nucleotides long, labeled with a 5' fluorescein molecule, that specifically binds the amplified target DNA. Next the hybridization mixture is applied to the electrode surface. The biotin molecule at the 5' end of the each amplified DNA target strand binds the NeutrAvidin molecules (an avidin analogue) coating the surface of the electrode.

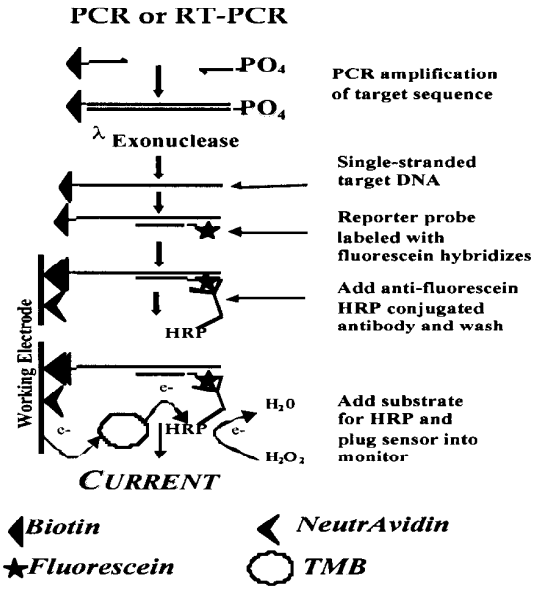

Fig. 1. Flow diagram detailing the PCR Detect Method.

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The result is a sandwich or "bridge" structure in which the fluorescein molecule attached to the reporter probe is brought into close proximity to the electrode surface (Fig. 1).

The binding of the reporter probe is done in a 50 to 500 mM salt hybridization solution. The lower the salt concentration, the more stringent the hybridization conditions. The appropriate salt concentration needed for specific binding of each reporter probe must be determined empirically. After a 15 min. incubation, the unbound
reactants are rinsed away using a wash solution. An anti-fluorescein antibody bound to horseradish peroxidase (HRP) is then added to the surface of the electrode [13]. The antibody rapidly binds the reporter probe's fluorescein moiety. The excess HRP-antibody complex is removed by briefly washing in a buffer containing the same salt concentration as the hybridization solution. Finally, a commercially available solution containing 3,3',5,5'-tetramethyl-benzidine (TMB) and hydrogen peroxide (H_2O_2) is brought to room temperature and added to the surface of the electrode. The electrode is then inserted into the monitor to obtain a reading. The catalytic HRP activity is measured as follows:

HRP reduces H_2O_2, and in the process becomes oxidized to form (HRP_{ox}).

$$H_2O_2 + 2H^+ + HRP_{red} \rightarrow HRP_{ox} + 2H_2O \quad (1)$$

The enzyme is then regenerated by TMB, which becomes oxidized after donating an electron to the oxidized HRP:

$$2TMB + HRP_{ox} \rightarrow 2TMB^+ + HRP_{red} \quad (2)$$

When the oxidized TMB^+ contacts the surface of the electrode it picks up an electron.

$$TMB^+ + e^- \rightarrow TMB \quad (3)$$

The AndCare monitor uses intermittent pulse amperometry to apply millisecond pulses of potential at −100 mV (vs. Ag/AgCl reference electrode) and measures the catalytic reduction current as electrons pass from the surface of the electrode through TMB to HRP to H_2O_2. If the solution contains sufficient concentrations of both H_2O_2 and TMB, current flow is proportional to the activity of the HRP that generates the TMB^+. Consequently, the more reporter bound, the higher the current. Conventional wisdom holds that the closer the reporter probe binds to the biotin binding site, the more likely the assay is to work well. Experimental studies are in progress to determine if secondary structures formed by RNA or single-stranded DNA are as important, or perhaps more important, in determining how successfully a reporter probe binds. Binding efficiency will ultimately determine the sensitivity of an assay.

For these initial studies, an amount of genomic DNA equivalent to that found in 10 cells of either the Cryptoperidiniopsoid sp. or *P. piscicida* was amplified using a set of conserved primers. These primers will amplify the 5' end of all known dinoflagellate 18S genes. PCR Detect assays were then performed by hybridizing various amounts of the amplified DNAs with each of two reporter probes specific for the Cryptoperidiniopsoid sp. and two probes specific for *P. piscicida.*

RESULTS AND DISCUSSION

Typical PCR Detect results obtained by hybridizing varying amounts of amplified 18S DNA from *P piscicida* and the Cryptoperidiniopsoid sp. with P. *piscicida* Probe 1, are shown in Fig. 2. Concentrations greater than 5 ng of amplified *P. piscicida* DNA product produced a measurable signal using this probe. The Cryptoperidiniopsoid sp. DNA did not cross react with this probe. Similarly, the Cryptoperidiniopsoid-specific probes only detected the Cryptoperidiniopsoid sp. DNA and not the *P. piscicida* DNA (Fig 3). The other two probes tested gave similar species-specific signals. These results indicate that the PCR Direct Detection method will work as well as other PCR-based techniques to detect low numbers of PLO species present in a sample. Investigations to establish the actual limits of detection using water column and sediment samples spiked with different densities of HABS are underway.

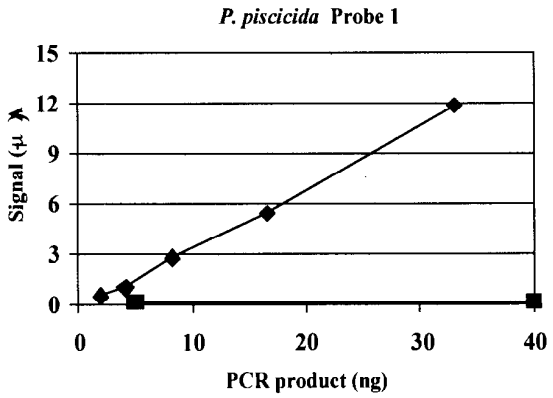

Fig. 2. Results of PCR Detect assay using a capture probe specific conserved region in the 5' portion of the 18S gene, *P. piscicida* probe 1, and varying amounts of PCR product obtained by amplifying the 5' portion of the 18S gene of *P. piscicida* (♦) or Cryptoperidiniopsoid sp. (■).

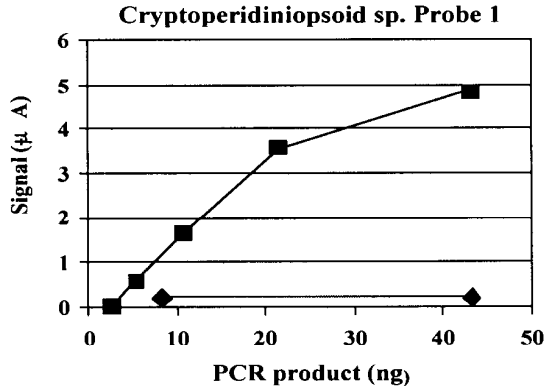

Figure 3. Results of PCR Detect assay using a capture probe specific to a conserved region in the 5' portion of the 18S gene, Cryptoperidiniopsoid sp. probe 1, and varying amounts of PCR product obtained by amplifying the 5' portion of the 18S gene of Cryptoperidiniopsoid sp. (■) *P. piscicida* (♦).

The PCR detect assay has several advantages. The assay is relatively fast. Up to 30 samples can be processed in an hour. The electrodes are prefabricated and inexpensive and the assay process can be automated, further increasing sample throughput. Despite these advantages, an assay that could measure the concentration of harmful algal RNA directly, without a PCR amplification step, would be potentially more quantitative and easier to perform. Such an assay also is being developed at AndCare and is referred to as the "Direct Detect Method".

The Direct Detect Method is similar to the PCR Detect method except that the PCR amplification step is omitted. In this case, RNA is extracted from a sample and simultaneously incubated with a 5' biotin-labeled capture probe and a 5' fluorescein reporter probe specific for the coding strand of the target RNA. Both the capture and reporter probes bind the target RNA at the same time the 5' biotin on the capture probe binds the NeutrAvidin coating the surface of the electrode. After a 20 min. incubation, the excess reactants are washed away, leaving a sandwich analogous to that found in the PCR Detect method. After the sandwich is formed, the remainder of the assay is the same as shown in Fig 1. The sensitivity of PLO RNA detection using regular biotin-labeled oligonucleotide probes has proven fairly low in initial tests. One modification to the assay that may provide increased sensitivity to the direct detect methods is the use of peptide nucleic acid (PNA) capture and reporter probes. PNA probes are synthetic molecules that mimic DNA. They contain the same nucleotide bases as DNA but have a neutral peptide backbone rather than a charged sugar-phosphate backbone. PNA probes have a number of advantageous properties. DNA/PNA complexes have a significantly higher thermal stability than comparable DNA/DNA hybrids, PNAs bind 50,000 times faster than regular oligonucleotide probes, and even a one bp mismatch between a probe and a target sequence will prevent the PNA probe from binding. Thus PNA probes will allow even one base pair differences in target sequences to be reliably detected. Furthermore, PNAs are not degraded by proteases or DNAses. The actual levels of RNA that can be detected using these probes are currently being investigated.

Caveats Designing Molecular HABS Assays

There are a number of caveats that should be considered when developing molecular assays, including electrochemical detection methods. First, for any assay to be accurate, there must be a uniform and substantial recovery of DNA or RNA from the cells in the sample being assayed. Unequal lysis will bias quantification and should be measured by inclusion of known numbers of a test organism as a positive control. Second, if any PCR amplification or *in situ* hybridization is required, the primers used in the assay have to be carefully designed and tested for specificity. Nonspecific amplification will bias the results leading to erroneous conclusions about the occurrence and abundance of the target organism. When designing nucleic acid probes, it is therefore important to systematically test for cross-reactions. Sometimes these cross-reaction problems can be identified by comparing the putative probe sequence against the know sequences in various databases. However, in most instances, there is insufficient sequence information available. Hence, a more reliable method of testing specificity is to extract DNA or RNA from both related species, and from non-related species that are likely to be abundant in the sample. These extracted DNA and RNA samples can then be used to test assay for specificity. If cross-reactivity is absent, the probe is likely to be specific. Third, RNA levels within a cell vary greatly with growth stage [14]. Hence using RNA as a basis for either quantifying or determining presence or absence of a particular HABS in a sample is likely to prove less precise than if the assay is based on detecting DNA. Finally, no one molecular or non-molecular technique will work quantitatively for all HAB species in all environments. When at all possible, multiple approaches such as direct counting, HPLC, antibody staining, etc. should be used in addition to the molecular techniques to determine if the methods agree. Only by using the molecular techniques in conjunction with solid field data will the full potential of molecular assays be realized.

CONCLUSIONS

The electrochemical detection technique, though in early stages of development with HABS, holds promise for estimating abundances in natural phytoplankton assemblages. If successful, this technique can be adapted to a wide variety of HAB species or strains and will relatively fast and easy to perform. In the future as more genes are identified for toxin production, there may be enough genetic variation within these genes to make species specific or group specific toxin production detectable through electrochemical means as well.

ACKNOWLEGEMENTS

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ARBITRARILY PRIMED PCR FINGERPRINTING OF RNA OF *ALEXANDRIUM TAMARENSE* GROWN UNDER VARYING NITROGEN/PHOSPHORUS RATIOS, AND IDENTIFICATION OF THE PERIDININ-CHLOROPHYLL *A*-BINDING PROTEIN GENE.

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ABSTRACT

We have conducted a study on arbitrarily primed PCR fingerprinting of mRNA of the dinoflagellate *Alexandrium tamarense* (Gonyaulacales, Gonyaulacaceae) cultured under varying N/P ratios, with the aim of identifying genes wich are differentially expressed due to differences in the nutrient concentration and balance. We have used the RT-PCR procedure and gel electrophoresis to detect the corresponding bands. Several differential bands have been detected, cloned and sequenced. The first identified band is a 450 bp fragment of the peridinin-chorophyll *a*-binding protein (PCP) gene. The derived protein sequence has a 100% homology with PCP of *Gonyaulax polyedra*. This protein is unique to dinoflagellates, and its gene fragment might serve as a nuclear probe to identify dinoflagellate presence in natural waters. The identified gene fragment has higher expression level under balanced than under unbalanced nutrient conditions.

INTRODUCTION

HAB events are presently widespread around many of the world coastal areas. The approaches to better knowledge of HAB species, their physiology and ecology will permit to find the ways to avoid the unwanted consequences of their blooms. In recent years, the tools of Molecular Biology have started been applied to the study of these organisms.

One of the current hypothesis to explain the bloom developing of HAB dinoflagellates, as well as the increase in per cell toxin amount is the shift in the N/P ratio in the waters where these algae dwell. Phosphorus deficiency has been shown to increase saxitoxin production in *A. tamarense* related to nutrient sufficient or N-deficient cells [1]. This is not the only hypothesis trying to explain this phenomenon, but is one that deserves attention, since the change in the main nutrient ratio must contribute to change the algal metabolism, in such important functions as photosynthesis, respiration, nitrate and phosphate uptake, etc. Relative increase in N versus P can drive the algal cell to accumulate nitrogenated compounds, among which is saxitoxin and its derivatives.

We have undertaken the study of differences that appear in the mRNA of *Alexandrium tamarense* when they are cultured under different N/P ratios. The differential cDNA fragments synthesized by reverse transcription of mRNA are sequenced and compared with known sequences that code for functional cell proteins. Many differences appear, that are dependent upon the difference in nutrient ratios.

Differential display (DD) of mRNA is a modification of the RAPD-PCR technique, which is based on the ability of the PCR to generate a reproducible array of products when the annealing step is performed at low stringency [2]. The technique we have used, Arbitrarily Primed PCR fingerprinting of RNA[3] is very similar to DD, except in thet it uses only one arbitrary primer for the PCR, instead of two (the arbitrary primer plus a poly-T primer). RNA is extracted and reverse-transcribed using arbitrary primers to yield cDNA, which is subsequently amplified by PCR. However, we started from mRNA instead of total RNA. The products of this reaction are then resolved by gel electrophoresis. Each cDNA fingerprint is dependent on the sequence of the arbitrary primers. mRNA fingerprints are obtained in this way, that permit to detect differential gene expression of the same cells under different culture conditions.

MATERIALS AND METHODS

1.-Cultures and growth conditions

A clone of *Alexandrium tamarense* KAC02 (from the University of Kalmar culture collection, Sweden) was grown and maintained in Pyrex culture tubes (25x150 mm) containing 25 ml of f/2 medium without silicate [4], using 0.22 µm filtered seawater from Ría de Vigo (Northwest Spain) (35.5 ‰ salinity). Illumination was on a 14:10 h LD cycle (at ca. 70 $\mu E\ s^{-1}\ m^{-2}$ irradiance provided by day-light fluorescent tubes) and temperature 15±1 °C.

Six nutrient treatments (see Table 1) were randomly assigned to replicate tubes and grown in the same light and temperature conditions. These cultures were subject to at least three transfers before the sampling took place. After the acclimatation period, cultures were grown in 10 L Pyrex flasks with areation. During the exponential phase, cells were harvested by filtration through10 µm mesh and pellets were kept at –80°C until their use.

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TABLE 1: Nutrient concentration in each treatment.

Nitrate (μM)	Phosphate (μM)	N/P Ratio
12	7.5	1.6:1
48	7.5	6.4:1
120	7.5	16:1
120	3	40:1
120	0.75	160:1
883	36.3	24:1 (f/2)

Six nutrient treatments (see Table 1) were randomly assigned to replicate tubes and grown under the same light and temperature conditions. These cultures were subject to at least three transfers before the sampling took place. After the acclimatation period, cultures were grown in 10 L Pyrex flasks with areation. During the exponential phase, cells were harvested by filtration through10 μm mesh and pellets were kept at –80°C until their use.

2.-Analytical methods

mRNA extraction: mRNA was directly extracted with a poly-T column: (QuickPrep micro mRNA purification kit from Pharmacia) from the cells previously broken by mechanical grinding in cold in the extraction buffer. This was done with conical mini-pestles in Eppendorff vials, in cold, applying short strokes. (This method proved to yield higher amount and quality of RNA when applied to total RNA, as compared with other disruption methods).

RT-PCR: Reverse transcription (RT) reaction was performed on a 50 μl assay containing: 0.01 μg mRNA extract, 50 ng random hexamers, Super Script buffer x1, 10mM DTT, 500 μM dNTPs, 40u RNase Block (Stratagene), and 400 u Super-Script II (Gibco). One tenth of the RT reaction product was used for the PCR, that was performed with. 100 μM dNTPs, 1.25 mM Cl_2Mg, Buffer x1 and 5 u Taq polymerase, and was primed with 0.5 μM of the arbitrary 10-mer oligonucleotide 5'-d(CTGCTGGGACRT). Thermocycler conditions were: 1 min at 94C followed by 35 cycles at 94 C (30 sec), 40C (2 min) and 72C (7 min) with a final elongation step of 7 min at 72C.

Electrophoresis and silver staining: The amplified fragments were electrophoresed in a 0.5 mm 12% acrylamide gel (Excel Gel, Pharmacia), in a Multiphor II (Pharmacia-BioRad) apparatus refrigerated at 15 C and silver-stained with the Bio-Rad kit, according to the manufacturer's instructions. A characteristic banding pattern appeared for each sample and primer. Image obtention and storage and band analysis were performed with Fluor Imager and the software Quantity One (Bio-Rad). Differentially displayed cDNA bands were excised from the gel with a sterile surgical razor together with the acrylamide and silver stain and eluted by heating at 60 C in sterile water. The eluted bands were reamplified by PCR in the same conditions as before.

Cloning and sequencing: Cloning of selected purified fragments was done with pCR-Scrip™ Amp SK(+) Cloning Kit [5, 6]. Bacterial colonies containing plasmids with an insert were analyzed through extraction of plasmid DNA by alcaline lysis [7]. The plasmid was digested with restriction enzymes such as SmaI and PvuII and then electrophoresed on agarose gel. When a fragment of the adequate size was found it is subsequently sequenced using primers T3 and T7. Sequencing was performed in an automated sequencer with fluorescent labelling.

Sequence analysis: The sequences obtained were introduced in the DNAman software, which translates it to the three posssible ORFs (Open Reading Frames) , and these, in turn, are compared with those in the genebank BLAST 2.0 (Basic Local Alignment Search Tool) at NCBI using the Basic Search. This tool gives the scores with the most similar sequences archived and the organisms to which they belong. The genbank ENTREZ (at NCBI) provides the most complete sequence found for these organisms. In this way, we can perform future comparison with the extended sequence of our *Alexandrium*

RESULTS

Differential Bands detected by RT-PCR and silver-staining: Most of the bands detected were common to all samples, but some of them were unique to one or some of the different N/P ratios used, or were expressed with clearly visible higher intensity. Figure 1 shows some differential bands of RT-PCR on acrylamide gel. Bands C, K and J have been cloned, sequenced and searched for homologies. Other differential bands are in the process of cloning or sequencing.

Identified sequence: band J: Peridinin-chlorophyll a-binding protein (PCP) gene fragment: Band J is a fragment of PCP. It is 440 bp long and was differentially expressed, its intensity being maximum in the cells cultivated under N/P ratios of 16:1 and 40:1, minimally expressed under P deficiency (N/P=140:1) and weakly expressed under N deficiency (N/P=1.6:1).

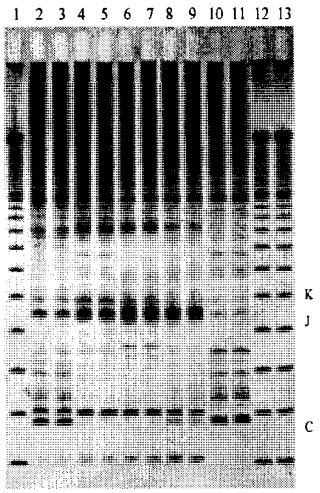

Figure 1.- Differential Display of mRNA of *A. tamarense* KAC02 cultured under several N/P ratios. Lanes:1,12,13:molecular weight marker; 2,3: N/P=1.6; 4,5: N/P=6.4; 6,7: N/P=16; 8,9: N/P=40; 10,11: N/P=160. All RTs performed with PN6. (Acrylamide gel). The differential cDNA bands labelled C, J and K, in duplicate, have been extracted for DNA cloning and sequencing. Band J is a fragment 440 bp long of the peridinin-chlorophyll protein gene. Bands C and K have also been cloned and sequenced.

The comparison of the sequence found with the genBank ENTREZ using the software DNAman yielded a homology of 100% with the peridinin-chlorophyll a-binding protein (PCP) gene of *Gonyaulax polyedra* and *Amphidinium carterae*. The highest homologies found are shown in Table 2.

The PCP gene of *Alexandrium tamarense* had not been sequenced before. The identified fragment J will be subject to primer extension, to obtain the whole gene, that will be registered in the GenBank.

Other differential bands: Bands labelled K and C have have been cloned and sequenced. Their introduction in the GenBank has not given any known homology higher than 36% We only consider as significant homologies higher than 75%. cDNA fragments will be extended to get longer fragments of the corresponding genes, and registered in the GenBank.

TABLE 2.- Sequences producing significant aligments With protein sequence of *A. tamarense* translated from Band J

SEQUENCE	SCORE (bits)
PCPprecursor, *Gonyaulaxpolyedra*	100
PCP 1 precursor *Amphidinium carterae*	100
PCP precursor, *Symbiodinium* sp	99
PCP 2 precursor, *A. carterae*	98
Chain M, PCP A.*carterae*	96
n-terminal region PCP precursor, *A. carterae*	73

DISCUSSION

The RT-PCR method allows us to better kow the gene regulation of cell events related to algal physiological states of nutrient deficiency or repletednes. Some of the bands that appear are expected to code for stress proteins, some of which will be useful in the study of stress syndrome of cells. In toxic dinoflagellates, such as *A. tamarense* clone KAC02 these could be related to toxin production.

Dinoflagellates have developed a water-soluble antenna with a high carotenoid:chlorophyll ratio, Most dinoflagellates have peridinin as their predominant carotenoid, enabling them to capture blue-green light (470-550 nm), which is inaccesible by chlorophyll alone [8]. Peridinin-chlorophyll *a*-binding protein (PCP) is unique to dinoflagellates and has no sequence similarity with other known proteins [9]. The first reportd sequence of PCP was that of *Symbiodinium* sp [9]. PCP of Gonyaulax polyedra has been identified and sequenced by [10].

Genetic regulation in dinoflagellates has been mostly studied in luciferin-binding protein of Gonyaulax polyedra . Its synthesis is under circadian translational control [11] and it had previously been suggested that translational control could be the main mechanism of circadian regulation in dinoflagellates [12]. However, in *Amphidinium carterae*, it has been found that the two major classes of light-harvesting proteins, LHCs (the major a/c containig intrinsic light harvesting proteins) and PCPs were under transcriptional control by light intensity [13]. Proteins related to toxigenicity in *Alexandrium fundyense* are also transcriptionally regulated through the cell cycle, and their genes are up- or down-regulated during G1 phase [14]. Our results show that PCP mRNA in *Alexandrium tamarense* is down-regulated in situations of nutrient-unbalance, with

deficiency of either nitrogen or phosphorus. This can be interpreted in terms of stress situations. mRNA levels drop under nutrient stress. To our knowledge, this is the first report on transcriptional regulation by nutrient ratio in dinoflagellates

The results for homology searching indicate that PCP of *Alexandrium tamarense* has not been cloned and sequenced before. The cloned and sequenced cDNA band can be used as a molecular probe for detecting the presence –in natural assemblages- of very low amounts of *A. tamarense* or related species. The homologies found suggest that the PCP might have a highly conserved sequence within Gonyaulacales or even dinoflagellates. Quantitative PCR will confirm the differential expression of PCP mRNA under the different nutrient ratios used, and Southern hybridisation with DNA of other dinoflagellate species will determine if this cDNA serves as a probe for a more or less restricted group of these algae. Primer extension to obtain the whole PCP gene sequence is under way, and the 3' end has already been sequenced.

ACKNOWLEDGEMENTS

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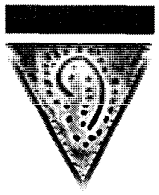
TOXIN DETECTION METHODS

Harmful
ALGAL
BLOOMS



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TASMANIA
2000

AZASPIRACID POISONING (AZP): A NEW SHELLFISH TOXIC SYNDROME IN EUROPE

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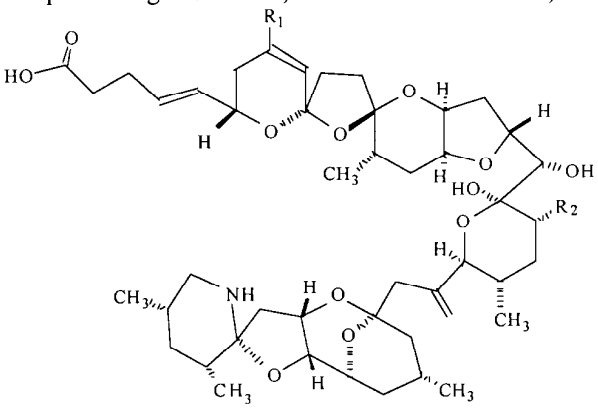
ABSTRACT

Human intoxications, following the consumption of Irish mussels (*M. edulis*), have occurred in The Netherlands (1995), Arranmore Island (1997), France (1998) and Italy (1998). The contaminated shellfish were cultivated in four different regions encompassing the entire west coastal region of Ireland. These intoxications have now been attributed to a new family of shellfish toxins. Three toxins have been isolated and structurally elucidated and they contain a novel spiro ring assembly. The major toxin, has been named azaspiracid (AZA) but substantial amounts of its methyl and demethyl analogues, AZA-2 and AZA-3, respectively, were also present in shellfish. The aetiology of azaspiracids and toxin dynamics in shellfish have been studied using liquid chromatography-mass spectrometry methods, especially LC-MS^3, and strong evidence has been obtained that azaspiracids are produced by a dinoflagellate. Azaspiracids are persistent in shellfish and have been found in mussels as long as eight months after the initial intoxication. In several instances, toxin levels in oysters (*C. gigas*) have been comparable to the levels found in mussels from the same cultivation area. Since azaspiracids are toxicologically and chemically different from previously identified groups of shellfish toxins, a new toxic syndrome has been declared and named, azaspiracid poisoning (AZP).

INTRODUCTION

In November 1995, mussels from Killary Harbour, Ireland, caused severe gastrointestinal disturbance in The Netherlands (>8 cases). The associated human symptoms consisting of nausea, vomiting, severe diarrhoea and stomach cramps were similar to diarrhetic shellfish poisoning (DSP) toxins. However, although intoxication of mussels by DSP toxins occurs nearly every year in Ireland, only insignificant levels of okadaic acid (OA) and dinophysistoxin-2 (DTX-2) were detected on this occasion. Also, the known toxic phytoplankton were not observed in water samples collected at that time. Toxicity persisted in shellfish from Killary for at least eight months, which is an unusually long period for a toxic shellfish episode. In the DSP mouse test, a slow progressive paralysis was observed using extracts of mussels and these neurotoxic symptoms were quite different from those typical of the DSP toxins. However, the prolonged intoxication period gave the opportunity to acquire bulk shellfish material for toxin isolation in sufficient quantities for characterisation. This toxin, which was tentatively named killarytoxin-3 (KT-3), was purified from acetone extracts of shellfish tissue using multiple chromatographic separation steps [1]. Structurally, this toxin is characterised by a trispiro assembly, a rare azaspiro ring structure, fused with a 2,9-



dioxabicyclo[3.3.1] nonane, and a carboxylic acid. It was therefore re-named azaspiracid [2].

Figure 1. Structures of azaspiracids: Azaspiracid (R_1=H; R_2=Me), Azaspiracid-2 (R_1=R_2=Me), Azaspiracid-3 (R_1=R_2=H),

Azaspiracids differ from all of the previously known nitrogen-containing toxins from shellfish or dinoflagellates, including, prorocentrolide [3] pinnatoxin [4], gymnodimine [5] and the spirolides [6]. These have an imine functionality, which is an essential pharmacophore, and reduction of these compounds to the corresponding amine results in a total loss of toxicity.

A second major toxic incident in 1997 in Arranmore Island, Ireland, was also caused by azaspiracid. However, two new analogues were also isolated from mussels, azaspiracid-2 (AZA-2) and azaspiracid-3 (AZA-3) which are 8-methylazaspiracid and 22-demethylazaspiracid, respectively [7]. Azaspiracids are probably produced by a dinoflagellate

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because of the highly oxygenated polyether structure, the pattern of methylation and seasonal occurrence.

Mouse symptoms, caused by extracts of mussels, were characterised by a slow and progressive paralysis and, at low doses, mice died within 2-3 days which is distinctly different from diarrhetic shellfish toxins [1]. The main pathological injuries caused by azaspiracids include necroses in the lamina propria of the small intestine, thymus and spleen. Lymphocyte injury and fatty changes in the liver were also observed [8]. The differences from DSP toxins are such that the poisonings constitute a new shellfish toxic syndrome that we have named Azaspiracid Poisoning (AZP).

METHODS

Shellfish samples

Shellfish tissue (6 g), *M. edulis* or *C. gigas*, were homogenised with acetone (25 ml). Acetone was evaporated under nitrogen and the residual aqueous medium was extracted with ethyl acetate (2 x 10 ml). Solvent was evaporated, reconstituted in acetonitrile (1 ml) and 5 µl was used directly for LC-MS analysis.

Liquid chromatography-mass spectrometry

LC-MS analysis was carried out using Hewlett Packard HPLC system (HP 1100 series) linked to a Finnigan MAT LCQ tandem mass spectrometer (Thermoquest, San Jose, USA). The MS was equipped with an electrospray ion-spray (ESI) interface.

Chromatographic separations were performed using a reversed phase column, C_{18} Luna(2), 5 µm, 150 x 2.0 mm, Phenomenex, Macclesfield, UK) at 40°C, under isocratic conditions with a mobile phase of acetonitrile-water (65:35 or 70:30) containing 0.05% trifluoroacetic acid (TFA) at a flow rate of 0.2 ml/min. Standard azaspiracids used for LC-MS method development were isolated from mussels [2], [7]. Using a flow injection at 3 µl/min, the mass spectrometer was tuned using azaspiracid standard solution (1 µg/ml) and the optimised capillary temperature was 220 °C with a voltage of 30 V. Full-scan collision-induced dissociation (CID) spectra were obtained using the protonated molecule for each toxin, AZA (*m/z* = 842), AZA-2 (*m/z* = 856), AZA-3 (*m/z* = 828) and the collision energy was set at 40%. The optimum collision energies for LC-MS^{2-6} experiments were in the range 25 –35%.

RESULTS AND DISCUSSION

Following the structural elucidation studies, the availability of standard toxins allowed us to develop LC-MS methods for the determination of azaspiracids in shellfish and phytoplankton. This now permits the implementation of control measures to protect public health and eventually should lead to identification of the biogenetic origin of these unusual toxins. The three main azaspiracids can be separated by HPLC and identification and determination is possible using several different mass spectrometric (MS) detection modes. An LC-MS method was first developed using the Finnegan LCQ ion-trap instrument using electrospray ionisation (ESI) in positive ion mode. The typical chromatograms for the analysis of mussel extract from Arranmore Island, without sample clean-up, are shown in Figure 2.

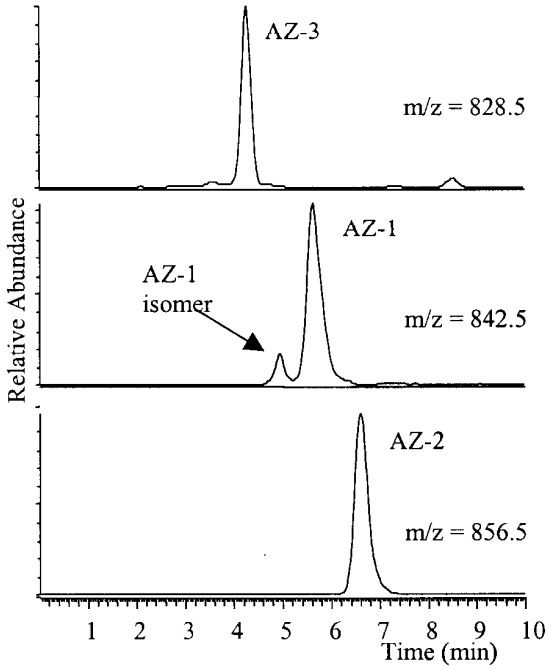

Figure 2. ESI-LC-MS of a mussel extract from Arranmore Island, Ireland.

The relative composition of the azaspiracids was AZA (46%), AZA-2 (34%) and AZA-3 (20%). In most shellfish samples AZ-1 was the predominant toxin. Evidence for the presence of an isomer of AZ-1 was also obtained using LC-MS (Figure 2).

Further evidence for the identity of azaspiracids may be obtained by using full-scan collision induced dissociation (CID) LC-MS. Using a collision energy of 40%, each of the three main azaspiracids fragmented similarly to produce signals due $[M+H]^+$, $[M+H-H_2O]^+$ and $[M+H-2H_2O]^+$. Thus, a mussel extract containing AZA-3 produced signals at 828.5, 810.5 and 792.5 (Figure 3). Multiple loss of water molecules during CID fragmentation is typically observed with other polyether shellfish toxins including OA analogues [9]. The first micro liquid chromatography-tandem mass spectrometry (µLC-MS-MS) method for the identification of azaspiracid in shellfish has recently been published [10] which used selected reaction monitoring (SRM) of the protonated molecule and three product ions, $[M+H-nH_2O]^+$ with n = 1-3. LC-MS methods were used to confirm that azaspiracids were responsible for two incidents of human intoxications from the consumption of Irish mussels in 1998 (Table 1).

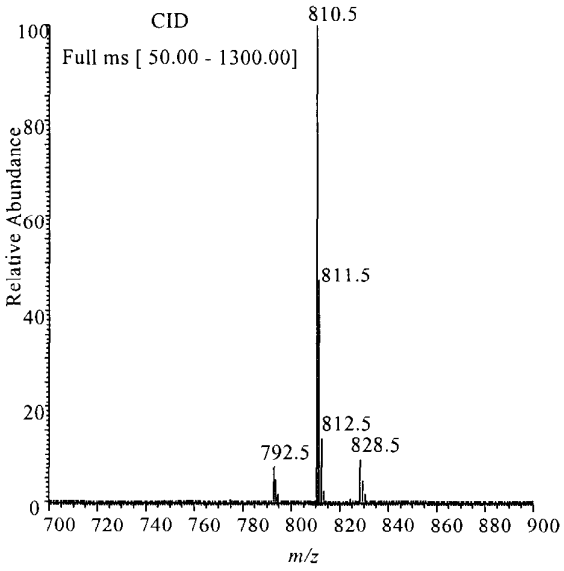

Figure 3. CID-LC-MS of AZA-3, $[M+H]^+$, *m/z* = 828.5, using a 40% collision energy.

The first incidents of AZP in Ireland occurred in regions that were remote and produced small amounts of mussels. However, during 1998-2000, most of the large shellfish producing areas had periods of contamination by azaspiracids. Sensitive and specific LC-MS[3] methods were developed and applied to the determination of azaspiracids in shellfish throughout the entire west coast of Ireland (1995-2000).

Table 1. Confirmed human intoxications from the consumption of mussels containing azaspiracids.

Date	Cultivation Region	Human Intoxications
Nov. 1995	Killary Harbour	The Netherlands
Oct. 1997	Arranmore Is.	Ireland
Sept. 1998	County Cork	France
Sept. 1998	Clew Bay	Italy

A major concern for human health was that the human toxic events involving Irish shellfish occurred during the operation of a DSP monitoring programme using 24 h mouse bioassays. Although the DSP mouse bioassays can detect azaspiracids, at high levels, shellfish that were negative using this assay caused human intoxications.

To establish the reasons for the inconsistent mouse bioassay results we undertook studies that established the following:

1. The variation in azaspiracid levels between individual mussels can be as much as 500%.
2. DSP mouse bioassay protocols result in the extraction of only 5-40% of total azaspiracids present in mussels.
3. Azaspiracids are unstable and they decompose in certain solvents, basic pH and with some chromatographic phases.

However, the most remarkable finding, that has serious implication for the implementation of effective monitoring programmes for AZP, were obtained from a study of seven mussel cultivation sites within the same bay in south-west Ireland. This is summarised in Figure 4. Not only were the toxin levels very variable between sites, but the AZP toxins were distributed throughout the mussel tissue and were not confined to the hepatopancreas. DSP toxins, OA analogues and pectenotoxins, concentrate in the hepatopancreas with only 2% in the remaining shellfish tissue. Thus, the shellfish from site #5 would pass the official DSP mouse bioassay since only the hepatopancreas is used for testing.

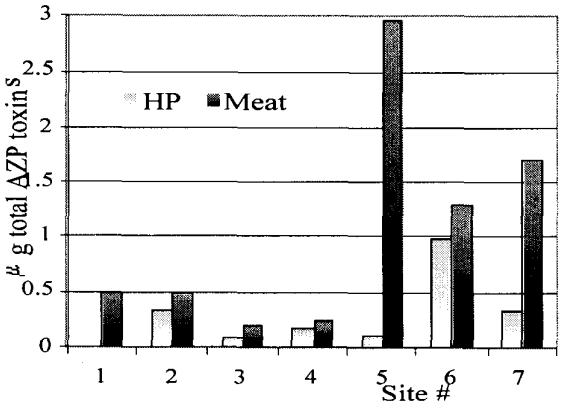

Figure 4. Distribution of azaspiracids (μg total AZP toxins/g) in mussel tissues (hepatopancreas, HP, and remaining meat) from seven sites in the same cultivation region in Co. Cork, Ireland.

We have also recently identified azaspiracids in mussels from England and Norway. Samples which were positive by DSP mouse bioassay, but chemical analysis showed insufficient DSP toxins to account for this toxicity, were examined for azaspiracids. The three main azaspiracids, AZA, AZA-2 and AZA-3 were identified and the toxin profiles were similar to those found in Irish shellfish with AZA predominant. In view of the fact that only a small number of shellfish samples have so far been examined for the presence of azaspiracids, it is likely that intoxication of European shellfish by azaspiracids is more widespread than has previously been appreciated.

Unlike DSP toxicity in Ireland due to OA/DTX2, cultivated oysters (*C. gigas*) were affected by azaspiracids as much as rope cultured mussels. This is surprising in view of the fact cultivation methods for these shellfish are different.

Another difference from DSP toxicity is that natural depuration of azaspiracids can be very slow and high toxin levels have persisted in mussels for 7-8 month periods after initial intoxications in three regions of Ireland in 1995, 1997 and 1999. The fact that azaspiracids migrate from the hepatopancreas to other shellfish tissues could account for this slow rate

of depuration compared with DSP toxins. Prolonged AZP intoxication periods have serious implications for the economic viability of the shellfish industry.

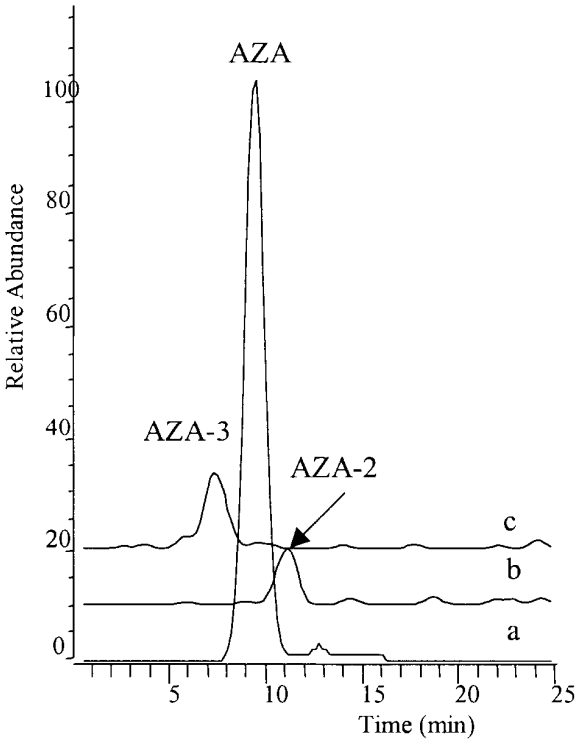

Figure 5. Chromatograms from the LC-MS^3 analysis of an extract from a marine phytoplankton sample collected from Co. Cork in 1999.

An unrelated research programme designed to collect bulk phytoplankton, rich in *Dinophysis acuta*, produced a sample which contained mainly OA and DTX2. However, the three main azaspiracids were also identified using LC-MS^3 analysis (Figure 5). Chromatogram a-c were obtained by targeting the protonated ions $[M+H]^+$ of AZA, AZA-2 and AZA-3, fragmentation to produce product ions, $[M+H-H_2O]^+$ and finally, spectra characteristic of each toxin (not shown). The relative composition was AZA (84%), AZA-2 (7%) and AZA-3 (9%). This phytoplankton sample contained mainly the dinoflagellates, *Ceratium* sp. and *Dinophysis* sp., but these were eliminated as potential sources of azaspiracids. Although the progenitor has not yet been identified, these data are the first experimental evidence that azaspiracids are produced by dinoflagellates.

ACKNOWLEDGEMENTS

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MIST Alert™: A RAPID ASSAY FOR PARALYTIC SHELLFISH POISONING TOXINS

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ABSTRACT

Jellett Biotek Ltd. has developed a rapid field test kit to screen for paralytic shellfish poisoning (PSP) toxins. The new test, called MIST Alert™, provides a qualitative (positive/negative) indication of the presence of PSP toxins in less than 20 min. It is designed as a screening method for regulatory labs to eliminate negative samples, thereby leaving a smaller number of positive samples to be tested with more sophisticated and time-consuming quantitative methods. Due to its simplicity and speed, MIST Alert™ can also be used in other applications such as shellfish harvest management and toxin research. The test is based on easy-to-use lateral flow immuno-chromatographic (LFI) test strips. The sensitivity of the test to several analogues of saxitoxin (STX) and neosaxitoxin (NEO) was investigated. The toxin analogues STX, GTX2/3 and C1/2 epimeric mixtures, B1 and decarbamoyl saxitoxin (dc-STX) were detectable at concentrations of approximately 200 nM. The test was somewhat less sensitive to the N1-hydroxy derivatives NEO and GTX1/4, requiring concentrations of 400 and 600 nM, respectively, to give a substantially positive test. All toxins were detectable within or close to the regulatory limit of 80 µg saxitoxin dihydrochloride equivalents per 100 g of tissue (= 1075 nM STX) using the AOAC extraction procedure.

INTRODUCTION

Variations of the intraperitoneal mouse bioassay, now standardized by the AOAC [1] procedure, have been used to detect paralytic toxins in shellfish for more than sixty years. Some alternative assays have been developed but none, until now, were well suited for field use. Antibody methods offered the best approach for the development of an easy-to-use, reliable and inexpensive test kit for PSP toxin detection. One approach to implementing an immunoassay is by lateral flow immuno-chromatography (LFI). In such an assay, all the components are incorporated into a test strip, so that it is only necessary to add a sample extract to initiate the sequence of reactions. As a result, LFI tests require no special expertise or laboratory equipment in their use. Consequently, this technology has found many applications, such as in home pregnancy test kits. The use of LFI for PSP toxin detection, however, presented some unique problems. Antibodies must recognize about twenty analogues of saxitoxin (STX) in a variety of complex matrices extracted from different shellfish tissues and species. Furthermore, in naturally contaminated shellfish a wide range of concentrations, from 0 to $>10^5$ µg saxitoxin equivalents (STXeq) per 100 g of tissue, may be encountered, rather than a simple presence or absence of toxic analyte. An ideal assay should indicate a positive result at or above the regulatory limit of 80 µg STX(2HCl)eq per 100 g of tissue and a clear negative below this value. To develop and test a new method for detecting PSP toxins an adequate supply of the purified toxins was essential, especially one involving antibodies where toxin-protein conjugates were required for both immunizations and to form capture lines on the test strips. Furthermore, the production of LFI test strips with reliable and uniform responses required specialized equipment to spray membranes with conjugated antibodies and toxins. Component concentrations were adjusted to give an average response to different analogues for each batch of several thousand test strips. Different capture line conjugates and mixtures of antibodies raised against different analogues give different responses so that test strips can be designed to suit toxin profiles found in different regions. In this publication we describe the response of a batch of LFI test strips to purified PSP toxins to illustrate some characteristics of this product.

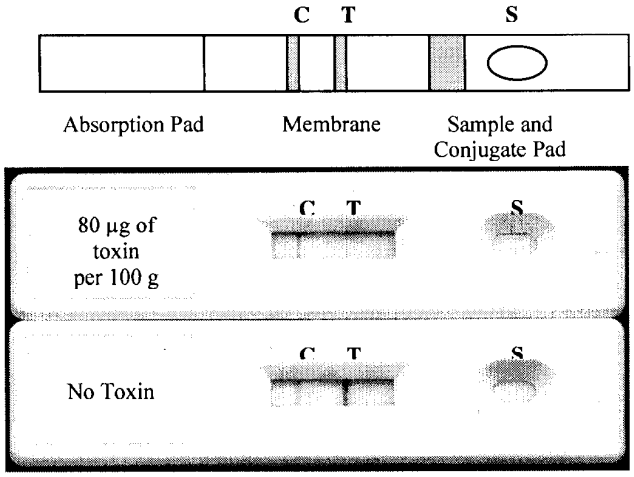

Fig. 1. Diagram of the MIST Alert™ test strip. The components are aligned with two cassettes containing developed test strips. Strips consist of an absorption pad, a membrane striped with a mixture of toxin analogues (the T line on the right) and an antibody detection reagent (the C line on the left) a sample pad and a conjugate pad containing the antibodies. The visible T line indicates absence of toxin in the sample and no line indicates presence of toxin. The C line indicates that the sample fluid has sufficiently resuspended and mobilized the antibody colour complex.

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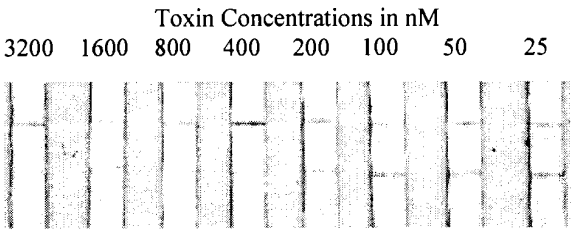

Fig. 2. Dilution series for neosaxitoxin. Eight test strips were attached to a card and sample pads dipped into microtitre plate wells containing 100 µL of running buffer with different concentrations of toxin.

MATERIALS AND METHODS

PSP toxins were purified from toxic strains of *Alexandrium* by methods described previously [2]. Toxin concentrations were measured by capillary electrophoresis [3] and calibrated with certified calibration solutions (National Research Council of Canada). Complete test strips were mounted in plastic cassettes (see Fig. 1) or glued on cards for dilution series experiments. Sample extracts were diluted to 1/5 with Jellett Biotek's commercial running buffer solution. Test strips mounted on cards were spaced so that the sample pads fitted into microtitre plate wells. The first well in a row contained 200 µL of buffer solution with 3200 nM toxin. The other seven wells across the plate contained 100 µL of buffer. Serial dilutions were made by transferring and mixing 100 µL from each well into the next giving a dilution series from 3200 to 25 nM. To represent the results graphically each card of strips was scanned and intensities of test lines were integrated using a BioRad Model-GS-690.

RESULTS

When a solution is added to the sample pad of a test strip, antibody and coloured particles are carried along the membrane and, in the absence of free toxin, bind to the toxin conjugate at the T line. Any free toxin present in the sample solution blocks antigen-binding sites so that the antibodies cannot bind to the conjugated toxin at the T line. If the toxin concentration is high enough, the T line is not visible. There is no competition of free toxin at the C line which will always appear, providing antibodies and coloured particles have properly bound and moved forward. Non-specific interference is indicated by the absence of a C line and can be caused by interfering compounds in shellfish extracts or factors such as salt content or pH effects. A visible C line shows that the test performed properly. Therefore, decrease in intensity of the T line compared to a negative control indicates the presence of PSP toxins in the sample. The concentration at which this occurs is different for each toxin. In Fig. 3 it can be seen that STX eliminated the T line at concentrations greater than 200 nM, whereas for NEO and GTX1/4 (equimolar epimeric mixture) concentrations in excess of 400 and 600 nM, respectively, were necessary to eliminate the T line. Due to interference from compounds in extracts of shellfish tissues it was necessary to dilute them to at least 1/5 with the running buffer. This also allowed for pH and salt concentrations for optimal test performance. The samples can consist of shellfish extracts, phytoplankton extracts or solutions of pure toxins diluted in the running buffer. An aliquot of 100 µL is applied to each test strip and the sample liquid resuspends the antibodies and colour complex located in the sample and conjugate pads, whereupon liquid migrates along the membrane through the capture lines and into the absorbant pad. In the presence of free PSP toxin a weak T line often forms but it gradually disappears by competition between free and bound toxin.

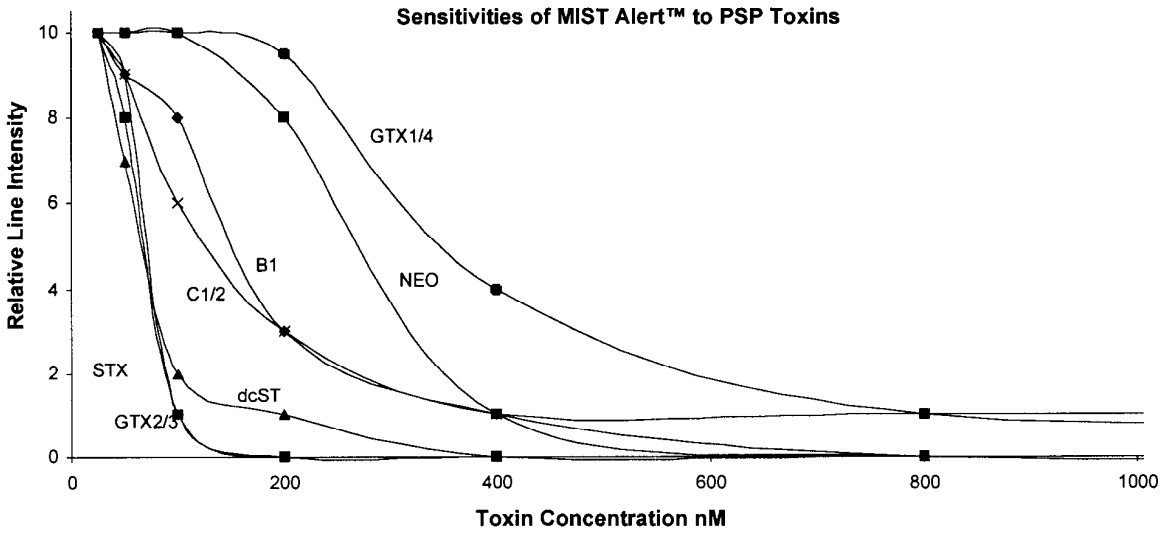

Fig. 3. Plots of test line intensities of purified PSP toxins from dilution series experiments.

DISCUSSION

Mist Alert™ is designed primarily to provide a rapid test for screening shellfish extracts to reduce the number of more accurate, quantitative analyses, most of which are negative. MIST Alert™, therefore, should not be used to replace analytical methods. Although the cost of mice is relatively low, testing laboratories have to charge according to the overall costs of an analytical laboratory. Concerns over the use of whole mammal bioassays as subjects for seafood testing are increasing and restricting the use of mice in routine screening programs for shellfish toxins. Other methods for PSP toxin analysis, such as liquid chromatography, are even more expensive than the mouse bioassay, particularly for small numbers of samples.

The MIST Alert™ test strips can be made relatively inexpensively in thousands using equipment designed for the production of LFI test strips. Production in large batches also ensures their reproducibility. For routine testing each test strip is mounted in a cassette (Fig. 1) and about 100 µL of extract diluted with a buffer solution is added to the sample well. This simple operation can be made without laboratory equipment and an eye-dropper can be used. After twenty minutes, persistence of a strong T line indicates that any PSP toxins in the sample are at concentrations less than the regulatory limit. Toxin concentrations at or above the equivalent of 80 µg STX(2HCl) per 100 g tissue (1075 nM for STX) in an AOAC extract eliminate the toxin line and further testing should be done on those samples.

The response of the test to different PSP toxins is unequal, due to specificities of the antibodies and competition for the bound toxin on the T line. Experiments with T lines of mixed toxin conjugates showed that this approach to detecting the entire family of PSP toxins was not as effective in eliminating the line as using a blend of antibodies. Cross-reactivities of our antibodies were generally similar to those reported by other groups [eg. 4, 5] in that antibodies against NEO recognized NEO and its analogues and those raised against STX recognized STX and its analogues, with good recognition of the respective GTX derivatives within the two groups. Fig. 3 shows that sensitivities of the test strip to STX, GTX2/3, dcSTX, C1/2, and B1 were around 200 nM, allowing for a five fold dilution to obtain test concentrations of 1075 nM, equivalent to an AOAC extract of shellfish tissue at the regulatory limit of 80 µg STXeq per 100 g. Responses to NEO and GTX1/4 were less than for STX analogues, indicating that a higher proportion of anti-NEO antibodies is needed for optimal response.

The slope of the intensity-concentration curve for GTX1/4 in Fig. 3 shows that the intensity of the T line gradually decreased with increasing toxin concentration. In fact, for all of the analogues that were tested, the slopes increased with higher sensitivity. The non-N1-hydroxy derivatives STX, GTX2/3 and dcSTX yielded the sharpest distinction between a line and no line, whereas as shown in Fig. 2, the T line persisted over a wider range in response to different concentrations of NEO. Interpretation of a single test result is difficult where a line remains visible, even though it may be faint in comparison to that of a control test. Such ambiguities occur near the detection limit of the strip assay for those analogues, but usually not at concentrations significantly above the regulatory limit.

Sensitivities of the assay to the N-sulfo-carbamoyl epimers C1 and C2, and B1 (GTX5) were intermediate between those for STX and NEO. This result is different from that previously reported for immunoassays in which cross-reactivities were very low to sulfamate toxins [4, 5,]. Because sulfate is easily lost from the N-21 position of sulfamate toxins, care was taken to ensure there were no detectable amounts of carbamate toxins present. Analysis by capillary electrophoresis showed no detectable contamination with STX or GTX2/3; if present, these components were well below the detection limits of the strip assay. Although toxicities of sulfamate toxins are relatively low compared to the carbamate group, they are easily converted to more toxic carbamate analogues. Therefore, the ability to detect sulfamate toxins is an advantage, especially in a screening test.

Test strips are currently being produced using the antibody mixture yielding cross-reactivities shown here for purified toxins. The composition of the solutions that are provided with the kit for dilution have been developed for different applications and are propriatary. Because of the differing affinities of the antibody mixture for the individual analogues it is not possible to provide a definitive detection limit for the mixed toxin profiles in shellfish tissues. Depending on the *average* toxin profile in any geographic area, the detection limit of the test units produced with the current antibody mixture will be in the range of 100-800 nM which corresponds to 7-60 µg STXeq per 100 g tissue. Jellett Biotek will endeavour to produce an antibody mixture with more uniform cross-reactivities among the analogues for future versions. MIST Alert™ is currently undergoing field trials in several locations, including Alaska and Great Britain, with comparisons to HPLC data and mouse bioassays. The results of this survey will be the subject of a future publication.

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APPLICATION OF THE MOUSE NEUROBLASTOMA (MNB) ASSAY TO THE STUDY OF PSP TOXINS FROM DINOFLAGELLATES AND CYANOBACTERIA; A COMPARISON OF DATA GENERATED BY THE MNB ASSAY TO PRE AND POST COLUMN HPLC

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ABSTRACT

Paralytic shellfish poisons (PSPs) are potent neurotoxins produced by dinoflagellates and some cyanobacteria species. A mouse neuroblastoma (MNB) assay was developed for the detection of sodium channel blocking toxins such as PSPs and was previously applied to the analysis of bacteria and shellfish. In this study we have used the MNB assay to screen and quantify the sodium channel blocking (SCB) activity of PSP producing dinoflagellates and cyanobacteria from Portuguese waters. *Gymnodinium catenatum* Graham presents the highest total toxicity value on a per cell basis (7.80±0.69 pg STXeq/cell) when compared with the toxic strain of *A. lusitanicum* Balech (0.25± 0.02 pg STXeq/cell) and the cyanobacteria *Aphanizomenon* sp. ($74x10^{-3}$pg STXeq/cell). This is the first report of the use of the MNB assay to detect PSPs in cyanobacteria from the freshwater environment and in evaluating the toxicity of *G. catenatum*. Results are presented which show that the MNB assay is a sensitive method for screening PSPs in dinoflagellates and cyanobacteria with different toxin profiles.

The results of MNB assay were also compared to HPLC data from pre and post-column analysis.

INTRODUCTION

Paralytic shellfish poisons (PSPs) are a complex of potent neurotoxins which act by blocking sodium channels in nerve axons [1]. These may cause severe human poisoning due to consumption of contaminated shellfish. PSPs also occur in freshwater reservoirs representing a health risk to both human and animals when reservoirs are used as a source of drinking water [2]. PSPs are produced by marine dinoflagellates, but some freshwater cyanobacteria are also capable of this function. In Portuguese waters, strains of species of the dinoflagellates *Alexandrium lusitanicum* Balech, *Gymnodinium catenatum* Graham and of the cyanobacterium *Aphanizomenon* sp. isolated from the marine and freshwater environment, respectively, were shown to produce PSPs by HPLC [3,4,5]. However, the biological activity of these strains has not previously been examined. The most frequently used method for this purpose is the mouse bioassay [6] but its use is considered unethical in several countries. A possible alternative is a cell culture system, the mouse neuroblastoma (MNB) assay, which detects PSPs based on their pharmacological action.

In this study, the feasibility of using the MNB assay to determine the total toxicity of dinoflagellates and cyanobacteria was assessed, using the strains detailed above, and the results compared to data from HPLC.

MATERIALS AND METHODS

Cell culture and toxin extraction

Dinoflagellates

Several dinoflagellates strains were cultured at 19-21°C on a 14:10 LD cycle. The media used was ASP7 [3] and f/2 [7] for *Alexandrium lusitanicum* Balech, clone LME 18-1 strain I and II, respectively, and GPM for *G. catenatum* Graham, clone LME 348 [4]. The non-toxic dinoflagellate *Alexandrium tamarense* (Lebour) Balech, clone PML 173a maintained in f/2 in the same culture conditions as above was used as a negative control for the MNB assay. For toxin analysis cultured cells were harvested in exponential growth phase (between 16 and 24 days, depending on the strain), collected by centrifugation at 2000g, suspended in 0.5N acetic acid (between 1-10 ml, depending on the pellet size) and disrupted by mechanical action with glass balls. Cell pellets were kept frozen at -20°C until toxin extraction.

Cyanobacteria

Lyophilised cells (10mg) of a clone of the toxic cyanobacterium *Aphanizomenon* sp., clone LME CYA 31, cultured in Z8 medium, were extracted with 2 ml of 0.5N acetic acid and methanol:chloroform (1:1) as reported in [5]. *Microcystis aeruginosa* Kuetzing emend. Elenkin, clone LME CYA 7, a non PSP producer, was used as a negative control for the MNB assay.

Toxin analysis

MNB assay

The MNB assay was carried out according to the method described by Gallacher & Birkbeck [8] with the following amendments: the concentration of ouabain

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Intergovernmental Oceanographic Commission of UNESCO 2001

and veratridine was determined by performing a checkerboard assay (3 wells per dilution) using concentrations of ouabain from 0 to 0.2 mM and veratridine from 0 to 0.04 mM in the absence of STX. The concentration of ouabain and veratridine which caused maximum cell death was subsequently used in assays. The exception was when matrix effects were assessed, in this instance extracts were added to cells alone. The concentration of saxitoxin equivalents (nM STXeq) in the unknown sample was determined by comparing the absorbance of the sample to that of a STX (STX, National Research Council, Canada) dose-response curve ranging from 2.5 to 320 nM. The value obtained was subsequently divided by the number of cells contained in 1 ml of acetic acid and total toxicity expressed in picogram of STX equivalents per cell (pg STXeq/cell) for comparison with HPLC. The MNB assay was performed in triplicate (using three separate assays) for each dinoflagellate and once for the cyanobacterium.

HPLC analysis

Precolumn LC-FLD analysis

Aliquots of cell culture extracts were passed through a SPE-C18 cartridge clean-up system. The resultant extract was derivatized with both hydrogen peroxide and periodate as oxidants and then analysed according to the procedure described by Lawrence *et al.* [9]. The column used was a Supelcosil LC-18 (150 x 4.6mm, 5 µm, Supelco, USA).

Cleanup of the *G. catenatum* extracts was improved by using SPE-COOH cartridges which achieved highest selectivity for most of PSP toxin groups. Optimised conditions for the oxidation reaction as described in [10] were used for the oxidation of all PSP toxins in order to obtain the highest sensitivity for the fluorescence response and consequently to obtain an accurate identification of PSP analogues. Identification and quantification of the toxins were performed using PSP standards from NRC, Halifax, Canada.

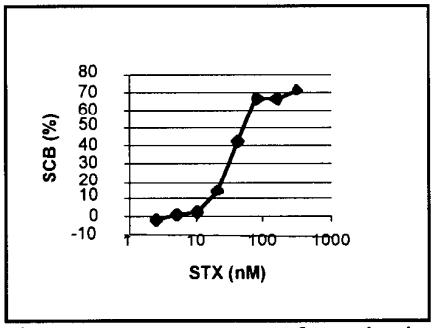

Fig. 2. Dose response curve for saxitoxin (STX) using the MNB assay.

Post-column LC-FLD analysis

Post-column oxidation was performed using the method of Oshima [11]. Three mixtures of PSP standards containing toxins of the STX group (neoSTX, dcSTX and STX), gonyautoxins (GTX1-5) and N-sulfocarbamoyl toxins (C1-4), kindly provided by Prof. Oshima, were used for identification purposes. The chromatographic separation was performed by reverse phase C8 column (Hypersil, MOS-5µm, 4.6x150mm, Supelco, USA). Toxin determination was performed in triplicate for dinoflagellates.

RESULTS

Response of the MNB cells to ouabain, veratridine and saxitoxin

Over the range of ouabain and veratridine concentrations maximum cell survival was approximately 20%. However, the concentration of ouabain and veratridine which gave this response varied and was dependent on the number of sub-cultures performed on the cell line (Figure 1).

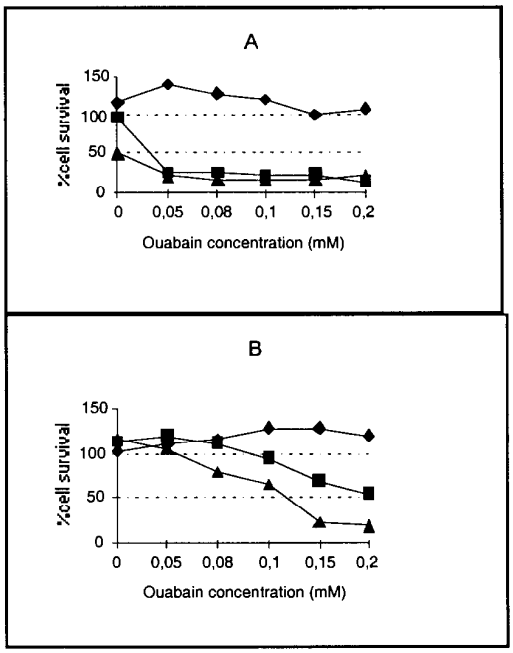

Fig.1. Effect of ouabain and veratridine on the viability of mouse neuroblastoma cells, after 3 sub-cultures (A) and 19 sub-cultures (B). Key to symbols: -◆- 0mM veratridine; -g-0.025 mM veratridine; -▲- 0.04 mM veratridine.

For routine use with saxitoxin, the combination of ouabain and veratridine which killed 80% of cells was chosen. Ouabain and veratridine were optimised for each assay and adjusted accordingly. A sigmoidal dose response curve was obtained when 2.5 to 320 nM of STX was added to MNB cells along with an otherwise lethal combination of ouabain and veratridine (Figure 2).

MNB assay: cytotoxic effects and comparison of MNB toxicity values to data obtained by HPLC

In some instances the dinoflagellate extracts caused cell death in the absence of ouabain and veratridine. This was due to unknown cytotoxic substances within the cells and could be removed by diluting the extract by 1/32 in tissue culture medium. Therefore, subsequent MNB assays using ouabain and veratridine were performed at 1/32 dilution or more. Further controls using non-toxic cell extracts of *A. tamarense* PML 173a for dinoflagellates and *M. aeruginosa* for cyanobacteria were incorporated into each assay. The toxicity values, on a per cell basis, obtained by MNB assay for two dinoflagellate species *A. lusitanicum* and *G. catenatum* and the cyanobacterium *Aphanizomenon* sp. are shown in Table 1. The dinoflagellate *G. catenatum* was significantly more toxic than the cyanobacterium. Sodium channel blocking activity was not detected in *A. lusitanicum* I although low level toxicity was detected in *A. lusitanicum* II.

Table 1- Quantities of SCB toxins produced by dinoflagellates and cyanobacteria

Organism	Cells/ml	MNB (pgSTXeq/cell)	HPLC (pgSTXeq/cell)
A. lusitanicum I LME 18-1	4.08×10^5	nd	nd
A.lusitanicum II LME 18-1	15.69×10^5	0.25 ± 0.02	1.06 ± 0.00
G.catenatum LME 348	4.24×10^5	7.80 ± 0.69	5.52 ± 0.36
Aphanizom. sp. *LME CYA 31*	21.58×10^7	74.04×10^{-3}	3.64×10^{-3}

nd= not detected

Initial screening of PSP toxicity, using precolumn HPLC, showed that *A. lusitanicum* strain II produced exclusively gonyautoxins (GTX1-4), whereas the toxic profile of *G. catenatum* was more complex, with several PSP analogues present (dcsTX, GTX2-3, B1, B2, C1, C3, C4 and dcGTX2). *Aphanizomenon* sp. produced mainly toxins from the STX group (dcSTX and STX) and GTX5. Recent improvements in the cleanup of extracts using SPE-COOH prior to prechromatographic HPLC analysis, resulted in an improvement in the separation performance for some PSP analogues, particularly for sulfocarbamoyl toxins (C1-4) present in the *G. catenatum* extract. The use of optimised conditions for the oxidation of toxins and particularly for the STX group, where the pH value has a significant influence, was applied in particular to the analysis of the cyanobacterium extract.

Toxin profiles by post-column HPLC confirmed the data obtained from pre-column HPLC for both dinoflagellates and cyanobacterium strains tested. The absence of toxins in *A. lusitanicum* strain I was confirmed by both pre and post-column HPLC.

The concentration of toxins determined by the MNB assay and post-column HPLC was compared (Table 1). PSPs were not detected by either technique in *A. lusitanicum* strain I. Both assays ranked the strains in the same order of toxicity.

G. catenatum extract showed the highest toxin value (7.80 and 5.52 pg STXeq/cell) as determined by MNB and post-column HPLC, respectively.

DISCUSSION

At the outset of the work some observations were made on the performance of the MNB assay. Of most relevance was the reduction of sensitivity of the cells to ouabain and veratridine as the number of sub-cultures in the cell line increased. If left unchecked, this adversely affected the response of the cells to STX by reducing the assay sensitivity. As a consequence the performance of the cell line was constantly monitored and the number of sub-cultures kept to a minimum.

Salts are known to interfere with the sodium channel response [8], which can lead to false positive data. Incorporation of the appropriate non-toxic cell extract controls and high dilution of the sample circumvented this.

The effect of dinoflagellate extracts on the MNB assay has previously been studied using *A. excavatum* and *A. fundyense* [12]. Out of the ten extracts tested two had a lytic effect on the MNB cells in the absence of ouabain and veratridine. This cell lysis was also observed in a number of shellfish extracts and the authors attributed it to unknown material co-extracted from the sample. This is a different effect to that of sodium channel blocking agents which are reliant on the presence of ouabain and veratridine. In this study the lytic effect was observed in all the dinoflagellates tested (including the negative control) but could be removed by diluting the sample without adversely affecting the sensitivity of the assay.

Previous work has shown that the MNB assay can detect PSPs in *A. excavatum* and *A. fundyense* cell extracts. In the present study, we have shown that the MNB assay can also detect PSPs in *A. lusitanicum*, *G. catenatum* and *Aphanizomenon sp.* To our knowledge, this is the first evaluation of the toxicity of these organisms by the MNB assay. However, differences in the exact toxin content reported for the MNB assay compared to HPLC were observed. The values obtained for *G. catenatum* and *Aphanizomenon* sp. by the MNB assay were higher than those obtained by HPLC, whereas, the toxin content of *A. lusitanicum* strain II was lower by the MNB assay compared to HPLC. Although HPLC comparison trials have been undertaken comparing toxin values obtained with those from the mouse bioassay [11], detailed validation of the methodology has not been undertaken with the matrices used in this study against the MNB assay. According to Jellet *et al.* [12] the tissue culture bioassay provided results virtually identical to those obtained with the mouse bioassay, and moreover, was considerably more sensitive. The results gained from HPLC analysis of the same study were less consistent when compared with

the results from both bioassay methods. Therefore which technique is the most accurate at determining toxicity in the organisms studied here is currently unknown and is the topic of further investigation, although some general observations can be made. The MNB assay estimates total toxicity, based on sodium channel blocking activity equated to STX equivalents using a STX dose response curve. Whereas, HPLC provides absolute toxin concentrations of individual PSPs from quantification of co-eluting compounds with toxic standards. The total toxin content reported for HPLC, expressed as saxitoxin equivalents, is calculated from application of conversion factors to the individual toxin concentrations as determined by the assessment of the toxicity of each compound by mouse bioassay. Different conversion factors are reported, depending on the mouse test and purity of standards, thereby introducing some inaccuracy in reporting toxicity values (i.e STX equivalents) by HPLC. Additionally, if HPLC is unable to detect any compound for which sodium channel activity exists, including compounds such as tetrodotoxin which are not part of the PSP group, the toxin values obtained by the MNB assay will be higher than for HPLC. We propose that this may have occurred in the case of *G. catenatum* where analysis by LC-MS [12] suggests that dcGTX2, 3 is present. This compound could not be quantified by HPLC due to the unavailability of standards but would give a sodium channel response in the MNB assay.

Conversely, the MNB is prone to salt interference which can cause inaccurate evaluation of toxicity values [8]. In this study this was deemed not to be an issue due to the high dilution of samples used and the inclusion of a negative control at each dilution.

Nevertheless, the differences in toxin content reported for the above strains by HPLC and the MNB assay does not detract from the observation that both techniques ranked the toxicity of the organisms in a similar order. Therefore, we conclude that the MNB assay responds to a range of PSPs toxin profiles and is a sensitive method for the screening of sodium channel blocking activity in dinoflagellates and cyanobacteria.

ACKNOWLEDGEMENTS

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A COMPARISON OF HPLC WITH ELECTROCHEMICAL OXIDATION, HPLC WITH CHEMICAL OXIDATION, AND THE MOUSE BIOASSAY FOR THE ANALYSIS OF PSP TOXINS IN SHELLFISH.

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ABSTRACT

High performance liquid chromatography (HPLC) is a powerful tool for the analysis of PSP toxins found in shellfish. The PSP toxins are chemically oxidized to form a fluorescent derivative prior to detection. We have previously shown that this derivative can also be formed electrochemically. Here we compare the results obtained from HPLC using the traditional post column chemical oxidation (PCRS), HPLC with electrochemical oxidation (ECOS) and the mouse bioassay of 40 toxic samples from giant scallops (*Placopectin magellanicus*) and 25 toxic samples from geoduck clams (*Panopea generosa*). Both shellfish types contained mostly gonyautoxins, with $GTX_{2/3}$ accounting for the bulk of the total toxicity. Scallop samples presented a much dirtier matrix and required clean up on a solid phase extraction cartridge prior to analysis using the ECOS system. Both HPLC-PCRS and HPLC-ECOS accurately predicted the mouse bioassay results using scallop samples. The geoduck samples were assayed directly without prior clean up on a solid phase extraction cartridge. For these samples, the PCRS accurately predicted the mouse bioassay results whereas ECOS tended to under-estimate the total toxicity as determined by the mouse bioassay. Most of the variation was contributed by samples whose total toxin content was less than 100 µg STX eq. per 100g fresh weight. With proper sample preparation, both HPLC-PCRS and HPLC-ECOS provided a viable alternative to the mouse bioassay with the ECOS system simpler to purchase, maintain and operate.

INTRODUCTION

HPLC is a powerful tool for the analysis of PSP toxins that occur in shellfish and algae. The saxitoxin ring system lacks a natural chromophore and must be modified to form a fluorescent derivative. Wong *et al.* [17] were the first to show that heating the saxitoxin ring system in alkaline peroxide resulted in the formation of a fluorescent pyrimido-purine (Fig. 1). This chemistry was used by Buckley [3], Sullivan [1, 14], Oshima [9, 10] and coworkers to form the basis of the current post column reactor system for the HPLC analysis of PSP toxins (Figure 2a). While this system offers significant advantages over the mouse bioassay, it also has problems. The oxidation of the saxitoxin ring system is subject to changes in the pH, temperature, flow rate, and age of the mobile phase and post-column reagents. Furthermore, the system requires two additional HPLC pumps to deliver the post column

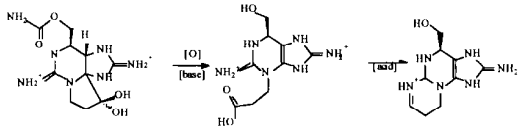

Figure 1. Degradation of saxitoxin to a pyrimido-purine derivative. This chemistry, with the modifications suggested by Quilliam *et al.* [11], forms the basis of the PCRS approach currently used for HPLC analysis of PSP toxins.

reagents and a heated reaction coil. This post column reactor can be replaced by an electrochemical cell [6, 7]. Termed the electro-chemical oxidation system or ECOS (Figure 2b), this approach has several advantages over the PCRS approach. ECOS is both less expensive to set up and easier to operate than the PCRS since it eliminates the need for the post-column pumps and heated reaction coil. A simple power supply is now available to replace the expensive electrochemical detector used in previous ECOS reports [2, 6, 7]. We have previously shown that both ECOS and PCRS give very similar values for PSP toxin content in algal samples [2]. Here we compare the two systems using more complicated shellfish matrices.

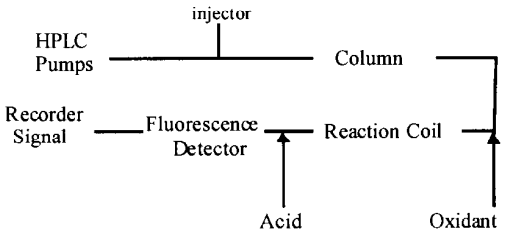

Figure 2a. The Post Column Reactor System (PCRS).

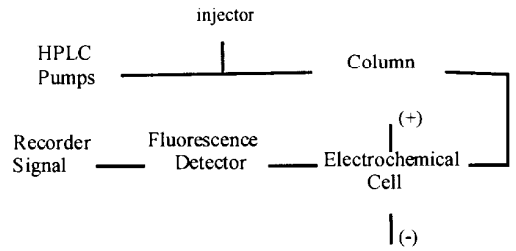

Figure 2b. The Electrochemical Oxidation System (ECOS). An electrochemical cell replaces the reaction coil and reagent pumps.

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MATERIALS AND METHODS

The HPLC systems used here are illustrated in Figure 2. Toxins were separated on an Inertsil C8 or C18 column (5μ, 150 mm x 4.6 mm) at 0.8 ml min^{-1} using the isocratic solvents of Oshima *et al* [10]: STX and neoSTX: 2 mM sodium heptane sulfonate in 30 mM ammonium phosphate (pH 7.1) with 5.7% acetonitrile; Gonyautoxins: 2 mM sodium heptane sulfonate in 10 mM ammonium phosphate (pH 7.1). In the PCRS, a 1 ml Kratos reaction coil in a 50°C heating block was installed after the column. Periodic acid (7 mM in 50 mM sodium phosphate (pH 9.0) at 0.8 ml min^{-1}) was added before the reaction coil and acetic acid (0.5 M at 0.4 ml min^{-1}) was added after the coil. For the ECOS, the reaction coil was replaced with a Guardstat™ power supply and model 5020 guard cell (ESA Inc., Chelmsford, MA, USA). Fluorescence was detected in both the PCRS and ECOS at 330 nm excitation and 390 nm emission. Shellfish extracts of geoduck (*Panopea generosa*), prepared using the AOAC protocol, were obtained from Kelly Curtis (University of Washington, Seattle WA, USA). Extracts of scallops (*Placopectin magellanicus*) were obtained from Kats Haya (St. Andrews Biological Station, St. Andrews, NB, Canada). Mouse bioassays were determined at the time of collection using the standard AOAC assay. Samples were then shipped to Syracuse NY and stored frozen at -20° C until HPLC analysis. Scallop samples (2 ml) were centrifuged at 8,000 x *g* for 15 min, then filtered through a C18 solid phase extraction (SPE) cartridge (Waters C18 Sep-Pak™ *lights*) previously conditioned with 5 ml 100% methanol and 5 ml 0.05 N acetic acid. Geoduck samples were centrifuged at 8,000 x *g* then analyzed directly without further purification. HPLC results were calibrated against PSP standards obtained from the toxin standards program at National Research Council, Canada, and converted to mouse units as described in [1]. The detection limits in pmol injected on column for the individual toxins using the ECOS system were: 0.063 pmol STX, 2.90 pmol NeoSTX, 4.64 pmol GTX_1, 0.28 pmol GTX_2, 0.16 pmol GTX_3 and 1.82 pmol GTX_4 [2]. Full details for the set up and maintenance of the ECOS system are presented in a technical report available from the authors (GLB) or from New York Sea Grant [4].

RESULTS AND DISCUSSION

Analysis of Shellfish Samples

A representative HPLC trace showing the gonyautoxin fraction from a crude extract of giant scallops is shown in Figure 3. In contrast to the PCRS, the ECOS system was more sensitive to fluorescent impurities present in the extracts. These could be removed by solid phase extraction using a C18 cartridge [8, Goddard and Boyer, in preparation] or detected by comparing chromatograms with the electro-

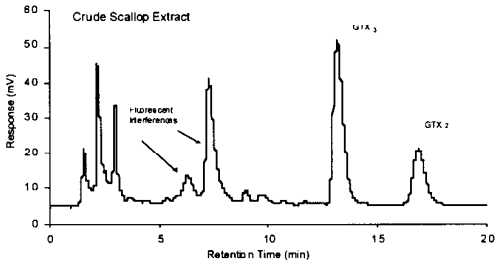

Figure 3. A representative HPLC-ECOS trace of the gonyautoxin fraction from a scallop digestive gland extract. The fluorescent impurities indicated in these samples could be removed by treatment with a C18 solid phase extraction cartridge. Solvent system was Oshima B [10].

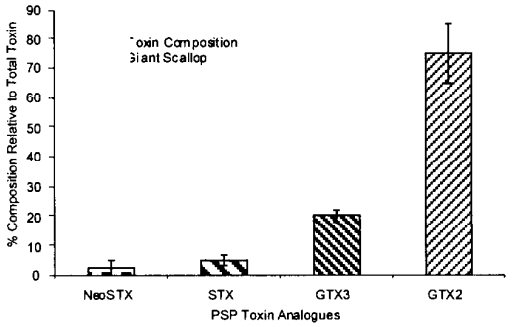

Figure 4. The toxin composition of *Placopectin magellanicus* as determined by HPLC-ECOS. (± s.d. n=40)

chemical cell turned on and off. The toxin composition in both extracts of giant scallops and geoduck clams predominately consisted of GTX_2 and GTX_3 with lesser amounts of saxitoxin and neosaxitoxin (Figure 4).

The relationship between the total toxin content determined using the mouse bioassay and by HPLC using either PCRS or ECOS oxidation for 40 giant scallop or 25 geoduck clam samples is shown in Figures 5 and 6. The regression line obtained comparing the post-column reaction system (PCRS) versus the mouse bioassay for giant scallops showed an R^2 of 0.94 with a slope of 1.0. A similar comparison of the HPLC-ECOS assay versus the mouse bioassay for scallops gave and R^2 of 0.92 and a slope of 0.91. Geoduck clams also showed excellent agreement when the HPLC-PCRS assay was compared to the mouse bioassay (R^2 = 0.99; slope = 0.92). Statistical analysis using a one-sided t-test at the 95% confidence interval indicated the slope of the regression line was not significantly different from 1.0 in all three cases. In contrast, a comparison of the HPLC-ECOS assay versus the mouse bioassay for geoduck clams gave a excellent correlation (R^2 = 0.96) but the slope of the regression line (0.66) was significantly less than 1.0.

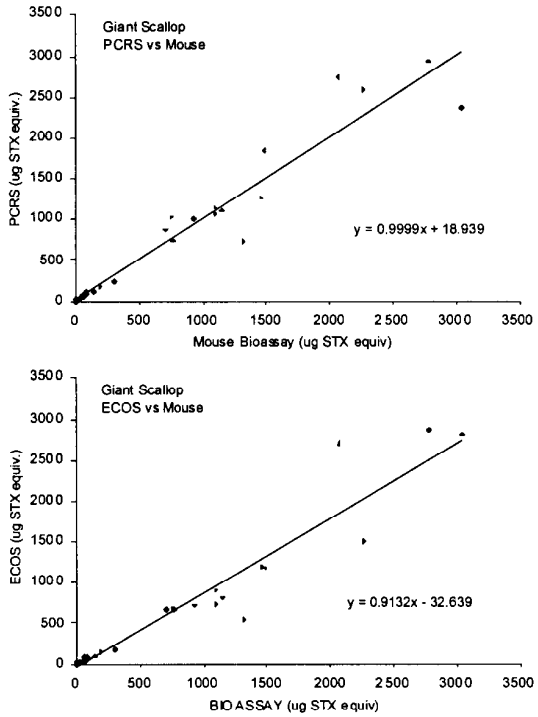

Figure 5. The relationship between total toxin, as determined using HPLC-PCRS (top) or ECOS (bottom), and the mouse bioassay in giant scallops (n=40).

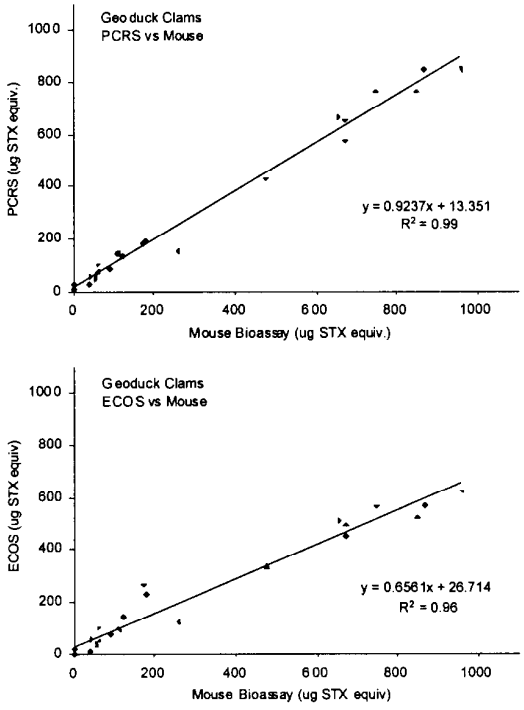

Figure 6. The relationship between total toxin, as determined using HPLC-PCRS (top) or ECOS (bottom), and the mouse bioassay in geoduck clams (n = 25).

DISCUSSION

The post column reaction system (PCRS) coupled with HPLC is a well-established system for the separation and analysis of PSP toxins. Correlation with the mouse bioassay is generally excellent. Previous comparisons have reported R^2 values ranging from 0.83 (n=86) for soft shell clams [12], 0.88 (n = 19) for scallops [8], and 0.95 (n = 95) for mussels [12]. The results reported here for the HPLC-PCRS analysis are in excellent agreement with these previous studies. Furthermore, the slope of the regression line was not significantly different than one, indicating that HPLC-PCRS should accurately estimate the mouse toxicity in these shellfish samples.

Despite these observations, an interlaboratory trial sponsored by NRC Canada found unacceptable variation in the PCRS approach (M. Quilliam, personal communication). One possible reason for this variation was the difficulties associated with the PCRS. The electrochemical oxidation of PSP toxins using ECOS may offer a viable replacement for the chemical oxidation of the PCRS approach. Detailed analysis of 40 scallop and 25 geoduck samples showed good correlation with the HPLC-PCRS analysis and the mouse bioassay. The differences in concentrations obtained with the two oxidation approaches were well within the average percent error (_ 10-20 %) for the HPLC or mouse bioassay methods. As expected, much of the variation was observed in samples with less than 80 µg STX equivalents per 100 g fresh weight. The ECOS approach proved to be less expensive and easier to operate than the PCRS. ECOS also had other advantages in that the electrochemical cell was easily turned on or off, providing an excellent tool for the detection of naturally fluorescent compounds [13] that may co-elute with known PSP toxins (Figure 3).

The ECOS system still has limitations. The electrochemical cell was prone to clogging with some matrices (i.e. scallops) and cell cleaning introduced an additional time and labor requirement [4]. While we were able to analyze dinoflagellate and cyanobacteria samples directly without any further clean-up [1, 3], a similar analysis of the 25 geoduck samples resulted in a systematic loss in sensitivity that caused the ECOS system to underestimate the mouse toxicity. This problem did not occur in scallop samples that were first filtered through a C18 solid phase extraction cartridge to remove nonpolar material. A similar matrix effect on the HPLC analysis of PSP toxins in shellfish has been reported by others [12].

In this study, the shellfish samples contained predominately saxitoxin, neosaxitoxin and $GTX_{2/3}$. All extracts were initially boiled in 0.1 N acetic acid as part of the AOAC extraction protocol and this treatment would have converted any N-sulfo or "C" toxins to their saxitoxin or gonyautoxin analogs. The direct analysis of the N-sulfo or "C" toxins by ECOS adds additional

complications. The ECOS system is compatible with all three commonly used solvent schemes for the analysis of PSP toxins, namely the Sullivan and Wekell binary gradient [14], the Oshima "A" and "B"isocratic systems [8-10], and the Thielert *et al.* binary step gradient [5, 16]. Unfortunately the "C" toxins are poorly separated using these neutral solvent systems and are best resolved using acidic mobile phases such as "Oshima C" [10]. Electro-chemical oxidation of the saxitoxin ring system does not occur at pH <6 [4]. Since the mobile phase in the ECOS approach also controls the oxidation pH, the pH of the mobile phase must be adjusted to >7 after the column but prior to the electrochemical cell for the analysis of C-toxins. This requires an additional pump and connections [7]. This pH adjustment is not necessary for routine monitoring programs using the AOAC extraction protocol. For those circumstances, the ECOS method would provide a robust and viable alternative to the mouse bioassay or HPLC-PCRS for the analysis of PSP toxins.

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This work was supported by New York Sea Grant. We would like to thank Dr. Kats Haya (St. Andrews Biological Station, Fisheries and Oceans - Canada, St. Andrews, NB) for the collection and bioassay of the scallop samples and Dr. Kelly Curtis (Fisheries Center, University of Washington, Seattle WA) for the collection and bioassay of the geoduck samples.

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TOTAL PRODUCTION OF C2 TOXIN BY *ALEXANDRIUM TAMARENSE*

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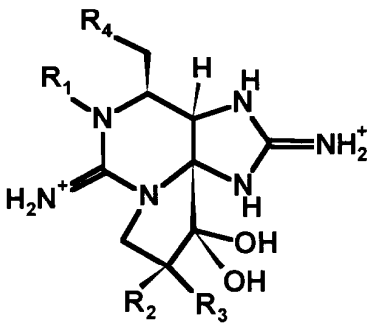
ABSTRACT

Milligram amounts of C2 toxin (C2T) and gonyautoxin 3 were produced for metabolism studies from a South China Sea strain of *Alexandrium tamarense* (ATCI01) that produces C2T as a predominant member of the paralytic shellfish toxin family. The toxic dinoflagellate was cultured batch wise in Fernbach flasks in artificial seawater K-medium. Large quantities of C2T were found to be present extracellularly in the culture medium. This was confirmed by HPLC-FLD and the mouse neuroblastoma assay. As much as 55 μg/L of C2T was found in the culture medium with an additional 45 μg/L found intracellularly. The presence of such large quantities of toxin extracellularly suggests that paralytic shellfish toxins may be classified as exotoxins.

INTRODUCTION

Harmful algal blooms (HAB) are a serious public health, economic and environmental problem in Hong Kong. On average, there are over 20 outbreaks annually of HAB in Hong Kong waters [1]. Recent and historical data indicate that paralytic shellfish toxins (PST) (Fig. 1) are one of the prevalent HAB toxins found in Hong Kong. In order to obtain sufficient amounts of pure PST for metabolism and other studies, our efforts have been focused on the production of PST by toxin-producing strains of *Alexandrium tamarense*.



R_1	R_2	R_3	R_4:-OCNH$_2$	R_4:-OCNSO$_3$
H	OSO_3	H	GTX2	C1
H	H	OSO_3	GTX3	C2

Fig. 1. Generic chemical structures of paralytic shellfish toxins.

Nutritional and environmental factors influencing algal growth and toxin production in batch cultures were investigated, and milligram quantities of PST were produced under a set of optimized conditions. In carrying out these optimization studies using artificial seawater, PST were consistently found in the culture medium as extracellular toxins.

MATERIAL AND METHODS

A toxigenic strain of *Alexandrium tamarense* (ATCI01) isolated from the South China Sea was used throughout this study. Stock cultures were maintained in natural seawater medium (NSW) at 23°C with 5000 lux and a 14/10 hour light cycle. The production cultures received a 1% innoculum from stock cultures in their late exponential phase with approximately 6000 cells/ml. Production cultures were grown in 1.5 L of artificial seawater (ASW) [2] supplemented with K-medium contained in 2.8 L Fernbach flasks under the same incubation conditions as for stock cultures for 21 days.

At regular time intervals, samples were taken to measure pH, cell number, total sodium channel blocking activity, and toxin content. Cell counts were done manually using an inverted microscope. Cells were harvested by centrifugation of 10 ml of culture samples at 3000 rpm for 5 min. Intracellular toxins were extracted by sonication of harvested cells. Extracellular toxins were extracted and concentrated from culture medium by activated charcoal and eluted using a mixture of acetic acid: ethanol: water (1:20:79). The sodium channel blocking activity was measured using a mouse neuroblastoma assay (MNB) modified from the method of Manger *et al.* [3] by using XTT instead of MTT for the endpoint determination. Toxins were determined by HPLC-FLD with post-column derivatization following the Oshima method [4] and using a column temperature of 30°C and a reaction coil temperature of 45°C as modified by Anderson *et al.* [5]. Production of gonyautoxins (GTX) was performed by immersing a microcentrifuge tube containing C-toxins in 0.1M HCl into boiling water for 10 min. C-toxin standards were generously provided by Prof. Y. Oshima, Tohoku University, Japan. GTX standards were purchased from NRC, Canada.

RESULTS AND DISCUSSION

The initial profile of PST produced by ATCI01 in a late log phase batch culture is shown in Fig. 2. C2 toxin (C2T) was found to be the predominant member of the toxin family. This profile is consistent with

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those found by other investigators of the same species isolated from the same region [5]. However, it should be noted that the toxin profile of this strain gradually changed with time such that at the end of these experiments, C2T was almost exclusively produced with very little GTX production.

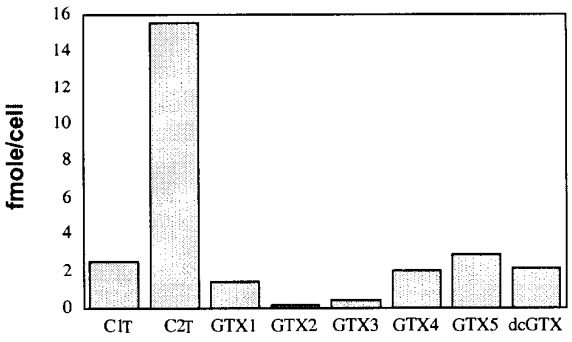

Fig. 2. The initial profile of toxins produced by ATCI01 in natural seawater.

In this study, ASW was used because it was chemically defined and was more conducive to optimization of culture conditions. The growth and toxin production curves of a typical run in ASW are shown in Fig. 3. Toxin production appeared to be growth-associated. Sodium channel blocking activity measured by MNB correlated very well with HPLC data (Fig. 4). Correlation between MNB and HPLC data was very good with R^2=0.951.

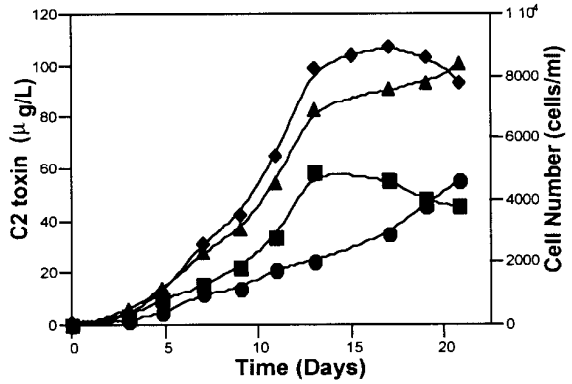

Fig. 3. The growth and toxin production curves of ATCI01 in a batch culture in ASW. (■)intracellular toxin, (●)extracellular toxin, (▲)total toxin, (♦)cell number.

Upon direct HPLC injection of both the ASW-medium and NSW-medium from cultures in the exponential and stationary phases, significant quantities of putative C2T was detected. The extracellular toxin was found in the culture medium from day 2 onward. During the exponential phase, intracellular C2T peaked at 58 μg/L with 24 μg/L in the medium. However, C2T in the culture medium reached 55 μg/L in the late stationary phase with an additional 45 μg/L found intracellularly. Initially, only C2T was found in the medium. However, as incubation continued, C1 toxin (C1T) was also found in the culture medium, presumably due to natural epimerization of C2T to C1T facilitated by the alkalinity of the medium.

In order to confirm that these were indeed C-toxins, the toxins were extracted and concentrated by binding to activated charcoal. The concentrated toxin extracts were subjected to acid hydrolysis, which converts C-toxins quantitatively to GTX. Extracts before and after hydrolytic conversion were analyzed by HPLC (Fig. 5).

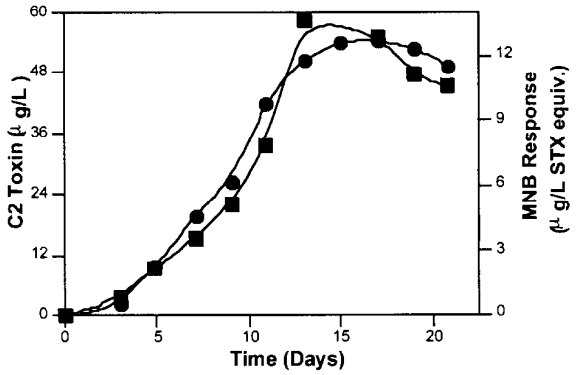

Fig. 4. Comparison of C-toxins produced by ATCI01 in a batch culture in ASW, as measured by MNB and by HPLC. (■)HPLC, (●)MNB.

Analyzed with C-toxin buffer, C2T was present before hydrolysis but absent after hydrolysis. Analyzed with GTX buffer, GTX3 was absent before hydrolysis but was present after hydrolysis. The extracts were also subjected to MNB with the anticipated increase in toxic potency as observed. These two tests confirmed that C2T was definitely present in the culture medium. These results suggest that C2T had passed, either actively or passively, through the cell membranes into the medium.

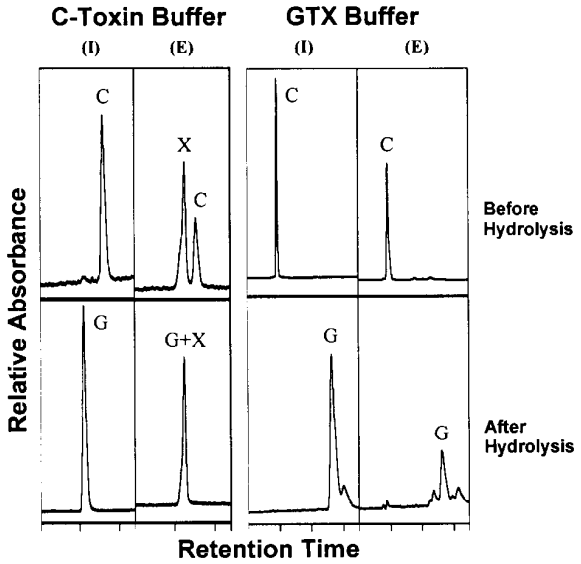

Fig. 5. HPLC chromatograms showing C2T(C) and GTX3(G) before and after acid hydrolysis at 100°C. (I)=intracellular, (E)=extracellular, (X)=unidentified.

The total production of C2T per cell was determined by adding together the intracellular and the extracellular toxin values (Fig. 6). It can be seen that toxin production was highest from the earliest time point in the exponential phase and gradually decreased with time. Reporting of the intracellular toxin production alone would underestimate the toxin production per cell by up to 50% depending on the stage at which the cells were harvested.

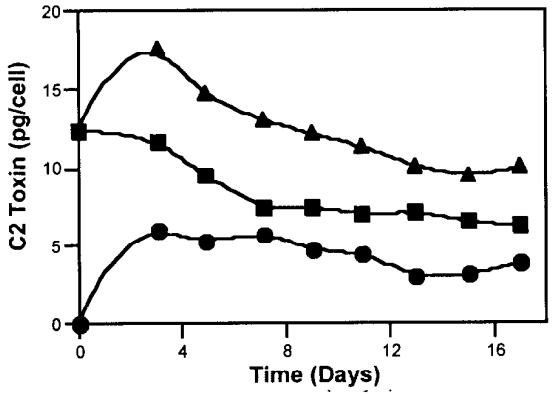

Fig. 6. Kinetics of intracellular and extracellular toxins production in a 21 day batch culture. (■)intracellular toxin, (●)extracellular toxin, (▲)total toxin.

The production kinetic data indicate that extracellular C2T was passed into the medium at a uniform rate until in the late exponential or early stationary phase (Fig.7). The rate increased from the onset of the stationary phase, during which cell numbers decreased only slightly. The extracellular toxin continued to increase but was not due to cell lysis alone. This suggests that the 'leakiness' of the cell membranes increased during the stationary phase. These results would support the argument that PST excretion could act as a defense mechanism or, as suggested by Wyatt and Jenkinson [6], as signalling molecules. During the late stationary phase of stock cultures (data not shown), the concentration of toxins in the medium exceeded 200 μg/L.

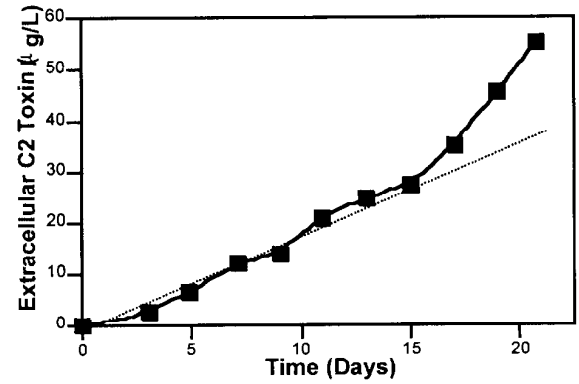

Fig. 7. Extracellular C2 toxin found in the medium.

CONCLUSION

The ATCI01 strain of *Alexandrium tamarense* produced predominantly C2T, a large proportion of which was found in the culture medium throughout a 21-day batch culture. Therefore, PST may also be considered as exotoxins under certain conditions. As exotoxins, they may have different environmental health implications compared to being endotoxins. This culture was useful in accumulating milligram amounts of C2T and GTX3 for other studies.

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EXOTOXINS PRODUCED BY THE TOXIC DINOFLAGELLATE ALEXANDRIUM MINUTUM CHARACTERISATION BY RADIORECEPTOR AND NEUROBLASTOMA ASSAYS DURING THE GROWTH CYCLE

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ABSTRACT

Endocellular Paralytic Shellfish Poisons (PSP) and exocellular toxicity of the growth medium were investigated in the red-tide dinoflagellate *Alexandrium minutum* over its growth cycle in 15L batch cultures. Total PSP activity of the endocellular medium (HPLC) and saxitoxin-like activity (SLA) of the exocellular medium (neuroreceptor binding assay) as well as toxicity of the cell-free medium to *Artemia* were examined. Peaks in toxicity of the exocellular medium coincided with peaks in the endocellular concentration of PSPs, both occurring in early to late lag phase and declining with culture age. SLA of the medium was found to be approximately 20 times higher (4-6 pg STX equivalents $cell^{-1}$) than the endocellular PSP toxicity (0.17- 0.29 pg STX equiv. $cell^{-1}$). Toxicity of the *A. minutum* cell-free medium towards *Artemia* did not appear to be correlated with exocellular SLA or endocellular total PSP toxicity. Also, a brevetoxin-like substance was detected by neuroblastoma tissue culture assay in the exocellular medium although no brevetoxin-like activity was detected by neuroreceptor binding assay (site 5 specific). It is concluded that, in addition to a saxitoxin-like agent, *A. minutum* produces an exotoxin capable of killing *Artemia*, which is heat labile (reduced toxicity to *Artemia* above 80°C), but is not a GTX or Brevetoxin.

INTRODUCTION

Of the genera of phytoplankton causing harmful algal blooms (HABs) the dinoflagellate *Alexandrium* contains the largest number of toxic species [1]. Although some species have been found to produce exotoxins [2, 3] these toxic principles have never been chemically characterised. Phytoplankton have been found to release up to 16% of their total fixed CO_2 as exudates [4] and algal exudates comprise a portion of the diet of clams (*Venus verrucosa*) [5]. Although evidence exists to suggest that filtered water from an *A. minutum* bloom is toxic to fish (Y. Halim, pers. comm.), evidence for the release of PSPs into the surrounding medium by dinoflagellates is limited [6].

The following work attempts to determine and correlate the endocellular (using HPLC) and exocellular toxicity (by neuroreceptor binding assay, neuroblastoma tissue culture assay and total toxicity to brine shrimp) of *A. minutum* in batch culture, over the entire growth curve of the dinoflagellate. Heat stability of the exocellular toxic principle(s) were also investigated.

MATERIALS AND METHODS

Algal Cultures

Three large 15L cultures (0.2µm filtered, 33ppt, GSe seawater) of *A. minutum* (AMAD-06, isolated from Port River, Adelaide, Australia by J. Cannon/S. Blackburn) were grown in sterile 20L polycarbonate (Nalgene) bottles. A forth bottle was filled with sterile GSe seawater (33ppt, 0.2µm filtered) medium only, as a control. Cultures were left to grow for 14 days (17°C, 12hr L/12hr D, 80µ$Em^{-2}s^{-1}$ light) After 14 days, 100ml subsamples were removed from the batch cultures, every second day for 48 days then every 3-4 days for the remainder of the experimental period. All sampling took place at midday. A 4ml aliquot was taken from the sample and fixed with glutaraldehyde (to 2%) and used for cell counting in a Sedgwick-Rafter counting chamber.

Endo/Exocellular toxicity

The remainder of the sample was gently gravity filtered through GF/F filters (0.45µm pore size). The filtrate was analysed with neuroreceptor binding assays [7] for site 1 specific, saxitoxin-like activity (SLA) and site 5 specific, brevetoxin-like activity (BLA) (carboy culture 3 only) [8]. A neuroblastoma tissue culture assay which responds to biotoxins (eg. STX, TTX, PbTx) was also utilised [9], although this data is not shown here. An attempt was also made to measure, in samples from the same days, the concentration of any exocellular PSPs by first adsorbing the PSPs on activated charcoal [10] and Carbograph columns (Alltech, Australia), eluting the toxins and analysing for PSPs by HPLC [11]. The exocellular medium was also assayed for toxicity to *Artemia* [3]. Endocellular PSP toxins were measured using HPLC [11].

Heat stability of exocellular toxins

Three separate, one litre cultures of *A. minutum* were also grown each with a corresponding control of sterile seawater replete with nutrients (GSe). Cultures were grown to the mid/late exponential phase and harvested. Cells were separated from the growth medium again via gentle gravity filtration and the cellular fraction was discarded. Each culture and control was divided into 11 equal aliquotes and subjected to temperatures of 17, 20, 30, 40, 50, 60, 65, 70, 80, 90, and 100°C for one hour. The 17°C cell-free culture was not heated but used as a

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control and kept at the normal growing temperature of *A. minutum*. Acidification of the medium was not carried out as total medium toxicity was being measured and acidification would have affected *Artemia*. Once at room temperature, treated medium and controls were used to challenge *Artemia*.

RESULTS

Culture Growth and Endocellular PSP

A long lag phase (data not shown) was observed in all batch cultures, but not in small, 250 ml cultures (of the same strain) and appears to be a phenomenon of large cultures of this alga only. A small culture inoculum is likely to have contributed to the long lag phase also. Peak cell concentrations for cultures 1, 2 and 3 were 7, 10 and 17 x 10^7 cells L^{-1} respectively, while maximal growth rates achieved were 0.4, 0.7 and 1.1 divisions per day, respectively. Growth in culture 1 was slower than cultures 2 (Fig 1A) and 3 (only results of carboy 2 are given here). Peak cell concentrations in culture 1 at the beginning of the stationary phase (day 115-146) were just over half those of culture 2 and over a third of those in culture 3. The highest endocellular PSP toxin concentrations of 0.17, 0.29 and 0.14 pg STX equivalents $cell^{-1}$ (cultures 1, 2 (Fig. 1B) & 3, respectively) were found in the day 73 (mid/late lag phase) samples. Only GTX (1-4) toxins were detected in the *A. minutum* cultures.

Exocellular toxicity in carboy cultures

The time course of exocellular SLA per cell in the *A. minutum* cultures (Fig. 1C) appeared to follow that of endocellular PSP but at a concentration per cell approximately 20 times higher. The highest exocellular SLA measured as pg STX equiv. $cell^{-1}$ for cultures 1, 2 (Fig. 1C) and 3 were 3.7, 5.5 and 1.8, respectively, also on day 73. Generally, as the cultures aged the SLA declined and continued to decrease into the death phase. No GTXs were detected in the cell free, filtered culture media after concentration on charcoal and Carbograph columns and subsequent HPLC of washings.

The toxicity to *Artemia*, of the cell free culture medium from cultures 1, 2 and 3 was compared with the same medium's SLA expressed in STX equivalents L^{-1} (Fig. 1D).

Dinoflagellate-free culture medium was always toxic to 4-day old *Artemia* resulting in tD50 values ranging from 4.5 to 12 hours. Peak cell-free medium toxicities occurred at day 133 (mid stationary phase) in cultures 1 and 2 (Fig. 1D) but earlier in culture 3 on day 104 (early exponential phase). No toxic response was observed in the controls. The toxicity of the cell-free culture medium did not therefore correlate with the concentration of saxitoxin-like substances in the medium.

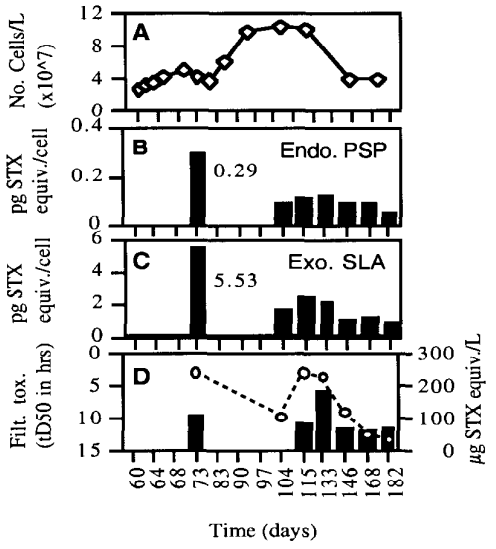

Figure 1: *A. minutum* dinoflagellate numbers (A), Total endocellular PSPs (HPLC) (B), total exocellular saxitoxin-like toxins (neuroreceptor binding assay) (C) and toxicity of exocellular medium to *Artemia* (D) in carboy culture 2. Note reverse scale in D for exocellular toxicity to *Artemia* (short tD50 means high toxicity). Total exocellular SLA (µg STX equiv.) (neuroreceptor binding assay) calculated as per litre of culture medium are superimposed (dashed line) in D.

Neuroblastoma tissue culture bioassay also confirmed the presence of saxitoxin-like substances in the cell-free culture medium of *A. minutum*. However, it also indicated the presence of an brevetoxin-like substance that confounded the SLA results. A high degree of brevetoxin-like activity (BLA) was found in the cell-free culture medium of *A. minutum*, particularly in cultures 2 and 3. Brevetoxins are powerfull sodium channel activating toxins and therefore their presence was further examined using a site 5 specific neuroreceptor binding assay similar to that used for the detection of site 1 specific, saxitoxin-like toxins. No BLA was detected in any of the samples from culture 3 and hence a similar assay of cultures 1 and 2 was not pursued.

Heat stability of exocellular toxins

Heat stability of the exocellular toxic principle(s) appeared to be affected by temperature in a non-linear manner (Fig. 2). *Artemia* bioassays on control, cell-free medium (*A. minutum* culture with cells removed) that had been held at 17°C produced a tD50 of 2.3 ± 0.6 h. A significant decrease in toxicity did not occur until 90°C (tD50 = 9-12h). Although there appear to be two previous small oscillations in toxicity (between 30°C and 65°C), these lows and highs more likely represent a high variability or fluctuations in the "noise" level in the

bioassay data, particularly given the apparent errors. Control culture media appeared unaffected by any of the heat treatments and were non-toxic to *Artemia* in all exposures.

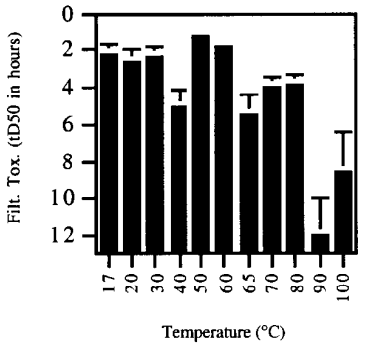

Figure 2: Time to death of 50% of *Artemia* (tD50) exposed to cell-free *A. minutum* growth medium after one hour heat treatment at different temperatures. Note use of reverse scale (short tD50 = high toxicity).

DISCUSSION

Endo/Exocellular toxicity

Not only a positive correlation in timing, but a 20 times difference was observed between the concentration of PSPs within dinoflagellate cells and the concentration per cell of SLA in the exocellular medium. It is difficult to extrapolate from data obtained from *in vitro* studies to natural algal blooms. Also the difference in method used for examining endo- (HPLC) and exo- (neuroreceptor assay) cellular toxins could posibly account for some of this disparity. However, this observation suggests that in the midst of a bloom of *A. minutum* the majority of SLA may exist in the exocellular medium or the water column, rather than inside the cell as previously thought. This release of activity into the surounding water is consistent with a substance that is allelopathic or defensive in nature, although this and other possible explanations remain to be examined in more detail. What is surprising, is the possible presence of another substance acting not unlike NSP toxins (but not brevetoxin) and which has not previously been reported from any species of *Alexandrium*.

The death of *Artemia* (presented as tD50) from *A. minutum*'s cell-free growth medium was completely independent from the concentrations of both endocellular PSPs and exocellular SLA. Similarly, there was no correlation between growth stage and the highest toxicity of the medium towards *Artemia* although this peak in toxicity was always in the exponential/stationary phase when cellular density was at its peak. This was also the case with the concentration of exocellular SLA on a per cell basis when compared to cell density. Again, although the toxicity of some PSP toxins is affected by heat, the effect is to increase toxicity and not a decrease as observed here.

Effect of heat on exocellular toxicity

A significant reduction in the toxicity of *A. minutum* cell-free medium to *Artemia* was observed when heated above 80°C. This is in contrast to what one would expect if the medium contained N-sulfocarbomyl PSP toxins. Oshima *et. al.* [12] found that heating weak N-sulfocarbomyl toxins (with a weak acid) produced the much more potent carbamate and decarbamoyl-GTXs 1-4 with a 10-100 fold increase in toxicity. Similarly, if we hypothesised the presence of GTX toxins, then we would expect toxicity to remain relatively stable when heated, not the decline witnessed in this study. Furthermore, pure PSPs (GTXs 1-4) in solution do not kill *Artemia* (data not shown). Hence, the reduction in toxicity above 80°C observed here reinforces the suggestion that the toxic principle(s) in the exocellular medium is not a PSP.

Conclusions

The aetiology of the toxicity of the *A. minutum* medium to *Artemia* has yet to be clarified. PSPs in solution do not kill *Artemia* and do not produce pathological tissue damage. Algae that produce sodium channel activating (SCA) toxins, such as brevetoxins [13] and ciguatoxins [14] do kill *Artemia*. Other SCA producing algae, such as *Chattonella* (brevetoxins), also cause pathological tissue damage in fish [15]. The neuroblastoma tissue culture assay conducted in this study suggested the presence of a brevetoxin-like toxin, however, a site 5 specific neuroreceptor binding assay did not detect any brevetoxin-like activity. These observations suggest that another unknown toxic principle(s) is active in the exocellular medium of *A. minutum*.

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MONOCLONAL ANTIBODY-BASED ENZYME IMMUNOASSAY FOR DOMOIC ACID BY USING HAPTEN-PROTEIN CONJUGATES OBTAINED AT THE NANOMOLAR LEVEL IN A REVERSED MICELLAR MEDIUM.

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ABSTRACT

A competitive enzyme-linked immunosorbent assay (ELISA) to measure domoic acid (DA) has been developed. DA-protein conjugates were prepared via the mixed anhydride method of Erlanger performed in a reversed micellar medium using 0.32-0.64 µmol of DA in a 100-fold molar excess relative to protein. Two specific monoclonal antibodies (MAbs), 1D12 and 3E1 were produced by hybridoma technology following immunization of a BALB/c mouse with a DA-bovine serum albumin conjugate. No significant cross-reactivity was observed with either glutamic, aspartic or kainic acids or proline. MAb 1D12 enabled the accurate and reproducible detection of DA levels in spiked mussel extracts pre-cleaned through a solid phase extraction column. A very good correlation (r^2 = 0.96; n = 6) was observed between the actual amounts of DA added and amounts detected. The working range achieved with this ELISA (0.3 - 3 µg/g of original mussel tissue) strongly suggests its potential as alternative assay for routine monitoring of shellfish (Maximum Permitted Level = 20 µg / g of shellfish tissue).

INTRODUCTION

Domoic acid (DA), a potent neuroexcitatory amino acid, is the causative agent of amnesic shellfish poisoning (ASP), a severe syndrome that occurred after the consumption of contaminated shellfish on the east and west coasts of North America. Since the eastern Canadian incident in 1987 [1-3], the primary source of DA was identified to be the marine diatom *Pseudo-nitzschia pungens* forma *multiseries* [4]. The wide spread occurrence of *Pseudo-nitzschia* species together with recent observations of DA in plankton and shellfish of many temperate regions has raised global awareness to DA.

For these reasons, several methods to quantify DA have been developed. Among them are the mouse bioassay [2] and more specific analyses based upon either instrumental methods (gas or liquid chromatography coupled to mass spectrometry and capillary electrophoresis), or pharmacological and immunochemical techniques [5]. Although specific polyclonal antibodies have been produced [6-12], their routine use is hampered by limited availability and requires batch standardization.

This work describes the production and characterization of monoclonal antibodies (MAbs) to DA using fully characterized DA-carrier conjugates we have produced [12] according to a rapid and simple procedure recently designed [13]. This is the first documentation of the production of MAbs to DA using very tiny amounts of haptenic material (100-200 µg) in a reversed micellar medium and their potential use in routine monitoring of shellfish prior to human consumption.

MATERIALS AND METHODS

Preparation of Conjugates

Conjugates were prepared as previously described [12] using the mixed anhydride coupling method of Erlanger *et al.* [14] modified by Pauillac *et al.* [13]. All chemical reagents, unless otherwise stated, were purchased from Sigma Chemicals Co (St. Louis, MO).

Briefly, 0.321 or 0.642 µmol of DA dissolved in 20 µL dry dimethylsulfoxide (DMSO) were converted to a mixed anhydride (yield > 95%) by adding a 10-fold molar excess of tributylamine and isobutyl chlorocarbonate. The conjugation of activated DA to carriers, was carried out in the system of reversed micelles of aerosol OT (AOT or sodium bis(2-ethylhexyl) sulfosuccinate) in octane [13]. Two carriers were used throughout: bovine serum albumin (BSA) and ovalbumin (OVA) providing an initial hapten/protein molar ratio around 100:1. Conjugates were recovered by acetone precipitation, resuspended in 1 mL distilled water, filter sterilized (0.22 µm), dispensed into sterile tubes then freeze-dried overnight and stored at -20°C until use. Carrier haptenization was assessed spectrophotometrically on PBS resuspended samples using the previously reported extinction coefficient for DA at 242 nm (ε = 83.83 mg^{-1} cm^2) and considering total carrier content as reported earlier [13]. The composition of the phosphate buffered saline solution (PBS) was as follows: 0.01 M KH_2PO_4/K_2HPO_4-150 mM NaCl, pH 7.2.

Mouse Immunization

Two female BALB/c mice (7 weeks of age) were immunized by intraperitoneal (i.p.) injections of 50 µg DA-BSA conjugate emulsified in 100 µL PBS/adjuvant mixture (1:1). Primary injection was made in Freund's complete adjuvant (FCA) and the following five secondary boosters were administered in Freund's incomplete adjuvant (FIA) at 3-4 week-intervals. Mice were sequentially bled from the retro-orbital venous system and individual sera were tested for anti-DA antibody activity.

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MAb Production and Selection.

One month after the last i.p. booster, the best responder received an intrasplenic injection (20 µg immunogen in PBS) under ketamine base anesthesia. Three days later the mouse was sacrificed under ether anesthesia and the spleen removed. Hybridomas were obtained by fusion of sensitized spleen cells with P3-X63-Ag8.653 myeloma cells at a ratio of 5:1 using the ClonaCell-HY kit (Stemcell Technologies Inc., Canada). Hybridomas were grown in a methylcellulose-based semi-solid selecting medium and clones were transferred after 15 days in 96-well tissue culture plates filled with normal medium. Specific antibody-secreting hybridomas were screened by indirect enzyme-linked immunosorbent assay (ELISA) employing DA-OVA as target antigen and other non-relevant control antigens. Selected hybridomas were cloned by plating onto semi-solid normal medium and maintained in tissue culture plates. Cells from two selected clones were grown as solid tumor in FIA-primed BALB/c mice and MAbs were purified from ascite fluids through protein A-Sepharose 4B affinity column. MAb subtyping was performed by means of an ELISA commercial kit from Boehringer-Mannheim (Germany).

Competitive ELISA.

Flat-bottomed polystyrene 96-well Maxisorp Immunoplates (Nunc, Roskilde, Denmark) were coated with 100 µL of DA-OVA conjugate (250 ng/mL) in PBS by incubation for 1 h at 37°C then overnight at 4°C. They were then washed five times with PBS and blocked with 5% skim milk powder in PBS (250 µl). All subsequent incubation steps, unless otherwise stated, were performed with 100 µL of reactant and carried out 1h at 37°C then followed by three washings with PBS containing 0.1% Tween 20 (PBS-T). Optimum dilution of crude (culture supernatants or ascites) or purified antibody preparations made in PBS-T containing 1% skim milk powder and 1% BSA (PBS-TMB) were preincubated with an equal volume of various concentrations of inhibitors (DA, proline or kainic, glutamic or aspartic acids). Samples of these mixtures were transferred onto DA-OVA-precoated plates. The antibodies associated with the plates were revealed by incubation with goat anti-mouse IgG peroxidase conjugate (GAM-PO) followed by the addition of o-phenylenediamine (OPD). After 30 min at 37° C, the enzymatic reaction was terminated by adding 50 µL of 2 M H_2SO_4 and the absorbance at 492 nm was measured using a Multiskan Plus Titerteck (Flow Laboratories, Puteaux, France).

DA Recovery in Rapid Mussel Extracts

Rapid extraction of blue mussels (*Mytilus edulis*) was performed according to the method developed by Quilliam *et al.* [15]. Extracts (1g tissue equivalent) were used either without additional treatment (crude extract) or pre-cleaned by passing through a strong anion-exchange solid phase extraction column, evaporated to dryness under nitrogen atmosphere then reconstituted in 5 ml of MeCN-H_2O (1:9). Finally, 1 ml samples of these crude or pre-cleaned suspensions (0.2 g tissue equivalent) were spiked with 4 µg of DA analytical standard (DACS-1C, NRC-CNRC, Institute for Marine Biosciences, Halifax, Canada) to generate concentrations ranging from 4,000 to 2 ng/ml by serial dilutions in MeCN-H_2O (1:9). The same dilutions of DA analytical standard were directly prepared in MeCN-H_2O (1:9) to serve as calibration solutions. These spiked samples or calibration solutions were mixed with an equal volume of MAb 1D12 dilution in PBS-TMB and transferred (50 µL/well) onto Covalink NH microplates (Nunc, Denmark) on which DA has been covalently immobilized via N-hydroxysuccinimide and carbodiimide-mediated amide bond formation. DA levels were determined in triplicate, using the competitive ELISA format as described above.

RESULTS AND DISCUSSION

DA-protein conjugates preparation and analysis

DA is a strong chromophore exhibiting an absorption peak at 254 nm (Fig. 1a), therefore its contribution in the conjugate can be easily determined without interference with the protein carrier. Fig. 1b shows the comparative UV absorption spectra of DA-BSA conjugate and a control reaction (BSA plus non-activated DA). These DA-protein conjugates prepared at the nanomolar level in a reversed micellar medium were found to contain around 10 and 20 hapten molecules per OVA and BSA carriers, respectively, as deduced from spectrophotometric measurements. This simple rapid and low cost one-step synthesis procedure [13] was found very efficient for preparing conjugates using small amount of haptenic starting material. On the one hand, these well-characterized conjugates were found to be highly immunogenic, since specific polyclonal antibodies were raised in mice immunized against DA-BSA [12]. On the other hand, they have proven to be stable to storage at -20°C for at least six months as evidenced by consistent performance of DA-OVA in immunoassay.

MAb production and characterization

Following completion of the immunization schedule, 285 hybridomas were produced in a single fusion experiment, 26 out of them (9.12 %) were selected on the basis of culture supernatant reactivity to DA-OVA and absence of cross-reactivity with control solid-phase antigens including the native carriers (BSA and OVA) and their conjugates with a non-related hapten (cholesterol). However clear inhibition in the competitive inhibition ELISA was observed for only two hybridoma supernatants using 1 µg DA per assay as inhibitor. Hybrids in positive wells were cloned and injected to FIA-primed BALB/c mice to induce ascite production.

Two purified MAbs of IgG2a,κ subclass (1D12 and 3E1) were studied in greater detail. None of these MAbs reacted with kainic, glutamic and aspartic acids or proline (data not shown) even at the highest concentration tested (100 µg/ml).

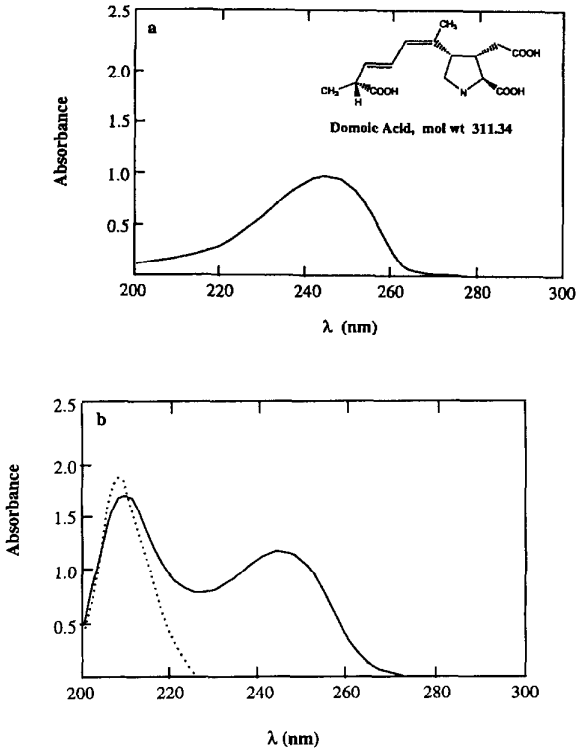

Fig. 1 Spectrophotometric measurements. (a): UV spectrum of DA in PBS/dioxan (99:1). (b): comparative UV absorption spectra. DA-BSA conjugate (solid line); Control reaction (dashed line).

DA recovery in spiked samples

Recovery test using commercially available mussels was carried out using sample extracts spiked with increasing amount of DA standard (Fig. 2).

The concentration of DA in pre-cleaned extract was accurately determined to within less than 5% in the diluent buffer in the range 31 and 306 ng/ml which corresponds to 0.3 - 3 µg/g of original mussel tissue. By contrast crude extract exhibited a strong matrix effect (data not shown). Usually the matrix effects involve interactions of the matrix, the analyte and the technology base of the test method. Regarding the matrix itself, the procedure used has proved to be highly efficient (recovery of DA greater than 90% even at trace levels) [15]. Regarding the analyte and dosage, the interassay coefficient of variation (n = 6) at various concentrations varied between 3-10%.

Owing to the regulation limits of 20 µg DA / g of shellfish tissue, these extraction, clean up and assay procedures should provide a useful complement to the standard HPLC analytical technique currently employed in monitoring DA in shellfish tissue. Although nine isomers of DA have been identified [16], they have not been tested in this study as they are known to play a minor role in ASP. As another important concern is the early warning of developing toxic algal bloom, this assay must be evaluated for DA determination in algal cultures and in seawater collected from the field.

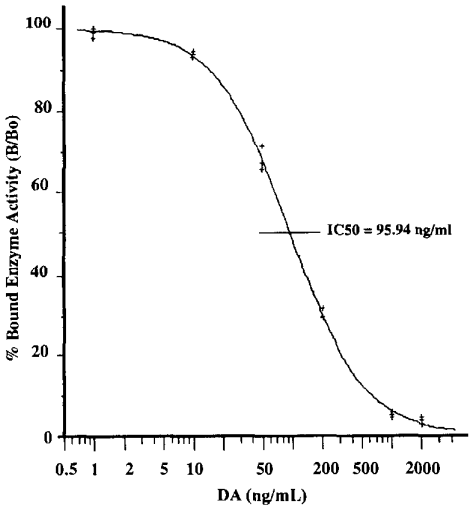

Fig. 2 Competitive ELISA standard curves for DA. DA dilutions were prepared in spiked mussel extracts pre-cleaned through a solid-phase extraction column.

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A NEW FLUORIMETRIC HPLC METHOD FOR THE DETERMINATION OF POLYETHER ACIDIC TOXINS IN MARINE PHYTOPLANKTON

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ABSTRACT

A new rapid, sensitive HPLC method was developed for the determination of acidic diarrhetic shellfish poisoning (DSP) toxins and this has been applied to the determination of toxin profiles in phytoplankton. Extracts from phytoplankton samples were reacted with the fluorimetric reagent, 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (BrDMEQ) and 10% diisopropylethylamine at 50°C for 20 min. Linear calibrations, R^2 = 0.995, in the typical analytical range of 1 - 60 ng OA injected and detection limits of 50 pg OA injected were obtained. OA, DTX-2 and pectenotoxin-2 seco acids (PTX-2SAs) were determined in phytoplankton as their DMEQ derivatives and toxin identification was confirmed using LC-MS. In a comparative study with the reagent, 9-anthryldiazomethane (ADAM), which was applied to the analysis of toxins in phytoplankton, the BrDMEQ method proved slightly more sensitive. However, the superior HPLC resolution of the polyether toxin DMEQ derivatives was the major advantage. Although, both OA and DTX-2 were found in all phytoplankton samples that contained *Dinophysis* sp., PTX-2SA compounds were detected in only a small number of samples.

INTRODUCTION

Polyether carboxylic acids, analogues of okadaic acid (OA), have been shown to be responsible for most outbreaks of diarrhetic shellfish poisoning (DSP) (Figure 1). This syndrome was first reported in Japan [1] and was attributed to dinophysistoxin-1 (DTX-1) which was isolated from mussels [2] that had been grazing on marine phytoplankton belonging to *Dinophysis* sp. Subsequently, two other groups of polyether toxins, pectenotoxins [3] and yessotoxins [4], were identified in shellfish associated with DSP. A number of isomers of OA have been isolated in recent years, including DTX-2 [5,6], DTX-2B [7] and DTX-2C [8]. Dinophysistoxin-2 (DTX-2), was the predominant toxin in major DSP episodes in Ireland [9,10] and this toxin has also been reported in Spain and Portugal [11-13].

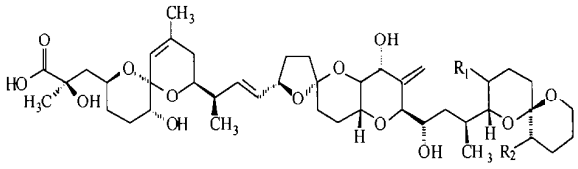

Figure 1. Structures of okadaic acid & analogues
Okadaic acid, OA, (R_1=CH_3, R_2=H)
Dinophysistoxin-2, DTX-2, (R_1=H, R_2=CH_3)
Dinophysistoxin-1, DTX-1, (R_1=R_2=CH_3)

Analogues of pectenotoxin-2 (PTX-2), which have an open chain rather than the large lactone ring, have recently been reported in mussels from New Zealand and from *Dinophysis acuta* in Irish waters. Three isomers have been identified and two have now been fully structurally elucidated, pectenotoxin-2 seco acid (PTX-2SA) and 7-*epi*-PTX-2SA [14,15]. These polyether compounds have a free carboxylic acid moiety (Figure 2) and have a similar chromatographic behaviour to the OA analogues. A 50-fold variation in level of DTX-1 in *Dinophysis fortii* was observed in a recent study in Japan [16].

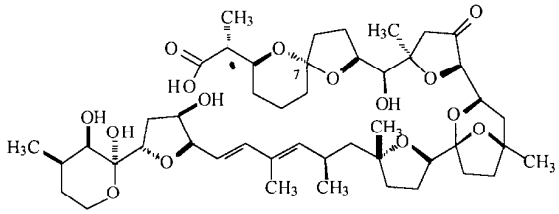

Figure 2. 7-*epi*-pectenotoxin-2 seco acid

A selected-reaction-monitoring micro-LC-MS-MS method was recently developed for the determination of four OA analogues in shellfish tissue [17] as well as a method for the determination of a wide range of DSP toxins, including PTX2SAs in shellfish [18]. However, liquid chromatography with fluorescence detection (LC-FLD), after pre-column derivatisation of acidic DSP toxins, remains the most widely used method for the determination of acidic polyether DSP toxins. The first LC-FLD method for the analysis of the OA class of toxins was reported in 1987 [19] and used 9-anthryldiazomethane (ADAM). Although an

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unstable reagent, the analytical protocol using ADAM is very sensitive and there have been several attempts to improve the robustness of this method [20-22]. A number of other derivatising reagents have been used for the determination of DSP toxins and this topic has recently been reviewed [23].

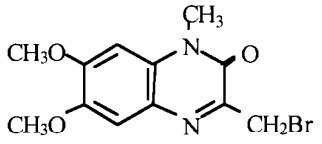

Figure 3. Fluorimetric derivatising reagent, BrDMEQ

We now report the development of a new analytical protocol for the analysis of acidic DSP toxins, including OA and PTX-2SA analogues, in marine phytoplankton using the stable reagent, 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone (BrDMEQ).

METHODS

Materials & chemicals

Purchased chemicals included diisopropylethylamine and 3-bromomethyl-6,7-dimethoxy-1-methyl-2(1H)-quinoxalinone, BrDMEQ (Aldrich, Gillingham, UK), 9-anthryldiazomethane, ADAM (Serva Feinbiochemica, Heidleberg, Germany), okadaic acid (95%, Sigma, Gillingham, UK) and dinophysistoxin-1(Calbiochem-Novabiochem, Nottingham, UK). OA solutions in methanol were calibrated with a certified OA standard solution (OACS-1, National Research Council, Halifax, Canada). A certified standard lyophilised mussel material containing two DSP toxins (MUS-2, National Research Council, Halifax, Canada) was reconstituted in methanol immediately prior to use to gave a mixture with 2.5 µg total toxins/ml (2.29 µg OA, certified, and 0.21 µg DTX-1/ml, uncertified). Dinophysistoxin-2 (DTX-2) was isolated from contaminated mussels [24] and 7-*epi*-pectenotoxin-2 seco acid was isolated from phytoplankton biomass that was predominantly *D. acuta* [15], as described previously. Solvents were HPLC grade (Labscan, Dublin, Ireland).

Phytoplankton sample collection and preparation

The phytoplankton samples were collected from the subsurface (5-10 m depth) in the coastal areas of Glandore, County Cork, Ireland in 1996 and 1999. Phytoplankton samples were obtained using a double phytoplankton net (590 x 120 cm) with an outer net of 50 µm and an inner net of 108 µm mesh. Typically, a 1ml sample of this phytoplankton concentrate contained 2 - 12 x 10^3 cells of *D. acuta* and, after freeze-thawing to disrupt cells, this was extracted with chloroform (2 x 3 ml) and the volume was brought to 10 ml. An aliquot (100 µl) was taken and evaporated prior to derivatisation using ADAM or BrDMEQ.

Derivatisation procedure and HPLC conditions

Using evaporated phytoplankton extract or OA, DTX-2 and 7-*epi*-PTX-2SA standards (0.1-1.0 µg), BrDMEQ in acetone (0.2% w/v, 200 µl), and diisopropylethylamine in acetone (10 % v/v, 200 µl) were mixed, sonicated for 5 min and heated at 50° C for 20 min in amber vials (2 ml). Solvent was removed under nitrogen and the residue was reconstituted in CH_2Cl_2/hexane (50:50 v/v, 2 x 0.5 ml) and transferred to a silica solid phase extraction (SPE) cartridge (3ml, Supelco, Gillingham, UK). The cartridge was then washed with CH_2Cl_2/hexane (50:50 v/v, 6 ml) followed by CH_2Cl_2:ethyl acetate (50:50 v/v, 9 ml). Target derivatives were eluted with CH_2Cl_2/CH_3OH (90:10 v/v, 6 ml). After evaporation to dryness under nitrogen, the residue was reconstituted in methanol (200 µl). A 20µl aliquot was analysed by reversed phase HPLC with a fluorimetric detector (RF-551, Shimadzu).

ADAM derivatives: Isocratic LC, flow 0.5 ml/min, using $CH_3CN/CH_3OH/H_2O$ (80:10:10 or 80:5:15) was performed with a Prodigy C_{18} column (250 x 3.2 mm, 5 µm, Phenomenex), a precolumn (Prodigy C_{18}, 30 x 3.2 mm, 5 µm); fluorimetric detection (λ_{ex} 365 nm, λ_{em} 412 nm).

BrDMEQ derivatives: Isocratic LC, flow 0.5 ml/min, using CH_3CN/H_2O (47:53) was performed using a Discovery C_{18} column (250 x 2.1 mm, 5 µm, Supelco), a precolumn (Security Guard, C_{18}, 4 x 3 mm, Phenomenex), fluorimetric detection (λ_{ex} 370 nm, λ_{em} 450 nm) derivatives.

RESULTS AND DISCUSSION

We previously showed that the collection of phytoplankton biomass, rich in *Dinophysis acuta*, was valuable for the isolation of rare the DSP toxins, DTX-2 and PTX-2SAs. The DSP toxin profiles in phytoplankton extracts can be determined using the ADAM fluorimetric HPLC method but the separation of derivatised toxins can be difficult. Quinoxalinone reagents have been developed for HPLC analysis and they have been shown to produce highly fluorescent derivatives [25]. The stable quinoxalinone reagent, BrDMEQ, was therefore examined for the derivatisation of acidic polyether toxins. Method development was carried out using OA standards and the reaction conditions and the solid phase extraction (SPE) clean-up were optimised.

Toxin standards, or extracts from phytoplankton samples, were reacted in acetone with BrDMEQ in the presence of diisopropylethylamine catalyst under mild reaction conditions, 50°C for 20 min. Sample cleanup was achieved using solid phase extraction (SPE) with a silica cartridge. After transfer of the reaction mixture to the cartridge, which was washed with two solvent mixtures, the derivatised toxins were eluted with

dichloromethane/methanol (90:10). The SPE was optimised for okadaic acid (OA) and the recovery was

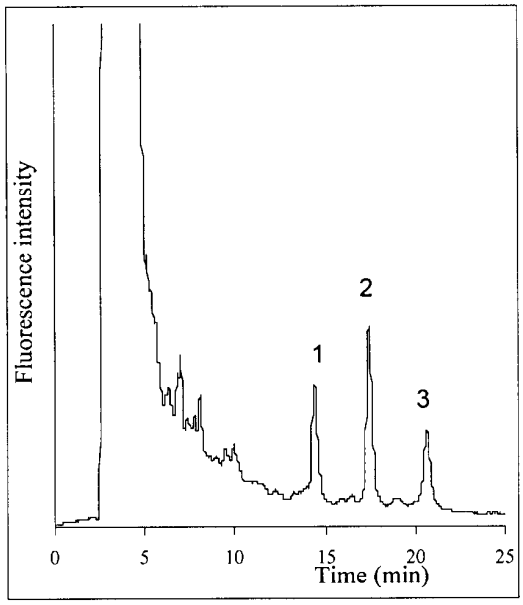

Figure 4. Fluorimetric HPLC of the DMEQ derivatives of OA (#1, 1.5 ng), DTX-2 (#2, 2.1 ng) and 7-*epi*-PTX-2SA (#3, 1.3 ng).

83 ± 2%. Isocratic reversed phase HPLC was carried out using acetonitrile/water with fluorimetric detection. The HPLC conditions were optimised to achieve a good resolution of the polyether toxins, OA, DTX-2 and 7-*epi*-PTX-2SA (Figure 4). The chromatographic separation of the isomers, OA and DTX-2 was examined using different C_{18} columns and the Discovery column gave best peak shapes and a very good resolution. The DMEQ derivatives of OA and DTX-2 were separated by > 3.5 min. Calibrations were linear (R^2 = 0.995) in the typical analytical range of 1 - 60 ng OA injected. Studies were carried out to determine the limit of detection for OA (S/N = 3), which was 50 pg on column. In a comparative study with the ADAM method, the sensitivity using DMEQ was improved by 20%. However, the main advantages of using the BrDMEQ reagent are that it is stable, unlike ADAM, and the good HPLC resolution of both OA and PTX-2SA analogues. Figure 5 shows the chromatogram obtained from the ADAM derivatisation of a phytoplankton (mainly *D. acuta*) extract. The ADAM derivative of 7-*epi*-PTX-2SA is difficult to resolve as it elutes close to the ADAM-OA derivative.

The same sample, analysed using the DMEQ method, gave a chromatogram in which the 7-*epi*-PTX-2SA derivative elutes well after DTX-2 (Figure 6). PTX-2SA isomer (#4) was the predominant pectenotoxin in this phytoplankton but it is unstable and cannot be isolated in a pure form as it spontaneously converts to 7-*epi*-PTX-2SA [18].

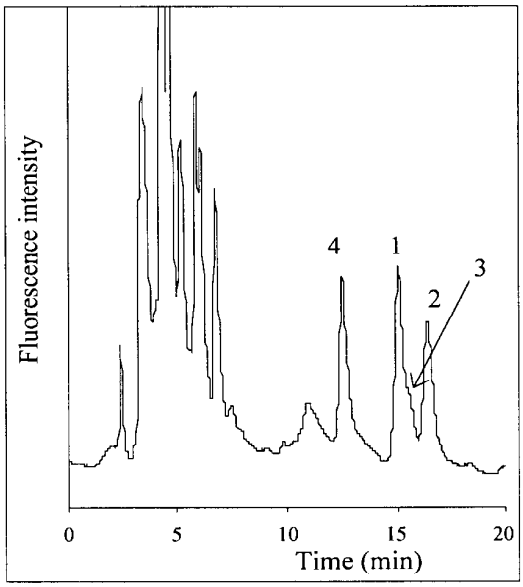

Figure 5. Chromatogram of the ADAM derivatives of toxins extracted from *D. acuta* (#1 OA, #2 DTX-2, #3 7-*epi*-PTX-2SA, #4 PTX-2SA isomer.)

This conversion of PTX-2SAs occurs during storage even at –20°C in both phytoplankton and shellfish. For confirmation of the identities of derivatised compounds, LC-MS analysis was also carried out using a Finnegan LCQ mass spectrometer with an electrospray interface, in positive ion mode. Thus, the expected $[M+H]^+$ signal for the DMEQ-OA derivative was obtained at *m/z* 1059.5 together with the main fragmentation ion at *m/z* 1037.0 for $[M+H-H_2O]^+$.

The DSP toxin profiles from *D. acuta* collected from the same coastal region of Ireland in 1996 and 1999 were different. Whereas pectenotoxin-2 seco acids were present in all *D. acuta* samples collected in 1996, these compounds were rarely observed in samples collected in 1999. A large variation in the DTX-1 level in *D. fortii* in Japan has been reported [16] but there is very limited information on PTX-2SA compounds as they have only recently been identified in *Dinophysis* sp. [14, 15].

The DMEQ procedure should prove to be a useful method for carrying out studies of the DSP toxin profiles in marine phytoplankton. This method is also applicable to the determination of DSP toxins in mussels (*M. edulis*) and other shellfish and a validation study is in progress.

ACKNOWLEDGEMENTS

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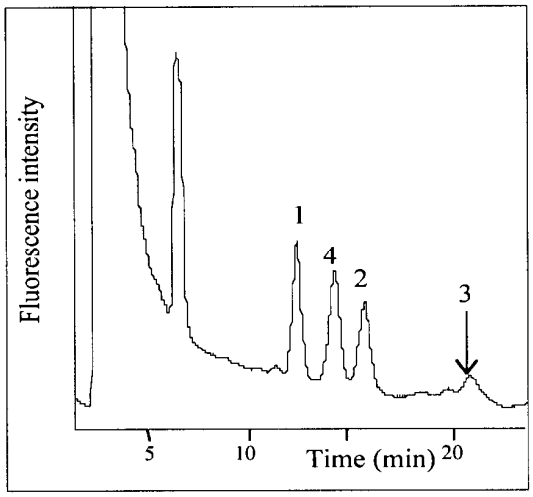

Figure 6. Chromatogram of the DMEQ derivatives of toxins extracted from *D. acuta* (#1 OA, #2 DTX-2, #3 7-*epi*-PTX-2SA, #4 PTX-2SA isomer.)

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PRODUCTION AND CHARACTERIZATION OF A MONOCLONAL ANTIBODY TO TYPE-2 BREVETOXINS

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ABSTRACT

Two murine monoclonal antibodies (MAbs 1A11 and 3A12) directed against brevetoxin PbTx-3 were selected from fusion of P3-X63-Ag 8.653 myeloma cells with BALB/c mouse immune spleen cells sensitized to a PbTx-3-bovine albumin conjugate. In competitive inhibition enzyme-linked immunosorbent assay (ELISA) experiments, both MAbs exhibited strong cross-reactivity (≥ 100%) to other PbTxs-2-type toxins (PbTx-2 and -9) but low cross-reactivity (9%) to a PbTx-1-type toxin (PbTx-1). The apparent dissociation constant (K_D) for the interaction of these MAbs with free PbTx-2-type toxins was in the 10^{-6}-10^{-7} M range. The limit of detection (≈ 5 ng/well) and the working range (8 - 150 ng/well) achieved with this ELISA coupled with adequate extraction methods would provide an alternative screening method to the intraperitoneal mouse bioassay for routine shellfish monitoring. Owing to the minute hapten quantities involved in conjugate preparation (0.446 µmol of PbTx-3) and the well-known advantage of monoclonal over polyclonal antibodies, this entire procedure appears most valuable for the development of immunoassays to highly potent marine polyether toxins.

INTRODUCTION

The marine planktonic bloom-forming dinoflagellate *Gymnodinium breve* (formerly *Ptychodiscus brevis*), responsible for Florida's red tides is known to produce brevetoxins (PbTxs), a class of 10 trans-fused polyether ladder neurotoxins. Based upon their polyether backbone structure, a nomenclature called the PbTxs series [1] has been used to classified them as PbTx-1-type (10 rings) and PbTx-2-type (11 rings) toxins. During bloom episodes, PbTxs concentrate in filter-feeding molluscs [2] and cause damage to fish, mammals and birds [3-5]. In humans, the toxins induce a bronchoconstrictor response when inhaled along the marine coastal environment [6] and upon ingestion of toxic shellfish produce a syndrome called Neurotoxic Shellfish Poisoning (NSP). More recently, other PbTx derivatives involved in human illness [7] have been isolated from New Zealand shellfish [8,9].

During the past decade, concerns have been expressed regarding safety of shellfish products and the detrimental effect on the shellfish industries, therefore much effort has been directed towards the development of precise, sensitive, reproducible and specific methods for PbTxs, as alternatives to the conventional mouse intraperitoneal bioassay [10]. The advent of antibody-based assays (radio- or enzyme-immunoassay, RIA or EIA, respectively) for PbTxs [11-18] has raised the hope of a promising approach for designing a routine mass screening method for shellfish prior to consumption. However the implementation of such a program requires high throughput sample screening with great specificity and sensitivity combined with simplicity and low cost, all requirements that could be potentially met with enzyme-linked immunosorbent assays (ELISA). To date, only polyclonal antisera to PbTxs have been produced. Although they exhibit high affinity, they are usually available in limited supply and their use requires batch standardization and continual supply of antigen for booster injections.

In two preceding reports [19, 20], we reported on the production of polyclonal antibodies specific for PbTxs-2-type toxins using PbTx-3-carrier conjugates prepared at the nanomolar level (400 µg of haptenic material) in a reversed micellar medium [21]. This three-step miniaturized conjugation procedure previously designed for cholesterol as a model for hydroxylated haptens [22] was further applied here to the first production and characterization of mice MAbs to PbTx-3. These results appear most promising for the development of MAb-based assays to other poorly available marine polyether-type potent neurotoxins such as ciguatoxins (CTXs) produced by the benthic dinoflagellates *Gambierdiscus spp.* [23] and transferred to various tropical reef fishes via the food chain.

MATERIALS AND METHODS

Materials

PbTxs type 1 or 2 were obtained from Latoxan (Rosans, France). [^{3}H]PbTx-3 (15 Ci/mmol) was obtained from CEA (Service des Molécules Marquées, Saclay, France) by chemical reduction of PbTx-2 with [^{3}H] sodium borohydride, according to previously published procedures [1, 11]. All other chemical reagents, unless otherwise stated, were purchased from Sigma Chemicals Co (St. Louis, MO, USA). High quality grade solvents from Prolabo (France) were dried according to standard procedures. Thin-layer chromatograms (TLC) were run on silica gel 60 precoated aluminum plates from Merck (Darmstadt, Germany).

Preparation of PbTx-3-protein conjugates and Immunization

The three-step procedure (Fig. 1) for conjugates preparation is reported elsewhere [20], it is based upon a modified version of the mixed anhydride coupling method of Erlanger *et al.* [24] performed in reversed micellar medium [21, 22].

A single female BALB/c mice (8 weeks of age) was immunized by five intraperitoneal injections of 70 µg of PbTx-3-BSA conjugate administered every 14 days in 100 µL saline solution/adjuvant mixture (1:1). The composition of the phosphate buffered saline solution (PBS) was as follows: 0.01 M KH_2PO_4/K_2HPO_4-150 mM NaCl, pH 7.2. Primary injections were made in Freund's complete adjuvant (FCA) and the following four secondary boosters were given in Freund's incomplete adjuvant (FIA). The mouse was sequentially bled from the retro-orbital venous system and individual sera were tested for anti-PbTx-3 antibody activity (see below).

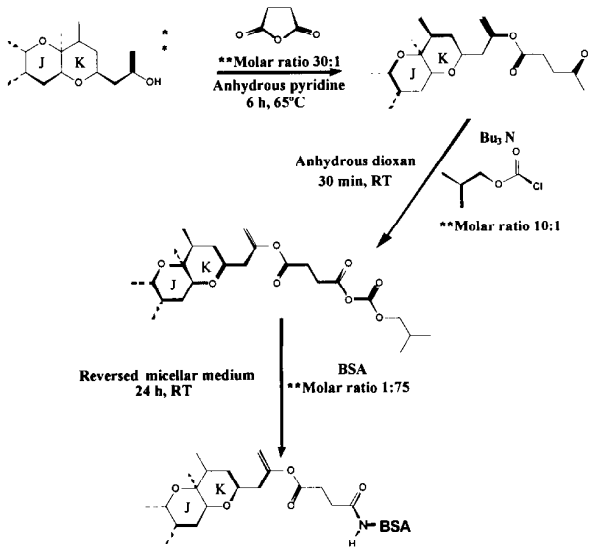

Fig. 1 Chemical coupling of PbTx-3 to carrier proteins
* Terminal reactive part of the PbTx-3 molecule (J-K ring).
** Molar ratio relative to hapten amount.

Monoclonal antibody production and selection

Three weeks after the last i.p. booster, the mouse received an intrasplenic injection (20 µg immunogen in PBS) under ketamine base anesthesia. Three days later the mouse was sacrificed under ether anesthesia and spleen cells were prepared and fused at a ratio of 5:1 with the P3-X63-Ag8.653 myeloma cells using the ClonaCell-HY kit (Stemcell Technologies Inc., Canada). Hybridomas were grown in a methylcellulose-based semi-solid selecting medium and clones were transferred after 15 days in 96-well tissue culture plates filled with normal medium. An indirect ELISA employing PbTx-3-OVA as target antigen and non-relevant control antigens was used to screen for hybridomas secreting anti-PbTx-3 antibodies (see below). Selected hybridomas were cloned by plating onto semi-solid normal medium and maintained in tissue culture plates. Cells from two selected clones were grown as solid tumor in FIA-primed BALB/c mice to produce ascitic fluid. Total protein concentration was evaluated by the method of Bradford (1976) using BSA as standard. Immunoglobulin concentration was determined by acetate gel electrophoresis of a fluid sample followed by scanning densitometry of the Red Ponceau stained banding pattern according to standard technique. MAb subtyping was performed by means of an ELISA commercial kit from Boehringer-Mannheim (Germany).

Antibody screening assay

All immunochemical reagents, unless otherwise stated, were purchased from Boehringer Mannheim (Indianapolis, IN., USA).

Hybridoma supernatants and ascitic fluids were first screened for antibody production to PbTx-3 by indirect ELISA. Flat-bottomed polystyrene 96-well Maxisorp Immunoplates (Nunc, Roskilde, Denmark) were coated with 100 µL of $(PbTx\text{-}3)_{10}$-OVA conjugate (250 ng/mL) in PBS by incubation for 1 h at 37°C then overnight at 4°C. Plates were washed five times with PBS and remaining active sites were blocked by addition of 250 µL of PBS containing 5% skim milk powder. Following three washes with PBS containing 0.1% Tween 20 (PBS-T), 100 µl of culture supernatant or ascitic fluid dilutions in PBS-T were added (1h at 37°C). Plates were washed again with PBS-T and antibodies activity was detected using a fluorescence-based amplification signal by the successive addition of goat anti-mouse ß-galactosidase conjugate (GAM-ßGZ: 1:4,000 dilution) and 4-methyl umbelliferyl ß-D-galactoside (4-MUG) at saturation in 0.1 M phosphate buffer, pH 7.2. After incubation for 30 min, fluorogenic substrate change was measured using a Dynatech Microfluor Reader with excitation at 365 nm and emission at 450 nm. Recorded values are expressed as relative fluorescence units.

Antibody specificity was further assessed by competitive inhibition ELISA. After blocking and washings of antigen-coated plates as in the indirect ELISA procedure, 25 µl optimum dilution of culture supernatant or ascitic fluid was added to each well followed by the addition of an equal volume of various concentrations of inhibitors (PbTx-3, PbTx-2; PbTx-9, PbTx-1). After incubation and washing steps the antibodies associated with the plates were revealed as described above.

RESULTS

Preparation of immunogen and target antigen

PbTx-3-protein conjugates prepared at the nanomolar level (400 µg or 0.446 µmoles of PbTX-3) in a reversed micellar medium (Fig. 1) were found to contain around 10 or 20 hapten molecules per OVA or BSA carriers, respectively. At the three stages involved, optimal conditions for their preparation were based upon previous work done with cholesterol [22]. On the one hand, these well-characterized conjugates have been found to be highly immunogenic, since PbTx-2-type specific polyclonal antibodies have been raised in two mice and a single rabbit immunized against such PbTx-3-BSA conjugates [20]. On the other hand, they have proven to be stable to storage at -20°C for at least six months as evidenced by consistent performance of PbTx-

3-OVA in immunoassay. It is noteworthy that the excess of unreacted PbTx-3 HS from BSA conjugation experiment (0.312 µmoles; 70% of the hapten input) was used for indirect epitope density determination (chromatographic method) and for the preparation of the target antigen (PbTx-3-OVA), so that only 400 µg of PbTx-3 could serve for both immunization and ELISA screening.

Primary screening of hybridoma supernatants

Following completion of the immunization schedule, in a single fusion experiment, 284 hybridomas were obtained in a semi-solid selecting medium from which 266 supported the transfer into 96-well tissue culture plates. Five out of them (1.9 %) were selected on the basis of culture supernatant reactivity to PbTx-3-OVA and absence of cross-reactivity with control solid-phase antigens including the native carriers (BSA and OVA) and their conjugates with a non-related hapten (cholesterol).

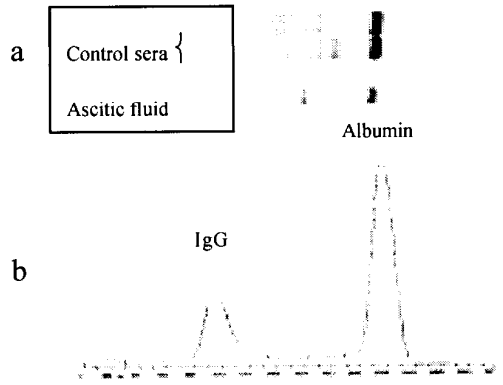

Fig. 2 Electrophoretic analysis of two different control sera and ascitic fluid 1A11. (a): Red Ponceau stained cellulose acetate gel electrophoregrams. (b): scanning densitometry of ascitic fluid 1A11 banding pattern

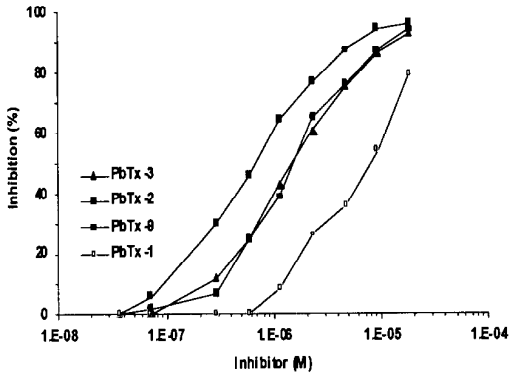

Fig. 3. Competitive inhibition of monoclonal antibody 1A11 binding to PbTx-3-OVA coated plates by free type-1 and type-2 PbTxs. Each value represents the mean of triplicate assays. Error bars are omitted (variation lower than 5 %). See text for other details.

Monoclonal antibodies specificity

Only two hybridoma supernatants (1A11 and 3A12) showed a clear specificity for PbTx-3 in competitive inhibition ELISA experiments. Hybrids in these positive wells were cloned, expanded and injected to FIA-primed BALB/c mice to induce ascite production. Each clone produced about 28 mg of MAb per ml of fluid (Fig. 2). Both MAb were found to be of IgG2a,κ subclass. The results of competitive inhibition of MAb 1A11 binding to PbTx-3-OVA coated plates by various inhibitors are reported in Fig. 3. A strong inhibitor effect was recorded with two PbTx-2-type toxins exhibiting high cross-reactivity values (132 and 100 % for PbTx-2 and -9, respectively). By contrast, only low cross-reactivity values were observed for PbTx-1 (9 %).

The apparent dissociation constant (K_D) for the interaction of this MAb with free PbTx-2-type toxins was in the 10^{-6}-10^{-7} M range. The limit of detection (LOD ≈ 5 ng/well) and the working range (8 - 150 ng/well) were calculated using the four parameter logistic fitting model provided by the Multicalc package program (EG & G-Wallac).

DISCUSSION

Although numerous antibodies to PbTxs have been raised in animals such as goats [11-14, 17, 18, 25], rabbits [16, 18], mice [25] and sheep [26], their production have required either high PbTx-3 (alcohol) quantities (2.5-5 mg) or minute amount (100 µg) of PbTx-2 (aldehyde). In this study we have produced the first MAbs to PbTxs using small amount of PbTx-3 (400 µg; 0.446 µmol) in a reversed micellar medium [21, 22]. The main improvements afforded by this new coupling procedure are: i) a moderate input hapten/protein molar ratio, ii) a good recovery of both conjugate and hapten in excess (removal of toxic material before animal injections), and iii) a reuse of unreacted hapten to prepare the testing antigen. This procedure opens the way towards the production of MAbs to other poorly available hydroxylated polyether-type toxins [20, 21].

Our mice MAbs appear mostly directed against the PbTx-2-type structural backbone (only 9% cross-reactivity to PbTx-1). These results confirm our earlier report on mice and rabbit polyclonal antibodies [20]. The lower affinity exhibited by our MAbs compared to other studies performed with polyclonal antibodies might be related to difference in immunization schedule or simply reflects individual serum variation.

As previous studies by Melinek et al. [17] and Poli et al. [18] have demonstrated the existence of at least 3 distinct epitopes in the PbTxs molecules, it can be reasonably postulated that a two-site sandwich ELISA format must fullfill requirements to shellfish monitoring [17]. Such format would employ a set of MAbs directed towards the invariant part of PbTxs molecules (the last 3 rings common to the two toxin types) as capture antibodies and another set of MAbs directed to the toxic head. Moreover this immunometric procedure would improve both specificity and sensitivity of the assay. Therefore precise epitope recognition by these two MAbs is currently underway.

Using competitive immunoassays, the antibody class(es) active over a specified ligand concentration range make(s) that polyclonal antisera to PbTxs behave like MAbs [17, 18]. Therefore, in order to avoid the variation in binding characteristics of polyclonal antibodies and to have a continuous source of well-defined antibodies that could be adequately mixed, MAb production and characterization must be actively pursued.

Although PbTxs immunodetection in shellfish extracts is poorly documented, promising work has been done by Poli and Hewetson [15] who found no significant interference when samples are used at sufficient dilutions. Therefore, the sensitivity achieved with this assay (LOD ≈ 5 ng/well) makes it very attractive for routine shellfish monitoring.

This work provides the first demonstration of the production of MAbs to PbTxs using minute amounts of toxins. A logical extension of this achievement is the development of a shellfish extraction protocol and the implementation of a MAb-based assay that would allow for easy and precise toxin detection in the field.

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The authors thank Philippe Cruchet for excellent HPLC technical assistance and Dr. Allan Cembella (NRC, Halifax, Canada) for the critical reading of the manuscript and for the linguistic revision of the text. This work was supported by grants from the Government of French Polynesia (Tahiti) and Pasteur Institute (Paris, France).

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STRATEGY FOR THE DEVELOPMENT OF ANTIBODIES AGAINST CIGUATOXINS: BREVETOXINS AS A MODEL FOR POLYETHER HYDROXYLATED COMPOUNDS

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ABSTRACT

As a model for hydroxylated polyether toxins, brevetoxin PbTx-3 was converted into a hemisuccinate derivative (PbTx-3 HS) and covalently linked to bovine serum albumin (BSA) and ovalbumin (OVA). By reacting a minute amount of PbTx-3 (0.446 µmol) with a 30-fold molar excess of succinic anhydride in dry pyridine, PbTx-3 HS was synthesized with a 100% yield (as verified by HPLC), then covalently coupled to the carriers via the mixed anhydride method performed in a reversed micellar medium. Conjugate analysis, by a combination of chromatographic and radioisotopic techniques showed highly concordant results. Using the optimal conditions for BSA-hapten formation (an input hapten/carrier molar ratio of 75), epitope density of the conjugates (n = 7) was between 15-24 (24-30% yield). In competitive inhibition enzyme-linked immunosorbent assay (ELISA) experiments, immunized mice and rabbits produced PbTx-2-type specific antibodies with apparent affinity constant (K_D) in the 10^{-6}-10^{-8} M range. No significant cross-reactivity was observed with PbTx-1-type toxin. Due to the minute hapten quantities involved, this entire procedure appears most valuable for the preparation of immunogen with poorly available polyether toxins like ciguatoxins (CTXs).

INTRODUCTION

Brevetoxins (PbTxs) and ciguatoxins (CTXs), trans-fused polyether ladder neurotoxins, are respectively produced by the planktonic bloom-forming dinoflagellates *Gymnodinium breve* (formerly *Ptychodiscus brevis*) and the benthic dinoflagellate *Gambierdiscus spp.* [1, 2]. Both toxins type share the same specific biological activity (a reflection of their closely related chemical structure) on the voltage sensitive sodium channel. In human, these compounds are toxic after consumption of contaminated shellfish (PbTxs) or fish (CTXs) and induce syndromes called Neurotoxic Shellfish Poisoning (NSP) and Ciguatera Fish Poisoning (CFP), respectively. Due to their adverse effects on human health and negative impacts on socioeconomic activities, these marine toxins have drawn much scientific attention but improved immunochemical detection methods are still required.

As an alternative to the conventional mouse intraperitoneal bioassay [3], immunoassays for PbTxs have been developed using goat, rabbit or sheep antisera [4-13]. However, in these studies, relatively large amounts of PbTx-3 (2-5 mg) have been used. The limited supply of pure CTXs and their molecular nature and complexity have greatly hampered the production of specific antibodies to these toxins. Despite the early claim of polyclonal [14] and monoclonal [15] antibody production to a CTX congener (probably CTX-1B) and their subsequent uses in different format assays, there is no effective screening immunoassay commercially available for ciguatoxic fish.

In this work, the production of mouse and rabbit antisera to PbTx-3 using hapten-carrier conjugates prepared at the nanomolar level in a reversed micellar medium [16] are described according to the method developed using cholesterol as a model for hydroxylated haptens [17]. The demonstration of specific antibodies utilizing only 400 µg of PbTx-3 strongly suggests that specific and sensitive analogous immunoassays are possible for detecting other potent marine polyether neurotoxins such as CTXs. This study extends preliminary results reported earlier [18].

MATERIALS AND METHODS

Materials

Type 1 or 2 PbTxs were obtained from Latoxan (Rosans, France). [^{3}H]PbTx-3 (15 Ci/mmol) was produced by CEA (Service des Molécules Marquées, Saclay, France) by chemical reduction of PbTx-2 with [^{3}H] sodium borohydride as previously described [4, 19]. All other chemical reagents, unless otherwise stated, were purchased from Sigma Chemicals (St. Louis, MO, USA). Analytical grade solvents from Prolabo (France) were dried according to standard procedures. Thin layer chromatograms (TLC) were run on silica gel 60 precoated aluminum plates from Merck (Darmstadt, Germany).

Synthesis and purification of PbTx-3 hemisuccinate

The optimal conditions for synthesis of PbTx-3 hemisuccinate (PbTx-3 HS) were explored by checkerboard experiments as previously described [17]. The most efficient condition is described hereafter. Crystalline PbTx-3 (400 µg; 0.446 µmol) was converted into a hemisuccinate derivative (PbTx-3 HS) by adding a thirty-fold molar excess of succinic anhydride solubilized

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in 50 µL of anhydrous pyridine. After incubation (6 h at 65°C) the solvent was evaporated under a stream of nitrogen. Finally, the residue was redissolved in 200 µL of CH_2Cl_2/MeOH (50:50) and succinylation was verified by TLC using $CHCl_3$/MeOH/ CF_3COOH (10:10:1) as developing solvent [9] and 30% aqueous sulfuric acid as spray reagent. After heating, PbTx-3 and PbTx-3 HS appeared as brown spots with Rf values of 0.44 and 0.34, respectively. PbTx-3 HS was quantified and purified using an HPLC system equipped with a UV monitor set at 210 nm and a chromatointegrator for peak area calculation (Kontron instrument, France). The column was a 250 mm x 4.6 mm i.d. ODP 50 reversed-phase (Asahipak Shodex, Prolabo, France) and separation was carried out under isocratic conditions with 80 % aqueous MeCN at a flow rate of 1 ml/min. PbTx-3 HS containing fractions were pooled and dried under nitrogen. The purity of the product was verified by a second HPLC run.

Preparation of PbTx-3-protein conjugates

Conjugates were prepared according to a modified version of the mixed anhydride coupling method of Erlanger *et al.* [20] performed in a reversed micellar medium [16]. Briefly, considering a overall yield for the chemical reaction and purification process near 100%, PbTx-3 HS (≈0.446 µmol) was converted into a mixed anhydride by adding a 10-fold molar excess of tributylamine and isobutyl chlorocarbonate as $1/12^{th}$ dilutions in dry peroxide-free dioxan. After 30 min at room temperature, the reaction yield was greater than 95% as verified by TLC. R_f values for PbTx-3 HS and its activated derivative were 0.32 and 0.96, respectively. The subsequent coupling reaction to the carriers occurred in a reversed micelle system of aerosol OT (AOT or sodium bis (2-ethylhexyl) sulfosuccinate) in octane using an initial hapten/carrier molar ratio of approximately 75:1. Bovine serum albumin (BSA) and ovalbumin (OVA) were used for immunization and antibody screening, respectively. Conjugates were recovered by acetone precipitation, resuspended in 1 mL distilled water, filter sterilized (0.22 µm), dispensed into sterile tubes then freeze-dried overnight and stored at -20°C until use.

Conjugate analysis

Carrier-hapten coupling was assessed by radioactivity measurements when [^{3}H]PbTx-3 (0.05 µCi) was added as tracer or by a chromatographic method in all cases [17]. Fig. 1 illustrates the outline of these procedures.

Radioisotopic method. The radioactivity of 25-30 µl aliquots of either pooled acetone supernatants recovered from conjugate precipitation (method B) or conjugates in PBS (method C) were determined by liquid scintillation spectroscopy. Aliquots were solubilized in 4 ml scintillation cocktail (formula-989, Packard), incubated 1 h, and counted in a LKB-Wallac liquid scintillation counter with a [^{3}H] efficiency of 45%. Samples were counted for a sufficient period of time to yield counting errors of 5-10%. The composition of the phosphate buffered saline solution (PBS) was as follows: 0.01 M KH_2PO_4/K_2HPO_4-150 mM NaCl, pH 7.2.

Chromatographic method. The pooled acetone supernatants recovered from conjugate precipitation were evaporated to dryness and the residues redissolved in 200 µL CH_2Cl_2/MeOH (99:1). First, AOT was removed by rapid chromatography technique on a Sep-Pak Plus silica gel cartridge (Waters-Millipore, Milford, MA, USA) as previously described [17]. Second, unreacted PbTx-3 HS was purified by HPLC (method A) as described above.

Using both methods, after molar conversion and calculations to restore the true values in the original samples, the degree of hapten conjugation was expressed by the hapten/carrier ratio (i.e. epitope density of the conjugates). For these calculations, total carrier content was considered as their recovery in control (carrier plus non-activated hapten) and assay tubes were previously found to be > 95% [16].

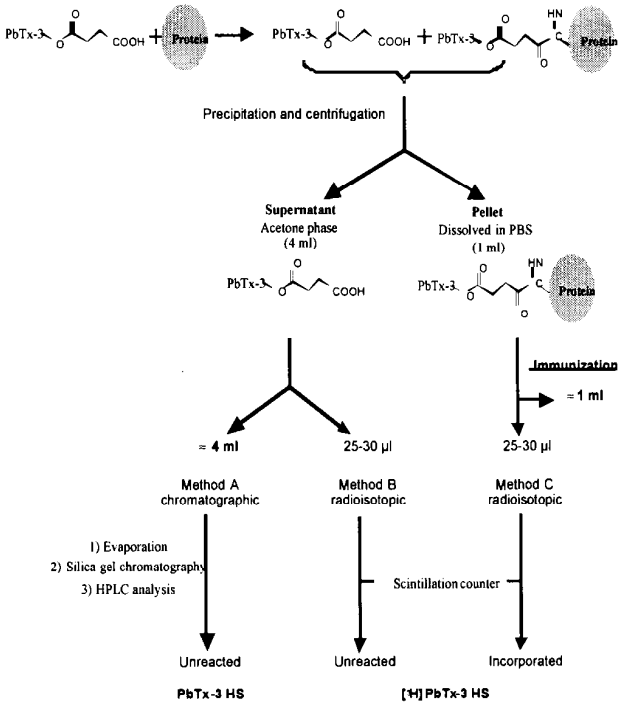

Fig. 1. Outline of the procedure for epitope density determination.

Immunization

Two female BALB/c mice (8 weeks of age) were immunized by five intraperitoneal injections of 70 µg of PbTx-3-BSA conjugate administered every 28 days (mouse #1) or every 14 days (mouse #2) in 100 µL PBS/adjuvant mixture (1:1). Primary injections were made in Freund's complete adjuvant (FCA) and the following four secondary boosters were given in Freund's incomplete adjuvant (FIA).

A male New Zealand White rabbit was immunized four times with 90 µg of PbTx-3-BSA conjugate. Subcutaneous injections were made every 4 weeks with 250 µl of conjugate in PBS/FCA mixture (1:1) for priming and in PBS/FIA (1:1) for secondary boosters.

One week after each injection, all animals were bled to test for the presence of specific antibodies.

Enzyme Immunoassays

All immunochemical reagents, unless otherwise stated, were purchased from Boehringer Mannheim (Indianapolis, IN, USA). Rabbit and mouse sera were first tested for antibodies against PbTx-3 by indirect enzyme-linked immunosorbent assay (ELISA).

Flat-bottomed polystyrene 96-well Maxisorp Immunoplates (Nunc, Roskilde, Denmark) were coated with 100 µL of $(PbTx\text{-}3)_{10}$-OVA conjugate (250 ng/mL) in PBS by incubation for 1 h at 37°C then overnight at 4°C. After washing five times with PBS, remaining active sites were blocked by addition of 250 µL of PBS containing 5% skim milk powder. Following three washes with PBS containing 0.1% Tween 20 (PBS-T), serially diluted immune or control sera (1:100 - 1:1,000,000 dilutions in PBS-T) were applied to the plates at 100 µL/well. Bound antibodies were revealed using a fluorescence-based amplification signal by the successive addition of goat anti-mouse or anti-rabbit IgG ß-galactosidase conjugate (GAM-ßGZ or GAR-ßGZ: 1:4,000 dilution) and 4-methyl umbelliferyl ß-D-galactoside (4-MUG) at saturation in 0.1 M phosphate buffer, pH 7.2. Following a 30 min incubation, fluorogenic substrate change was measured using a Dynatech Microfluor Reader with excitation at 365 nm and emission at 450 nm. Recorded values are expressed as relative fluorescence units.

The specificity of the antisera was further assessed by competitive inhibition ELISA. Sera (1:16,000 final dilution) were first preincubated overnight at +4°C with an equal volume of various concentrations of inhibitors (PbTx-3, PbTx-2; PbTx-9, PbTx-1). Samples of these mixtures were transferred (50 µL/well) onto PbTx-3-OVA-precoated plates. After incubation and washing steps the antibodies associated with the plates were revealed as described above.

RESULTS AND DISCUSSION

PbTx-3-protein conjugates

Conjugates were prepared at the nanomolar level in a reversed micellar medium according to the procedure described in Materials and Methods. At the two stages involved, optimal conditions for their preparation were based upon previous work done with cholesterol [17].

First, PbTx-3 HS was prepared according to the optimized protocol then analyzed and purified by HPLC. Second after conjugation, substitution ratio were carefully analyzed using various methodology (Fig. 1). Table 1 reports the comparative results obtained by radioisotopic and chromatographic analysis of PbTx-3-BSA conjugates. Whereas direct radioisotopic procedure (method C) was performed using the acetone-purified conjugate, both indirect procedures involved either chromatographic determination (method A) or radioactive counting (method B) of the unreacted PbTx-3 HS in the acetone phase. These three methods yielded an acceptable level of agreement and concordant results in all cases; the epitope density of the conjugates (n=7) fell between 15-24 and the overall conjugation efficiency was in the range 24-30%. Using the same coupling and analysis procedures, epitope density of OVA conjugates was found to be about 10.

Hapten	Exp. N°	Method for epitope density determinaton			Yield (%)[4]
		A	B	C	
[^{3}H]PbTx-3	*1*	*18*	*20*	*15*	*24*
	2	*19*	*24*	*18*	*27*
	3	*22*	*20*	*17*	*27*
PbTx-3	4	23	-	-	30
	5	19	-	-	25
	6	21	-	-	28
	7	19	-	-	25

Table 1. Determination of the epitope density of various PbTx-3-BSA conjugates. A: Chromatographic quantitation of unreacted hapten in the acetone supernatant. B: Radioactive counting of unreacted tritiated hapten in the acetone supernatant. C: radioactive counting of incorporated tritiated hapten in the conjugate.

In similar previous reports, PbTx-3-BSA conjugates have been prepared from either 5 mg [4] or 2.5 mg of PbTx-3 [9] using toxin hemisuccinate derivatives and standard carbodiimide condensation procedures. However, Levine and Shimizu [10] were able to prepare a new immunogen using minute amount (100 µg) of PbTx-2 (aldehyde) by a probable Michael type 1,4-addition reaction mechanism. Therefore these coupling procedures were found to be inapplicable to poorly available hydroxylated haptens. Moreover, in these studies, lower epitope densities were obtained (8-14) and their determination required the use of a radioactive tracer. In the present study, the main objective was to minimize the amount of toxin usually required for antibody production while preserving a good hapten coupling efficiency and immunogenicity. Consequently, using a modified version of the mixed anhydride coupling method of Erlanger *et al.* [20] performed in a reversed micellar medium [16], toxin-protein conjugates were obtained with only 400 µg of PbTx-3. In addition we have confirmed previous results indicating that the availability of a radioactive probe is not a prerequisite to monitor antigen preparation at the nanomolar level [17].

Production and characterization of antibodies against PbTx-3

An indirect ELISA was used to monitor the production of anti-PbTx-3 antibodies in mice and rabbit sera (data not shown). The reactivity of individual sera was assessed in

competitive ELISA experiments using various PbTx congeners as inhibitors. The apparent association constant (K_D) values of antibodies binding to PbTx-3 were 4 x 10^{-8} M, 1 x 10^{-6} M and 1 x 10^{-7} M for mouse #1, mouse #2 and rabbit, respectively (Fig. 2). Using serum from the best responding mouse (#1), the limit of detection (LOD) and the limit of quantitation (LOQ) of this assay were around 3 x 10^{-9} M (0.13 ng/well) and 1 x 10^{-8} M (0.45 ng/well), respectively. Although this assay appears less sensitive than previous immunoassays using goat, rabbit or sheep antibodies [4, 8-10, 12, 13], the LOD is still several orders of magnitude lower than that of the conventional mouse bioassay [21, 22].

For all antisera (from mice and rabbit), cross-reactivity values for PbTx-2-type toxins (PbTx-2, -3 and -9) were equal to or greater than 100% (data not shown). However moderate cross-reactivity (6-15%) was observed with a PbTx-1-type toxin (PbTx-1, data not shown); similar results with the other congeners of this type (PbTx-7 and -10) are to be expected since only minor changes in the terminal side chain of molecules having the same polyether backbone structure differentiate congeners within a type.

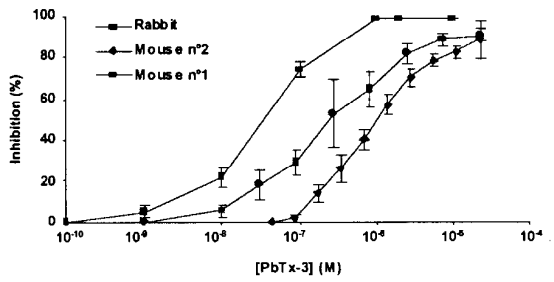

Fig. 2 Competitive inhibition of mouse and rabbit antisera binding to PbTx-3-OVA coated plates by free PbTx-3. Each value represents the mean of triplicate assays, with errors bars indicating ± SD. See text for other details.

The demonstration of the specificity and the affinity of these new mice and rabbit antibodies strongly suggests the feasibility of this immunochemical approach for other rare marine polyether potent neurotoxins like CTXs produced by the benthic dinoflagellates *Gambierdiscus spp.* [2]. However, the disadvantage of a polyclonal approach is that for long-term antibody production, a continual supply of antigen is required for booster injections. Therefore monoclonal antibody (MAb) production, which can provide a continuous supply of specific antibodies, is currently underway in our laboratory. Some preliminary results on PbTx-2-type specific MAbs production and characterization are also presented in this symposium [23]

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THE REVERSED MICELLAR MEDIUM AS A UNIVERSAL TOOL FOR THE DEVELOPMENT OF ANTIBODY-BASED ASSAYS TO MARINE PHYCOTOXINS USING SMALL AMOUNT OF TOXIC MATERIAL

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ABSTRACT

The production of specific antibodies to marine phycotoxins is hampered by considerations such as toxicity, scarcity or chemical nature of these compounds. A rapid, simple and low cost procedure for preparing minute amount of hapten-protein immunogenic conjugates in a reversed micellar medium was succesfully applied to purified toxins (0.32 - 0.64 µmol) such as brevetoxin (PbTx-3) and domoic acid (DA) and the synthetic JKLM ring fragment of ciguatoxin (CTX). Epitope density of conjugates ranging from 15 to 25 was determined using a combination of chromatographic, spectrophotometric, chemical and radiochemical techniques, depending upon the hapten. Following mouse immunization with the corresponding conjugates, highly hapten-specific polyclonal and monoclonal antibodies were produced, with apparent dissociation constant (KD) values in the range of 10^{-6}-10^{-9} M. These results confirmed the potential for preparing immunogen with very rare haptens whose low detection level still remains problematic.

INTRODUCTION

Marine phycotoxins encompass a large class of usually low molecular weight and chemically different compounds, ranging from polyethers to alkaloids and peptides or even amino acids [1]. Data from human intoxication incidents and laboratory tests, including mouse bioassays using highly purified standards, have contributed to help regulatory authorities recommend a maximum permitted limit (MPL) for each toxin above which seafood products must be banned for safety reasons [2]. However, for the ciguatoxins (CTXs) responsible for ciguatera food poisoning (CFP), the absence of sufficient standards has led to a more complicated situation. Therefore, a consensus has been established only on the basis of the most potent derivative (CTX-1B) which fortunately represents the major toxic compound in carnivorous fish from the Pacific Ocean [3-5]. Among the detection methods developed for marine phycotoxins, enzyme immunoassay (EIA) still appears as one of the most promising technique for a cost-effective seafood product monitoring program. The challenge is to detect low quantities of several families of closely related compounds having different toxicity and accumulated in various amount in seafood products. The importance of such an immunochemical approach has been recently emphasized in New Zealand [2] where scientists, industry and regulators have to face the unique situation of testing for the presence of toxins associated with amnesic, diarrhetic, neurotoxic and paralytic shellfish poisoning (ASP, DSP, NSP and PSP, respectively).

The first step towards toxin immunodetection is the definition of the main targets upon which most effort must be focused. Such a task involves: i) the analysis of the toxin profile of many seafood samples responsible for human intoxication, ii) the identification of the chemical structure of the toxic compounds involved iii) the use of functional assays (receptor binding or cytotoxicity assays) to tentatively correlate structure-function relationship with intoxication symptoms. Once the main targets are defined, the best source of relevant toxins must be found. A commercial source is preferred, when possible. Alternatively chemically generated toxin fragments [6] or synthetic fragments common to the more toxic compounds [7] can be used.

Here we review the application of a micro-coupling procedure performed in a reversed micellar medium [8] for the production of polyclonal and monoclonal antibodies (MAbs) against commercially available toxins such as domoic acid (DA) and a brevetoxin congener (PbTx-3), or a synthetic tetracyclic ring fragment (JKLM) of CTX-1B [9]. This procedure employing less than 0.7 µmol of hapten was found to be very simple and efficient. Moreover, micro-analytical techniques for epitope density determination have been designed.

MATERIALS AND METHODS

Immunogen construction

The preparation of the conjugates and their subsequent analysis have been outlined elsewere in detail [7, 10-13].

The micro-coupling procedure employed throughout is based upon a modified version of the mixed anhydride method of Erlanger et al. [14]. It was originally devised using a chromophoric carboxylic molecular model [8] then optimized using cholesterol [12] and later succesfully applied to non-chromophore hydroxylated or carboxylic toxins.

Briefly, a minute amount (less than 0.7 µmol) of carboxylic toxins (DA or JKLM ring fragment) or the hemisuccinate derivative of a hydroxylated toxin (PbTx-3 HS) were activated by adding a 10-fold molar excess of tributylamine and isobutyl chlorocarbonate for 30 min at room temperature. The conjugation of activated hapten to carriers was carried out in a system of reversed micelles of aerosol OT (AOT or sodium bis(2-ethylhexyl) sulfosuccinate) in octane [8]. Two carriers were used throughout: bovine serum albumin (BSA) and

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ovalbumin (OVA) providing an initial hapten/protein molar ratio around 75:1 or 100:1. Conjugates were recovered by acetone precipitation, resuspended in 1 mL distilled water, filter sterilized (0.22 µm), dispensed into sterile tubes, then freeze-dried overnight and stored at -20°C until use. For subsequent analysis, conjugates were resuspended in phosphate buffered saline (PBS: 0.01 M KH_2PO4/K_2HPO_4-150 mM NaCl, pH 7.2) and the acetone supernatants were pooled. Carrier-hapten formation was assessed either by direct methods (spectrophotometry or radioactive counting) using aliquots of the conjugates or indirect methods (HPLC or radioactive counting) using aliquots of the unreacted haptens in the acetone phase [10-13].

Antibody production and characterization

Mice or rabbits were immunized as previously reported [7, 10, 11, 13] using 50-90 µg of the corresponding conjugates administered with Freund's complete adjuvant (FCA) for priming and Freund's incomplete adjuvant (FIA) for 4-5 subsequent boosters (Table 2). Monoclonal antibody (MAb) production and selection was performed as described in this symposium [11, 13]. Antibody specificity was checked by enzyme-linked immunosorbent assay (ELISA) using the corresponding immunoconjugates and various amplification systems as previously described [7, 10, 11, 13].

RESULTS AND DISCUSSION

Enzyme immunoassay (EIA) formats applied to the detection of biologically relevant haptens such as marine polyether toxins relies mainly on: i) the availability of sufficient amount of purified pure toxins or toxic derivatives ii) the preparation of well-defined immunogenic hapten-carrier conjugates, iii) the judicious selection of antibody preparations exhibiting adequate concentration of binding sites, high affinity and specificity, and iii) the use of rapid but efficient extraction procedures minimizing matrix-effects generally associated with assays in biological samples.

Much of our effort over the past seven years has been devoted to the development of efficient, practical (i.e. economic) and simple hapten-carrier coupling methods for poorly available natural products like marine phycotoxins. Owing to the scarcity of polyether toxins, we have used monensin, a commercially available polyether antibiotic as molecular model [14]. Relatively bulk quantity of this hapten (500 mg; 0.721 mmol), was fully converted into an hemisuccinate derivative. Then 0.25 mmol portions of this latter were covalently linked to BSA or OVA using various coupling methods from which the mixed anhydride method of Erlanger et al. [15] was found far more efficient. Such procedures were performed in a semi-organic medium (i.e., mixture of a water-miscible organic solvent in aqueous buffer) and attempts at decreasing the reactant amounts were accompanied by a decrease in coupling yield. The minaturized adaptation [8] of the reversed micellar coupling procedure [16] opens the way to the easy preparation of imunogenic toxin-carrier conjugates.

The reversed micellar conjugation procedure

The formation of reverse micelles (Fig. 1) by mixing a small volume of aqueous solution with an appropriate detergent (e.g. AOT) in organic solvent is strongly dependant upon the degree of hydration of the system [17]. This physico-chemical parameter is of paramount importance to allow a chemical reaction to occur in the very limited size domain provided by the vesicle, which acts like a microreactor [16]. In any hapten, the optimized procedure we designed [12] always yielded a valuable miniaturized haptenization of various carrier molecules, which was compatible with immunogenicity and antibody detection. Such success must be related to two essential reasons. First, the protein is protected from the organic environment by encapsulation into the reversed micelle. Second, the activated hapten is protected against hydrolysis until the coupling reaction takes place inside the vesicle.

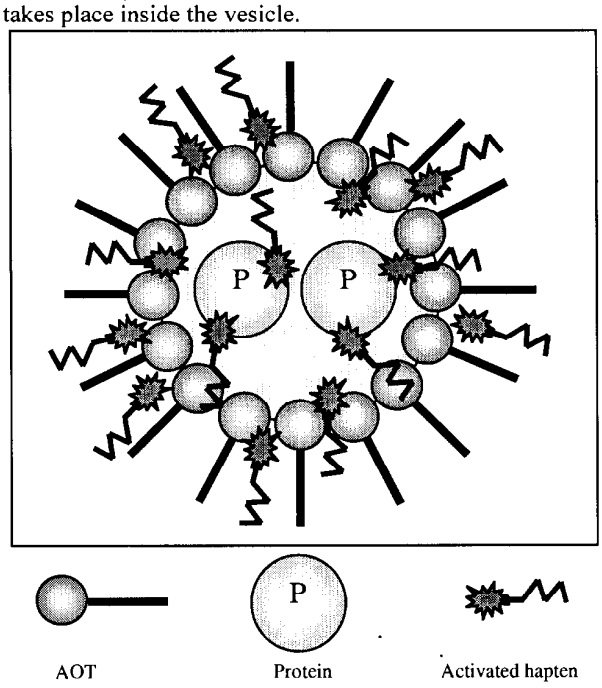
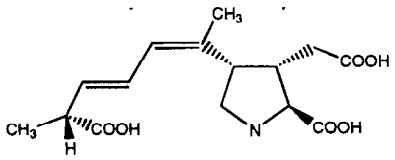


Fig. 1 Schematic representation of the reversed micellar conjugation procedure. The vesicle (microreactor) is delimited by a monolayer of AOT molecules with their polar heads facing the inner aqueous phase and their hydrocarbon tails projecting into the bulk organic phase.

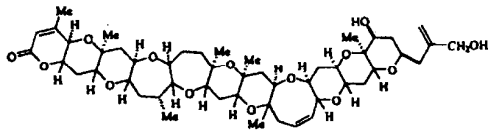
Application to toxic material

The relevance of this coupling procedure has been demonstrated by the production of mouse and rabbit antibodies to: i) (DA), responsible for ASP [10, 11], ii) PbTx-3, responsible for NSP [13], iii) JKLM ring fragment of CTX-1B, the most potent CTX responsible for CFP [7]. The chemical structures of these haptens are reported in Fig. 2. The main improvements afforded by the entire coupling procedure are: i) a moderate input hapten/protein molar ratio, ii) a good recovery of both

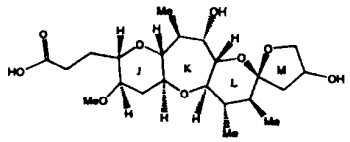
conjugate and hapten in excess (removal of toxic material before animal injections), and iii) a reuse of unreacted hapten to prepare the testing antigen. Furthermore, the first mouse MAbs to these compounds have been produced using very tiny amounts of haptenic material (200-400 µg) in this reversed micellar medium.



DA (MW 311.34)



PbTx-3 (MW 896)



JKLM (MW 444.52)

Fig. 2 Chemical structures of DA, PbTx-3 and JKLM ring fragment.

Table 1 Application to toxic material

Hapten	Amount	Reactive group	Conjugate analytical method
PbTx-3	400 µg	OH	Radioactive counting[a] HPLC or radioactive counting[b]
DA	200 µg	COOH	Spectrophotometry[a]
JKLM	300 µg	COOH	Trinitrophenylation[a]

(a): direct method; (b): indirect Method.

Our strategy was to utilize as coupling site either the primary alcohol function in PbTx-3, to synthesize a hemisuccinate derivative via succinic anhydride condensation, or by direct coupling via the carboxylic function of DA and JKLM ring fragment (Table 1). Epitope density of conjugates ranging from 15 to 25 was determined using a combination of chromatographic, spectrophotometric, chemical and radiochemical techniques, depending upon the hapten used.

Table 2 Antibody production to toxic material

		Apparent dissociation constant (K_D)		
Hapten	Antigen dose	M. PAbs[a]	M. MAbs[b]	R. PAbs[c]
PbTx-3	75 – 90 µg	4×10^{-8} M	1×10^{-6} M	1×10^{-7} M
DA	50 µg	4×10^{-8} M	4×10^{-8} M	ND
JKLM	50 – 90 µg	7×10^{-9} M	1×10^{-7} M	2×10^{-8} M

(a): Mouse antibodies; (b) Mouse MAbs; (c) Rabbit antibodies

In either case, the reactive end of these haptens is located at the terminal end of the molecules (Fig. 2), thus enhancing antigenic exposure during animal immunization (Table 2). After mouse or rabbit immunization with the corresponding conjugates, highly hapten-specific antibodies were produced with apparent dissociation constant (KD) values in the range of 10^{-6}-10^{-9} M.

The present work demonstrates the feasibility of production of polyclonal antibodies and MAbs to very rare haptens and their successful use in EIA formats.

Concluding remarks

It is noteworthy that EIA results may be less representative of the composite potency of food samples compared to functional assays as the response is based on the percentage of epitope cross-reactivity rather than on the measurement of true biological effects.

With antibodies, depending upon the toxin family, two general situations can arise and must be precisely evaluated to avoid a number of false positive reactions or false negative reactions (worse from a public health viewpoint). First, only one compound is highly toxic, so the other members of the family should not be recognized (e.g. DA is highly toxic compared to its isomers and kainic acid) [18]. Second, several compounds are more or less toxic but accumulate at significant level in seafood (e.g. CTX-1B, CTX-2B1, CTX-2B2, CTX-2A2, CTX-3C and CTX-2A1) whereas some exhibiting both very low specific toxicity and concentration (e.g. CTX-2C, CTX-3B and CTX-4A) do not represent a potential threat to public health [19]. Therefore it would be necessary to use a mixture of several antibody preparations (polyclonal or monoclonal) directed to distinct epitopes associated with the most potent toxins identified by functional assays. In this context, our improved micro-coupling procedure appears most valuable to rapidly obtain such antibodies using a small amount of toxic material. An alternative method would be provided by a pharmaco-immunological approach where toxins would be first captured by their natural receptor, then detected by antibodies. Such approach requires distinct sites for receptor binding and antibody interaction, so would not be suitable for very small haptens (e.g. DA).

Finally, as the principal requirements for toxin immunodetection in food matrices are sensitivity and specificity, effort must be also made to develop rapid and efficient extraction procedures. Towards this goal, a very promising work has been done by a New Zealand group who designed a common extraction protocol applicable to ASP, DSP, NSP and PSP toxins [2].

In our work with DA antibodies [10,11] we have successfully applied the procedure developed by Quilliam et al. [20] for the accurate and reproducible detection of low toxin level in spiked mussel extracts. In addition recent experiments with PbTxs-specific antibodies open the way to the detection of toxins in various matrices (shellfish extracts, seawater and mammalian serum and urine) without additional purification, concentration and/or dilution (Naar et al., submitted for publication).

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MICROCYSTIN DETERMINATION USING HPLC COUPLED WITH ULTRA-VIOLET AND MASS SPECTROMETRIC DETECTION INCORPORATING SIMULTANEOUS CID-MS AND MS-MS

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ABSTRACT

A new liquid chromatography method (LC-UV-MS) has been developed which meets the requirement for both high specificity and sensitivity for microcystins by coupling photodiode array ultra-violet (PDA-UV) and mass spectrometric (MS) detectors. An optimised solid phase extraction (SPE) method was evaluated for the recovery efficiencies for four of the most common microcystins at the concentrations, 1.0 and 10 μg/l. Six commercially available microcystins and nodularin were efficiently separated by reversed-phase LC using an acetonitrile/water gradient. Microcystins have a unique β-amino acid moiety, Adda, which gives a fragment ion at *m/z* 135 that is characteristic and distinguishes microcystins from other classes of toxins. After UV detection, both CID and MS/MS experiments were carried out simultaneously using electrospray ion-trap instrumentation.

This method was applied to the analysis of lake water samples from Ireland that were positive following screening by protein phosphatase inhibition assays. Both known and unidentified microcystins were confirmed in samples with detection limits better than 0.1 μg/l.

INTRODUCTION

Microcystins (MCs) are the most abundant toxins from cyanobacteria and they are also the most complex group of cyanobacterial toxins that can contaminate water supplies [1]. Microcystins have been implicated in both animal and human intoxications throughout the world [2]. Microcystins are produced by a variety of cyanobacteria including, *Microcystis*, *Oscillatoria* and *Anabaena* sp. [3,4]. MCs are characteristic cyclic heptapeptides composed of γ-linked D-glutamic acid (D-Glu), D-alanine (D-Ala), β-linked D-erythro-β-methylaspartic acid (D-MeAsp), N-methyl-dehydroalanine (Mdha), a unique C_{20} β-amino acid, 3-amino-9-methoxy-2, 3, 8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda), and two variable L-amino acids. The conjugated diene moiety on the Adda chain provides a useful chromophore for their detection during chromatographic analysis and also a useful fragment ion for identification in the CID mass spectrum of MCs [5]. The acute toxicity values, LD_{50} (i.p. mouse) of most microcystins are in the range 50-600 μg/kg [6] and the lethal dose, LD_{min}, for MC-LR is 0.47 mg/kg [7], resulting in the death of a mouse within 1-3 h. MCs are inhibitors of serine/threonine protein phosphatases PP2A, PP3 and PP1 in decreasing order of potency [8,9]. They are also potent tumour promoters [10] and a large number of human fatalities in a haemodialysis unit in Caruaru, Brazil has been attributed to MCs [11]. The World Health Organisation has recently recommended a guideline limit for drinking water of 1 μg MC-LR/l [12]. Nodularin (NOD) is a cyclic pentapeptide and contains the same three amino acids as microcystins, D-MeAsp, Adda and D-Glu. In addition to these, the ring structure consists of L-Arg and Mdhb (N-methylamino-β-dehydrobutyric acid). Nodularins show significantly less variation than microcystins with only seven different nodularins being characterised to-date [13,14]. Nodularins penetrate hepatocytes more easily than microcystins and have been shown to be liver carcinogens [15].

Analytical methods for the analysis of MCs in drinking water have recently been reviewed [16] and HPLC with diode-array UV detection remains the most commonly used protocol [17]. Immunosorbent methods or solid phase extraction (SPE) for sample cleanup, followed by FAB liquid chromatography-mass spectrometry (LC-MS) has been successfully applied to the trace analysis of MCs in water [18,19].

We now report the development of a HPLC method for the determination of MCs using photo-diode array ultra-violet (PDA-UV) detection together with collision induced dissociation (CID) and MS/MS experiments which were carried out simultaneously using electrospray ion-trap instrumentation. This method was validated using six microcystin standards and nodularin.

METHODS

Materials & chemicals

Purchased chemicals included 4-nitrophenyl phosphate (NPP), DL-dithiothreitol, ethylene glycol-bis-(3-aminoethyl ether), N,N,N',N'-tetraacetic acid (EGTA), Tris-HCl and bovine serum albumen (BSA) and trifluoroacetic acid (Sigma-Aldrich, Gillingham, UK); protein phosphatase 2A (PP2a, Upstate Biotechnology, Lake Placid, NY, USA); Microcystin-RR, Microcystin-YR, Microcystin-LR, Microcystin-LA (Calbiochem, Nottingham, UK); Microcystin-LW, Microcystin-LF (Alexis Corporation, Nottingham, UK). Solvents were HPLC grade (Labscan, Dublin).

LC-UV-MS Instrumentation

LC/UV/MS/MS studies on microcystins were carried out on a HP 1100 series HPLC system with PDA-UV detector (G1315A, Hewlett Packard) linked with a Finnigan MAT LCQ tandem mass spectrometer (Thermoquest, San Jose, USA). The mass spectrometer was equipped with an electrospray ion-spray (ESI) interface. Using a flow injection

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at 3 µl/min, the mass spectrometer was tuned using MC-LR and the optimised capillary temperature was 220 °C with a voltage of 3.0 V. The optimum collision energy for MS-MS experiments was determined for each toxin and this varied from 27% for MC-LA to 39% for NOD. Separation of the microcystins was achieved on a C_{18} Luna(2) column (5 µm, 150 x 2.0 mm, Phenomenex, Macclesfield, UK) at 40°C (5 µL injection). A gradient elution of acetonitrile/water (30/70 with 0.05 % TFA) to 100% acetonitrile (with 0.05 % TFA) over 42 min was used at a flow rate of 0.2 ml/min.

Colourimetric protein phosphatase assays

Phosphatase assays were carried out using a procedure based on that developed by MacKintosh et al. [9] and Tubaro et al. [20]. Dilution buffer (A) was prepared by mixing solutions of Tris-HCl, EGTA and BSA with DL-dithiothreitol as described previously [21]. Enzyme assay buffer (B) was prepared using Tris-HCl, $MgCl_2$ and EDTA solutions with DL-dithiothreitol. Protein phosphatase 2a (PP2a, 10 units/25 µl glycerol) was dissolved in dilution buffer A (5 ml) and divided into 0.5 ml batches which were snap frozen in liquid nitrogen and stored at -80°C. Just prior to an assay, a vial was thawed and dilution buffer A (2 ml) was added. Assays were carried out in microtitre plates using 50 µl of this solution (equivalent to 0.02 units PP2A per reaction).

Standard MC-LR solutions (0.5-10 ng/ml) were prepared for calibrations and assays were carried out using the following: assay buffer B (100 µl), standard or sample solution (50 µl), PP2a diluted enzyme solution (50 µl) and 4-nitrophenyl phosphate solution (NPP, 141 mmol, 50 µl). Absorbances were measured after 1 h and the average absorbance value for controls (N = 4), determined using only assay buffer B (200 µl) and NPP solution (50 µl), was subtracted from assay data. Typical IC_{50} values corresponded to 3 ng MC-LR/ml which represented 0.15 ng/ well.

HPLC analysis of cyanobacteria samples using PDA-UV and protein phosphatase detection

A freeze dried cyanobacteria sample (5-10 g) was extracted with methanol (2 x 50 ml) and an aliquot (1 ml) was evaporated to dryness and made up to 100 µl in water.

This was then injected onto the HPLC equipped with PDA-UV detection. An Atlantis C_{18} column (250 x 3.2 m, Phenomenex) was used with a solvent flow of 0.5 ml/min using the same gradient elution as previously described. Fractions were collected every 30 s and were evaporated to dryness, reconstituted in assay buffer B and analysed, following appropriate dilution (1:100 – 1:1000), by the PP2a assay. A chromatographic profile was then generated by plotting the % PP2a activity against the fraction number.

Recovery study of MCs using SPE

Four stationary phases were used in this study: Envirosep C_{18}, (Supelco, 3ml, 500mg); Discovery C_{18}, (Supelco, 3ml, 500 mg); Bakerbond C_{18} polar plus, (J.T. Baker, 3ml 500 mg); Oasis HLB, (Waters, 3ml 60 mg). The SPE procedure was based on that developed by Lawton et al. [22]. SPE was conditioned with 5 ml MeOH (0.1% TFA), 20 ml MeOH and 20 ml water. The SPE was loaded with the 250 ml spiked sample and washed with 10 ml each of 10% MeOH, 20% MeOH and 30% MeOH. Finally, elution of MCs using 6 ml MeOH (0.1% TFA) which was evaporated to dryness, reconstituted in water (1 ml) and 100 µl was then analysed by HPLC with PDA-UV detection.

Table 1.
Percentage recovery (% RSD) for microcystins from spiked lake water and LC grade water using Bakerbond polar plus SPE.

Toxin	1µg/l lakewater	1µg/l LC water	10 µg/l lakewater	10 µg/l LC water
MC-RR	37 (20)	67 (15)	73 (8.9)	88 (8.5)
MC-YR	78 (6.4)	83 (16)	82 (5.3)	89 (11)
MC-LR	83 (11)	88 (14)	86 (6.8)	93 (7.6)
MC-LA	96 (0.7)	92 (5.8)	93 (2.8)	94 (8.6)

RESULTS AND DISCUSSION

A major problem in the development of any analytical method for the trace analysis of microcystins in water is the efficiency of the cleanup and concentration procedures [22,23]. A lake water sample (250 ml) and HPLC grade water (250 ml) were each spiked with four standards (MC-RR, MC-YR, MC-LR, MC-LA) at concentrations of 10 µg/l and 1 µg/l. These were then applied to each of four SPE columns. Three sets of data were recorded for each and average recoveries and the relative standard deviation (% RSD) values were calculated. The importance of optimising an SPE method for microcystins is illustrated by the fact that recoveries varied from 30% for MC-RR using an Envirosep cartridge to 99% for MC-LA using the Oasis

HLB phase. With the exception of MC-RR at 1 µg/l, the best and most consistent recoveries for microcystins were obtained with the Bakerbond polar plus SPE cartridge, which has a high carbon loading (Table 1).

Six commercially available microcystins and nodularin were separated by reversed-phase LC using an acetonitrile/water gradient and PDA-UV detection (Figure 1). Toxic microcystins all have a unique β-amino acid moiety, Adda, and the α-cleavage of the methoxy group produces a fragment ion at *m/z* 135 which is characteristic of a microcystin.

After UV detection, both CID and MS/MS experiments were carried out simultaneously using electrospray ion-trap instrumentation (Figure 2). The CID spectra (not shown) for the microcystin standards, MC-YR, MC-LR, MC-LA, MC-LW, MC-LF, contained the molecular ion $[M+H]^+$ at *m/z* 1045, *m/z* 995, *m/z* 910, *m/z* 1025 and *m/z* 986 respectively, MC-RR showed mainly the $[M+2H]^{2+}$ ion at *m/z* 520 and all gave the expected Adda fragment ion at *m/z* 135.

MS/MS experiments revealed the $[M+H-H2O]^+$ was the major fragment ion for each of the microcystins and an improved signal/noise was observed when compared with LC-MS. This is a typical advantage of an ion-trap mass

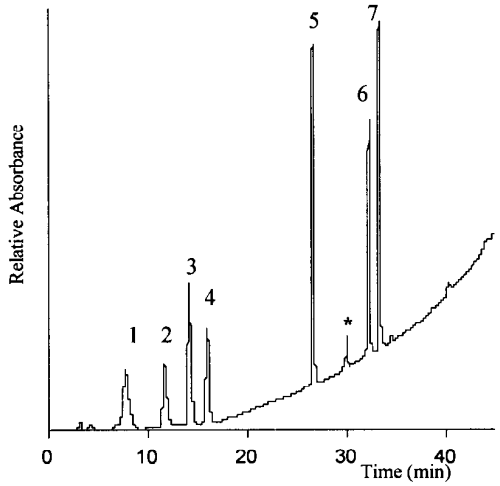

Figure 1. HPLC-UV of six MCs and NOD; 1. MC-RR, 2. NOD, 3. MC-YR, 4. MC-LR, 5. MC-LA, 6. MC-LW, 7. MC-LF; 25ng of each toxin on-column; (*contaminant MC from the MC-LA standard.).

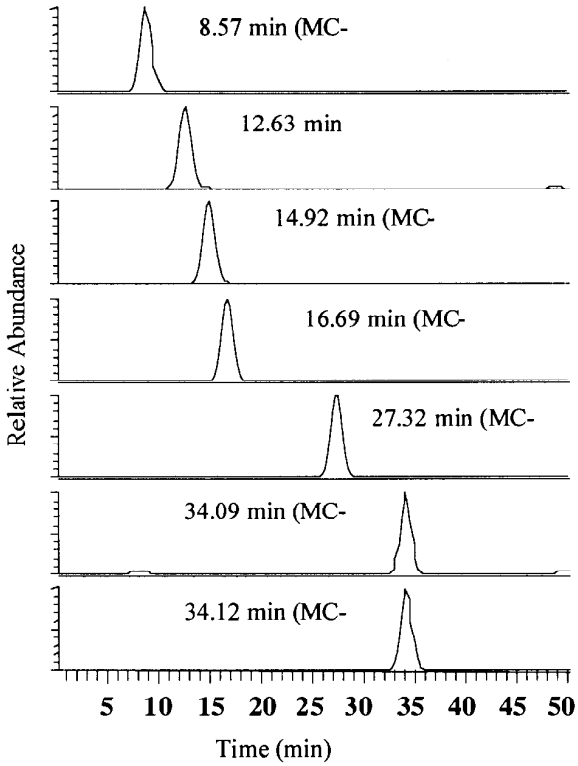

Figure 2. LC-MS-MS of a mixture of six MCs and NOD using electrospray ionisation in positive mode.

spectrometer but one disadvantage is the low mass cut-off. However, the abilility to perform simultaneous 'up-front' CID as well as MS/MS experiments effectively nullifies this disadvantage as the CID allows the detection of the characteristic fragment ion at *m/z* 135 due to the Adda moiety. The detection limits for each of the seven toxins examined in this study were better than 0.1 µg/l.

This method was applied to the analysis of lake water samples from Ireland that were positive by protein phosphatase assays.The chromatogram (Figure 3A) was produced from a lake sample, Co. Cavan, Ireland, using HPLC and with the assay of an aliquot of each fraction using PP2a. When the fractions that strongly inhibited PP2a were analysed by HPLC-UV-MS/MS, MC-RR, MC-YR and MC-LR were identified as well as two unidentified MCs. Figure 3B shows the spectrum produced by 'up-front' CID MS of MC-LR in this sample with its characteristic fragmentations.

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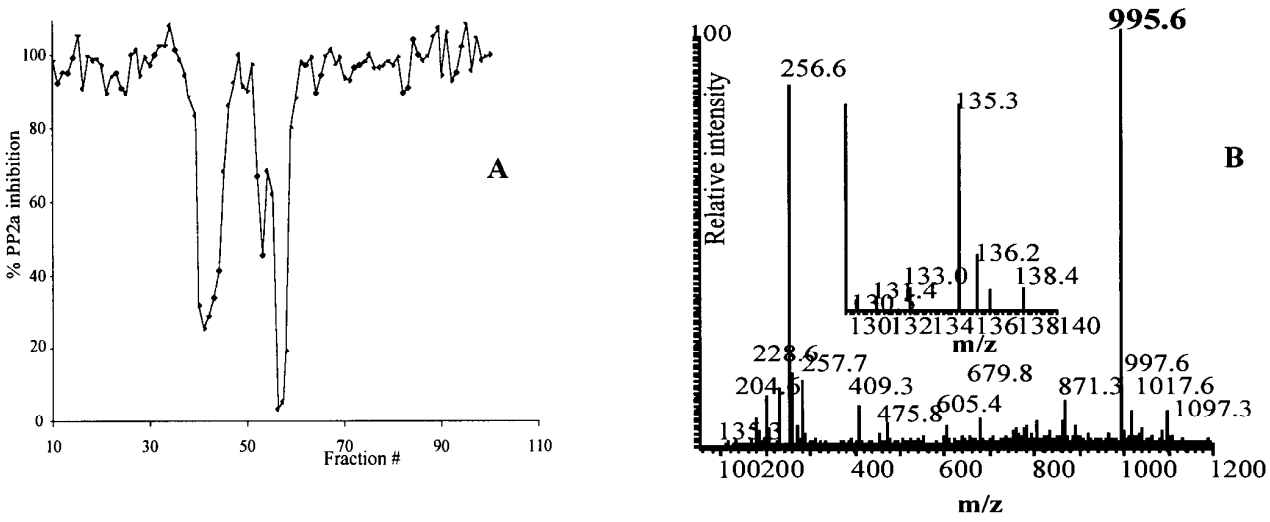

Figure 3. A) HPLC analysis of a cyanobacterial extract from Ireland, with detection using PP2a assays; B) CID-MS of MC-LR in this extract showing the fragmentation ions characteristic of Adda (insert).

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EVALUATION OF A BIOLUMINESCENCE ASSAY FOR DETECTION OF NODULARIN AS AN ALTERNATIVE TO HPLC AND PROTEIN PHOSPHATASE INHIBITION ASSAY

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ABSTRACT

The brackish water cyanobacteria *Nodularia spumigena* produces the hepatotoxic cyclic pentapeptide nodularin.
For the quantitative determination of this toxin we applied a simple, cost efficient and robust bioluminescence inhibition assay with the bacterium *Vibrio fischeri*. The obtained results were evaluated by comparing the data with established analytical methods (protein phosphatase inhibition assay [PP1] and high performance liquid chromatography [HPLC]).
The results obtained with the bioluminescence assay showed good agreement with those from the PP1 assay and HPLC analysis. The detection limit in the bioluminescence assay was determined to be 250 ng nodularin (abs.) with standard deviations of about ± 10 %. The bioluminescence assay is well suited for a fast and reliable screening, i.e. if no exact quantification is needed. Handling procedures to achieve optimal test conditions within the bioluminescence assay are presented.

INTRODUCTION

One important group of toxin producing phytoplankton are cyanobacteria such as *Nodularia spumigena* [1]. This species is omnipresent in the Baltic Sea but can also be found in brackish water lakes and estuaries. Nodularin, the toxic agent, has been described as hepatotoxic [2]. Human and animal intoxications arise when water reservoirs become contaminated with these potentially toxic cyanobacteria [3].
Due to the toxic effects of nodularin we assumed that nodularin also inhibits the bioluminescence of the bacterium *Vibrio fischeri*. So far reliable data on bioluminescence inhibition by this compund and an established standardised handling procedure to screen for its presence in water samples were not available. We used a test kit for waste water analysis [4] and adopted it to nodularin determination in seawater. The obtained data were evaluated and compared with those from a protein phosphase inhibition assay (PP1) [5,6] and with high performance liquid chromatography (HPLC) [7,8].

MATERIAL AND METHODS

Seven laboratory grown batch cultures isolated from Baltic Sea strains of *Nodularia spumigena* were used: KAC (Kalmar Algae Collection) 10, KAC 12, KAC 66, and KAC 68. The cultures were placed in a temperature controlled room (16 °C, 12 h daylight) in 1 L glass flasks.
Additionally, we included cultures grown under phosphate deficient conditions to enhance the number of samples for statistical evaluation and in this state not to interpret the data in relation to toxin production under various nutrient conditions.
Defined volumes of the cultures (50 mL during the exponential growth phase) were filtered through 0.45 µm glass-fibre filters in triplicate. The filters were extracted using 2 mL of methanol/water (70:30, v:v) and ultrasonic treatment for 20 min. The supernatant (centrifugation, 12,000*g, 4 min) was filtered through 0.45 µm disposable polyethylene-terephthalate syringe filters. The resulting extraction solutions were used for both the PP1 assay and HPLC analysis.
The bioluminescence assay requires organic solvent free media. Therefore, the algal extract had to be re-dissolved in 2.5 % aqueous NaCl solution. In this study the extracts from about 2,500,000 cells were evaporated to dryness under a stream of nitrogen and slight heating of the vial (45 °C) and re-dissolved in 0.5 mL 2.5 % NaCl solution. This solution was transferred to a glass cuvette and 0.5 mL of the *Vibrio fischeri* test solution was added (test kit from DrLange, Düsseldorf, Germany, Order Nr.: NRRL-B-11177). After incubating the cuvette for 30 min. (dark, 16 °C) the decrease of bioluminescence in comparison to a blank (2.5 % NaCL solution) was measured with a spectrophotometer (490nm).
The PP1 assay was carried out according to Ward et al. [5] using PP1A from Calbiochem, LaJolla, USA.
For HPLC analysis with ultraviolet (UV) detection the method of Lawton et al. [7] was used. In addition to this HPLC method we used HPLC with mass-spectrometric (MS) detection carried out according to Hummert et al. [9,10].

RESULTS

The experiments performed in this study clearly show that increasing nodularin concentrations diminish the bioluminescence of the bacterium *V. fischeri*.
A linear response in the bioluminescence assay was obtained for nodularin concentrations of up to 4 ng μl^{-1} (Fig. 1). The blank induced a 4 % inhibition which can,

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however, be easily explained by a bioluminescence decay during the incubation period.
The inhibition values of the seven cyanobacterial extracts ranged from 16 % to 37 % (Fig. 2). According to the standard curve this covers the range from 1 ng nodularin per µl up to 2,5 ng nodularin per µl. It can be re-calculated that 4.6 to 17.2 ng nodularin per µg carbon can be found in the investigated samples. The detection limit was determined to be 250 ng nodularin absolute. Each sample was measured twice and each measurement was done in triplicate. The standard errors range between 8 and 12%. The measured toxin values with the bioluminescence assay and with HPLC and PP1 are summarised in table 1.

Sample	nodularin [ng *µg carbon^{-1}] **HPLC-UV**	nodularin [ng* µg carbon^{-1}] **PP 1 assay**	nodularin [ng* µg carbon^{-1}] **biolum.assay**
KAC 10	13.4 +/- 0,7	9.8 +/- 0,5	12.2 +/-1.3
KAC 12	6.2 +/- 0,3	3.2 +/- 0,2	10.6+/-1.1
KAC 66	3.4 +/- 0,2	4.1 +/- 0,2	9.4+/-0.9
KAC 68	5.3 +/- 0,3	3.7 +/- 0,2	4.6+/-0.5
KAC 10 P-lim	9.6 +/- 0,5	7.6 +/- 0,4	5.7+/-0.6
KAC 12 P-lim	7.3 +/- 0,4	6.7 +/- 0,3	17.2+/-1.7
KAC 66 P-lim	9.0 +/- 0,5	6.9 +/- 0,3	12.6+/-1.3

Table 1 Nodularin content in *Nodularia spumigena* samples measured with HPLC/UV, PP1 assay and bioluminescence inhibition assay (mean values from triplicate analysis, +/- indicate standard deviation)

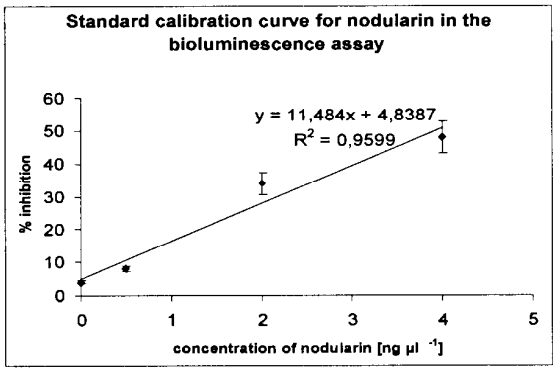

Fig. 1 Standard calibration curve for nodularin in the bioluminescence assay. The regression line is shown. Mean values of triplicate analysis, error bars indicate standard deviation.

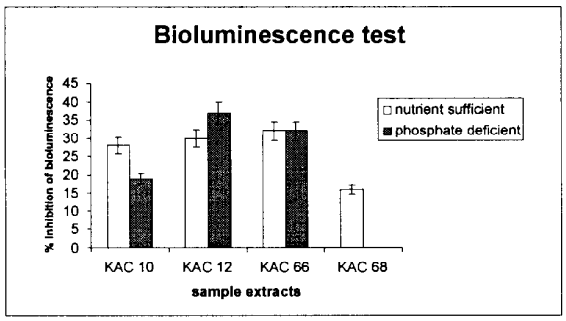

Fig. 2 Results from the bioluminescence test (mean values of triplicate analysis are shown, error bars indicate standard deviation). Each sample extract used in the test is composed of app. 2.500.000 *Nodularia spumigena* cells.

DISCUSSION

Our study showed that the bioluminescence assay is suitable for semi-quantitative determination of nodularin. The measured nodularin contents from the bioluminescence assay were in a good agreement (R^2 ~ 0.85) with those determined with the PP1 assay and by HPLC/UV and HPLC/MS (MS-data not shown). However, the sensitivity and selectivity within this assay towards nodularin is low and the standard errors were around ± 10 % which is twice as high as in the PP1 assay or HPLC, respectively. The data presented here are based on laboratory grown strains of *Nodularia spumigena*. We also applied the bioluminescence assay to evaluate the toxicity of a cyanobacteria bloom in the Banter See in Wilhelmshaven, Germany. A microscopic determination of the phytoplankton composition showed a clear dominance of *Nodularia spumigena* and extracts from the biomass clearly showed a significant inhibition of bioluminescence. Posterior HPLC analysis proved the presence of nodularin in these samples.
It can therefore be stated that the bioluminescence inhibition assay is acceptable as a pre-screening of phytoplankton with advantages in cost efficiency (about 30 USD for one series), simplicity and the possibility for field measurements.
For posterior evaluation of the data we decided to express the toxin content relative to carbon. The carbon content per cell was determined prior to each toxin analysis (data not shown).

CONCLUSION

The described simple screening with a bioluminescence inhibition assay can be used on its own for a first detection and even for a semi-quantitative determination of nodularin during *Nodularia spumigena* blooms or to evaluate the toxicity of laboratory cultures. If this test is positive, it is highly desirable that HPLC analysis is carried out for an exact determination of the compounds in question.

ACKNOWLEDGEMENT

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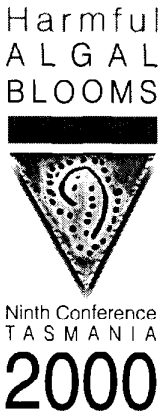
PHYSIOLOGY OF TOXIN PRODUCTIION



SPIROLIDE PRODUCTION AND PHOTOPERIOD-DEPENDENT GROWTH OF THE MARINE DINOFLAGELLATE *ALEXANDRIUM OSTENFELDII*

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ABSTRACT

The effects of physiological status on spirolide production were studied in nutrient-replete batch cultures of a toxic strain of the dinoflagellate *Alexandrium ostenfeldii*. Although complete cell synchronisation was not achieved by dark adaptation, the concentration of motile vegetative cells apparently increased in the light and decreased in the dark. The concentration of extracted chlorophyll *a* followed the same trend as the cell concentration, with no apparent shift in the amount of chlorophyll *a* per cell in relation to the light/dark (L/D) phase. Analysis of spirolides by liquid chromatography coupled with mass spectrometry (LC-MS) showed that the toxin profile did not vary significantly over the L/D cycle, and consisted primarily of a des-methyl-C derivative (>90% molar), with minor constituents C, C3, D, D3 and des-methyl-D. The total spirolide concentration per unit culture volume was directly related to the concentration of cells and chlorophyll *a*, but there was a dramatic increase in cell quota of spirolides at the beginning of the dark phase and a corresponding decrease in the light. The biosynthesis of these polyketide-derived metabolites is apparently governed by light-dependent events during the cell division cycle.

INTRODUCTION

The marine dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech *et* Tangen has been recently identified as the source of toxic spirolides [1]. These potent macrocyclic imines were first isolated and characterised from shellfish viscera [2,3], and later identified in the plankton from Nova Scotia, Canada [4,5]. New rapid and highly sensitive methods to quantify spirolides in only few plankton cells by liquid chromatography-mass spectrometry (LC-MS) [6] have been applied to the analysis of a spirolide-producing *A. ostenfeldii* clone [1].

The biosynthesis of other toxic metabolites by *Alexandrium* spp. is known to be regulated by a complex interplay of environmental and intrinsic genetic factors (reviewed by [7]). Typically, changes in environmental variables, such as light, salinity, turbulence, temperature, and macronutrients, influence the cell quota of toxin (Q_t), either by direct effect or via a feedback interaction with cell growth rate. As the cell divides, Q_t is partitioned between the daughter cells [7,8]. The synthesis of PSP toxins in *Alexandrium* occurs during vegetative growth in the G1 phase of the cell cycle [8], thus any prolongation of G1 phase (decrease in growth rate [μ]) may result in higher Q_t even if the rate of toxin synthesis is constant. Physiological studies on dinoflagellate production of tetrahydropurine neurotoxins (e.g., saxitoxin derivatives) [7] and polyether toxins [9] have generally indicated that the toxin composition is characteristic of the strain, and that the toxin profile is rather refractory to change [10,11], except under extreme environmental stress.

In photoautotrophic dinoflagellates, the photoperiod influences many diurnal physiological processes, including cell division, nutrient assimilation, vertical migration and bioluminescence rhythms. The direct dependence of cellular processes on light/dark (L/D) cycles can be exploited to phase or synchronise the cell division cycle. Dark-induced synchronisation followed by entrainment on a defined L/D cycle has been previously used to study the cascade of events involved in toxin production in the dinoflagellates *Alexandrium fundyense* [8] and *Prorocentrum lima* [12].

There are few studies on the effects of photoperiod and cell division cycle events on the production and accumulation of polyketide-derived metabolites. We attempted to use dark-induced synchronisation of *A. ostenfeldii* cultures to determine the effects of photoperiod on the cell quota of spirolides through successive cycles of cell division. Such studies are a prerequisite to establish the links between toxin biosynthesis and discrete stages of the cell division cycle. Furthermore, these data can be used to determine the optimum photoperiod for maximum growth and to quantify the effects of light induction on other cellular processes, such as chlorophyll synthesis.

MATERIALS AND METHODS

Experiments were conducted on a clonal isolate of *Alexandrium ostenfeldii* (AOSH1) from Ship Harbour, Nova Scotia in unialgal batch cultures using aseptic techniques. Stock cultures (1.0 L) in exponential growth phase were inoculated into 12 L of L1 growth medium in triplicate 15 L Belco glass carboys. Cultures were grown with gentle aeration to maintain homogeneity at 15±1 °C under a 14:10 light/dark (L/D) photocycle at an ambient photon flux density of 260 µmol m^{-2} s^{-1}. After 106 h of dark adaptation, culture samples were collected by sterile syringe at 2 h intervals throughout three L/D cycles for measurements of chlorophyll *a* (extracted and *in vivo*), cell number, cell size and spirolide concentration. During the dark period, samples were collected under red light (<0.1 µmol m^{-2} s^{-1}) to avoid photo-induction.

Growth of cultures prior to dark adaptation was monitored by optical microscopic counts (125X). During the experiment, cell concentrations were determined using a Coulter Counter (Multisizer II). *In vivo* chlorophyll *a* fluorescence of whole cultures (10 ml) was measured by fluorometry (Turner Designs Model 10). Particulate

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chlorophyll samples were filtered (Whatman GF/C) and extracted in darkness with 90% acetone (72 h) at –20 °C for quantitation by fluorometry.

Spirolides were analysed from three culture fractions: cells, filtrate (cell free medium), and whole culture. Duplicate samples of whole culture were filtered through 0.5 ml spin-cartridges (Millipore Ultrafree-MC, 0.45 µm) by centrifugation at 500 x *g*. The filtered cells were extracted by spin-filtration with 1 ml of 100% methanol [5]. Extracellular spirolides in the cell free medium were determined by direct injection of the filtrate. Spirolides were analysed by liquid-chromatography with ion-spray mass spectrometry (LC-MS) (PE-SCIEX API-III) [6] using purified standards.

RESULTS

After inoculation of stock cultures into fresh growth medium, *A. ostenfeldii* cells remained in lag phase for one week. Dark adaptation for 106 h was initiated after Day 8, when the mean cell concentration had reached 800 ± 150 (n=3) cells ml^{-1}. During dark adaptation, the mean cell concentration declined substantially to 468 ± 109 cells ml^{-1}. After transfer to the 14:10 L/D cycle, the cell concentration oscillated with the photoperiod, decreasing in the dark and increasing in the light phase (Fig. 1). This variation in cell concentration between the light and the dark phase was maintained throughout the experiment, for three L/D cycles. Non-motile cells, resembling pellicular cysts, accumulated on the bottom of the culture vessel, particularly during the dark phase. Based upon cell counts of motile vegetative cells alone, the net growth rate, calculated from T=0 to the end of the experiment, was low (μ = 0.18 div. d^{-1}).

The concentration of particulate chlorophyll *a* (ng ml^{-1}) in the cultures exhibited the same trend as the cell concentration (Fig. 2). As for the cell concentration, the amplitude of the oscillation in chlorophyll *a* between the light and dark phases increased with each successive L/D cycle through the experiment. There was no apparent shift in chlorophyll *a* per cell related to the L/D phases.

Analyses by LC-MS showed that the sum of spirolides extracted from the cellular fraction, plus that found in the cell-free culture medium, was similar to that extracted from the whole culture. Leakage or excretion of spirolides from healthy vegetative cells accounted for <3% of the total spirolide content of the *A. ostenfeldii* cultures. Total spirolide concentration per unit culture volume (whole culture) fluctuated in response to the L/D cycle, similar to the pattern exhibited by the cell number and chlorophyll *a* concentrations (Fig. 3). Spirolide levels in the culture peaked at the end of the light phase and plummeted by as much as 20% early upon entry into darkness. However, in contrast to the pattern of cellular chlorophyll *a*, there was a dramatic increase in cell quota of spirolides at the beginning of the dark phase, peaking by the middle of the dark phase, and a corresponding decrease in the light (Fig. 3). The variation in the cell quota of spirolides over the last two L/D cycles was >50%, when the increase was calculated from the middle of the light period to the maximum in the dark. Variation in total concentration of chlorophyll *a* and spirolides over

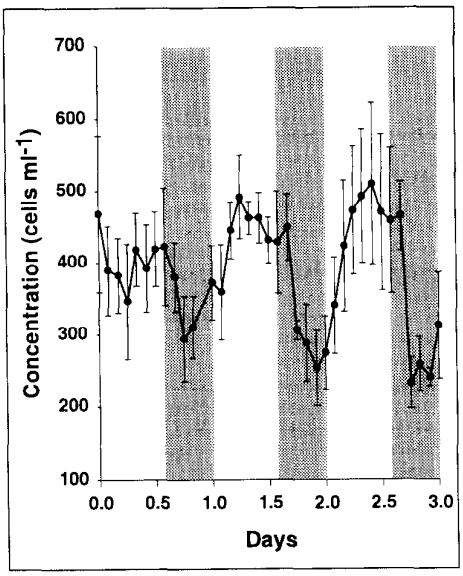

Fig. 1. Variation in cell concentration of *A. ostenfeldii* AOSH1 over several photocycles. Dark bars denote the darkness periods.

the L/D cycle was not due to cell size differences; mean cell diameter in the light was 27.9 (± 0.4 s.d.)µm, compared to 26.5 (± 0.6 s.d.)µm in darkness.

The spirolide profile of this isolate was very stable, and no substantial variations were noted in response to the photoperiod. Des-methyl-C comprised >90% of the total toxin on a molar basis, whereas derivatives C, C3 and des-methyl-D were minor components.

DISCUSSION

We report here the first evidence that extrinsic environmental factors, specifically photoperiod, can influence the rate of production and cell quota of macrocyclic imines in marine dinoflagellates. Toxin production in dinoflagellates is also known to be subject to genetic regulation [7,8,9], but physiological mechanisms and regulatory control of the biosynthetic pathways of toxin production are poorly understood.

Cell Growth and Photoperiodic Events

Compared with other *Alexandrium* isolates, this *A. ostenfeldii* strain is fastidious and less robust in mass culture. Even under recently optimised growth, reducing the light from 250 to 70-100 µmol m^{-2} s^{-1}, cells appear healthy, but growth rates remain <0.2 div. d^{-1} (A. Cembella, unpublished data). Long term acclimation to higher than optimal light intensity followed by prolonged dark exposure to achieve cell synchronisation may account for the apparent decline in cell numbers in darkness and the subsequent low net growth rate.

To reduce the deleterious effects of turbulence on cell growth, diffuse aeration was supplied at a level only sufficient to maintain roughly homogeneous distribution of motile cells and to minimise sedimentation. Prior to the experiment, samples were collected simultaneously

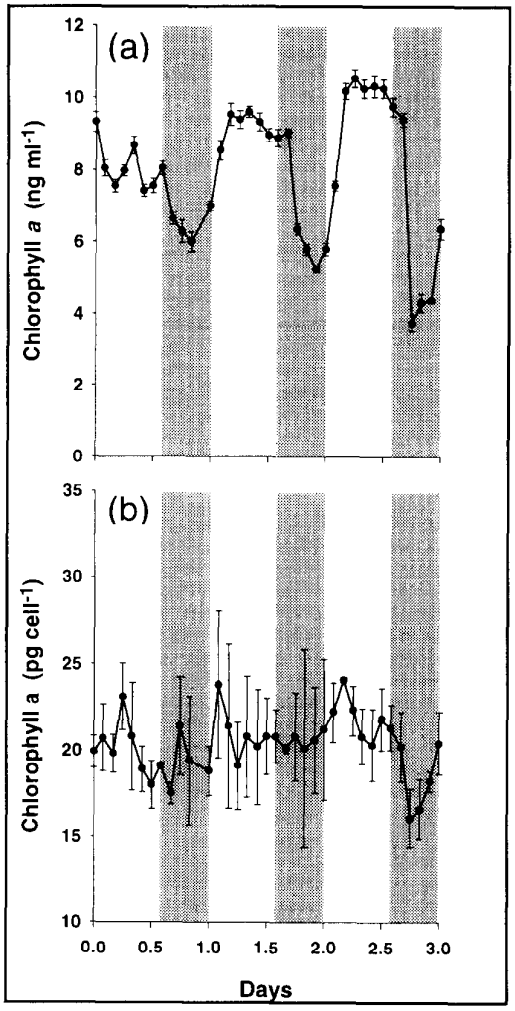

Fig. 2. Variation in chlorophyll *a* per unit culture volume (a) and per cell (b) over several photocycles. Note different scaling of the Y-axes.

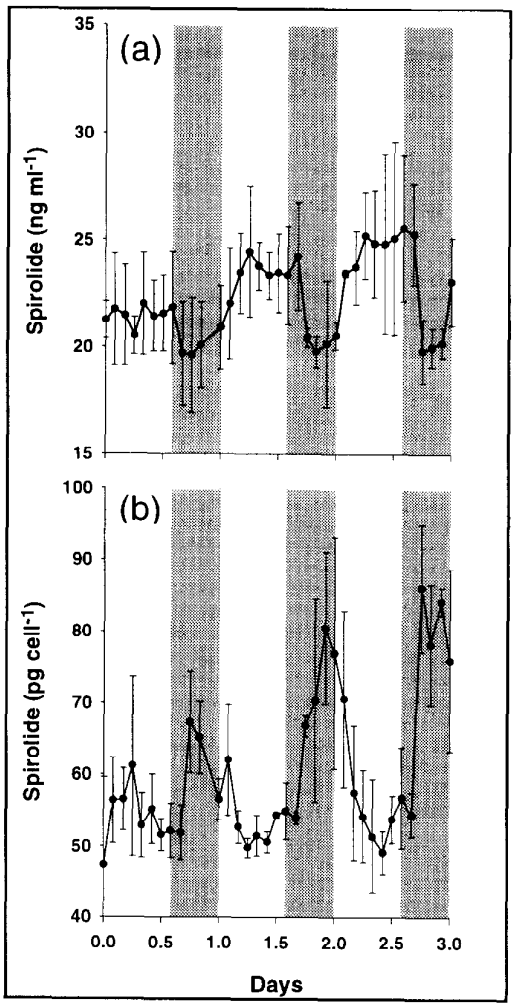

Fig. 3. Variation in total spirolide concentration per unit culture volume (a) and per cell (b) over several photocycles. Note different scaling of the Y-axes.

from several locations in the carboy to confirm homogeneity. The inlet port for cell sampling was situated several centimetres off the bottom of the carboy to ensure that motile (and presumably cycling) cells were primarily selected. The phasing of the cell concentration with the photoperiod, following a pattern of increasing cell concentration in the light and decreasing in the dark phase could be correlated with the vertical migration of motile cells. Higher deposition of dead cells, cell debris and pellicular cysts on the bottom during the dark period, and regeneration of vegetative cells from pellicular cysts in the light, might also account for the apparent growth kinetics. Nevertheless, visual observations confirmed that turbulence was sufficient to prevent layer formation of motile cells even in the dark. This phenomenon of vertical migration and encystment (pellicular cyst formation) during the dark phase has been described for *Alexandrium taylori* [14]. In this species, pellicular cysts give rise to motile cells at the beginning of the light phase, indicating that excystment and encystment may be controlled by light and regulated via the cell cycle.

For *A. ostenfeldii*, the rate of chlorophyll *a* production was approximately in balance with the cell division rate, as evidenced by the coupled oscillation in the total amount of chlorophyll *a* and cell numbers in the culture over the L/D cycles. If the cells were synchronised, chlorophyll *a* concentration should increase in the same stepwise manner as the cell concentration (see [13]). For *Prorocentrum lima*, Pan *et al.* [13] showed a L/D period-dependent increase and decrease of chlorophyll *a* cell quota, at least in the period before the cultures became asynchronous. For *A. ostenfeldii*, there was no shift in the chlorophyll *a* per cell in relation to the L/D phases. This might be due to the low cell division rate and/or to poor synchronisation.

Numbers of motile cells collected were insufficient for statistically valid identification of the cell cycle phases using nuclear DNA staining and flow cytometry. Since we were unable to attain a high level of cell synchronisation with *A. ostenfeldii* via dark acclimation, we did not observe the typical pattern of stepwise

increases in cell concentrations, as for *A. fundyense* [8] and *Prorocentrum* spp. [13].

Production of Spirolides

The dramatic increase in total spirolide per cell at the beginning of the dark period and the decrease during the light periods showed that spirolide biosynthesis is affected by light-dependent metabolic events. The >50% increase in the cell quota of spirolides after the L/D shift through several photocycles indicates a coupling of spirolide production to the photoperiod and cell cycle. By comparison, in *Prorocentrum lima*, the cell quota of the polyketide-derived DSP toxins increased in the light, but also extended through several phases of the cell cycle [12]. In contrast, although PSP toxin production by *A. fundyense* occurred in the light, synthesis was restricted to the G1 phase [8].

The transition of a fraction of the motile vegetative cells to pellicular cysts and the formation of dead cells may account for the variation in total spirolide concentration per unit culture volume. Pellicular cysts are a temporary quiescent stage produced through ecdysis of vegetative cells [14]. Since pellicular cysts are arrested in G_o-phase, maintaining only basal metabolism, these recurrent cells should have approximately the same cell quota of spirolides as vegetative cells before ecdysis. The maximal cell quota observed primarily from motile vegetative cells at the end of the dark phase is explicable as net spirolide production if this period also represents the late mitotic phases G2+M, just prior to cytokinesis.

The consistently low concentration of spirolide found in the medium (<3% of total spirolide of the whole culture) tends to indicate that leakage and excretion of spirolides from healthy vegetative cells, pellicular cysts and cell debris is not an important cycling mechanism. There is some preliminary evidence (M. Quilliam, unpublished data) that spirolides may be somewhat unstable in water at pH >5, although the decomposition rates in buffered seawater are unknown. Thus although it is conceivable that decomposition could account for low ambient spirolide levels in the medium, this is counter-indicated by the relative consistency in the spirolide profile (major derivative des-methyl-C) found in both the cellular fraction and the medium.

It is still unclear if spirolide biosynthesis is directly light-dependent, or if biosynthesis, intracellular transport and excretion are indirectly mediated via the effects of light on enzymes and other functional metabolites. In any case, the apparent lack of any photoperiod-dependent shift in spirolide composition indicates that the cascade of events leading to biosynthesis of the various spirolide analogues is on a time-scale shorter than that of the sampling intervals. By comparison, in *Prorocentrum lima,* the production of DTX4 derivatives was initiated in G1 phase and continued into S phase, whereas other derivatives, such as OA and DTX1, were produced later in S and G2 phases [12].

This study has provided significant insights into the light-dependence of spirolide production, but little information is available on the biosynthesis of polyketide-derived metabolites by dinoflagellates. Further effort will be directed towards the use of cell synchronisation techniques coupled with studies of gene expression of putative biosynthetic genes for spirolides.

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UNDERSTANDING THE EFFECTS OF NUTRIENTS ON TOXIN PRODUCTION IN *ALEXANDRIUM TAMARENSE*

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ABSTRACT

A series of experiments (batch and semi-flow-through cultures) were conducted to try to understand how the variation of nutrient (N, P) levels affect toxin production in a locally (Hong Kong) isolated strain of *Alexandrium tamarense* (ATHK). Batch culture experiments revealed that cellular toxin contents reached their peak at the beginning of log phase and then decreased gradually under N and P replete conditions. Nitrogen limitation had minor effects on toxin content at the beginning of log phase but caused a decrease in toxin content in later growth stages. Low concentrations of P kept toxin levels constant and slightly increased later. Semi-flow-through experiments indicated that N limitation related to low toxin production and P limitation caused high toxin production. The algae produced medium level toxins under turbid-static condition. The mechanism of effects of N and P on toxin production is discussed. The fact that toxin content is closely related to arginine content in the cell implied that N impacted on toxin production by affecting arginine level directly, while P affected toxin production through regulating N metabolism in algal cells.

INTRODUCTION

Many researches have focused on toxic HAB (harmful algal bloom) organisms and toxins in the past two decades, of which PSP (paralytic shellfish poisoning) toxins are of most concern. Some species in the dinoflagellate genus *Alexandrium*, *Gymnodinium* and *Pyrodinium* can produce potent PSP toxins. Although toxin profile is a relatively conservative property of a particular toxic species or strain, it has been revealed that there are differences in toxin production properties (toxin content or toxin profile) among different toxic species, sometimes even among strains from different locations or at different times [1,2,3]. Many factors could affect toxin production of toxic algae, such as genetic characteristics, symbiotic bacteria and environmental factors. It has been reported that in batch culture experiment, toxin content of a particular species or strain can vary with growth stages [4,5,6]. Studies have been carried out to try to understand the relationship between toxin production and environmental factors, including salinity[7,8], temperature[9], light intensity[9], and nutrient levels [5,6,8,10,11]. According to studies, nutrients (including nitrogen and phosphorus sources) are the most notable factors affecting toxin production. However, it's still hard to explain the possible mechanisms between availability of nutrients and toxin production, which is important in prediction and mitigation of impacts of toxic species.

Alexandrium tamarense is a common toxic species in Hong Kong waters. However, there is limited knowledge concerning physiological characteristics of this species found in this area, especially on toxin production. PSP toxin contamination in shellfish has been reported in Hong Kong. This paper is trying to understand the possible role of nutrients in controlling toxin production in *Alexandrium tamarense* isolated from Hong Kong waters.

MATERIAL AND METHOD

Organism

Alexandrium tamarense (ATHK), which was isolated from Hong Kong waters, was a kind gift from Jinan University. The species had been cultured for more than one year before the experiment.

Algae culture and sample collection

F/2 culture medium was used for both batch culture and semi-flow-through experiments. Culture media was sterilized by filtration with 0.22 _m membrane. All glassware was autoclaved before use. Algae were cultured under 20 °C, 3500-4000 lux with light:dark ratio 12h:12h. Algal concentration was determined by cell counting with inverted microscope or by measurement of fluorescence *in vivo*.

Algal cultures were mixed gently before sampling. A fraction was taken for cell enumeration. Collected algae were concentrated with 10-_m mesh. The concentrated algae were then flushed into a 10 ml centrifuge tube and centrifuged (×3000 g). The supernatant was discarded and the pellet was transferred to a 4ml vial with 3ml 0.05N acetic acid solution. The mixture was sonicated immediately with a probe sonicator for 10 minutes to disrupt the cells (examined with microscope). After centrifugation (10,000 rpm) for 5min, the supernatant was collected and filtered with a 0.22 _m membrane filter and stored at -20_ for toxin analysis.

Batch culture experiment

Algae were inoculated into 2 L culture medium in 2.5 L flasks. The initial cell concentration was approximately 400 cells/ml. For N, P replete experiments, algae for inoculation were taken from mid log phase. For N, P deplete experiment, in order to

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eliminate the effects of stored N and P in the cell, algae for inoculation were cultured in special f/2 media without nitrate and phosphate for one week. The initial concentration of nitrate and phosphate in N, P deplete experiments were made according to Table 1. Algal samples were collected at 3-4 day intervals, beginning from the fifth day after inoculation. Around 500-1000 thousand cells were collected each time, determined by cell counting results and volume collected.

Table 1. Initial concentration of nitrate and phosphate in culture media for N, P deplete experiment

Treatment	Nitrate concentration (mol/L)	Phosphate concentration (mol/L)
L'NL'P	11.76	2.4
L'NLP	11.76	7.2
L'NMP	11.76	18
LNL'P	44.1	2.4
MNL'P	176.4	2.4
LNLP	44.1	7.2

L': extremely low L: low M: medium
N : nitrate P: phosphate

Semi-flow-through experiment

One 1iter treatments were used in a semi-flow-through experiment, and each treatment was duplicated. Nitrate concentration in medium for N limited experiment was 50 _mol/L; and phosphate concentration in medium for P limited experiment was 1 _mol/L. Five dilution rates were made for N and P chemo-static experiment: 0.07, 0.14, 0.21, 0.28 and 0.35. Culture media were renewed according to the dilution rate every day. Algae were not collected for analysis until the cell concentration become constant. For the turbid-static treatment, f/2 culture medium was used. The dilution rate was calculated according to the growth rate determined by cell counting each day. Cell concentration of turbid-static treatment was maintained around 3000 cells/ml.

Analytical methods

PSP toxin analysis

Both Thielert's method and Oshima's method were used in the experiment [12,13]. A significant modification was made to Thielert's method [14]. And a slight modification was made to Oshima's method: a WATERS Nova-Pak C18 column was used and the concentration of ion-pair reagent (heptanesulfonic acid) was raised to 3 mM for analysis of GTX toxins. Only GTX1-4, neoSTX, STX were analyzed with the two methods. The same sample was analyzed twice before and after acid hydrolysis to calculate C toxins, GTX5 and GTX6.

Toxin standards, including STX, neoSTX, GTX1, GTX2, GTX3, and GTX4 were purchased from the National Research Council Canada, Marine Analytical Chemistry Standards Program (NRC-PSP-1B), Halifax NS, Canada.

Amino acid analysis

Amino acid samples were collected in the same way as those for toxin analysis except that the collected cells were flushed twice with 3.1% NaCl solution before centrifugation. Collected algal pellets were boiled in double distilled water for 10 minutes to extract amino acids. Extracted samples were then analyzed (mainly for Arg, Gln and Glu) according to a previously published method [15].

Nitrate and phosphate analysis

Nitrate and phosphate in culture media were analyzed according to the methods introduced in Parsons et al., 1984[16].

RESULTS

Batch culture

(1) Toxin content

The initial concentration of N and P has remarkable effects on toxin content in cells grown in batch culture. Under N, P replete conditions, toxin content per cell reached the peak at the fifth day after inoculation, and then decreased gradually. At the end of the log phase, the toxin content decreased from around 20 fmol/cell to 5-8 fmol/cell. Despite the decreasing pattern of toxin content per cell, toxin content per volume correlated well with the growth curve (Fig.1).

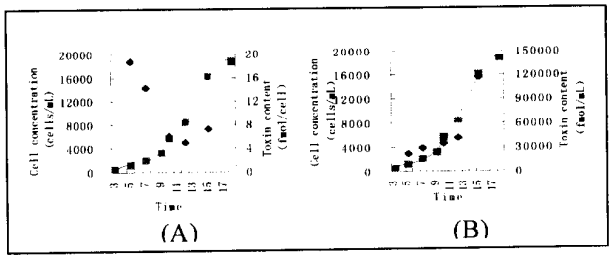

Fig.1. Variation of toxin content under N, P replete condition(—■— cell concentration; ◆ toxin content)
(A) Toxin content per cell
(B) Toxin content per volume

Under N, P limited conditions, when *Alexandrium tamarense* was mainly under stress of P limitation (MNL'P), toxin content increased over the whole exponential growth phase, and toxin content per cell at the stationary phase was 1.8 times higher than in cells at the beginning of exponential phase. However, when the cells mainly under stress of N limitation (L'NMP), toxin content decreased, and was nearly 4 times lower than in cells at the beginning of exponential phase.

In treatments of L'NLP and LNLP, the concentration of both N and P was not enough for the growth of algae, however, the depletion time of N was before the depletion time of P. There was a "V" shaped curve of toxin content per cell. The toxin content

decreased at first when N was depleted, and then began to increase slightly when phosphate in the medium was not available. On the contrary, in treatments of L'NL'P and LNL'P, the depletion time of P was before the depletion time of N, the toxin content increased at first when P was a limiting factor, and then decreased and remained relatively constant when N became a limiting factor as well.

The initial N:P ratio had little effect on toxin content at the beginning of exponential phase, which was between 14-20 fmol/cell.

(2) Toxin composition

ATHK strain contains 10 toxins: C1, C2, GTX1, GTX2, GTX3, GTX4, GTX5, GTX6, neoSTX and STX. There was no obvious variation in toxin composition among the different treatments. But compared with the toxin composition at the beginning of exponential phase, the ratio of GTX toxins increased and that of C toxins decreased at the stationary phase.

Semi-flow-through experiment

(1) Toxin content

Variation of toxin content in semi-flow-through experiments is shown in Fig. 2. Under N limited condition (50 _mol/L of nitrate), the toxin levels of algae increased from 13 to 30 fmol/cell as dilution rate increased, which indicated that the increasing availability of nitrogen source under N limited condition would elevate the toxin content per cell. While for algae cultured under P limited condition (1 _mol/L of phosphate), the toxin levels decreased from 104 to 29 fmol/cell as dilution rate increased, which indicated that the increasing availability of phosphorus source under P limited conditions would decrease the toxin content per cell. It was obvious, however, that toxin levels under P limited conditions were much higher than that under N limited conditions. Toxin content in algae cultured under turbid-static condition was between that of N-limited and P-limited conditions.

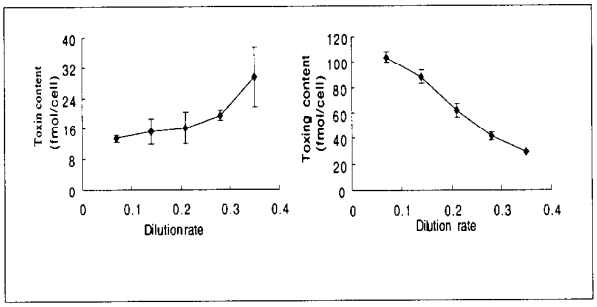

Fig.2. Variation of toxin content under different N, P limited conditions
(A) N limited conditions (B) P limited conditions

(2) Relationship between arginine content and toxin content

Good correlation was found between arginine contents and toxin contents in *A. tamarense* cells under both N and P limited conditions. Under N limited condition, the content of arginine was between 35-168 fmol/cell, while under P limited condition, the content of arginine was between 179-503 fmol/cell. Toxin content per cell also correlated well with arginine content, regardless of nutrient conditions (Fig. 3).

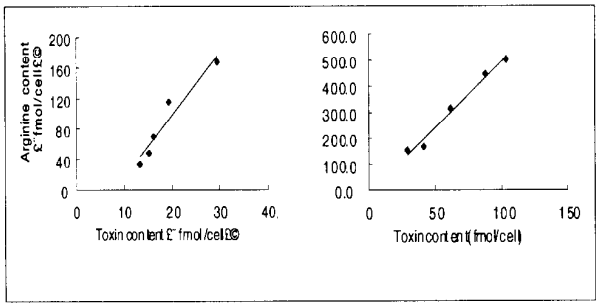

Fig.3. Relation between toxin content and arginine content under different N, P limited conditions.
(A) N limited conditions (B) P limited conditions

(3) Variation of Gln/Glu ratio

It was found that the variations of Gln/Glu ratios experienced similar trends to that of toxin contents under different N or P limited conditions to increase or decrease as the dilution rates increased from 0.14 (Fig. 4, also refer to Fig. 2). However, the Gln/Glu ratios at the lowest dilution rate of 0.07 did not follow the pattern above for unknown reasons. Under P limited conditions, the Gln/Glu ratios were much higher than those under N limited conditions and correlated well with arginine contents and toxin contents.

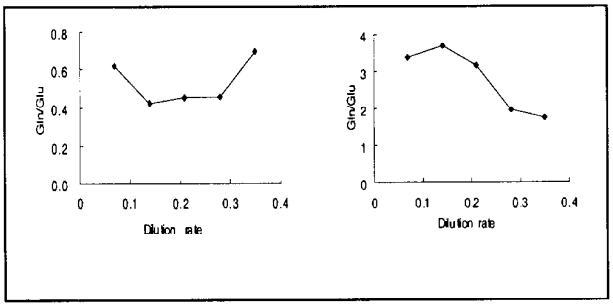

Fig.4. Variation of Gln/Glu ratio under different N, P limited conditions
(A) N limited conditions (B) P limited conditions

DISCUSSION

Many researches have studied the toxin production process and possible controlling factors. People have tried to relate the toxin content to growth rate, but the relationship is not always predictable, especially at the beginning part of exponential phase, or under P limited

condition. Anderson et al. suggested that the variation of toxin content could be divided into growth stage variability and environment-caused variability, including variability caused by temperature, salinity, light intensity, nutrients and so on [8]. The variation in toxin content of different growth stages could be well explained by different net toxin production rates, as suggested by Anderson. However, the toxin content variability caused by environmental factors is still not clear. Some contradictory results have been found in the following studies. For example, Anderson et al. found that the free arginine content varied as the mirror image of changes in toxin content in *Alexandrium fundyense*, but Flynn found that toxin content co-varied with free intracellular arginine in *Alexandrium minutum*[11]. In this experiment, however, arginine and toxin content under different conditions in semi-flow-through experiment exhibited a close relationship between the two compounds.

Flynn et al. suggested that Gln/Glu ratio c ould serve as a good indicator of N status in cells[17]. This experiment found, when the dilution rate was beyond 0.07, that the Gln/Glu ratio might be a good indicator of N status in *Alexandrium* cells: to increase with more N supply (higher dilution rate under N limited conditions) or with more potential of N redundancy (lower dilution rates under P limited conditions), which might be important to understand toxin synthesis in cells. However, we could not explain why the Gln/Glu ratios under the lowest dilution rate (0.07) departed from the pattern discussed above.

To some extent, the role of N in PSP toxin production is relatively easier to understand, because saxitoxin contains 33% N on a molecular weight basis. In our semi-flow-through experiment, both the arginine content and toxin content increased as the dilution rate increased (more N available), which indicated that N availability affects toxin synthesis directly. But the effects of P were still not clearly understood. In our experiment, however, the results suggested that P limitation might have caused N redundancy and arginine accumulation in *Alexandrium* cells, whether might through preventing the "normal" synthesis pathways ("block"), or stimulating *de novo* synthesis, which causes high toxin content per cell. According to the experimental results, a model hypothesis is proposed to explain the possible role of nutrients in controlling toxin production (Fig. 5). However, research is needed before the model can be confirmed.

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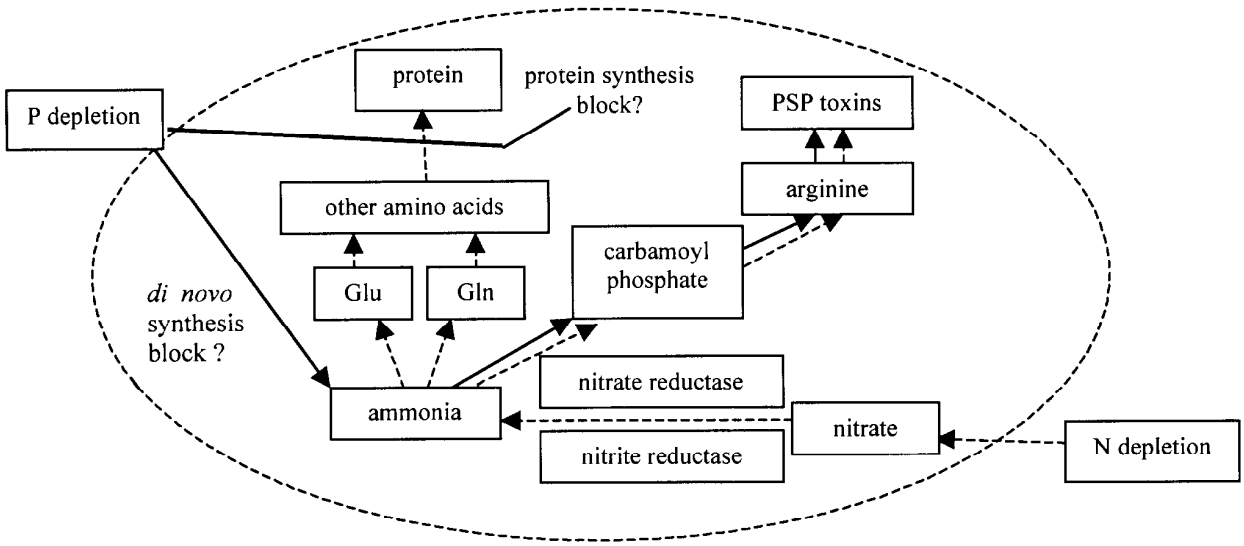

Fig. 5. A model to explain the possible roles of N, P in affecting toxin production
(The dashed arrow showing "normal" pathway of N metabolism)

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ENVIRONMENTAL FACTORS AFFECTING THE NEUROTOXIN PRODUCTION OF *CHATTONELLA ANTIQUA* (RAPHIDOPHYCEAE

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ABSTRACT

Neurotoxin production and ichthyotoxicity of *Chattonella antiqua* (Hada) Ono (Raphidophyceae) were investigated at different temperatures and light intensities under laboratory conditions. Variation in temperature had a pronounced effect on toxin profiles of the flagellate. The yields of CaTx-II (corresponding to PbTx-2) and CaTx-III (corresponding to PbTx-3) peaked at 15°C with 0.39 and 3.18 pg cell^{-1}, respectively. As the temperature increased, the amounts of CaTx-II and CaTx-III decreased gradually, but there was an increase in the amount of CaTx-IV (corresponding to oxidized PbTx-2). The sharp decrease in all the fractions was found at temperatures above 25°C where the organism showed a little growth. Light intensity showed comparatively less influence on toxin profiles of this species than the temperature. The yields of CaTx-I and III at 20 μE m^{-2} sec^{-1} were 0.86 and 1.84 pg cell^{-1}, respectively and decreased slowly with increased light intensity. The concentration of CaTx-II remained nearly constant between 20 and 100 μE m^{-2} sec^{-1} and decreased at high light intensities. Ichthyotoxicity showed the highest toxicity at 15°C and markedly reduced as the temperature exceeded 20°C. Cells cultured at 30°C were about 2 times less toxic than those cultured at 15°C.

INTRODUCTION

The microscopic planktonic algae play an important role in the environment as critical food for shellfish as well as the larvae of commercially important crustaceans and finfishes, but in some situations algal blooms can have a negative effect, causing severe economic losses to aquaculture and fisheries and having major environmental and human health impacts [1]. The problems associated with toxic algal blooms are no longer limited to the dinoflagellates and are becoming increasingly severe on a global scale [2].

The fish farming in different parts of Japan plays an important socio-economic role. With the development of industries along the coasts of seas and bays, Japan, aquaculture of yellowtail, red sea bream, oyster and laver have been suffering from outbreaks of red-tides. The raphidophytes present a serious problem for aquaculture in many parts of the world including Japan, North America, Asia and New Zealand [3]. The marine chloromonad, *Chattonella antiqua* (Hada) Ono, forms heavy red tides in different parts of Japan [4]. Along the coast of Harima Nada in the Seto Inland Sea, approximately 14 million cultured yellowtail were killed by this species in 1972 [5]. *Chattonella marina* (Subrahmanyan) Hara et Chihara, is capable of producing neurotoxic, hemolytic and hemagglutinating compounds [6, 7, 8]. Other species of Raphidophyceae, *C. antiqua*, *Fibrocapsa japonica* Toriumi et Takano and *Heterosigma akashiwo* (Hada) Hada, also produce different neurotoxic components [9-11].

The toxicity of red-tide producing phytoplankton is known to be influenced by some physiological or environmental factors. In cultures of *Gonyaulax catenella* Whedon et Kofoid, toxicity per cell increased when suboptimal temperatures were used for the growth [12]. However, there is a little information on the influence of environmental factors on the growth and toxicity of raphidophycean flagellates. In the previous study, we separated four neurotoxic components from the laboratory cultures of *C. antiqua* and found all the components to be fluctuated with the age and growth stage of the culture [9]. The present study describes the effects of temperature and light intensity on the growth and toxicity of *C. antiqua*.

MATERIALS AND METHODS

C. antiqua cells were isolated from the Yatsushiro Sea, Japan during the massive red-tide outbreaks in 1984 and stock cultures were maintained in tubes containing 10 ml of Provasoli's ES medium [13] at 25°C under light intensities of 60 μE m^{-2} sec^{-1} (12:12 h L:D cycle).

Growth was monitored at 5°C intervals from 10 - 30°C and light intensities at 20, 60, 100, 140 and 180 μE m^{-2} sec^{-1}. Cultures were pre-adapted for one generation and grown without shaking or aeration. All growth studies were done in triplicate using an inoculum of about 400-450 cells ml^{-1} from a culture in logarithmic growth phase. Cultures were examined every alternate day to observe the growth. The average number of cell divisions per day (K) for the 8-day growth period was calculated according to Guillard [14].

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Toxicity tests were conducted on the 8th day using the 3 - 4 month old juvenile red sea bream, *Pagrus major* Temminck et Schlegel (180-300 mg, 22-30 mm). Fishes were added in 300 ml culture medium of *C. antiqua* (temp. 23-24°C, dissolved oxygen 6.0-6.3 ppm) and observed for 24 h with aeration. For control experiments, ES medium was used. Toxicity was calculated from the survival time of the fish and was expressed in fish units (FU) [8, 9]. One FU represents the amount of toxin needed to kill a fish in 30 minutes. For example, one FU of brevetoxin (PbTx) standard here is equivalent to 3-5 $\mu g\ l^{-1}$ of PbTx-1, 10-15 μgl^{-1} of PbTx-2, 20-30 μgl^{-1} of PbTx-3 or 350-400 $\mu g\ l^{-1}$ of oxidized PbTx-2 [8]. Toxicity of the culture is reported as FU per 10^6 cells and calculated using previously described formula [9].

Neurotoxins were extracted from the mid logarithmic phase cultures (8th day) of *C. antiqua* at different temperatures and light intensities by a modification of the method of Baden and Mende [15]. After being partially purified on TLC toxins were applied to a C-18 reverse phase HPLC system and analyzed by comparison with brevetoxin standards. Toxins standards used were PbTx-1, 2, 3, 7, 9 and oxidized PbTx-2, cordially provided by Dr. D.G. Baden, University of Miami, USA.

RESULTS

Growth and toxicity of *C. antiqua* at different temperatures and at constant salinity (30 ppt), light intensity (60 $\mu E\ m^{-2}\ sec^{-1}$) and pH (8.2) are shown in Fig.1. The maximum growth rate was observed at 25°C with a division rate of 0.60 divisions day^{-1} which was significantly higher than at 20°C (0.55 divisions day^{-1}). Ichthyotoxicity of the cultured *C. antiqua* cells were distinctly influenced by the different temperatures maintained during the culture. Toxicity of cells cultured at 15°C was highest (0.21 FU 10^6 $cells^{-1}$), which decreased with increased culture temperature. The cells cultured at 25°C were about 2 times less toxic than those cultured at 15°C. At 30°C, growth was largely inhibited and the toxicity was lowered to only 0.08 FU 10^6 $cells^{-1}$.

Fig. 2 illustrates the growth rate and ichthyotoxicity of *C. antiqua* at different irradiances at a fixed salinity (30 ppt), temperature (25°C) and pH (8.2). The maximum growth rate occurred at 100 $\mu E\ m^{-2}\ sec^{-1}$ but there was not much difference in growth rate between 60-180 $\mu E\ m^{-2}\ sec^{-1}$ (0.603, 0.623, 0.60 and 0.603 divisions day^{-1} at 60, 100, 140 and 180 $\mu E\ m^{-2}sec^{-1}$, respectively). Ichthyotoxicity of the cultured cells was influenced by light intensities, but the influence was not as pronounced as it was in case of different temperatures. Cells were found to be most toxic at 20 $\mu E\ m^{-2}\ sec^{-1}$ with a toxicity of 0.16 FU 10^6 $cells^{-1}$ though the growth rate was greatly depressed at this light intensity. The toxicity decreased slowly with increased light intensity: 0.125 FU 10^6 $cells^{-1}$ at 60 $\mu E\ m^{-2}\ sec^{-1}$, 0.12 at 100 $\mu E\ m^{-2}\ sec^{-1}$, 0.11 at 140 $\mu E\ m^{-2}\ sec^{-1}$ and 0.09 at 180 $\mu E\ m^{-2}\ sec^{-1}$.

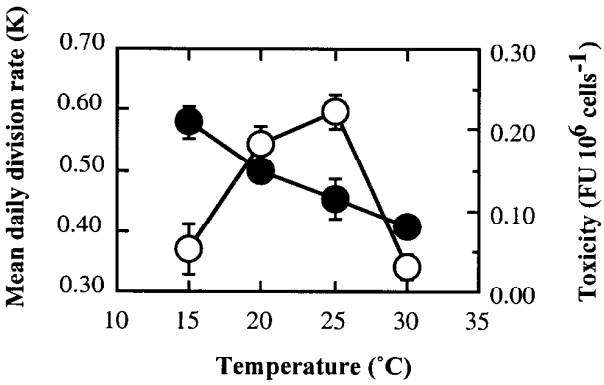

Fig. 1. The effect of temperature on ichthyotoxicity and growth rate of *Chattonella antiqua* (closed circles for toxicity and open circles for mean daily division rate (K).

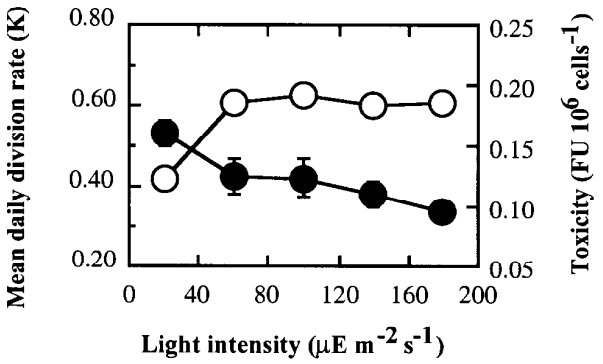

Fig. 2. The effect of light intensity on ichthyotoxicity and growth rate of *Chattonella antiqua* (closed circles for toxicity and open circles for mean daily division rate (K).

Variation in temperature had a major effect on toxin profiles of *C. antiqua*. Toxin levels at different temperatures, expressed on a per cell basis, are shown in Table 1. This species, when cultured at 15°C, produced highest amounts of CaTx-II (corresponding to PbTx-2) (0.39 pg $cell^{-1}$) and CaTx-III (corresponding to PbTx-3) (3.18 pg $cell^{-1}$) with the lowest yield of CaTx-IV (corresponding to oxidized PbTx-2) (1.29 pg $cell^{-1}$). The yield of CaTx-I (corresponding to PbTx-1) was found to be higher (0.94 pg $cell^{-1}$) at 20°C and decreased gradually with increased temperature, down to 0.20 pg $cell^{-1}$ at 30°C. At 25°C the amount of CaTx-III was about 2 times less than at 15°C, whereas that of CaTx-IV (corresponding to oxidized PbTx-2) was at its highest (2.48 pg $cell^{-1}$). The sharp decrease in all the fractions was found at temperatures above 25°C where the organism showed little growth.

Light intensity showed minor effect on the toxin profiles of the cultured cells. At 20 $\mu E\ m^{-2}\ sec^{-1}$ they grew very slowly but yielded highest amounts of CaTx-I (0.86 pg $cell^{-1}$), CaTx-II (0.45 pg $cell^{-1}$) and CaTx-III (1.84 pg $cell^{-1}$) and the lowest of CaTx-IV

(2.0 pg cell^{-1}), as compared to the other light intensities (Table 1). The amounts of CaTx-I and III decreased slowly with increased light intensity. The concentration of CaTx-II changed to a small extent between 20 (0.45 pg cell^{-1}), 60 (0.40 pg cell^{-1}) and 100 μE m^{-2} sec^{-1} (0.44 pg cell^{-1}) and then reduced at high light intensities (0.21 pg cell^{-1} at 140 μE m^{-2} sec^{-1} and 0.28 pg cell^{-1} at 180 μE m^{-2} sec^{-1}). The amount of CaTx-IV fluctuated as the light intensities altered

Table 1. Effects of temperature and light intensity on production of toxins (pg cell^{-1}) in *Chattonella antiqua*

Factors	CaTx-I (PbTx-1)	CaTx-II (PbTx-2)	CaTx-III (PbTx-3)	CaTx-IV (Oxidized PbTx-2)
Temperature (°C)				
15	0.79	0.39	3.18	1.29
20	0.94	0.16	1.93	1.45
25	0.80	0.34	1.69	2.48
30	0.20	0.14	0.92	1.11
Light Intensity (μE m^{-2}sec^{-1})				
20	0.86	0.45	1.84	2.00
60	0.80	0.40	1.69	2.48
100	0.77	0.44	1.74	2.18
140	0.67	0.21	1.34	2.46
180	0.78	0.28	1.16	2.34

*Names of corresponding standard toxins are shown in parentheses.

DISCUSSION

It has been known that different toxic phytoplankton species have different physiological responses. Temperature has an important effect on the growth and toxicity of red tide producing phytoplankton in both culture and nature [12, 16]. In the present study both growth and toxicity of *C. antiqua* were found to be strongly affected by a variation of temperature. This species showed its highest toxicity at 15°C with the lowest growth rate. The toxicity decreased with increased temperatures and the cells cultured at 25°C were about 2 times less toxic than at 15°C. On the basis of thermal effects it appears that slow growing cultures become more toxic than fast growing cultures. The tendency of decreasing toxicity at higher temperatures and vice versa at lower temperatures were observed by Ogata et al. [17], who reported that the toxin of *Alexandrium tamarense* (Lebour) Balech (= *Protogonyaulax tamarensis*) obtained from cells grown at low temperatures was higher than at high temperatures. A similar observation has also been made by Proctor et al. [12] when studying the relationship between temperature and toxicity of *Alexandrium catenella* (Whedon et Kofoid) Balech (= *Protogonyaulax catenella*). They observed that the toxicity of *A. catenella* became higher when the growth was poor at low temperatures. But the temperature effect on the toxicity of *C. antiqua* cells did not conform to our recent studies on another raphidophycean flagellate, *Heterosigma akashiwo* [18]. The toxicity of *H. akashiwo* increased significantly by increasing temperature up to 20°C and then decreased as the temperature went up further.

Light intensity is known to play an important role on the toxin production of the red tide producing phytoplankton [19, 20]. In our present observation the light intensity had a small effect on the ichthyotoxicity of *C. antiqua*. The toxicity decreased with increased light intensity as seen in *A. tamarense* (= *P. tamarensis*) [19] but this was not the case for *A. catenella* (= *P. catenella*) [20]. The highest toxicity of *C. antiqua* at 20 μE m^{-2} sec^{-1} (lowest light intensity among the tested range) does not resemble with that of *H. akashiwo* which showed its highest toxicity at 200 μE m^{-2} sec^{-1} (highest light intensity among the tested range) [18]. The light intensity effect on toxicity of these flagellates differs from species to species.

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INVOLVEMENT OF NADPH OXIDASE-LIKE ENZYME IN THE PRODUCTION OF SUPEROXIDE ANION BY *CHATTONELLA MARINA*

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ABSTRACT

Chattonella marina, a raphidophycean flagellate, is a highly toxic red tide phytoplankton which causes severe damage to fish farming in Japan. Recent studies have demonstrated that *Chattonella* spp. produce reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (·OH) under normal growth conditions. Since harmful effects of ROS have been well documented in various biological systems, ROS may be involved in the toxic mechanism responsible for mortality in fish exposed to *Chattonella*. In the present study, we found that the cell-free system prepared from *C. marina* cells showed NAD(P)H-dependent O_2^- generation, and this response was blocked by diphenyleneiodonium (DPI), a potent inhibitor of mammalian NADPH oxidase. When the cell-free extract of *C. marina* was analyzed by immunoblotting using antibody raised against the human neutrophil cytochrome b558 large subunit (91 kDa), a main band with approximately 100 kDa was detected. Based on these results, we propose that *C. marina* has a enzyme system analogous to neutrophil NADPH oxidase as a source of O_2^- production.

INTRODUCTION

Blooming of toxic phytoplankton species, *Chattonella* spp., have frequently caused massive mortalities of cultured fish, especially yellowtail *Seriola quinqueradiata* in Japan [1]. Although the precise mechanism of the toxic action of *Chattonella* spp. is still unclear, suffocation is generally supposed to be the direct cause of the fish death by this plankton species [2-3]. Recent studies demonstrated that a decrease in oxygen partial pressure of arterial blood is the earliest physiological disturbance observed in fish after exposure to *C. marina* [4, 5]. In addition, several lines of evidence suggested that excessive mucus on the gill surface, which probably induced by *C. marina*, may interfere with O_2 transfer, resulting in asphyxia [6, 7].

It has been shown that *Chattonella* spp. generate reactive oxygen species (ROS) such as O_2^-, H_2O_2, and ·OH radical [8-18]. Since ROS are generally considered to be toxic against living organisms [19, 20], the ROS generated by *Chattonella* spp. may be responsible for gill tissue injury leading to eventual fish death. This hypothesis may be supported by our previous finding that one strain of *C. marina* which produces very low level of O_2^-, was less toxic against yellowtail than the other strain with higher O_2^- producing ability [15]. We also have found that *C. marina* exhibited ROS-mediated toxic effect on a marine bacterium, *Vibrio alginolyticus* [10]. In addition to *Chattonella* spp., it has recently been reported that *Heterosigma akashiwo* also produces ROS, and shows ROS-mediated toxicity on rainbow trout [21]. Thus, it seems likely that the production of ROS is a common feature of raphidophycean flagellates [16, 18]. Based on these findings, it is conceivable that ROS are major causative factors responsible for the toxic action of *Chattonella* and other raphidophycean flagellates.

To gain the insight into the mechanism of ROS generation in *C. marina*, we examined the O_2^- generating activity in the cell-free system prepared from *C. marina* as well as in the intact flagellate cells.

MATERIALS AND METHODS

Chattonella marina isolated in Kagoshima in 1985 was generously provided by Kagoshima Prefectural Fisheries Experimental Station, Japan. Axenic clonal culture of this strain was established by repeated dilution culture and by treatment with antibiotics [10, 17]. The plankton cells were cultured at 26°C in sterilized ESM medium (pH 8.2) under 30 $\mu E \cdot m^{-2} \cdot S^{-1}$ illumination with a cycle of 12 h light and 12 h dark. Exponentially growing cells (1 to 3.5 x 10^4 cells ml^{-1}) were used throughout the experiments. The cell-free extract was prepared by the gentle agitation of flagellate cell suspension for 30 s at room temperature and subsequent centrifugation (3000 x g) for 1 min. The supernatant was concentrated to one-thirty of its original volume by ultrafiltration using the membrane (Amicon Model 8400) which passes molecules with molecular weight of less than 10,000. In chemiluminescence analysis for the detection of superoxide anion (O_2^-) produced by the flagellates or cell-free extracts, we used 2-methyl-6(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*] pyrazin-3-

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one (MCLA) as a superoxide-specific chemiluminescent probe as previously described [22].
For immunoblotting analysis, the cell-free extracts were diluted 1:1 with electrophoresis sample buffer (10 mM Tris-HCl, 20% glycerol, 1% SDS, 1% 2-mercaptoethanol, pH 6.8), and 10 μg protein was loaded on a 10% polyacrylamide gel. After electrophoretic separation and transfer to PVDF membrane (Roche), probing and detection of western blots were performed by chemiluminescence detection method (Tropix) by using antibody against human cytochrome b558 large subunit.

RESULTS AND DISCUSSION

A highly sensitive chemiluminescent assay method for detecting O_2^- has recently been developed by using MCLA as a O_2^--specific chemiluminescent probe [22]. Our previous studies have revealed that the luminescence response is proportional to flagellate cell concentration, and is inhibited by superoxide dismutase (SOD) to a background level. This luminescence response was strongly enhanced by the addition of glucose/mannose-specific lectin, concanavalin A (Con A) [17]. We also have found that other lectins with different carbohydrate specificity showed similar stimulatory effects on *C. marina* [17]. The responsiveness of the flagellate cells to lectins suggests that the flagellate cells have a signal transduction pathway connected between cell surface lectin-binding sites and O_2^- generation system similar to phagocytic cells in which the generation of O_2^- is stimulated by the binding of certain lectins on the cell surface [23]. In addition to the lectins, it has been shown that mucus substances derived from the gill lamellae of yellowtail and other fish species also cause increased O_2^- production by *Chattonella* [9, 24]. These results suggest the possibility that the generation of ROS by the flagellate cells might be amplified in contact with mucus on the surface of gill lamellae, which may lead to further aggravation of gill tissue injury.
In various biological systems, the generation of ROS is most likely to be controlled by oxidoreductases utilizing NAD(P)H as a source of reducing equivalents for the reduction of O_2 to O_2^-. NADPH oxidase located in the plasma membrane of leukocytes is a well-known enzyme complex which catalyzes the single-electron reduction of O_2 to O_2^- at the expense of NADPH [25]. In higher plant cells, it has been reported that there are some NAD(P)H oxidase activities capable of generating O_2^- in the plasma membranes, and ROS generation in plant cells exhibits many characteristics reminiscent of the so-called oxidative burst in mammalian phagocytic cells [26]. Our recent studies demonstrated that the chemiluminescence response of *C. marina* was inhibited

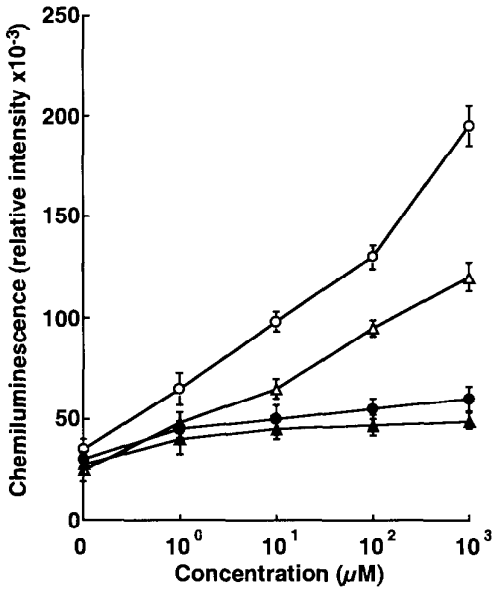

Fig. 1. Effects of various concentrations of NADPH (○), NADH (△), NADP+ (●), and NAD+ (▲) on MCLA-mediated chemiluminescence responses in the cell-free extract of *C. marina*. After simultaneous addition of MCLA and indicated concentrations of each compound to the cell-free extracts, chemiluminescence responses were measured at 27°C. Each point represents relative intensity of integrated emission during the first 30 s.

by the treatment with membrane impermeable protease, suggesting that a certain redox enzyme system located on the cell surface may be involved in the O_2^- generation [17]. This notion is supported by the finding that O_2^- was mainly detected on the cell surface of *C. antiqua* [27]. To gain clues to the underlying mechanism of O_2^- generation in *C. marina* cells, we prepared cell-free system from *C. marina* cells, and examined its O_2^- generating activity. Interestingly, we found that chemiluminescence response of cell-free extract was increased by the addition of NADPH in a dose-dependent manner, whereas NADH was less effective than NADPH, and NAD^+ and $NADP^+$ had no effect (Fig. 1). Furthermore, NAD(P)H-dependent chemiluminescence in the cell-free extract was blocked by diphenyleneiodonium (DPI), a specific inhibitor of mammalian NADPH oxidase [28]. On the other hand, allopurinol and KCN, which are inhibitors for other O_2^--generating enzymes [29], had no significant effect (Table 1). These results suggest that *C. marina* may have an enzyme system closely related to the neutrophil NADPH oxidase. The neutrophil NADPH oxidase consists of two plasma membrane proteins, gp91-phox

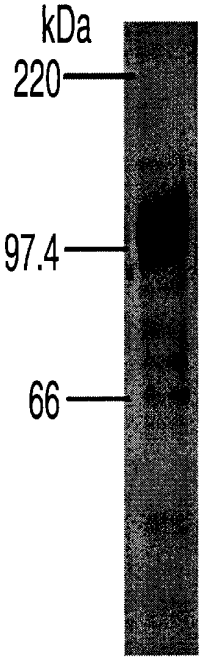

Fig. 2. Immunoblotting of *C. marina* cell extract with antibody to human neutrophil cytochrome b558 large subunit (gp91-phox). *C. marina* cell extract was electrophoretically separated in a 10% polyacrylamide-SDS gel, transferred to PVDF membrane, and immunostained with antibody to gp91-phox and subsequent peroxidase-conjugated secondary antibody.

and p22-phox, which together form heterodimeric flavocytochrome b558 and three cytosolic regulatory proteins [25]. To further investigate the similarity between the human neutrophil NADPH oxidase and the O_2^- generating system in *C. marina* cell, immunoblotting analysis of the cell-free extract of *C. marina* was carried out using antibody raised against the human neutrophil cytochrome b558 large subunit (gp91-phox). As shown in Fig. 2, the main band was detected in the 90-110 kDa molecular mass range that was similar in size to the human cytochrome b558 (91 kDa). These results suggest that immunologically related protein of the similar molecular weight as human cytochrome b558 is present in *C. marina*.

Table 1. Effects of various inhibitors on the MCLA-mediated chemiluminescence response in the cell-free system prepared from *C. marina* cells.
Chemiluminescence responses in the cell-free extract were measured in the presence of the indicated concentration of each compound. The results were expressed as percentage of relative intensity of integrated emission during the first 30 s in control cell-free extract.

Compound added	Chemiluminescence responce (% of control)
Control (None)	100.0 ± 2.0
+ DPI (100 mM)	40.0 ± 5.0
+ Allopurinol (100 mM)	102.0 ± 3.0
+ KCN (100 mM)	110.0 ± 4.0

In conclusion, our results provide evidence for the possible involvement of NAD(P)H oxidase, analogous to the neutrophil NADPH oxidase, in O_2^- generation in *C. marina*.

ACKNOWLEDGEMENTS

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EFFECT OF IRRADIANCE ON SUPEROXIDE PRODUCTION BY *CHATTONELLA MARINA* (RAPHIDOPHYCEAE) FROM SOUTH AUSTRALIA AND JAPAN

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ABSTRACT

Previous autecological studies demonstrated that Japanese and Australian strains of the raphidophyte *Chattonella marina* had similar tolerances for temperature and salinity, but substantial differences in irradiance requirements [1]. In the present work, differeing superoxide production and toxic effects on the zooplankton *Artemia salina*, and fish *Onchorynchus mykiss* are documented between geographic strains and light treatments. The fish killing mechanism of *C. marina* is suggested to be a synergistic effect between reactive oxygen species (ROS) and neurotoxins, and the results cannot be explained on the basis of these mechanisms of toxicity on their own.

INTRODUCTION

Fish mortalities caused by raphidophycean flagellates are a serious problem for aquaculture in the United States, Canada, New Zealand and Japan [2]. The first Australian report of a *Chattonella* associated fish mortality was in April 1996 in Boston Bay, South Australia [3] when approximately 1,700 tonnes of caged southern bluefin tuna (*Thunnus maccoyii*) worth $A45 M died coincident with a *Chattonella marina* bloom of 6.6 x 10^5 cells per litre. Blooms of *Heterosigma* and *Chattonella* have remained a problem to fin-fish aquaculture worldwide despite being widely researched in Japan for the last 2 decades [2], however, the killing mechanism of *Chattonella* blooms remains unclear. Initial theories for toxicity centered around anoxia, mucus production, respiratory, ionoregulatory and cardiovascular physiology of fish exposed to *Chattonella* blooms [4,5,6]. Histopathology showed severe changes in fish gill pathology when exposed to *Chattonella* [7]. Studies have also centered on the production of a toxin similar to breve toxins produced by *Gymnodinium breve* [8,9]. More recent studies have shown that some raphidophytes produce reactive oxygen species (ROS) such as superoxide ($\cdot O2^-$), hydrogen peroxide (H_2O_2) and the hydroxyl radical $\cdot OH$ [10,11] implicated in fish mortality.

In this study we investigate the role that irradiance may have on superoxide production by *C. marina*, and determine the differences between the Australian and Japanese strains in ROS toxicity to marine organisms. The relationship between ROS and neurotoxin production in fish mortalities is also discussed.

METHODS

Algal Culturing

Stock cultures of *Chattonella marina* from Japan (NEIS-118) and Australia (CMPL01) were isolated and maintained as per Marshall & Hallegraeff [1]. Treatment cultures were grown as follow; zooplankton bioassays - 125 ml conical flasks under cool white fluorescent tubes (150 µmol m-2s-1) or metal halide lamps (500 µmol m-2s-1); fish bioassay - aerated 3L glass or 20 L polycarbonate vessels under light conditions as above. Unless stated, experiments were conducted using early to mid exponential phase cultures.

Animal Bioassays

Four day old *Artemia salina* meta-nauplii were introduced to 3 ml of *C. marina* culture. Treatments of high and low irradiance as described above, and controls of GSe media and culture with 500 units/ml catalase (Cat) and 50 units/ml superoxide dismutase (SOD) were in triplicate. Observations of toxicity were assessed as death, response, and no response. Response was defined as paralysis or reduced respiratory movements. Rainbow trout smolts (*Oncorhynchus mykiss*: 18-61 grams) were conditioned to 25 psu salinity over a period of 7 d. Fish were exposed to Australian and Japanese *C. marina*, at cell densities of 5-7 x 10^6 cells L^{-1}, under high (> 200 µmol $m^{-2}s^{-1}$), low (< 50 µmol $m^{-2}s^{-1}$) irradiances, and darkness, with 1 hr preconditioning treatments (ethics approval 98081 - University of Tasmania). Australian *Chattonella* cell free extract was prepared through gravity filtration. Observations were taken until morbidity or death occurred. Surviving fish were sacrificed at 6 hrs. Gills were removed and processed using routine methods, stained with H&E and Alcian blue-periodic acid Schiff (PAS). Gill tissue was analysed by light microscopy to determine extent of lesions, epithelial separation and mucus cell disruption

Chemiluminescence Analysis

The oxygen radical superoxide (O2-) was measured using the luciferin analogue 2-Methyl-6-(*p*-methoxyphenyl)-3,7-dihydroimidazo[1,2-*a*]pyrazin-3-one (MCLA) as described by Lee et al. [12] at a concentration of 5 x 10-6M and standardised against 5 x 10-6 M superoxide dismutase (SOD) . Each measurement was made in triplicate.

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Intergovernmental Oceanographic Commission of UNESCO 2001

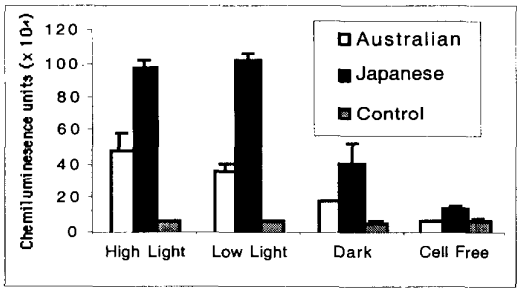

Fig. 1. Superoxide production of *C. marin* in response to different irradiances

RESULTS

ROS Production

The Japanese strain produced a higher level of superoxide under both high and low irradiances compared to the Australian strain, but with no significant difference between high and low irradiance treatments. There was a significant decrease between the high and low light exposures and the dark treatments in both the Japanese and Australian strains (Fig. 1). The Australian strain however exibits a significant decrease in superoxide production between the low irradiance treatment and the dark treatment, with the dark treatment insignificant from the control. The cell free extracts of *C. marina* showed superoxide production of 6-8 times less than whole cell samples, relative to each treatment and strain. Cell free production of superoxide was significantly higher ($p>0.05$) than the controls.

Artemia Bioassay

Death did not occur in the *Artemia* exposed to *C. marina* within the experimental time frame. Meta-nauplii of *Artemia* showed a maximum toxic response (paralysis, clumping or mucus) to *C. marina* at culture age 8 to 27 d (exponential phase). No toxic response was noted for an aged (90 d stationary stage) culture or in controls (data not shown).

Response of *Artemia* after 6 hrs exposure to high or low light cultures was not significantly different between either Australian and Japanese strains (Fig. 2), and no decrease in toxic response was noted with the addition of the enzymes SOD and catalase (data not shown). However *Artemia* showed a reduced response to the Japanese strain for the dark treatments and cell free treatments when compared to the Australian strain

Fish Bioassays

Initial excitability, loss of balance, rapid opercular movements and a decrease in activity were symptoms exhibited within 15 minutes exposure to *C. marina* (Table 1). There was no significant difference in fish mortality between high and low irradiance treatments, with most mortalities occurring within 2 hrs. The rate of opercular movements was highest in fish exposed to Japanese strains under high irradiance, and greater for both Australian and Japanese strains with increased irradiance (Table 2). Dark treatment enhanced fish survival, with only 1 mortality out of 4 occurring. There was no mortality in the cell free extract. Fish mucus was noted in all treatments except dark treatment, cell free extract and controls. A twisting of the posterior spine, thought to be due a neurotoxic effect, was noted in all treatments including the cell free extracts, but absent in the controls.

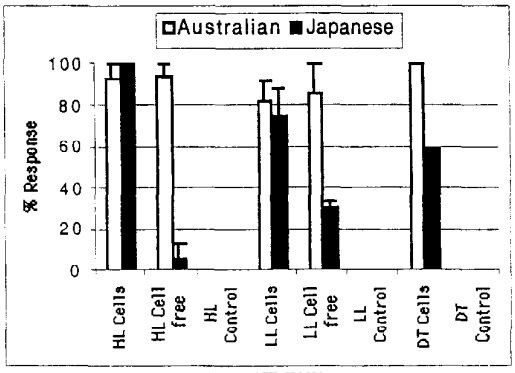

Fig 2: Toxic response of *Artemia salina* when exposed to *Chattonella marina.*

Histopathology revealed no significant damage in gill lamellae consistent with ROS. Although some effect was noted between the treatments and controls, there were no quantifiable histological changes.

DISCUSSION

Relationship between light intensity and ROS toxicity

Although the zooplankton *Artemia* showed no acute toxicity from *Chattonella* cells, paralysis of *Artemia* occurring in treatments with and without the enzymes SOD and Catalase indicate that 1) *Artemia* are not susceptible to ROS toxicity and 2) there is another toxic principle involved (most likely a neurotoxin) influencing *Artemia* behaviour, but not causing death. Medlyn [13] tested adult *Artemia* against 4 strains of *Gymnodinium breve* (Davis) extract which produces three toxic components, PbTx-2, PbTx-3 and PbTx9, lethal to *Artemia* after 72 hours exposure. These three toxic components are similar to those produced by *Chattonella marina* [9]. The raphidophyte *Heterosigma akashiwo* has also been found to have lethal toxicity to *Artemia* from both cultures and cell filtrates [14], and is known to produce relatively low levels of superoxide and hydrogen peroxide compared to *C. marina* [15]. The effect of the *C. marina* culture on *Artemia* is more likely to be of neurotoxic origin than from ROS'.

Artemia displayed the same paralysis response in both Australian whole cell and cell free extract, indicating that the toxicity component is present outside the cells. The significantly lower response in *Artemia* from the Japanese cell free and dark treatments suggests that the Japanese strain produces much lower amounts of the neurotoxins.

Table 1; Response of rainbow trout (*Onchorynchus mykiss*) when exposed to the ichthyotoxic flagellate *Chattonella marina*..

Treatment		n	Time until fish responses (mins)				
			Opercular movements (beats/minute)	Loss of balance	Production of mucus +low +++high	Involuntary flexion	Death
High Light	Control	4	0	0	0	0	0
	Aust	4	15	15	15 +++	15	95 ± 9
	Jap	4	45	30	15 ++	0	127 ± 45
Low Light	Control	4	0	0	0	0	0
	Aust	4	15	15	15 +++	0	75 ± 30
	Jap	4	60	0	15 +++	15	105 ± 64
Dark	Aust	4	ND	ND	1 @15 +	15	1 @ 140
	Jap	4	ND	ND	0	0	0
Cell Free	Aust	4	0	0	0	15	0

Table 2: Opercular movements per minute in *Onchorynchus mykiss* exposed to *Chattonella marina* under high and low irradiances (n=8).

Australian.	Japanese.	Control
High Irradiance (> 200 µmol $m^{-2}s^{-1}$)		
132±11.3	152±0	no data
Low irradiance (< 50 µmol $m^{-2}s^{-1}$)		
116	112	110±14.1

There was a moderate toxic effect of both Japanese and Australian *C. marina* upon rainbow trout. The fish exhibited symptoms consistent with the production of ROS such as mucus production, but not at the intensity as described by [16], and limited gill pathology indicates that the levels of ROS production were not lethal. However, the presence of superoxide was confirmed through the chemiluminesence assay. Opercular movements did increase under both high and low light intensity, indicating that the fish were in respiratory distress. This observation was also reported for bream (*Pargus major*) exposed to *C. marina* [6]. The symptoms displayed by fish exposed to cell filtrate compare with those described by Ahmed and Onoue [17], were fish where exposed to a crude toxic extractrom *C. marina*.

CONCLUSION

A synergistic effect of neurotoxins and reactive oxygen species toxicity by *C. marina* is suggested by the variation in toxic impact on different organisms under changing irradiances. There is also evidence that different strains (ecophenotypes) may simultaneously produce different toxic effects. Relationships between growth phase, temperature light iron, and both ROS and neurotoxin production have previously been reported [8,9,18]. The development of a bloom prediction model may need to incorporate not only traditional physical factors such as stratification, water temperature, and eutrophication, but also irradiance, as demonstrated in Fig. 3.

In monitoring fish mortality events, involvement of both ROS and "breve-like" toxins needs to be studied concurrently rather than in isloation.

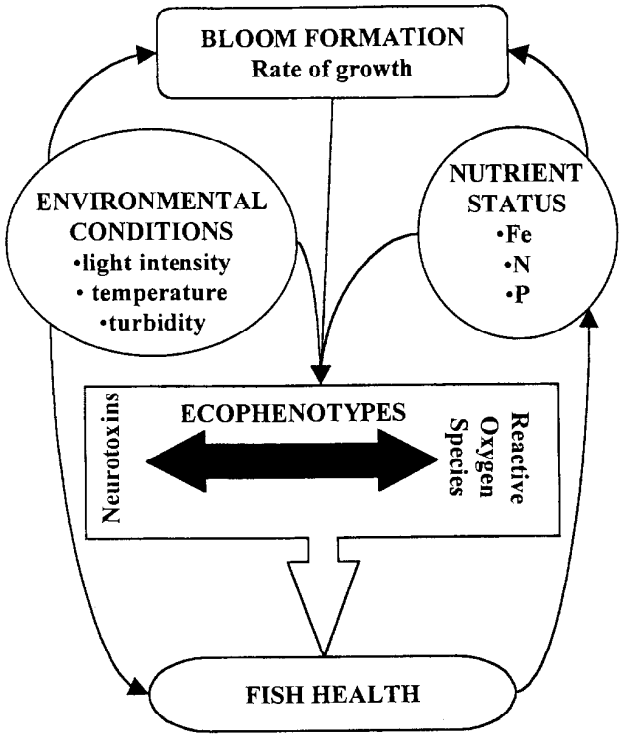

Fig. 3 Flow diagram demonstrating possible exogenous effects upon *C. marina* toxicity

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THE EFFECTS OF IRON ON DOMOIC ACID PRODUCTION BY *PSEUDO-NITZSCHIA MULTISERIES*

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ABSTRACT

Domoic acid (DA), the responsible agent for Amnesic Shellfish Poisoning, contains three carboxylic acid residues that could potentially bind trace metals such as iron. To investigate if DA production was affected by the iron status of the cell, replicate cultures of the diatom *Pseudo-nitzschia multiseries* were grown under trace-metal clean conditions containing 0, 0.12 µM and 11.7 µM added iron. All three iron treatments showed similar initial growth rates and resulted in stationary phase densities of $>10^5$ cells mL^{-1} after 10 days in culture. In contrast, DCMU-enhanced fluorescence indicated that the cultures without added iron were iron-limited by day 18, as evidenced by an Fv/Fm ratio of 0.2 as compared to the iron-replete cultures (11.7 µM Fe) with a ratio of 0.5 throughout the culture period. Cultures without added iron showed a marked drop in the cellular chlorophyll *a* content in stationary phase and never produced more than 5 pg DA $cell^{-1}$ (<1000 ng mL^{-1}). In contrast, iron-replete cells contained 5-10 times more chlorophyll *a* per cell, and DA production increased steadily throughout stationary phase to nearly 50 pg $cell^{-1}$ (5500 ng mL^{-1}). The intermediate level of added iron (0.12 µM) showed an intermediate response. These results indicate that the lack of available iron strongly inhibits the ability of *P. multiseries* to produce DA. The decrease in DA production, chlorophyll *a* synthesis, and photosynthetic efficiency suggest that DA is not produced as a chelator to increase the availability of iron to the cells. The cause of this decrease is unknown, but the biosynthesis of DA requires both nitrogen and energy, two resources that are likely to be limiting under iron-deficient conditions.

INTRODUCTION

The production of domoic acid (DA) by the pennate diatom *Pseudo-nitzschia multiseries* was first identified in 1987, when the consumption of toxic mussels (*Mytilus edulis*) in Atlantic Canada resulted in human death and illness [3]. Domoic acid is now known to be produced by a number of *Pseudo-nitzschia* species, including *P. multiseries, P. pseudodelicatissima, P. seriata, P. australis, P. pungens,* and *P. fradulenta* [3]. It has been implicated in the death of pelicans and cormorants in Monterey Bay, CA [4], and more recently in the death of sea lions from the same region [19].

The role of environmental factors in influencing DA production has been extensively studied [1]. Early studies with *P. multiseries* showed that the production of DA in exponential phase was extremely low. These levels increased by more than 100-fold as the culture entered stationary phase, depending on the strain. Similar results were observed for *P. seriata* [11]. In contrast, DA production by *P. australis* began in mid-exponential phase and remained constant or decreased as the cultures entered stationary phase [5]. Silicon is often a limiting nutrient for diatoms and DA production is inversely correlated with the amount of added silicon in both batch [15] and continuous culture [1]. Phosphorus-limited cultures also showed enhanced DA production, in both batch and continuous cultures [1, 14], suggesting that both nutrients may act by decreasing the growth rate relative to the potential maximum growth rate of the organism, rather than directly stimulating DA production [15]. Fewer studies have been done on the effects of nitrogen limitation, though it generally decreases the level of DA production [2].

Domoic acid is an analog of kainic acid and the presence of three carboxyl groups in its chemical structure immediately suggests that it should chelate or bind metal ions (Fig. 1). Numerous iron-binding compounds (siderophores) have been recently identified from marine sources. They play an important role in controlling the availability of iron [10, 20], and recent evidence suggests that coastal environments may be limited for this essential nutrient [9]. Here, we report on the effects of iron on growth and DA production by *Pseudo-nitzschia multiseries* in culture.

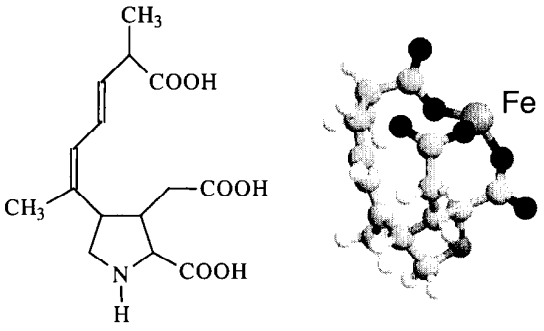

Fig. 1. A stick figure of domoic acid and the space-filling structure of the hypothetical DA-Fe complex. The DA-Fe structure was generated using MM2 molecular mechanics to draw the structure with the low energy minimum. The oxygen atoms of the carboxyl groups are indicated by the dark balls.

MATERIALS AND METHODS

Replicate cultures of *Pseudo-nitzschia multiseries* clone CLN-1 (isolated in January 1997, from a sexually reproducing mixture of clones KP-104 and KP-105 originally isolated from Cardigan Bay, Prince Edward Island, Canada) were grown in artificial seawater supplemented with f/2 nutrients [8]. Cells were grown at 20°C using a 10:14 h light:dark cycle at ~100 µmol photons m^{-2} s^{-1}. To remove contaminant iron, the salts and macronutrients used to prepare the artificial seawater were first passed through a Chelex 100 column [17] and 500 mL in 1000 mL acid-washed polycarbonate Nalgene flasks were sterilized in a "trace-metal-clean" steam autoclave. Iron-EDTA, at 0 (no added iron), 0.12 and 11.7 µM, was added by sterile filtration. An initial culture was started by inoculating cells into 500 mL of f/2 medium containing 0.12 µM Fe. That culture was grown for 6 days (exponential phase) in order to lower the amount of available iron prior to being used to inoculate (1%) duplicate 1000 mL flasks at the three concentrations of iron. Direct measurement by graphite furnace atomic absorption spectrometry estimated the contaminant iron, plus iron carried over in the inoculum, to be between 10-20 nM for the "no-added iron" flask, and insignificant for the higher iron additions.

Growth was determined by visual cell counts (triplicate). Chlorophyll *a* (duplicate) was measured fluorometrically after extraction with acetone [18]. *In vivo* fluorescence was measured using a Turner Designs 10 fluorometer before and after the addition of 10 µM DCMU (dichlorophenyl dimethylurea) to inhibit electron transport [7]. DCMU-enhanced fluorescence was calculated as $(F_{DCMU}-F_0) / F_{DCMU}$. Domoic acid was measured in the cells plus culture medium without filtration (total DA), after sonication, by HPLC after derivatization with FMOC [2, 16].

RESULTS

Iron and the growth of Pseudo-nitzschia multiseries

The effect of different levels of added iron on the growth of *Pseudo-nitzschia multiseries* in culture is shown in Fig. 2a. All three iron treatments resulted in similar exponential growth rates (~0.8 div d^{-1}). Only slight differences were observed in the yield (~10^5 cells mL^{-1}) among the treatments. In contrast, the chlorophyll *a* content per cell was markedly different among the three cultures. Stationary phase iron-replete cells (11.7 µM Fe) had between 2-5 times more chlorophyll *a* per cell than cultures grown without added iron (Fig. 2b). Similar results have been observed for other iron-limited diatoms [7]. Differences among the iron treatments were also observed in the DCMU-enhanced fluorescence. Cells grown with 11.7 µM added iron had a response near 0.5-0.6, typical of iron-replete cells [7]. In contrast, the DCMU-enhanced fluorescence of cultures without added iron dropped from 0.6 during exponential growth to below 0.2 in stationary phase. A similar decrease to a ratio of 0.2 has been reported for iron-limited cultures of the pelagophyte *Aureococcus anophagefferens*, the chlorophyte *Dunaliella tertiolecta* and the diatom *Phaeodactylum tricornutum* [7, 12]. Combined, these results suggest that cultures grown in the absence of added iron, but in the presence of the 10-20 nM contaminant iron, are stressed for iron, but not necessarily iron-limited in their growth. Cultures grown in the presence of normal f/2 iron (11.7 µM) did not show signs of iron stress. Intermediate levels of iron (0.12 µM) resulted in a condition where the chlorophyll *a* per cell and the DCMU-enhanced fluorescence cycled between the two values, at times resembling iron-replete cells and at times resembling cells without added iron; this cycling has not been observed in subsequent experiments.

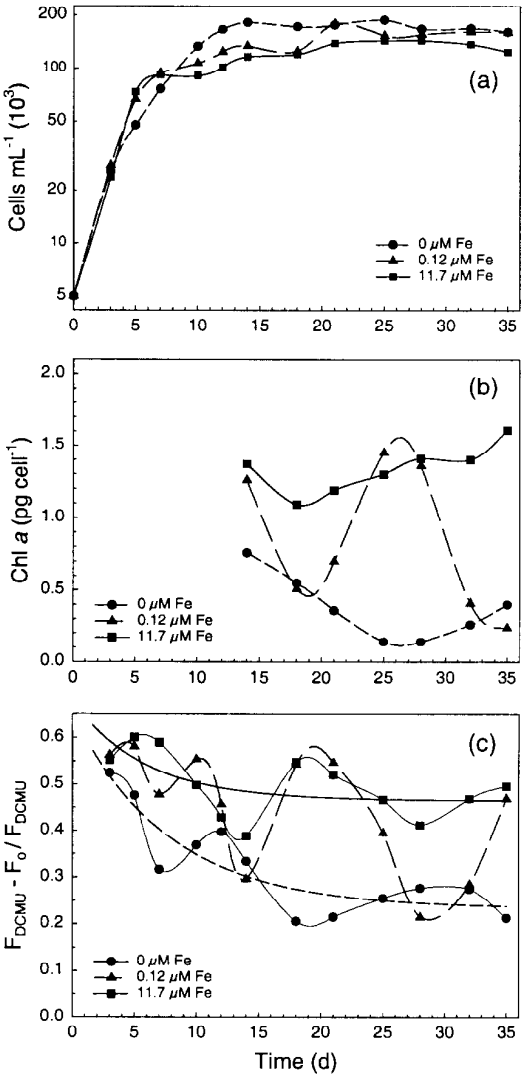

Fig. 2. The effect of three concentrations of iron on (a) growth, given in cells mL^{-1}; (b) mean cellular chlorophyll *a*, in pg chlorophyll *a* $cell^{-1}$, and (c) DCMU-enhanced fluorescence of *Pseudo-nitzschia multiseries* in culture.

The effects of iron on domoic acid production

The effects of iron on DA production are shown in Fig. 3a and 3b. For iron-replete cultures (11.7 µM Fe), the DA levels increased during the stationary phase to 50 pg cell^{-1} (5500 ng mL^{-1}). In contrast, iron-stressed cultures grown with no added iron showed very little increase in the DA level upon entering stationary phase, producing about 10 times less DA than the iron-replete cultures (5 pg DA cell^{-1}). Again, intermediate levels of iron (0.12 µM) resulted in an intermediate response.

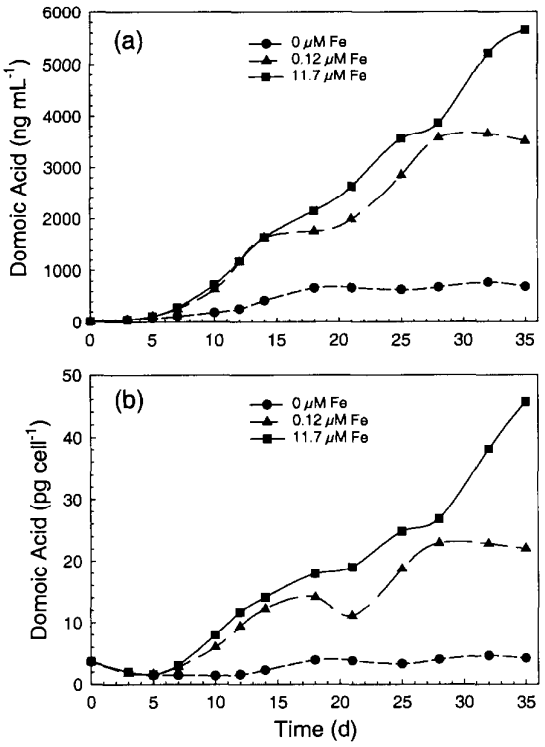

Fig. 3. The mean concentration of domoic acid in *Pseudo-nitzschia multiseries* expressed (a) per mL culture and (b) per cell for three concentrations of iron.

DISCUSSION

In contrast to silica or phosphorus limitation, DA levels markedly dropped in iron-stressed cultures relative to their iron-replete controls. These results are similar to what has been reported for nitrogen limitation, where nitrogen-depleted stationary-phase batch cultures of *P. multiseries* failed to show enhanced DA production until replenished with nitrate [1, 2]. There are several possible explanations why DA levels might be inversely correlated with iron. First, DA is an amino acid and nitrogen is an essential element for its synthesis [1]. Thus, any factor that decreases nitrogen uptake and/or assimilation may also affect DA biosynthesis. Iron is a key component of the enzyme nitrate reductase, a limiting step in the acquisition of nitrogen by these cultures, and in glutamine synthetase, a key enzyme for the conversion of ammonia into amino acids [6]. A decrease in available iron may have decreased the nitrogen incorporation into the cells. This would also account for the observed decrease in the chlorophyll *a* per cell. A second possibility is based on carbon flux. The precursors for the biosynthesis of DA come from two sources: acetyl CoA via geranyl pyrophosphate and from the citric acid cycle via oxoglutarate [1]. A decrease in the activity of aconitase in the TCA cycle, another iron-requiring enzyme, could limit the cell for oxoglutarate, resulting in both a decreased incorporation of nitrogen and a decrease in an essential precursor for DA biosynthesis.

A more intriguing possibility was put forth by Pan et al. [13], who argued that competition for cellular energy regulated DA biosynthesis. During exponential growth, there is a high demand for metabolic energy for primary metabolism. Consequently, the energy available for DA biosynthesis is low. As the culture enters stationary phase and growth slows due to silicon or phosphorus limitation, photosynthesis continues to produce ATP and reductant, which become increasingly available for DA biosynthesis. However, those cultures entering stationary phase because of iron stress appear to have very inefficient photosynthetic machinery as evidenced by the marked decrease in DCMU-enhanced fluorescence. They also have lower cellular chlorophyll *a* levels and are thus less capable of capturing photosynthetically active radiation. These stationary-phase cultures are therefore likely to be energy limited and without excess reductant and biosynthetic precursors to devote to DA biosynthesis. Similar results were observed in light-limited cultures, where a photon flux density of ~100 µmol photons m^{-2} s^{-1} was necessary to sustain DA biosynthesis in stationary- phase cultures [2].

The decrease in DA production with increasing iron stress argues against a role for DA in iron acquisition. Iron uptake mechanisms, using strategies of either iron reduction or chelation, are induced under low iron conditions. Our results show that photosynthetic efficiency, chlorophyll *a* synthesis, and DA production were still impaired during stationary phase at the lowest concentrations of iron. Thus while DA binds iron, as does citrate, glucuronate and other acidic cellular components, it seems unlikely that its function is to obtain iron for the metabolic requirements of *P. multiseries*. It may nevertheless serve as an iron storage molecule similar to ferritin. Its production during stationary phase would also cause cellular iron to become soluble during cell lysis and aid in its recycling into new biomass. Further studies on the bloom dynamics of *Pseudo-nitzschia multiseries* as well as the *in situ* chelation of iron by DA and other organic compounds may aid in understanding this problem.

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AMINO ACID PROFILES IN SPECIES AND STRAINS OF *PSEUDO-NITZSCHIA* FROM MONTEREY BAY CALIFORNIA: INSIGHTS INTO THE METABOLIC ROLE(S) OF DOMOIC ACID

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ABSTRACT

The phenomenology of domoic acid (DA) production by strains in the *Pseudo-nitzschia* species complex has received considerable attention, leading to general observations that DA accumulation is stimulated by growth limiting or stress conditions. Although DA is a structural analogue of proline, no direct evidence is available linking DA and proline metabolism in *Pseudo-nitzschia.* Analysis of proline metabolic pathways does, however, suggest a potential link between proline catabolism and DA biosynthesis. In order to assess the impact of DA production on amino acid metabolism, a method based on pre-column derivatization of amines with phenyl-isothiocyanate (PITC) was optimized for HPLC-UV detection of phenylthiocarbamyl (PTC) derivatives of DA and other free amino acids (FAA) in marine phytoplankton. HPLC profiles of PTC-FAA in 5 species and 20 strains of *Pseudo-nitzschia* spp. from Monterey Bay revealed unique features when compared to other phytoplankton species. DA accumulation varied by 2-orders of magnitude among independent isolates of *P. multiseries* and *P. australis*, with isolates of the latter species exhibiting consistently higher cellular yields of DA. Proline content was lower in cells accumulating high levels of DA (>1 fmole cell) consistent with the hypothesized function of proline as an upstream precursor of DA. All *Pseudo-nitzschia* species accumulated large pools of taurine (≥50% of total FAAs), a FAA not detected in other diatom species when grown in Monterey Bay seawater (34 ppt). As taurine content covaried with DA accumulation in *Pseudo-nitzschia*, it may provide a useful biomarker for potentially toxic bloom events.

INTRODUCTION

The phycotoxin DA has received international attention ever since its initial identification as the causitive agent of amnesic shellfish poisoning (ASP) following human intoxications on Prince Edward Island in 1987 [1,2]. Diatoms in the genus *Pseudo-nitzschia* were soon identified as the primary source of DA [3]. Vertebrate intoxication requires bioaccumulation and vectoring of this water soluble amino acid via the local food chain [4]. Members of the *Pseudo-nitzschia* species complex are widely distributed in the coastal waters of North America; several species are known to produce DA in sufficient quantities during blooms to cause wildlife deaths and force closure of shellfisheries in these regions [e.g. 5,6,7,8,9]. These events have prompted the development and routine implementation of methods for sensitive and accurate quantification of DA in water and tissue samples [10,11] as well as molecular probes for rapid identification and enumeration of potentially toxic species in local waters [12,13]. While these efforts have improved monitoring capabilities for this phycotoxin, our understanding of the biochemistry and physiological role of DA in marine algae has lagged far behind. Biochemical investigations of DA metabolism are of equal importance to monitoring efforts as they can provide a description of the physiological function of this amino acid and thereby lead to better predictions of the environmental conditions stimulating toxic blooms by *Pseudo-nitzschia* spp. Additionally, descriptions of the biosynthetic pathways leading to DA may provide insights for development of therapeutic strategies for mitigation of domoic acid poisoning.

The present report summarizes research efforts guided by the hypothesis that domoic acid metabolism is intimately associated with proline metabolism in *Pseudo-nitzschia* spp. by (1) substituting for the normal homeostatic role of free proline and/or (2) resulting from downstream catabolism of derivatives of this protein amino acid. Several lines of evidence suggest an interaction between DA and proline metabolism. While domoic acid and the related compound kainic acid act as glutamate receptor agonists, they also exhibit structural similarity to the proline family of amino acids being substituted at the 3 and 4 positions of the prolyl ring with acetate- and isoprenoid-like moeities, respectively. The terminal reaction scheme for DA proposed by Douglas and colleagues [14] based on *in vivo* isotopic labeling patterns of DA also suggests a direct coupling to proline metabolism (Fig. 1, *top*). A 3-hydroxy-glutamate like moiety is hypothesized to provide the amine constituent of DA [14], this compound is most-likely derived from oxidative degradation of 3-hydroxyproline (3-HYP, Fig. 1, *bottom*) a common cell wall constituent in diatoms [15]. Biochemical coupling of DA biosynthesis with proline catabolism is depicted by our proposed model (Fig. 1, *bottom*). This metabolic scheme incorporates the reported environmental stimuli associated with DA accumulation [16] and identifies several enzymatic targets which are the subject of on going research. Previous reports on the phenomenology of DA production support a functional similarity of DA and proline in that both are amino acids that accumulate in phytoplankton under a variety of growth-limiting environmental stresses [e.g. 16,17,18].

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The current report examines the interrelationships of amino acid pools involved in DA and proline metabolism through comparisons of variation in FAA composition within and between batch cultures of toxic and non-toxic species of phytoplankton. If DA is derived from or substitutes for the physiological function of proline, then the pool sizes of these two amino acids should be negatively correlated.

METHODS

Algal Cultures: Clonal isolates of various *Pseudo-nitzschia* species were derived from picks of net samples obtained at various locations in Monterey Bay. Species identities were established by SEM analysis and further supported by DNA probe hybridization technologies [13]. Cultures of other phytoplankton species were kindly provided by the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (CCMP). Stock cultures were maintained in 0.2 Ìm filtered, autoclaved Monterey Bay seawater enriched with f / 2 levels of nutrients [19] under a 14L:10D light cycle at 15 °C. Physiological differences among strains and species were examined using a "common garden" culture technique. Stock cultures were innoculated into aliquots of a common media stock and the subcultures were grown concurrently in the same incubator. Unless otherwise indicated, data presented are derived from cultures sampled at the end of log phase, when intracellular DA contents generally peaked.

Amino Acid Analysis: Cell samples were collected by centrifugation and media removed by vacuum aspiration. Cell pellets were either immediately extracted with 80% methanol in HPLC grade water or frozen in liquid nitrogen and stored at -75 °C. No difference in extraction efficiency for intracellular amino acids was observed between these two methods. The cells in methanol were heated at 50 °C for 30 min leaving de-pigmented cell pellets indicative of complete permeabilization of the cells.

Although the FMOC-Cl labeling method provides high sensitivity detection of DA in environmental samples [10], presence of reaction by-products precluded its use for routine analysis of proline and it derivatives without substantial sample clean-up. Therefore total amino acids were derivatized with PITC which efficiently labels both primary and secondary amines [20].

Methanolic extracts were rendered cell free by centrifugation. Subsamples of the supernatant were evaporated to dryness, resuspended in 10 ÌL 50% ethanol and labeled with 20 ÌL of a mixture of 90% ethanol : triethanolamine : PITC (7:2:1, by volume) for 30 min at room temperature. Phenylthiocarbamyl amino acids (PTC-AA) were evaporated to dryness and stored at -20 °C. PTC-AA were resuspended in 5% acetonitrile in 0.1M ammonium acetate, 0.2 Ìm filtered and separated on an Intersil ODS3, 2 x 150 mm column with a gradient profile of: 5% acetonitrile (0 to 5 min), 5-15% acetonitrile (5 - 8 min), 15 % acetonitrile (8 - 11 min), 15 - 60% acetonitrile (11 - 15 min) and 60 % acetonitrile (15 to 19 min) all in 0.1M ammonium acetate at a flow rate of 0.5 mL / min at 25°C. Detection was achieved by UV absorbance at 254 nm. Authentic amino acid standards were used to identify elution peaks and detection response factors. Peak identities in physiological samples were confirmed by co-elution with internal standards and elution times referenced to gamma amino butyric acid (GABA) added to all samples.

Detection of PTC-DA was in excellent agreement with isocratic UV (242 nm)-HPLC quantification of DA [5] both in calibration standards and phytoplankton extracts. A higher detection response factor for PTC-DA resulted in improved detection of low levels of DA (ca. 5 pmole / injection) in physiological extracts (Fig. 2). The PITC labeling method and optimized HPLC gradient permitted clear resolution of proline and its derivatives from DA as well as other amino acids involved in proline metabolism with an average %CV for peak elution times (RRT) of 0.81% (Table 1). PTC-DA exhibited higher UV response factors (RMR) than other PTC-AAs reflecting the presence of the isoprenoid side chain on DA. No discrimination in labeling efficiency between primary and secondary amines was observed.

Table 1. Relative rentention time (RRT) and UV detection sensitivity (RMR) of PTC-labeled amino acids separated by gradient HPLC on an Intersil ODS3 2x150mm column.

Amino Acid	*RRT*	*RM*
ASP	0.11	0.37
GLU	0.15	0.37
4-HYP	0.26	0.45
SER + ASN	0.44	0.47
GLY	0.49	0.49
GLN	0.52	0.51
B - ALA	0.61	0.37
3-HYP	0.64	0.45
HIS	0.71	0.47
TAU	0.79	0.50
THR	0.86	0.92
ARG	0.91	0.49
ALA	0.93	0.64
PRO	0.97	0.50
Domoic Acid	***1.00***	***1.00***
GABA	1.16	0.46
TYR	1.26	0.31
VAL + MET	1.27	0.88
CYS-CYS	1.29	0.61
ILE + LEU	1.32	1.23
PHE	1.36	0.49
ORN	1.37	0.66
TRP	1.39	0.73
LYS	1.40	0.46

RESULTS AND DISCUSSION

Monterey Bay is an ideal resource for comparative biochemical studies of domoic acid metabolism as at least six species of *Pseudo-nitzschia* are present seasonally and exhibit a range of toxicities [4].
An important step in the elucidation of DA biosynthetic pathways is to evaluate the potential for genetic determination of DA accumulation capacity. Surveys of independent isolates of *P. multiseries* and *P. australis* reveal substantial intra-specific variability in their capacity for DA accumulation (>100 fold, Table 2) by the end of log phase growth. Higher levels of DA accumulation are associated with lower cell yields in culture indicating a potential metabolic cost of DA biosynthesis.

Table 2. Variation in cell yield and domoic acid production by clonal isolates of two *Pseudo-nitzschia* species isolated from either the Santa Cruz Wharf (SCW) or the Monterey Coast Guard Wharf (MW) on Monterey Bay, CA. Data presented as mean (s.d.) n = 3.

Species	*Strain Code*	*Isolation Date, Location*	*Cell Yield* 10^3 mL^{-1}	*DA Content* *fmol cell*$^{-1}$
P. multiseries	*MU3*	8 - 8 / 97 SCW	281.7 (20.3)	0.027 (0.002)
"	*MU3b*	8 - 8 / 97 SCW	188.8 (15.8)	0.008 (0.002)
"	*MU4*	11 - 9 / 97 SCW	189.4 (21.1)	0.000 (0.000)
"	*MU5*	23 - 4 / 98 SCW	100.2 (16.9)	2.479 (0.041)
P. australis	*AU39*	27 - 4 / 98 SCW	29.9 (5.6)	25.866 (0.031)
"	*AU43*	8 - 5 / 98 MW	57.4 (11.2)	0.060 (0.041)
"	*AU44*	8 - 5 / 98 MW	1.2 (0.5)	1.034 (0.730)
"	*AU45*	8 - 5 / 98 MW	7.3 (2.1)	65.799 (0.288)
"	*AU47*	8 - 5 / 98 MW	10.4 (4.0)	93.057 (0.070)
"	*AU48*	8 - 5 /98 MW	7.9 (4.9)	40.137 (0.077)

Analysis of PTC-AA profiles of toxic and non-toxic phytoplankton revealed that *Pseudo-nitzschia* spp. are characterized by unique FAA compositions (Fig. 3). *Pseudo-nitzschia* spp. maintain large pools of FAA leading to proline biosynthesis (ARG, ORN), but low PRO pools. Hydroxy proline derivatives, if present, were a trace component (<0.1%) of the FAA pools in the species examined. Surprisingly, this analysis revealed that *Pseudo-nitzschia* are characterized by high, constitutive levels of the non-protein amino acid taurine, a compound that was not detected in other phytoplankton species.

The accumulation of the sulfo-amino acid taurine is commonly associated with osmotic stress in other organisms and taurine accumulation in Atlantic isolates of *P. multiseries* and *P. pungens* has been reported to be induced at high salinities [21]. The present observations modify this view by revealing that taurine is a normal constituent of *Pseudo-nitzschia* spp. when cultured at standard seawater salinities (33-34 ppt). We have not detected this amino acid in other phytoplankton genera including 10 representatives of *Nitzschia* spp. (data in prep.) suggesting that taurine may be a useful biomarker for *Pseudo-nitzschia*. Additionally, the correlation of intracellular taurine abundance with DA (Fig. 4) is consistent with the hypothesis that taurine may be involved in mitigating intracellular effects of DA. In contrast proline, which is often assigned a similar osmoregulatory role (in addition to its role in protein biosynthesis) exhibits a negative association with DA (Fig. 4), consistent with its hypothesized role as an upstream precursor to DA. Given the diverse metabolic demands on proline, it most likely represents a high turnover pool. In toxic strains of *Pseudo-nitzschia*, taurine and DA may fulfill the homeostatic roles normally ascribed to proline.

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NITROGEN OR PHOSPHORUS DEFICIENCY INCREASES ALLELOPATHY IN *PRYMNESIUM PARVUM*

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ABSTRACT

In this work we aimed to investigate if the marine haptophyte *Prymnesium parvum* known to produce a set of highly potent exotoxins (commonly called prymnesins) is able of allelopathy. Also, if the production of allelopathic substances is influenced by the nutrient conditions of the culture media. The reason is that recent studies have shown that the toxicity of *P. parvum* is enhanced when the cells are grown under N- or P-deficient conditions. In this study, three algal species (*Thalassiosira weissflogii*, *Prorocentrum minimum*, *Rhodomonas cf. baltica*), were incubated with cell-free filtrate of *P. parvum* cells grown under N or P deficient or sufficient conditions. All tested species had their growth inhibited by additions of cell-free-filtrates of *P. parvum* cultures grown under N- or P-deficient conditions. Addition of filtrates from the nutrient sufficient *P. parvum* cultures had no negative influence on the growth of any of the tested species. The filtrates of the nutrient deficient *P. parvum* cultures were highly toxic to *Artemia salina* nauplia while the nutrient sufficient were not. Thus, the results indicate that the released organic compounds by *P. parvum* are prymnesins suggesting that Prymnesium toxins may play an allelopathic role in nature.

INTRODUCTION

The brackish-water haptophyte algae *Prymnesium parvum* produces a set of exotoxins commonly called prymnesins [1, 2]. As blooms of *P. parvum* are usually mono-specific this gives an indication that the algae has a mechanism enabling them to outcompete other phytoplankton species [3]. As *P. parvum* has a moderate growth rate, the monospecificity of the blooms cannot be explained in this way [4, 5, 6, 7].

Excretion of extracellular organic substances (allelopathy) have been suggested to be an important factor affecting phytoplankton succession, by altering the competing capability between different algal species [8]. Toxic substances produced from different algal groups have been found to inhibit the growth of diatoms [9, 10]. Some flagellate species have also been found to secrete substances inhibiting the growth of coexisting phytoplankton species [11]. Already three decades ago, *P. parvum* was shown to be able to excrete toxic substances to the water [12]. However, allelopathic interactions between *P. parvum* and other phytoplankton species have not been reported yet. The release of toxic substances, suppressing the growth of algal competitors would be a selective advantage by the algal species capable of allelopathy.

The ability to compete successfully for the limiting nutrient is crucial for a phytoplankton species to become dominant. Allelopathy has been studied mainly in terrestrial plants, and these studies have shown that allelopathy is closely associated with competition for resources. The release of extracellular organic substances by some marine phytoplankton species under nutrient deficient conditions has been found by some authors [13, 10, 14].

In a previous work, we have shown that *P. parvum* toxicity (hemolytic activity) increases markedly when this algae is grown under N- or P-deficient compared to nutrient sufficient conditions [15]. Thus, production of toxins in this species suggests that there is a chemical response to low nutrient levels. Discovering that nutrient stress increases toxicity and that these broad activity toxins are excreted into the water, led us to speculate that the prymnesins may have an allelopathic role in the biology of *P. parvum*. In this work, we have studied the influence of *P. parvum* cultures cell-free filtrates on the growth of three phytoplankton species. The aims of the work were: (i) to investigate if toxic substances produced by *P. parvum* have an allelopathic effect on other algal species and (ii) if this (potential) allelopathic effect is influenced by the nutrient status of the media in which *P. parvum* was grown. The tested algae were: the diatom *Thalassiosira weissflogii*, the cryptophyte *Rhodomonas cf. baltica*, and the dinoflagellate *Prorocentrum minimum*. These species were chosen because: they occur together with *P. parvum* in natural marine phytoplankton communities; all species are easily grown in laboratory cultures and they represent three different important algal groups in marine systems.

METHODS

A toxic strain of *Prymnesium parvum* (CCMP 708) and the non-toxic algal species *Thalassiosira weissflogii* (KAC 24), *R. cf. baltica* (KAC 30) and *Prorocentrum minimum* (BAH ME 66) were initially grown in f/10 media [16] except vitamins which were added following the method of [17] at 7‰. All cultures were kept at 20 °C on a 16 h light:8 h dark cycle under an irradiance of 100 $\mu E \cdot m^{-2} s^{-1}$ provided by 36 W cool-white fluorescent lamps.

Three batch cultures of 2.5 L were used to cultivate *P. parvum* cells in order to obtain cell-free filtrates. *Prymnesium parvum* was grown under N and P sufficient or deficient conditions (Table 1).

Table. 1. Initial nitrogen and phosphorus concentrations and ratios, in the media used to cultivate *Prymnesium parvum*.

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Treatment	NO_3 µM	PO_4 µM	N/P-ratios atoms
Sufficient:	58	3.6	16:1
N-deficient	14.5	3.6	4:1
P-deficient	58	0.9	64.1

The other 3 algae were grown at nutrient sufficient conditions as 150 ml batch cultures in tissue culture flasks (200 ml, Nunclon, 12 bottles of each species). All bottles were randomly rearranged to minimize the effects of any light or temperature gradients end gently mixed twice a day.

Cell density and chlorophyll *a* were measured daily in the *P. parvum* cultures by sub-sampling 5 and 25 ml of each culture, respectively. The cells were preserved with acid Lugol's solution and counted (a minimum of 400 cells per sample), using an inverted microscope (Nikon TMS). The cell-free filtrates were collected during exponential phase for the nutrient sufficient (day 5) and stationary phase for the nutrient deficient (days 7 and 8) *P. parvum* cultures. The concentrations of NO_3^-, PO_4^{3-} and NH_4^+ in the filtrates were analyzed according to [18]. *Artemia* bioassay [19] was used to test the toxicity of the cell-free filtrates. The mortality was transformed into probit units [20] and plotted against log-transformed cell concentration. From the regression line, the 50 % mortality (24-h LC_{50}) was calculated. The filtrates from the different *P. parvum* cultures were then used for the allelopathic growth experiment.

From each of the tissue culture bottles, 90 ml were removed and replaced with the cell-free filtrates from the three different *P. parvum* cultures (three replicates of each treatment). Autoclaved seawater was used instead of filtrate as controls. Extra macro- and micronutrients (as in F/10) were added to ensure that the growths of the different species were not limited by nutrients. Every 12 h, 10 ml of each culture was sub-sampled for cell enumeration using a flow Cytometer (FacsCalibur, Becton Dickinson), which was calibrated by manual counting. The samples were preserved in this case with paraformaldehyde. At the end of the experiment, nutrients in each bottle were analyzed (as above).

To determine whether growth rate varied significantly among treatments within each species (N- or P-deficient versus sufficient treatments) ANOVA and Fishers PLDS analyses were used.

RESULTS

In the N- and P- sufficient cultures (cells harvested during the exponential growth phase), final cell densities were up to 40.4 10^3 cells ml^{-1}. For the N- and both NO_3^- and PO_4^{3-} were found in the nutrient sufficient cultures, while in the P and N-deficient the corresponding nutrients were below analytical detection at the time of filtration (in the nutrient sufficient treatment, these nutrients were in surplus amounts, however).

Filtrates from the nutrient sufficient *P. parvum* cultures were not toxic to *Artemia salina* while filtrates from the N- or P-deficient treatments were. The calculated LC_{50} values for the N- and P-deficient filtrates corresponded to 49.7 10^3 and 33.6 10^3 cells ml^{-1}, respectively.

A similar pattern after addition of the different filtrates on the growth of *Thalassiosira weissflogii*, *R. cf. baltica* and *Prorocentrum minimum* was found (Fig 1). All three species, rapidly decreased their cell densities when cell-free filtrates from *P. parvum* cultures grown under N or P-deficient conditions were added to the 3 tested algae. Since N and P were in excess in the media of all 3 tested algal species, the observed decrease in cell densities for these algae can not be attributed to nutrient depletion due to consumption by the algae. Filtrate additions from the nutrient sufficient cultures did not reduce the cell numbers in any of the tested species (Fig 1). Instead, these cultures resembled the controls showing a positive growth pattern in these treatments.

ANOVA was used to compare the growth rates measured for each species, indicating significant differences among the treatments for *Thalassiosira weissflogii*, *R. cf. baltica* and *Prorocentrum minimum* ($p < 0.0001$, 0.001 and 0.0001, respectively). The growth rates for the algae which were exposed to filtrates from N- or P deficient cultures were, in all cases, significantly different from those observed in cultures treated with filtrate from the nutrient sufficient or the control cultures. No statistical differences were found in the growth rates between the nutrient sufficient treatments and the control group for any of the 3 species.

DISCUSSION

In this study, we show for the first time that under N or P deficient growth conditions cell-free filtrates of *P. parvum* cultures inhibited the growth of *Thalassiosira weissflogii*, *Prorocentrum minimum* and *R. cf. baltica*. The observed decline in cell densities was related to excretion of toxic substances by *P. parvum*, indicating that the exudates play an allelopathic role. The cell filtrate toxicity was highest in the cultures grown under N- or P- deficient conditions. In contrast, cell-free filtrates of cultures grown under N and P sufficient conditions did not exert a negative influence on the growth of any of the tested species. This suggests that the production of allelopathic substances is regulated by the availability of nutrients. The filtrates of the nutrient deficient *P. parvum* cultures were highly toxic to *Artemia salina* nauplia while the nutrient sufficient were not. Thus, the results indicate that the released organic compounds by *P. parvum* are prymnesins suggesting that Prymnesium toxins may play an allelopathic role in nature.

In coastal areas phytoplankton cells are subjected to extreme variable nutrient conditions. Physiological adaptations to these conditions, such as the production of allelopathic substances is of no doubt important for the survival and competitiveness of the species capable to do so. There are several kind of microorganisms that are able to produce allelopathic compounds under nutrient stress [21]. Although there are several reports on the excretion of toxic substances by different algal species [11, 9, 22, 23], these reports are however not connected to the nutrient status of the cells. Another haptophyte, *Chrysochromulina polylepis* have been shown to inhibit the growth of the diatom *Skeletonema costatum* when grown under P-deficient conditions [10]. This suggests

that toxic haptophytes seem to have in common the excretion of toxic substances under nutrient deficient conditions. Thus, haptophytes growing without stress at their optimum rate may not be able to produce allelopathic substances, but when growth rate declines due to e.g. nutrient deficiency, the secondary metabolic processes become active.

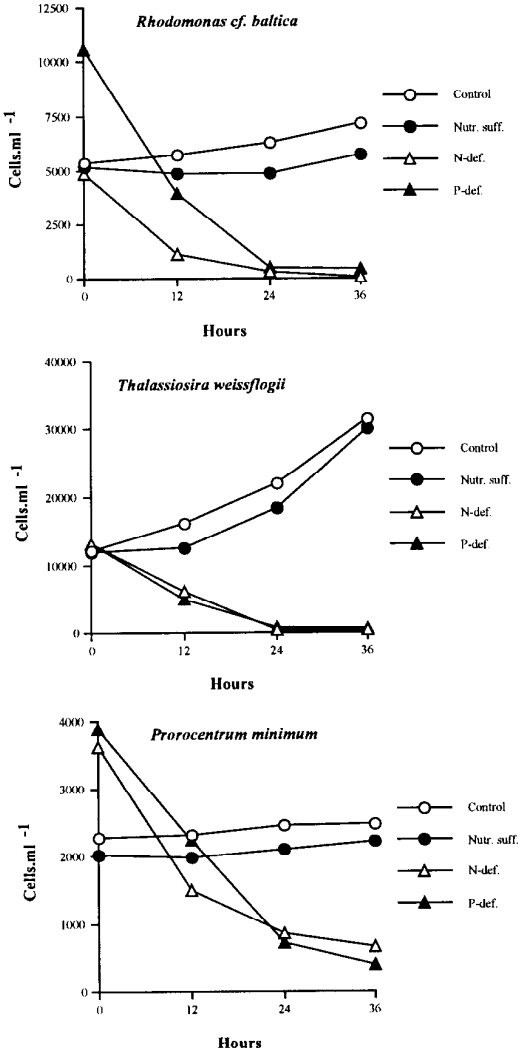

Fig. 1. Cell densities for 3 phytoplankton species after addition of filtrates from *P. parvum* cultures grown under N-deficient (N-def), P-deficient (P-def), and NP sufficient (Nutr.suff.) conditions. Instead of *P. parvum* filtrates, filtered seawater additions were used as controls.

When an algal species is able to effectively compete with other algal species for the deficient nutrient by its rapid growth under these conditions, secondary metabolites such as toxins e.g. are not produced. However, toxin production is switched on as a defense system when the organism is no longer able to compete for the limiting nutrient. *Prymnesium parvum*, cell-free filtrates from cultures grown under nutrient sufficient conditions in this study, did not, in contrast to nutrient deficient cultures, inhibit the growth of any of the tested algal species. Our results support the conclusion that only under conditions of limited growth allelochemicals are produced.

Microalgal allelopathy is not a concept generally accepted. One of the criticisms of the existing evidence concerns the use of unnaturally dense populations in the cultures tested with species that do not occur together in nature. In this study, *P. parvum* had final cell densities that varied between 40-77 10^3 cells ml^{-1}. This is well below cell densities reported by [24] for blooms of *P. parvum* in nature. All three species (*T. weissflogii*, *R. cf. baltica* and *P. minimum*) used in the present study are regular components of the plankton flora in the Baltic Sea have been found at the same location on several occasions [25].

Conclusions

Under N or P deficient growth conditions cell-free filtrates of *P. parvum* cultures inhibited the growth of *T. weissflogii*, *P. minimum* and *R. cf. baltica*. *Artemia* bioassay showed that the filtrates from the N and P deficient *P. parvum* cultures were toxic while the nutrient sufficient cultures were not. The observed decline in cell densities was related to excretion of toxic substances by *P. parvum*, indicating that the exudate play an allelopathic role. The cell filtrate toxicity was highest in the cultures grown under N- or P- deficient conditions. In contrast, cell-free filtrates of cultures grown under N and P sufficient conditions did not influence negatively the growth of any of the tested species. This suggests that the production of allelopathic substances is regulated by the availability of nutrients. Our findings demonstrate that *Prymnesium* toxins appear to be the exudate substances inhibiting the growth of several phytoplankton species supporting the hypothesis that *Prymnesium* toxins may play an allelopathic role in nature.

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EFFECT OF POLYAMINES ON GROWTH AND TOXICITY OF *CHRYSOCHROMULINA LEADBEATERI* (HAPTOPHYTE)

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ABSTRACT

The haptophyte *Chrysochromulina leadbeateri* dominated a phytoplankton bloom in the Vestfjord area (Norway) in May-June 1991, and caused a massive mortality of penraised Atlantic salmon. As part of a project examining the nutrient conditions that may cause the ichthyotoxic *C. leadbeateri* to outcompete other algal species, we conducted a series of experiments to test the influence of polyamines (1-1100 µM putrescine, cadaverine) on the growth and toxicity of non-axenic cultures of *C. leadbeateri* (CLTJ1). Exponentially growing cultures were incubated with or without polyamines under light and temperature controlled conditions. Cell abundance and toxicity (haemolytic and *Artemia salina* assays) were analysed after 24 and 48 h. Addition of polyamines (1-110 µM) to the culture medium stimulated growth and resulted in increased cell numbers in *C. leadbeateri* (+ 5-30 %). High concentrations of both polyamines (1100 µM) had a negative effect on *C. leadbeateri* cell numbers (i.e. up to 90-100 % lysed cells relative to the control cultures). Haemolytic substances were extracted both from the cells and the whole culture (cells+medium). Within the range 0-11 µM of putrescine addition, *C. leadbeateri* showed very low haemolytic activity (HA < 1 Seq µg ml^{-1}). Over the range 1-110 µM cadaverine and cadaverine + putrescine, and 110 µM putrescine, HA increased up to 10 Seq µg ml^{-1} in the particulate fraction after 48 h. The discrepancy between the HA of the particulate fraction and the whole water extracts of the same samples suggests that the haemolytic compounds released by *C. leadbeateri* cells were stable in the water phase although the cells were lysed. These results suggest that (i) polyamines may act as growth enhancing compounds, (ii) and as cofactors with haemolytic/ichthyotoxic substances forming a "haemolytic-polyamine complex" that could be stable in a colloidal phase and possibly "hypertoxic".

INTRODUCTION

Some haptophyte species (e.g. *Chrysochromulina Prymnesium*) are known to produce ichthyotoxic substances. Among the *Chrysochromulina* species, *C. polylepis* is considered to be the most ichthyotoxic in Scandinavian waters whereas *C. leadbeateri* shows lower toxicity during blooms [1] and often loose its toxicity in cultures.

In May-June 1991, a bloom of *C. leadbeateri* developed in the Ofotfjord-Tysfjord area and adjacent fjords, and killed 600 metric tons of farmed Atlantic salmon [2]. During the winter 1990-1991, more than 70 % of the total stock of Norwegian spring-spawning herring (1.5 x 10^6 metric tonnes) overwintered in the Ofotfjord, and 1 % of the stock presumably died in this fjord [3]. The herrings altered the chemical conditions in the water i.e. decrease of oxygen and increase of nitrate concentrations, release of organic substances which may have favored the *C. leadbeateri* bloom and its large toxicity. Herrings have polyamine-rich tissues that are decomposed by bacteria, and polyamines e.g. histamine, putrescine and cadaverine are rapidly released from decaying herring. These polyamines together with ichthyotoxic substances may have a synergistic effect, and form structural complexes [4] which could explain the unusual toxic nature of the *C. leadbeateri* bloom in 1991.

In this paper, we present new evidence of the effect of polyamines on the growth and toxicity of *C. leadbeateri*.

METHODS

Strain history and preparation of polyamines

Non axenic *Chrysochromulina leadbeateri* (ClTj1) was isolated from a toxic bloom in the Lofoten area (Northern Norway) [5] and obtained from the Oslo Culture Collection, Norway, and maintained in seawater medium (salinity = 33) with modified f/10 nutrients [6] corresponding to N/P ratio = 16:1 and 20 nM selenium at 17° C and 80 µmol m^{-2} s^{-1} (16:8 L:D cycle). Polyamines (putrescine, cadaverine, Sigma) were dissolved in saline (30 ‰) Milli-Q water and the solutions were sterile-filtered (0.2 µm). The polyamines stock solutions (22 mM) were tested for haemolytic activity (horse blood) and toxicity against the brine shrimp *Artemia salina* respectively before additions to the algal cultures.

Effect of polyamines on algal growth and toxicity

Polyamines were added to 10 ml cultures at 1, 10, 110, 1100 µM final concentration using putrescine, cadaverine or both putrescine and cadaverine. As a control saline Milli-Q water was used. All treatments (duplicates) were incubated for 24 or 48 h under similar conditions as the stock cultures, and repeated at two occasions during exponential growth. Initially (time = 0) and at the end of the incubation, subsamples were taken for microscopic observation of live cells, cell counts, haemolytic activity and *A. salina* acute toxicity assays.

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Analytical procedures

Cells were enumerated in Lugol's fixed samples with at least 400 cells counted. The effect of polyamines on growth was determined as cell number ratio = (number of cells grown with polyamines/ number of cells in the control) x 100. The haemolytic activity of algal culture extracts (chloroform-methanol-water = 13:7:5) was tested on horse blood as described by [7]. Extracts were obtained from algal cells retained onto a Whatman GF/C filter (all treatments) or from the whole culture (lysed cells + medium ; 1100 µM polyamines). In order to compare these two fractions, the results are expressed as saponin equivalents per culture volume (Seq µg ml^{-1}). The toxicity of *C. leadbeateri* against *A. salina* was examined on *A. salina* 48 h nauplii. Cysts of *A. salina* were hatched according to [8]. Algal cultures were diluted with filtered seawater (33 ‰) to give a dilution series of 1, 1.25, 2, 3.3, 5, 10, 20, 50, and 100 %. As a control filtered autoclaved seawater was used. Five hundred µl of each dilution was added to a 500 µl well (Falcon multiwell 96 wells) and 20 nauplii were transferred to each well, and duplicates were used for each culture dilution. The microwells were incubated under similar conditions as described above. After 24 h or 48 h, the mortality of nauplii at each cell concentration was determined, transformed into probit units [9], plotted against log-transformed cell numbers allowing to calculate a 24 h- or 48 h- LC_{50}.

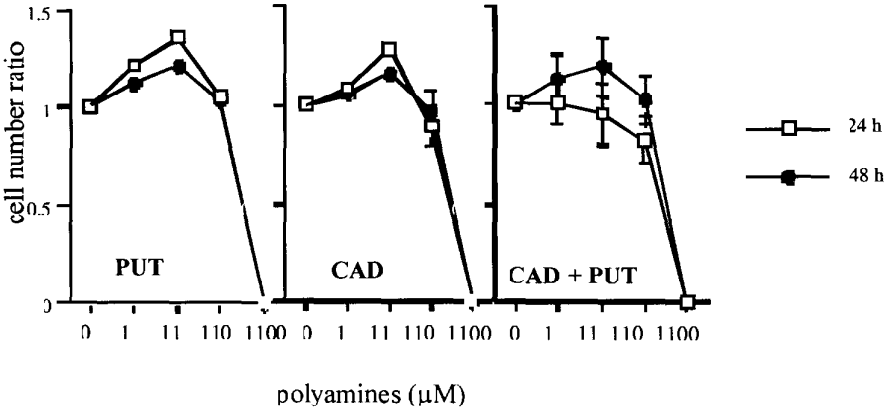

Fig. 1. Cell number ratios (number of cells with polyamines/number of cells in the control) in *C. leadbeateri* cultures grown with various concentrations of putrescine (PUT), cadaverine (CAD), cadaverine + putrescine (CAD + PUT) relative to control cultures. Mean ± SD, n = 4.

RESULTS

Cell numbers

In cultures of *C. leadbeateri* grown with 1-110 µM putrescine for 24 and 48 h, cell numbers increased relative to the controls (Fig. 1). In cultures with 1-110 µM cadaverine, or cadaverine + putrescine, cell numbers were higher than the controls after 24 h (5-30 %) and 48 h (+ 10-20 %). The largest increase in *C. leadbeateri* cell numbers (+ 30 %) was found with putrescine. The addition of either polyamines at the highest concentration (1100 µM) resulted in a massive cell lysis (> 95 % cell numbers) after 24 h. The few remaining cells were not able to swim, possibly due to the loss of their flagella. After 48 h, cell debris and aggregates only could be observed in these cultures.

Toxicity

Chrysochromulina leadbeateri was not found toxic to *A. salina* at any sampling occasion in all treatments.

During exponential growth, the haemolytic activity (HA) of *C. leadbeateri* was below detection in the polyamines stock solutions, the stock cultures and in the controls. In treatments with polyamines, HA was above detection (0.4-1 Seq µg ml^{-1}) after 24 h, and increased up to 10 Seq µg ml^{-1} after 48 h (Fig. 2). Cells of *C. leadbeateri* exposed to cadaverine or cadaverine + putrescine showed a higher HA at polyamines concentrations 1-110 µM in comparison to controls and cells exposed to putrescine 1-11 µM. Since *C. leadbeateri* cells were lysed after 48 h with either polyamines 1100 µM, HA was also measured on the whole water medium. The results showed a lower HA of the particulate fraction in comparison to the whole fraction (Fig. 3). This possibly explains the low HA at 1100 µM cadaverine and cadaverine + putrescine from Fig. 2.

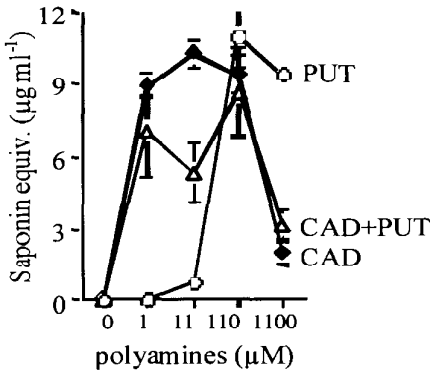

Fig. 2. Haemolytic activity (saponin equivalent µg ml^{-1}) of the fraction of *C. leadbeateri* cultures retained onto a Whatman GF/C filter. *C. leadbeateri* cultures were exposed to putrescine (PUT), cadaverine (CAD), cadaverine + putrescine (CAD+PUT) at various concentrations for 48 h during exponential growth. Mean ± SD, n = 3.

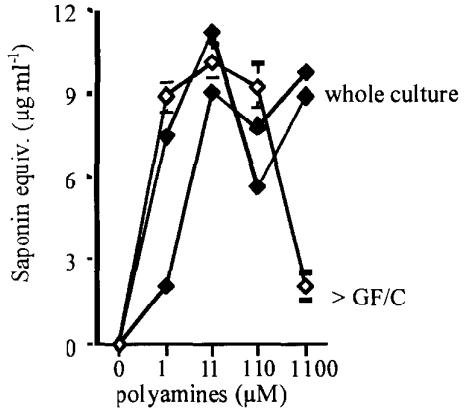

Fig. 3. Haemolytic activity (saponin equivalent µg ml^{-1}) of the extracts from the fraction of *C. leadbeateri* cultures retained onto a Whatman GF/C filter (> GF/C ; mean ± SD, n = 3) and the whole culture (n = 2). *C. leadbeateri* cultures were exposed to 1100 µM cadaverine for 48 h during exponential growth.

DISCUSSION

This study provides evidence that polyamines are growth enhancing substances and have a possible synergistic effect with ichthyotoxins (i.e. haemolytic) on the overall toxicity of *C. leadbeateri*.

Putrescine and cadaverine are found in every living cell, but there are also a decomposition product of proteins released by living organisms or through bacterial decomposition in the water column [10]. In seawater, putrescine concentrations are relatively low (50-250 nM) but the turnover times can be only a few hours [11]. Previous studies have reported that putrescine contributed from 70 % up to 300 % to bacterial production in oxic and anoxic waters respectively [11]. Polyamines are considered as growth regulators in higher plants [12] but little is known about the effect of polyamines on phytoplankton in seawater. Putrescine, spermidine and cadaverine have been showed to stimulate the growth, cell division and photosynthesis in *Dunaliella tertiolecta* [13], suggesting that these polyamines may act as growth regulators. Also the addition of polyamines to *D. tertiolecta* cultures during stationary phase stimulates growth, apparently alleviating nitrogen limitation (e.g. liberation of N during the degradation of the polyamines). The addition of < 5µM putrescine increases the growth rate (+ 30 %) of the dinoflagellate *Gymnodinium mikimotoi* [14]. In our study, the addition of polyamines increases *C. leadbeateri* cell densities (i.e. cell division over 24 and 48 h) during the exponential phase, indicating that polyamines may act as growth regulators as for *D. tertiolecta* and *G. mikimotoi* but seem to rule out their role as a source of extra nitrogen since nitrate was available in the *C. leadbeateri* cultures.

Ichthyotoxic activity requires the presence of cofactors such as divalent cations or polyamines, and spermine was found to enhance the ichthyotoxic activity of crude *Prymnesium* extracts [15]. This means that for the toxin to be active on a fish, the cofactors have to be present at the same time. In our study, the addition of polyamines increased the haemolytic activity of extracts of *C. leadbeateri* cells and of the whole water fraction containing lysed cells and bacteria. *C. leadbeateri* is usually not toxic, and we suggest that polyamines act as cofactors forming an active toxic complex as for *Prymnesium*. The dinoflagellate *Pfiesteria* i.e. "ambush-predator" is found toxic in the presence of unknown substances released by fish in the water [16]. At high polyamine concentrations (> 110 µM), *C. leadbeateri* showed erratic swimming behavior, aggressive behavior towards other cells (e.g. cannibalism), similar to senescent or oxidative stressed cells. During the development of the *C. leadbeateri* bloom in 1991, the large stock of overwintering herrings in the Ofotfjord-Tysfjord may have provided a large source of polyamines i.e. cofactors, resulting in the formation of a large pool of "active ichthyotoxic" complexes. Moreover, our results show that the complex polyamine-haemolytic substances is resistant to bacterial degradation over a short period (48 h). This might partly explain why the *C. leadbeateri* bloom remained toxic after its transport to the outer Vestfjorden with outflowing water [2].

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UPTAKE OF HUMIC SUBSTANCES BY THE TOXIC DINOFLAGELLATE *ALEXANDRIUM CATENELLA*

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ABSTRACT

Alexandrium catenella is a paralytic shellfish poisoning (PSP) dinoflagellate which blooms in organic-enriched coastal waters. As part of a long term project examining mixotrophy and toxin producing phytoplankton, we conducted a series of experiments to test whether *A. catenella* utilizes humic substances (HS) and other high molecular weight organic compounds. Humic utilization was tested by culturing cells in the presence of HS or nitrate and exposing them to radioactively labeled HS (^{3}H-HS; isolated from seawater using XAD and subsequently labelled by Amersham). ^{3}H-HS uptake was determined directly by isolating individual cells with micropipettes and measuring radioactivity using a liquid scintillation counter. *A. catenella* cells accumulated radioactivity, with HS-grown cells having greater ^{3}H-HS uptake rates compared to nitrate-grown cells. Fixed cells (controls) showed a linear increase in radioactivity, indicating passive adsorption of ^{3}H-HS, however dead cells had lower radioactivity at the end of the experiment compared to live cells. Moreover, intracellular accumulation of radioactivity was verified using microautoradiography. Cells in HS cultures were also tested for protein uptake using FITC-labelled caseine. After 48 h, approximately 60% of *A. catenella* cells contained fluorescent bodies, indicating caseine uptake. The similarity of these inclusions to those found in *A. catenella* cells exposed to FITC-labelled dextrans provides further evidence of organic uptake by this dinoflagellate, although the mechanism of uptake remains unclear.

INTRODUCTION

Dissolved organic nitrogen (DON) is the most abundant chemical form of N (other than N gas) in the surface ocean [1]. This pool of N comprises a diverse mixture of compounds with varying concentration, molecular weight, and structural complexity whose bioavailability to primary and secondary producers are still relatively unclear [2]. Low molecular weight (LMW) compounds such as amino acids and urea are rapidly assimilated [e.g. 3, 4], but these compounds are generally considered to be ≤20% of the DON transported by rivers to estuaries, with the bulk of the DON comprising principally uncharacterized, complex, high molecular weight (HMW) compounds [5].

Recent studies have indicated that some phytoplankton, including HAB species, are able to utilise DON compounds directly [e.g. 2, 4, 6, 7], while others are able to access the DON pool by use of exoenzymes [8, 9]. DON may also become bioavailable to phytoplankton following photochemical oxidation of DON which releases ammonium [10]. Additionally, indirect access to the DON pool by phytoplankton may arise from the assimilation of DON into bacterial biomass and/or regeneration of DON as ammonium [11, 12].

Alexandrium catenella is a toxic (PSP) dinoflagellate species with widespread distribution, being documented from temperate waters in north America (between California and Alaska), Japan, South Africa, southern Chile, Australia, New Zealand and Europe (Mediterranean) [13]. It produces potent neurotoxin (saxitoxin) which accumulates in filter feeding shellfish and poses a significant public health risk for human consumers. Previous investigations have shown that this dinoflagellate can take up HMW (2000 kDa) carbohydrate macromolecules [14], and that up to 40% of cellular N can be derived from humic substances (isolated by adsorption on XAD-8 resin) [15]. In this paper, we present new evidence for direct utilisation of organic compounds by *A. catenella.*

METHODS

Strain history and preparation of ^{3}HS

Toxic *A. catenella* (axenic strain CCMP1598) was obtained from the Provasoli Centre for Culture of Marine Phytoplankton, Maine, USA, and maintained at the Kalmar Culture Collection in enriched seawater medium (salinity = 26) with f/20 nutrients [16] at 18 °C and 80 µmol photons $m^{-2}s^{-1}$ (12:12 L:D cycle). HS were isolated using XAD-8 resin [17, 18], and tritium-labelling was achieved by Amersham. The specific activity of the ^{3}H-HS stock solution was 30 mCi.

Uptake of ^{3}HS

A. catenella cells were grown in seawater medium (salinity = 26) ammended with f/20 nutrients [16] and 10 µM of HS or NO_3^-. (Note that subsequent nutrient analyses showed a residual amount of NO_3^- present in HS cultures). Exponential phase *A. catenella* cells were subsampled (Fig. 1), and placed in 20 ml glass scintillation vials to which ^{3}H-HS was added (1

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μCiml^{-1} final concentration). Cells were incubated at 80 μmol photons m^{-2}s^{-1} (12:12 L:D cycle), with triplicate cultures being harvested at 24, 36 and 48 h. The incubation started at the beginning of the light period, so cells which were sampled at 24 h had experienced one light (12 h) and one dark (12 h) period. Those sampled at 36 h had experienced 24 h light and one dark period, and cells sampled at 48 h had experienced two light and two dark periods. When sampled, cells were fixed with 2% vv^{-1} (final concentration) glutaraldehyde in 0.25M sucrose [19]. They were then isolated by filtering onto 10 μm Nitex mesh, rinsed six times (3 x 30ml filtered seawater + 3 x 15ml Phosphate Buffer Solution) before being resuspended (by placing the mesh upside down) in a Sedgwick Rafter counting chamber filled with filtered seawater. Cells were isolated using a glass micropipette under 100x magnification and rinsed three times in filtered seawater (by isolating three times in succession into three different seawater drops) before being placed into Eppendorf tubes for counting. To estimate background radioactivity in the rinse medium, blank samples were also prepared using an equivalent volume of the last seawater rinse. One ml of scintillation fluid was added to the tubes before radioactivity (disintegrations per minute) was measured using a liquid scintillation counter (Wallac Winspectral 1414). Counts were made over 20 min to ensure a <2% counting error. Cellular radioactivity was calculated by subtracting the radioactivity in blank samples from paired cell samples and dividing by the total number of cells in each vial. Controls consisted of dead cells which were fixed in three different ways at the beginning of the experiment: (1) 2% vv^{-1} (final concentration) paraformaldehyde in 0.25M sucrose; (2) 2% vv^{-1} (final concentration) glutaraldehyde in 0.25M sucrose; and (3) 2% vv^{-1} saturated $HgCl_2$ solution [19,20]. Dead control cells were sampled at 0, 24, 36 and 48 h, fixed and isolated in the same manner as live cells and counted for radioactivity.

Microautoradiography

To check that accumulation of radioactivity by *A. catenella* was the result of active ^{3}H-HS uptake and not passive adsorption, cells were prepared for microautoradiography [21]. Cells from ^{3}H-HS incubations were filtered onto 10 μm Nitex mesh, resuspended in filtered seawater and filtered onto 0.2 μm membrane filters (Millipore). Filters were rinsed 3 times with 20 ml filtered seawater to remove excess radioactivity and air-dried. Once dry, filters were attached to microscope slides using a filter freeze-transfer technique [22], mounted with Permount solution, and exposed to Kodak emulsion NTB-2 in the dark. Filters were dried in the dark for 7 days before being developed in Kodak D19 and fixed in 1% acetic acid and Ilford rapid fixer. Slides were observed under 400x phase contrast, and photomicrographs were recorded on Ektachrome 400 ASA color slide 35mm film.

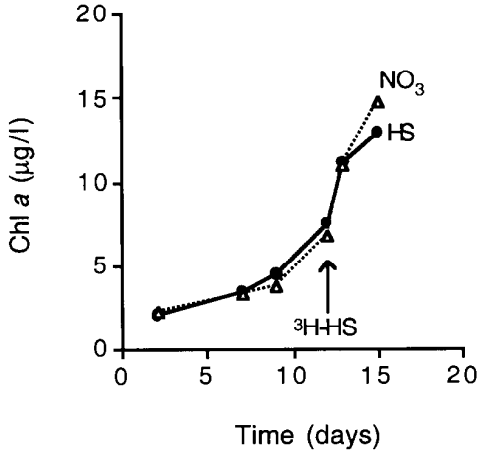

Fig. 1. Growth of *A. catenella* in the presence of nitrate (NO_3) or humic substances (HS); arrow indicates when cells were subsampled for ^{3}H-HS uptake experiments.

RESULTS

Uptake of ^{3}HS

HS and nitrate-grown *A. catenella* cells accumulated radioactivity, indicating ^{3}H-HS uptake (Fig. 2). After 48 h, radioactivity in *A. catenella* cells increased from 1 - 7 disintegrations per minute per cell [dpm cell^{-1}], with greatest accumulation during the first 24 h (including 1 light and 1 dark period). Nitrate-grown cells also accumulated ^{3}H-HS, with a delay compared to HS-grown cells. Fixed cells (controls) showed a low activity at time zero (~0.1 dpm cell^{-1}), which increased linearly with time to ≥4 dpm cell^{-1} (Fig. 3), indicating time-dependent passive adsorption of ^{3}H-HS. However microautoradiography confirmed active uptake of ^{3}H-HS by living *A. catenella* cells. The light coloured inclusions (similar to those observed during uptake of FITC-labelled dextrans; [14]) demonstrate intracellular radioactivity, whereas fixed control cells show no light inclusions (Fig. 4.). The halo-effect in non-living control cells suggests that the fixatives alter cell-surface properties which cause increased cell "stickiness" (particularly for $HgCl_2$ preserved cells), resulting in extracellular accumulation of radioactivity. Intracellular toxin concentrations were low (maximum PSP toxins = 0.2 pM cell^{-1}), with greater proportions of N-rich C-toxins compared to STX, NEO, and GTX toxins, but there was no apparent difference in toxin content between cells exposed to different N sources. In addition, C (300 - 430 pg) and N (50 - 90 pg) quotas of cells after 14 days were similar in the different N treatments, confirming HS utilisation by *A. catenella*. Flow cytometric bacterial counts confirmed that bacterial abundance was insignificant during the course of the experiment.

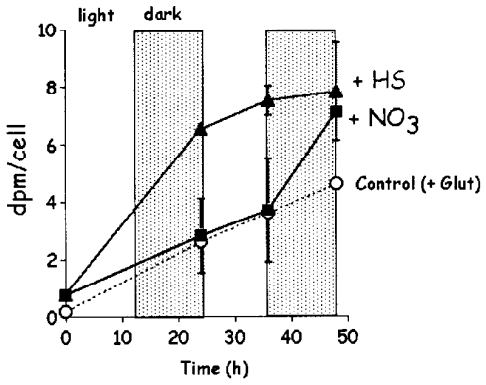

Fig. 2. Uptake of ^{3}H-HS (disintegrations per minute per cell; dpmcell^{-1}) by *A. catenella* in the presence of humic substances (+HS) and nitrate (+NO_3). Error bars represent standard deviation of triplicate cultures.

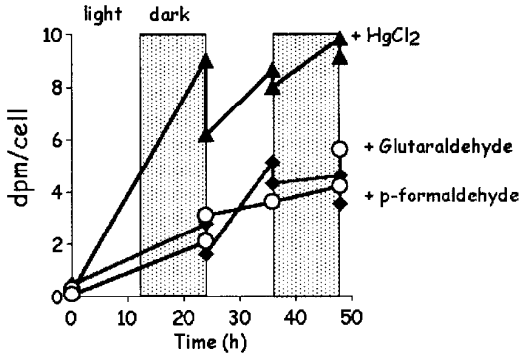

Fig. 3. Accumulation of radioactivity (disintegrations per minute per cell; dpmcell^{-1}) by dead control cells fixed (from time = 0 h) in 2% vv^{-1} saturated $HgCl_2$, glutaraldehyde and para-formaldehyde. Symbols represent values for independent duplicate cultures.

DISCUSSION

This study provides new evidence of organic uptake by the toxic dinoflagellate *A. catenella*, adding to previous studies which showed enhanced growth in the presence of HS [15] and direct uptake of carbohydrate macromolecules (FITC-labelled dextrans; [14]).

Direct utilisation of dissolved LMW organic nitrogen compounds such as urea and amino acids has been demonstrated for many algal species [2], however there is an increasing body of evidence which shows that HMW compounds are also available for phytoplankton uptake [4, 15]. While phytoplankton can access the DON pool indirectly through bacterial metabolism of DOM which produces ammonium, or via bacterivory [12], direct uptake of DON may be possible through the action of exoenzymes [7], or by pinocytosis (where the plasma membrane forms a vesicle enclosing the organic compounds; [23]). Such mechanisms have not yet been demonstrated in *A. catenella*, but the results of this study indicate that this species can take up organic macromolecules. Our experiments show that while there is passive adsorption of radioactivity by fixed control cells (due to a change in cell suface properties), active uptake of ^{3}H-HS does occur, forming intracellular inclusions similar to those observed after incubation with FITC-labelled carbohydrates [14] and protein (caseine; Legrand et al., unpublished data).

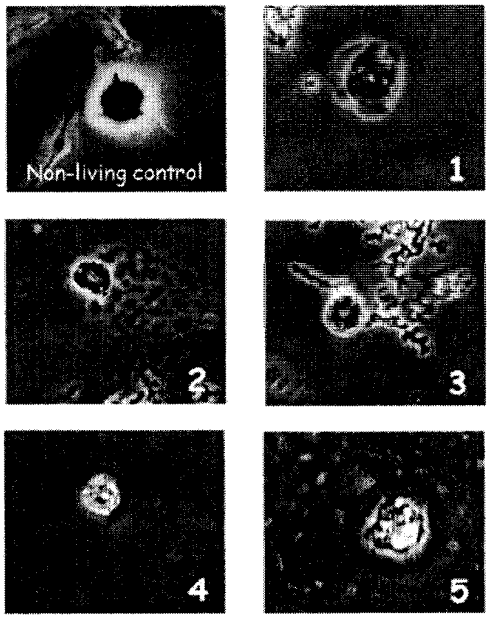

Fig. 4. Microautoradiographs of *A. catenella* cells after 48 h incubation with ^{3}H-HS; 1 - 5: cells with incorporated ^{3}H-HS (as indicated by white inclusions).

In a similar study, Segatto et al. [24] examined uptake of ^{3}H-HS by the dinoflagellate *Ceratium furca*. Single cells were manually isolated from a natural phytoplankton community in the Gullmar Fjord and exposed to ^{3}H-HS and/or HS and incubated under high and low irradiance. They found that *C. furca* took up ^{3}H-HS, but radioactive accumulation was much lower (dpmcell^{-1} $\approx$ 1.5) than in our experiment with *A. catenella* (dpmcell^{-1} $\approx$ 7).

A large proportion of N in isolated HS is loosely bound as ammmonium, amino acids or precipitated polypeptides which are readily utilized by bacteria [5]. Accumulation of ^{3}H-HS by *A. catenella*

demonstrated in this study may therefore represent uptake of smaller, more labile molecules associated with HS, rather than the condensed polyaromatic carbon structures which make up the bulk of HS [25]. Even though we cannot rule out that cells utilized only a small portion of these organic compounds, our results confirm that *A. catenella* cells can access the HS pool and may use this to support growth and biomass production. Furthermore, the delay in ^{3}H-HS uptake by nitrate-grown cells may indicate that prior exposure to DOM is a necessary prerequisite for uptake, however further experimentation is needed to confirm this. In summary, our experiments show that *A. catenella* can take up radioactively labelled HS, indicating that this organism utilises macromolecular organic compounds, presumably through pinocytosis.

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IRON NUTRITION IN THE BROWN TIDE ALGA, *AUREOCOCCUS ANOPHAGEFFERENS*: CHARACTERIZATION OF A FERRIC CHELATE REDUCTASE ACTIVITY

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ABSTRACT

Blooms of the pelagophyte, *Aureococcus anophagefferens,* are responsible for the brown tides that occur in the Peconic estuary on Long Island, NY. To understand the role of iron in controlling these blooms, the growth of *A. anophagefferens* was examined in artificial seawater supplemented with f/2 nutrients using trace metal clean techniques. Cultures grown on 10-100 nM added iron complexed with twice its concentration of NTA gave maximum cell yields and growth rates similar to that obtained in iron-replete (11 μM) cultures. Thus the iron requirement for maximum growth in *A. anophagefferens* was lower than 100 nM. The use of trace-metal buffered conditions did not significantly affect the growth of *A. anophagefferens* in culture. An estimated iron quota (Q_{Fe}) for these cultures was ~5 amol $cell^{-1}$. To understand how *A. anophagefferens* obtains its needed iron, the enzyme ferric chelate reductase was characterized from this organism. This constitutive enzyme showed a broad pH optimum, was bispecific for NADPH and NADH, and has a Vmax (0.56-1.3 μmol min^{-1} mg^{-1} protein) and Km (~400 μM) for ferric HEDTA typical of other eukaryotic algae. The observed rates of enzyme activity (average 10 - 20 nmol min^{-1} mg^{-1} protein using NADPH) are sufficient to support the maximum growth rate of the organism. Laboratory cultures showed no evidence for siderophore or chelator formation under low iron conditions. We conclude that *A. anophagefferens* CCMP1708 has a low iron requirement and the necessary mechanism to obtain that iron.

INTRODUCTION

The occurrence of unusual plankton blooms (brown tides) by the pelagophyte *Aureococcus anophagefferens* has been detrimental to the shellfish industry and eelgrass beds of the Peconic and Great South Bay estuary systems on Long Island NY. These blooms were first observed in 1985 and have reoccurred almost annually since that time. In some cases, i.e. 1991, these blooms are widespread throughout the entire estuary system. In other instances, the blooms are confined to selected embayments such as West Neck Bay [3, 13].

Environmental variables which may trigger or contribute to these brown tides include elevated salinity arising from drought conditions during the preceding winter, pulses of rainfall delivering organic or micronutrients to the embayments, reduced grazing, and restricted flushing of the bays [3]. Analysis of macronutrient concentrations (nitrate, nitrite and phosphate) did not shown a marked difference between pre- and post-bloom conditions, suggesting that these inorganic macronutrients do not explain the increased incidence of brown tides in these embayments. One of the more intriguing hypotheses has been the role of iron and chelators in promoting the growth of *Aureococcus* [4, 6]. Initial estimates of the minimal iron required for *A. anophagefferens* were 9 μM, a level that is very high in comparison to most marine species and well above the levels (<1 μM) expected to be found in most inshore marine ecosystems [2]. Addition of iron to natural waters containing a bloom of *A. anophagefferens* markedly increased their growth rate, suggesting these organisms may be iron-limited *in situ* [8]. Here, we have reinvestigated the role of iron on the growth of *A. anophagefferens* and characterized a ferric chelate reductase activity that may be important for iron uptake in this organism.

MATERIALS AND METHODS

Cultures of *A. anophagefferens* CCMP1708 were obtained from the Provasoli-Guillard culture collection and grown in artificial seawater supplemented with f/2 nutrients [11] and 1 μM selenium [4]. Cells were grown at 20°C using a 14:10 light:dark cycle at 180 μE m^{-2} sec^{-1}. Growth was determined by *in vivo* fluorescence using a Turner Designs 10AU fluorometer and by visual cell counts under phase contrast microscopy. Large cultures were grown in acid-washed 12 L carboys. Smaller cultures (Figure 1) were grown in 28 ml acid-washed polycarbonate tubes that could be inserted directly into the fluorometer without opening. To remove contaminate iron, the salts and macronutrients used to prepare the artificial seawater were first passed through a Chelex 100 column [12]. Each culture series was done in triplicate and transferred through 5 successive culture cycles to dilute internal pools of iron.

Ferric chelate reductase activity was assayed using a modification of the method of Dailey and Lascelles [5]. Cells were centrifuged at 16,000 x *g* and the resulting pellet ground with sea sand in 100 mM Tris maleate pH 5.0 buffer using a mortar and pestle. The extract was centrifuged at 10,000 x *g* to remove cell debris and the supernatant mixed with twice its volume of saturated ammonium sulfate. Ferric chelate reductase was allowed to precipitate for 10 min on ice, centrifuged at 10,000 x *g* and the pellet resuspended in Tris maleate buffer. The final FCR assay mixture contained Tris maleate buffer (66.7 mM), 0.1 mM ferric HEDTA as an iron source, and 0.4 mM ferrozine. The reaction was initiated by the addition of NADH or NADPH (0.1 mM final concentration) and the absorbance measured at 562 nm for 10 min. Protein concentrations were determined

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using the Pierce BCA assay or via the method of Bradford.

RESULTS

Iron and the Growth of Aureococcus.

Key to studying the iron metabolism of *Aureococcus* is the ability to grow it in defined artificial seawater. In contrast to previously published reports [4], *Aureococcus* required relatively little iron to achieve maximum growth rates greater than 1 div day^{-1}. *Aureococcus anophagefferens* cultures grown on as little as 11 nM added iron gave maximum cell numbers (4-9 x 10^9 cell L^{-1}), maximum fluorescence (~850) and cell yields (average 170 mg per carboy) similar to that obtained in iron-replete (11 µM added iron) cultures (Figure 1). Similar results were obtained using EDTA as a chelator in both standard (2x) and trace metal buffered (100 µM EDTA) cultures [1]. Direct measurement of the iron contamination in solution (<10 nM) by graphite furnace atomic absorption allowed us to estimate an iron quota of ~5 amol Fe $cell^{-1}$ for *A. anophagefferens* [1]. Maximal growth rates were observed for all iron concentrations greater than 10-20 nM with no significant differences between the treatments. High iron concentrations (11 µM), if anything, slightly inhibited growth of the organism.

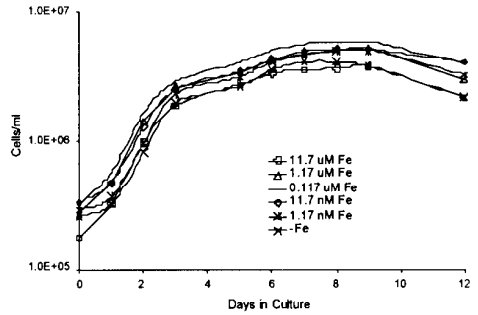

Figure 1. The growth of *A. anophagefferens* CCMP 1708 under different conditions of added iron using 2x NTA as a chelator. Points represent the average of triplicate cultures. Experiment illustrated was from the third transfer cycle through equivalent media. The coefficient of variance for the individual points ranged between 10-20%.

While little effect was observed on growth rate or cell yield under the differing amounts of added iron, marked differences were observed in the DCMU-enhanced *in vivo* fluorescence (Figure 2). Cultures containing less than 100 nM added iron showed a marked decrease in their DCMU enhanced fluorescence from a value of 0.6, characteristic of iron-replete cells, to a value near 0.3, characteristic of iron-deficient cells.

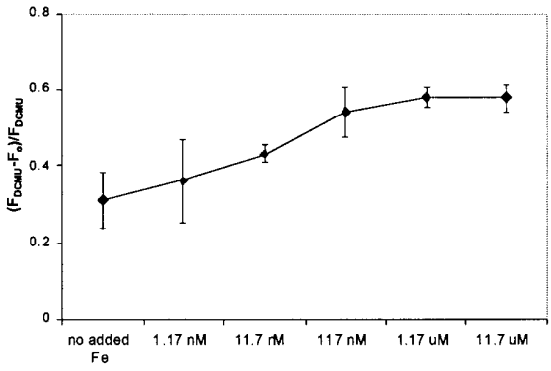

Figure 2. The DCMU-enhanced fluorescence for six different cultures of *A. anophagefferens* grown with different amounts of total iron. *In vivo* fluorescence was measured after a 30 min dark incubation, before (F_0) and after (F_{DCMU}) the addition of 10 µM DCMU to inhibit electron transport [6, 8]. DCMU enhanced fluorescence was calculated as (F_{DCMU}-F_0) / F_{DCMU} and is the average (± s.d.) of three replicates.

This difference was significant at the 95% confidence level. Similar results have been reported by Gobler and coworkers for iron-limited cultures and natural populations of *A. anophagefferens* [7] and by Greene *et al* for the iron-limited and iron-replete cultures of the chlorophyte *Dunaliella tertiolecta* and the diatom *Phaeodactylum tricornutum* [9].

Measurement of Ferric Chelate Reductase

The ability of *A. anophagefferens* to grow at maximal growth rates with low levels of added iron suggested that it might employ a highly efficient iron uptake mechanism. To determine how *Aureococcus* obtains its essential iron, we looked for both chelator (i.e. siderophore or strategy 2) and reductase (i.e. strategy 1) high-affinity iron-uptake systems [14]. No evidence was obtained for the presence of a siderophore-mediated system in iron-limited cultures using the Arnow assay for catechols, the Csaky assay for hydroxamate siderophores or an ^{55}Fe-binding assay (Boyer, unpublished). In contrast, both iron-replete and iron-limited cultures of *Aureococcus* showed strong ferric chelate reductase (FCR) activity (Figure 3). This activity was bispecific for NADPH and NADH with a Vmax of 0.56 (NADH) to 1.3 (NADPH) µmol min^{-1} mg^{-1} protein, and Km of ~400 µM for FeHEDTA and 10-20 µM for NAD(P)H. FCR from *A. anophagefferens* exhibited a broad pH optimum between 5.5 and 6.0 typical of most higher plant ferric chelate reductases [10]. It was not affected by nitrogen source, indicating it was distinct from the nitrate reductase activity previously characterized in cultures of *Aureococcus*. [Nichols and Boyer, submitted].

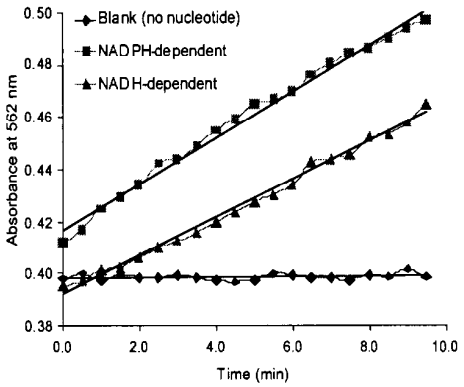

Figure 3. NAD(P)H-dependent ferric chelate reductase activity in iron-replete extracts of *A. anophagefferens*. Early experiments using crude extracts showed a strong non-enzymatic reduction of iron. This background reduction could be dramatically reduced by quickly precipitating the FCR activity with 67% ammonium sulfate prior to being assayed.

The effect of iron availability on FCR activity in a representative culture is shown in Figure 4. NADPH-dependent FCR was the predominate activity in iron-replete (i.e. > 1 μM) cultures. This activity showed only a slight to no increase under iron-limiting conditions. In contrast, NADH-dependent FCR was the minor activity under iron-replete conditions but increased 2-fold in cultures without added iron, often to a level greater than the NADPH-dependent form. These results suggest that there may be two distinct FCR activities present in *Aureococcus*: a constitutive NADPH activity and an inducible NADH-dependent activity. Further characterization of the role of these enzymes is currently in progress.

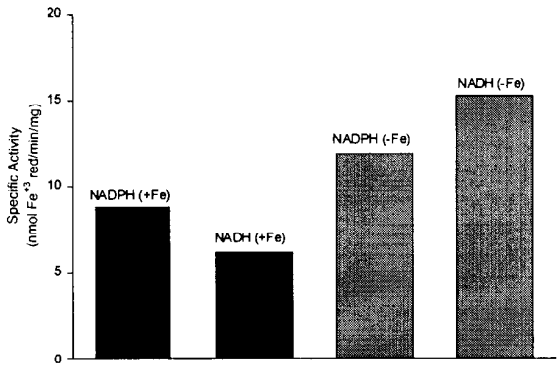

Figure 4. The effect of iron availability on the nucleotide specificity and FCR activity in *Aureococcus anophagefferens* CCMP 1708 under iron replete (>1 μM, +Fe) and iron-limited (no added iron, -Fe) conditions.

FCR activity in Natural Populations.

To test if FCR activity was also present in natural populations of *A. anophagefferens*, 1 L water samples were collected from four different stations during two separate brown tide events and shipped on ice overnight to Syracuse for analysis. The FCR activity was assayed in cell-free extracts (Table 1). The results from the 1998 and 1999 samples are not directly comparable due to a change in methodology between the two years. In 1999, the FCR activity was partially purified by precipitation with ammonium sulfate to eliminate or decrease background reduction. This purification led to a higher FCR activity per protein, and lower FCR activity per cell for the 1999 samples than was observed by direct assay of crude extracts in 1998. The 1998 bloom was also a spring event whereas the brown tide occurred during the winter of 1999. In both years, the FCR activity measured during bloom events was high relative to what was normally observed in culture.

DISCUSSION

Aureococcus can achieve maximum growth on iron concentrations as low as 100 nM. At concentrations below 100 nM, changes are observed in the Fv/Fm ratio, but not necessarily in the growth rate or cell yield. We conclude that *Aureococcus anophagefferens* CCMP 1708 has a requirement for iron similar to that observed in oceanic species (~ 5 amol cell^{-1}). Once cultures had been acclimated to different chelators, both NTA and EDTA readily supported growth. No stimulation using citrate was observed as previously reported [4].

Aureococcus has both a constitutive (NADPH) and an inducible (NADH) ferric chelate reductase activity similar to that observed in higher plants and other algae. We believe this activity is part of a high-affinity iron uptake mechanism to obtain the iron needed for growth (Figure 5). The presence of this activity explains our inability to iron-limit cultures using trace-metal buffered media containing 100 μM EDTA [1]. This high EDTA concentration would readily serve as a substrate for FCR, providing ample iron to maintain maximum growth rates in excess of 1 div day $^{-1}$.

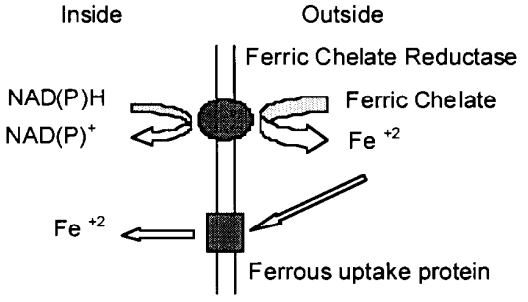

Figure 5. The role of ferric chelate reductase in a high affinity iron uptake mechanism. The ferrous uptake protein is postulated based on other algae and higher plants. It has not been established for *Aureococcus*.

Table 1. Ferric Chelate Reductase activity in natural populations of *Aureococcus anophagefferens*. Values are in nmol Fe reduced per min per mg protein or nmol Fe reduced per min per 10^9 cells.

Station (date)	Cells liter^{-1}	NADPH Activity		NADH Activity	
		nmol Fe mg protein^{-1}	nmol Fe 10^9 cell^{-1}	nmol Fe mg protein^{-1}	nmol Fe 10^9 cell^{-1}
180 (5/5/98)*	3.6×10^8	46.11	4.5	23.91	2.35
180 (5/18/98)	1.3×10^8	18.91	4.6	14.91	4.00
130 (12/22/99)*	1.6×10^9	435.9	0.070	0.17	0.000028
160 (12/22/99)	1.5×10^9	545.3	0.083	237.9	0.037
180 (12/22/99)	8.5×10^8	326.1	0.092	613.8	0.17
190 (12/22/99)	1.4×10^9	295.7	0.062	606.9	0.13

* 1998 experiments were run on crude extracts without ammonium sulfate precipitation. In 1999, the FCR was partially purified by precipitation with 67% ammonium sulfate prior to assay.

Using our estimated iron quota of 5 amol cell^{-1}, a bloom density of 10^9 cells L^{-1} would require ~5 nM iron. Given a division rate 1 div day^{-1}, a ferric chelate reduction rate of 0.003 nmol min^{-1} 10^9 cells^{-1} would be required to support this growth rate. The observed FCR rates (Table 1) are well in excess of this requirement and should easily supply sufficient iron to support growth at its maximal rates at low cell densities. It is unlikely that the initiation of brown tides is limited by iron. However, even with a low iron quota, the iron requirements of large blooms in excess of 10^{10} cells L^{-1} could be limited by the available iron concentrations in the Peconic system (100-200 nM dissolved Fe) [8]. Whether the maintenance and demise of brown tides is limited by iron depends on what iron is available to *A. anophagefferens* during the latter stages of bloom formation. Work in progress suggests that ferric chelate reductase from *Aureococcus* may have a broad substrate specificity (Szmyr, unpublished). This could potentially make iron available to the cell from a variety of natural sources, including particulate iron. It may also be important in recycling iron from natural sources.

ACKNOWLEDGMENTS

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EFFECTS OF IRON AND MANGANESE CONCENTRATION AND THEIR RATIO ON CELL GROWTH AND CYLINDROSPERMOPSIN PRODUCTION OF THE CYANOBACTERIUM *CYLINDROSPERMOPSIS RACIBORSKII*

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ABSTRACT

The control of toxic cyanobacterial blooms is of great importance to public and environmental health. *Cylindrospermopsis raciborskii* is one of the common potentially toxic cyanobacteria found in Queensland, Australia. In this study the effects of manganese (Mn) and iron (Fe) concentration and their concentration ratios on cell growth and production of the hepatotoxin cylindrospermopsin (CYN) by *C. raciborskii* were assessed in batch cultures.

Manganese was neither limiting nor inhibiting to growth and CYN production of *C. raciborskii* at concentrations ranging from detection limit (2 μg L^{-1}) up to 2.3 mg L^{-1}. Additionally, no Fe limiting conditions were induced at elevated Mn concentrations up to approx. 1.5 mg L^{-1} Mn. Growth limitation due to Fe limitation occurred at concentrations of about 10 μg L^{-1} or lower. A linear relationship existed with a slope of unity between the specific cell division rate constant (μ_c) and the specific CYN production rate constant (μ_{CYN}) when Fe was limiting. This finding suggests that CYN production was indirectly controlled by the effect of Fe limitation on the rate of cell division and not through any direct effect on metabolic pathways of CYN production.

INTRODUCTION

Cylindrospermospis raciborskii, a potentially toxic cyanobacterium is common in Queensland freshwater lakes and reservoirs. Little is known regarding the nutrient requirements of and factors affecting the production of CYN by *C. raciborskii*. Manganese and iron are essential nutrients at low concentrations but have the potential to be toxic at high concentrations. Mn and Fe have been found at high concentrations in waterbodies in Queensland [1] which has led to speculation regarding a potential link between abundance of these nutrients and *C. raciborskii* occurrence. Cyanobacterial toxins have also been considered as potential siderophores, this hypothesis regarding the role of cylindrospermopsin is examined here.

The objective of this study was to determine Fe and Mn requirements and tolerance of *C. raciborskii* and the effect of Mn and Fe concentrations and their ratios on cell growth and CYN production in *C. raciborskii* grown in batch cultures.

METHODS

Batch culture experiments were conducted with *C. raciborskii* (AWT 205/1 strain) grown in Jaworskii's Medium (JW) [2] with modification of Fe and Mn concentration. The cultures were grown at a constant temperature of 25(±2)°C, with continuous cool white fluorescent illumination of 81 (±3) μE $m^{-2}s^{-1}$. The study consisted of two experiments focussing on (i) the manganese tolerance of *C. raciborskii* and (ii) the effect of elevated Mn concentration (i.e. Mn toxicity) with respect to the Fe concentration on *C. raciborskii.*

1. Mn tolerance experiment

For the Mn tolerance test JW was modified to Mn concentrations of 0, 0.077, 0.193, 0.386, 0.772 and 1.93 mg L^{-1} Mn in the medium. Each sample (triplicates for each treatment) was inoculated with 1 mL of *C. raciborskii* stock culture resulting in a starting concentration of about 100 trichomes mL^{-1} of *C. raciborskii.* Three times per week sub-samples were collected for cell enumeration, CYN quantification and nutrient analysis.

2. Fe limitation and the effect of Mn toxicity with respect to [Fe] on C. raciborskii

The Fe limiting concentration of *C. raciborskii* was examined with particular emphasis on the effect of elevated Mn concentration on the Fe requirements. Modified JW was prepared in triplicate with Fe concentrations of 0, 6.8, 17, 34, 68 and 340 μg L^{-1}. Manganese was added resulting in Fe:Mn concentration ratios of 5.7, 11, 57 and 118. These media were inoculated to a final cell concentration of approximately 220 trichomes mL^{-1} of Fe and Mn starved *C. raciborskii.* Samples were collected twice weekly and the samples from the three replicates formed a composite sample which underwent cell enumeration, CYN analysis and nutrient analysis.

3. Cell enumeration, CYN analysis and Fe and Mn analysis

For cell enumeration samples (3 mL) were preserved in Lugol's solution, then dispensed into glass Sedgewick–Rafter chambers and counted using phase contrast microscopy. The exponential growth rate constant μ_C and doubling time (T_2) were calculated from

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Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J. (eds)
Intergovernmental Oceanographic Commission of UNESCO 2001

the slope of the linear regression of a plot of the logarithm cell number versus time.

For CYN analysis, 1 mL of sample was repeatedly frozen and thawed, subsequently filtered (0.22 μm) and analysed on a HPLC coupled with a tandem mass spectrometer, (LC-200 series pump, series 200 autosampler and API 300 MS/MS with turbo ionspray interface; Perkin Elmer Sciex Instruments, Thornhill, Ontario, Canada). The transition from the $(M + H)^+$ ion (416 m/z) to the fragment ion at 194 m/z was used for quantitation using the multiple reactant monitoring mode (method described in [4]). The CYN production rate constant μ_{cyn} and CYN production per cell (Q_{cyn}) for the log and stationary phase were calculated from the slope of a plot of ln [CYN] versus time and from [CYN]/cell concentration respectively.

For analysis of Fe and Mn concentration, 5 mL of culture media were filtered (0.45 μm) and analysed using Atomic Absorption Spectroscopy (AAS) (Z-8000 Zeeman, Hitachi Ltd Tokyo, Japan.)

RESULTS AND DISCUSSION

Mn tolerance experiment

For this experiment, Mn concentrations were adjusted from 0 (no Mn added to the media) to approximately 2 mg L^{-1}, these concentrations were confirmed by AAS. The Mn concentration in the media decreased from day 10 to day 20 of the experiment as can be seen in (Fig 1a), which corresponds with the end of the exponential phase and the onset of the stationary phase in all cultures (Fig 1b). The growth of all cultures, irrespective of the Mn concentration, were statistically similar and only two phases were observable (i.e. exponential phase, until day 12 and stationary phase from day 12–38). The specific cell division rate constant, μ_c during the exponential phase was 0.84–0.88 day^{-1}, giving a doubling time of 19–20 hrs.

CYN concentration over the experimental period followed similar trends in all Mn concentrations (Fig 1c). CYN concentration at the start of the experiment was below the detection limit but the concentration increased significantly at late exponential phase (day 7 to day 15). During this period the specific CYN production rate constant was 0.16–0.2 day^{-1} therefore the CYN concentration doubled every 3.5–4 days. After day 15 the CYN concentration continued to rise at a slower rate, to a mean CYN concentration of approximately 40–60 μg L^{-1} after 38 days when the experiment was terminated.

The data from the Mn tolerance experiment suggest that neither cell growth nor CYN production was inhibited by either Mn toxicity or limitation in any of the treatments.

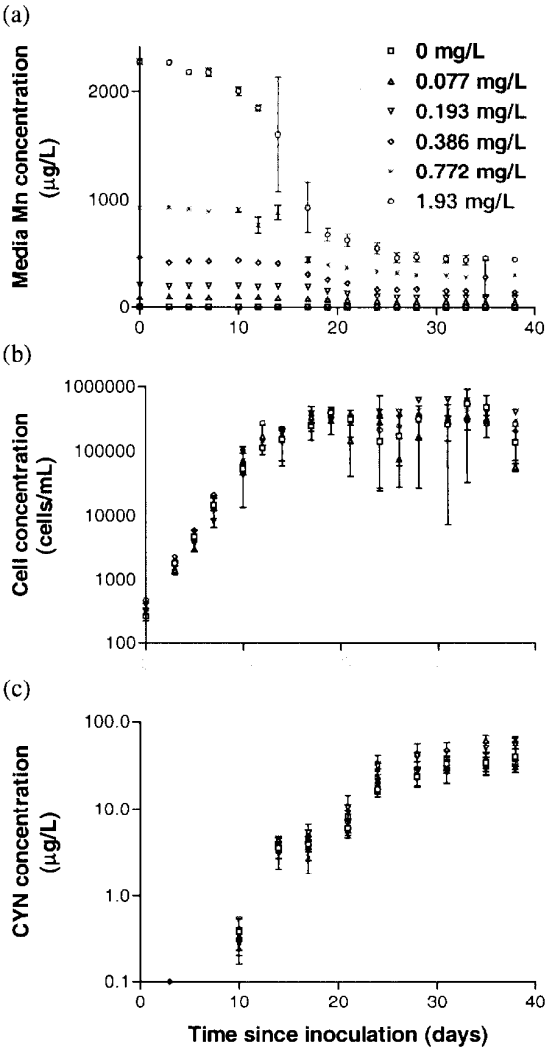

Fig. 1. (a) Mean Mn concentration in growth media, (b) mean cell numbers, and (c) mean CYN concentration for the Mn tolerance experiment with various Mn treatments, error bars indicate standard deviation.

In the treatment with no additional manganese, it is possible that ultra-trace amounts of this element were present in the media below the detection limit of 2μg L^{-1}, suggesting that the minimum manganese concentration required for growth is very low. This compares to a surface water [Mn] of 10–200 μg L^{-1} in a typical Queensland reservoir [5].

Previous research has shown that elevated manganese concentrations can inhibit growth of cyanobacteria[6,7,8]. These results demonstrate that Mn concentrations greater than 200 μg L^{-1} did not effect growth or biomass yield of *C.raciborskii* which is in contrast to findings for *Microcystis aeruginosa* which experienced a decreased growth rate with 200 μg L^{-1} of Mn [8].

During the exponential growth phase, the specific Mn accumulation rate in the highest Mn

treatment and the specific cell division rate constant were similar (0.8–0.9). During cell enumeration morphological differences between trichomes in different treatments were observed during the mid stationary phase. The trichomes growing in the higher Mn concentration media appeared relatively short and thickened when compared with trichomes growing in the normal media (i.e. 0.386 mg L^{-1} initial [Mn]). Also an increased number of akinetes (~4/trichome) and more pronounced cell pinching were observed in the media with elevated Mn concentration (Fig 2a) compared to those growing in normal JW media (Fig 2b).

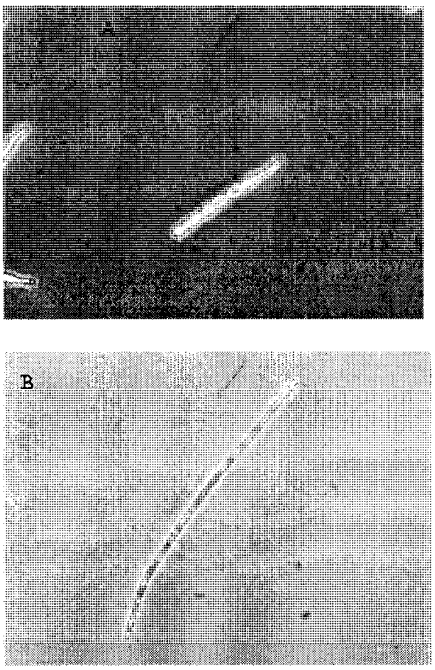

Fig. 2. Micrographs (both x400, phase 3 contrast) at day 21 of the experiment. (A) *C. raciborskii* trichome grown in 1.93 mg/L Mn treatment and (B) *C. raciborskii* trichome grown in 0.193 mg/L Mn treatment

It may be possible that the increased akinete formation is a stress response to elevated Mn as akinetes are often produced in large numbers by senescent populations. The factors responsible for akinete induction remain obscure, with induction in some species of cyanobacteria being triggered by phosphate deficiency or by a lack of light in other species [9].

Fe requirements of C. raciborskii

The Fe requirements were tested at initial Fe concentrations ranging from 0–68 μg L^{-1} plus a control, standard JW, [Fe] = 340 μg L^{-1}. The 0 added Fe and 6.8 μg L^{-1} Fe treatments were found to have an initial [Fe] of 9 and 11 μg L^{-1} respectively when confirmed by AAS. These concentrations fall close to the limit of detection of the method of 10 μg L^{-1}. Despite precautions such as using acid washed glassware and AR grade chemicals for media preparation some Fe carryover was inevitable.

As with the Mn experiment, the Fe concentration decreased sharply from the start of the stationary phase (day 12–15). Despite the fact that Fe was detectable at day 0 and up to about day 10 in the 0 Fe treatment, cell growth was substantially inhibited in this treatment from the start of the experiment. Cell numbers declined after day 4 and no cells were detected at day 10 in the 0 Fe treatment. Also in the 6.8 μg L^{-1} Fe treatment and to a lesser extend in the other low Fe treatments growth was slower and the stationary phase was reached later when compared with the control (Fig 3a). The doubling time during the exponential phase ranged from 18 hrs in the control treatment to about 29–35 hrs in the treatments with 17–68 μg L^{-1} to 44 hrs in the 6.8 μg L^{-1} treatment. The total cell yield at stationary phase in all treatments except the 0 Fe treatment were similar.

A more pronounced effect of Fe limitation was observed on the CYN production rather than cell growth. No CYN was detected in the 0 Fe treatment. A trend of increasing CYN production with increasing Fe supply at experiment start was indicated by the data (Fig 3b). The rate of CYN production was greater in expt. 2 when compared with expt. 1 with CYN doubling rates between 16 hrs in the control and 41 hrs in the 6.8 μg L^{-1} treatment.

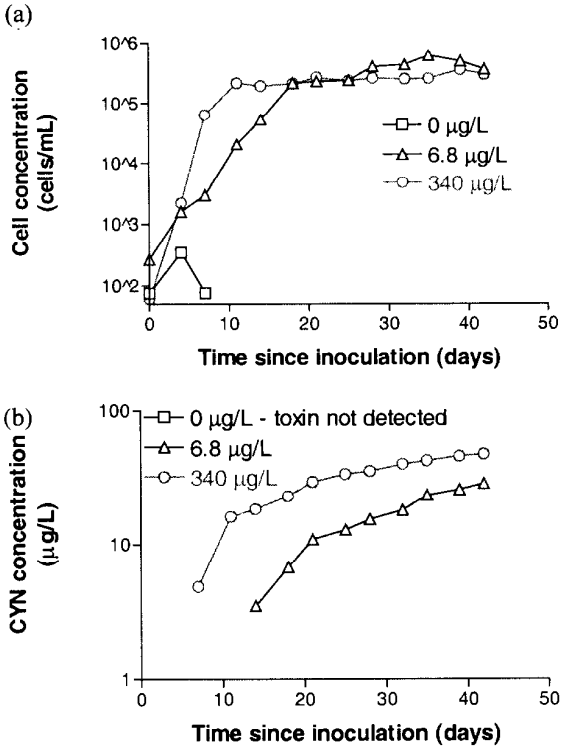

Fig. 3. Cell concentration versus time (a) and CYN concentration versus time (b) for the 0 and 6.8 μg L^{-1} Fe treatments and the control treatment (340 μg L^{-1} Fe).

A plot of the specific CYN production rate constant versus the specific cell division rate constant calculated from the Fe limitation study is shown in Fig.

4. The data indicates a linear relationship exists between μ_C and μ_{CYN}. A slope of close to 1 and an intercept close to 0 may be interpreted as a good indication that CYN production is controlled by cell production. Previous work on *M. aeruginosa,* [10] shows a similar relationship under nitrogen limiting conditions, and it was concluded by the authors that microcystin production in *M. aeruginosa* is a function of cell division under growth limiting conditions and therefore only indirectly influenced by environmental factors

CYN production was not stimulated by Fe deficiency as may be expected if CYN had the Fe accumulation function of a siderophore. Further support of this hypothesis was obtained from a direct chemical assay of CYN's Fe chelating ability, which demonstrated that the Fe chelating capacity of CYN is too low to be classified as a siderophore [11]. Additionally, CYN production was not directly inhibited under Fe limiting conditions which would be expected if Fe is directly involved in CYN synthesis.

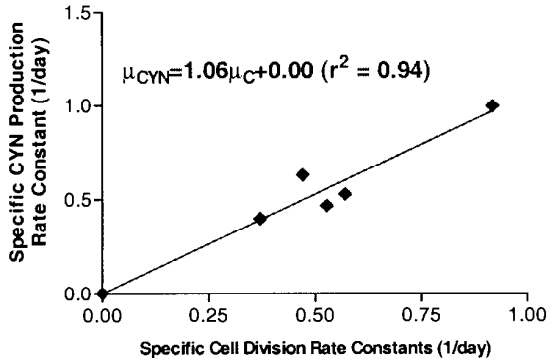

Fig. 4. Plot of the specific CYN production rate constant μ_{CYN} versus the specific cell division rate constant μ_C for the data from expt 2. (Note that μ_C and μ_{CYN} were obtained for the period with maximum growth and CYN production respectively).

Mn toxicity with respect to [Fe] on C. raciborskii

Previous studies have indicated that Mn is capable of inducing Fe deficiency symptoms in cyanobacteria by a competitive mechanism[12], thus it is important to study these metal requirements in parallel. To assess Mn toxicity in relationship to Fe limitations, treatments with 17 μg L^{-1} and 34 μg L^{-1} Fe were replicated, with Mn concentration increased by a factor of 10 (from 0.193 to 1.93 mg L^{-1}). No growth inhibition was observed in these treatments. In contrast, increased cell proliferation was observed at higher initial Mn concentrations (and Mn:Fe ratios) (Fig. 5), and CYN concentration followed a similar pattern (data not shown). As observed in expt. 1 morphological changes to *C. raciborskii* were evident at elevated Mn concentrations. Over the range of Mn:Fe ratios examined here the elevated Mn concentration was unable to induce symptoms of Fe deficiency, typified by growth inhibition.

Results of expt 1 did not show any inhibition of growth or CYN production *C. raciborskii* over the range studied <2μg L^{-1}–2 mg L^{-1} indicating that this species has wide tolerance limits for this trace element. The minimum Fe requirement, demonstrated by significantly decreased growth and decreased CYN

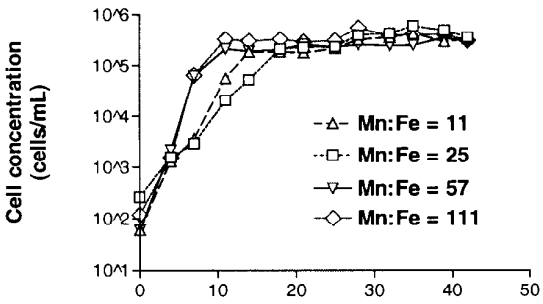

Fig. 5. Plots of cell concentration versus time for treatments with relatively low Fe concentration and low and elevated Mn concentrations.

production constant was 9 μg L^{-1}. Additionally these results support the hypothesis that CYN is not a siderophore and that CYN production is only indirectly controlled by environmental factors via their effect on cell growth.

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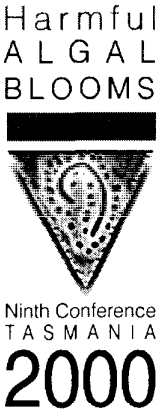
BIOTRANSFORMATION OF TOXINS



PARALYTIC SHELLFISH TOXINS IN THE DINOFLAGELLATE *ALEXANDRIUM TAMARENSE* AND THE MUSSEL *MYTILUS EDULIS* FROM CHINHAE BAY, KOREA IN THE SPRING OF 1996 AND 1997

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ABSTRACT

The toxicity and the toxin profiles of *Alexandrium tamarense* and the mussel *Mytilus edulis*, were investigated weekly at four stations in Chinhae Bay during the spring of 1996 and 1997. *Alexandrium tamarense* was more abundant at Stn M1 in 1996 and Stn C2 in 1997, while the density of total phytoplankton was always higher at Masan. The population of *A. tamarense* always comprised a larger proportion of the total phytoplankton at Chilchundo than at Masan. The relationship between the mussel toxicity and *A. tamarense* abundance was significant, although the time lag between the maximum in mussel toxicity and *A. tamarense* abundance shifted about 1-2 weeks. The toxicity of the mussel was always much higher in Chilchundo than in Masan. The cellular toxicity of *A. tamarense* in size-fractionated phytoplankton (10-100 μm) was higher at Masan than at Chilchundo in 1997. In addition, the toxicity of the mussels might be related to the relative proportion of *A. tamarense* abundance to the total phytoplankton abundance, and the ingestion rate of the *A. tamarense* by the mussels. The size-fractionated phytoplankton showed toxin profiles containing only C1-C2 at two stations in 1997, while the toxin profiles in *M. edulis* consisted of 10 components including C1-C2, GTX1-5, dcSTX, STX and neoSTX. These results show that the toxicity of *M. edulis* had clearly originated from *A. tamarense* blooming in Chinhae Bay, while biotransformation probably caused a relative increase in carbamate toxins in the mussels.

INTRODUCTION

In April 1986, a poisoning incident occurred, for the first time, in Kamchon Bay near Pusan due to the consumption of the mussel *Mytilus edulis* [1]. Since then, extensive routine surveys of PSP toxicity have been carried out [2,3]. Han *et al.* [4] first identified the occurrence of the toxic dinoflagellate *Alexandrium tamarense* in Chinhae Bay, and then, *A. tamarense* blooms have be observed to be seasonal, recurrent, and the most common cause of outbreaks of PSP in Chinhae Bay. The toxicity and toxin profiles of *A. tamarense* and mussels from Chinhae Bay were determined [5,6]. However, until now, most studies have been descriptive of the toxicity and toxin profiles of *A. tamarense* and mussels for only a short time. The objectives of the present study were to investigate the relationships between the toxicity and toxin profiles between *A. tamarense* and the mussel *M. edulis* at four stations in Chinhae Bay in the spring of 1996 and 1997, and to understand in detail the poisoning of the mussel *M. edulis* in Chinhae Bay.

MATERIALS AND METHODS

Stns M1 and M2 in Masan Bay were located at the inner part of Chinhae Bay. Many shellfish culture farms are established at Stns C1 and C2 at Chilchundo (Fig. 1). Weekly observations were conducted at Stn M1 and Stn C1 in Chinhae Bay from 15 March to 31 May 1996, and at Stn M2 and Stn C2 from 7 March to 29 May 1997.

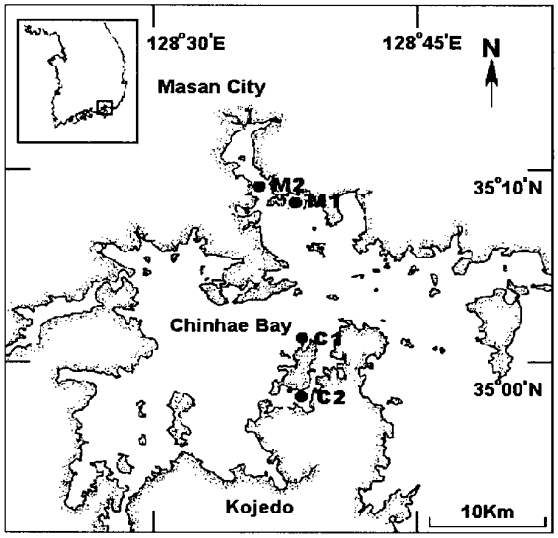

Fig. 1. Sampling stations in Masan Bay (Stns M1 & M2) and at near Chilchundo (Stns C1 & C2) in Chinhae Bay, Korea in the spring of 1996 and 1997.

Sampling of phytoplankton including A. tamarense

Phytoplankton samples including *A. tamarense* were collected weekly from a depth of 2 m using a Van-Dorn sampler. The samples (300 ml) were preserved with 3 ml of 25 % glutaraldehyde. Fixed samples were concentrated by settling, and cells of *A. tamarense* were counted. Dissection of thecal plates using a 5 % sodium hypochlorite solution under a biological microscope (Carl Zeiss, Axioplan) was used to identify *A. tamarense.*

Collecting the mussel M. edulis and size-fractionated phytoplankton

The mussel *M. edulis* (30 individuals on average) were collected weekly, from a depth of 2 m, at four stations during the period. The mussels were frozen, transported to the laboratory, and stored at –30 °C until analysis.

Water samples for size-fractionated phytoplankton were collected weekly, from a 2 m depth, at two stations

from 7 March to 29 May 1997. Twenty liters of seawater were fractionated between 10 and 100μm with nylon nets. The size-fractionated subsamples were harvested by centrifugation (VS - 4000, Vision Co.) and stored frozen (-20 °C).

Extraction of toxins

Extracts prepared from the digestive glands of the mussels were passed through a Sep-Pack ODS cartridge column which had been pre-washed with 10 ml of methanol and water, and the elute between 1.5 and 2.0 ml was applied to an ultrafiltration kit (Ultrafree C3LGC, Millipore) and a 5,000 g centrifugation.

The harvested fractionated phytoplankton was suspended in 0.5 N acetic acid (10 times the volume of the pellet volume of the harvested cells) and sonicated for 1 min. (Nihonseiki UH-50 sonicator). The supernatant obtained by centrifuging the cell suspension at 1,000 g was then applied to the ultrafiltration kit. Each 10 μl of the filtrate was then used for analysis.

Bioassay and toxin analysis

Mouse bioassay of mussel *M. edulis* extracts was performed with the modified method of Association of Offical Analytical Chemists (A.O.A.C) using ICR mice (male, 18-20 g). Toxin profiles of strains of *A. tamarense* were determined with a fluorometric HPLC system according to Oshima [7] with slight modifications. HPLC consisted of a high pressure pump (Vintage 2000 LC, OromTech, Korea), C18 Column (Rainin, U.S.A.), a double head reaction pump (Tsp, U.S.A.), and a fluorescence detector (Orom Tech FL-300, Korea).

RESULTS

Toxicity of M. edulis and the number of A. tamarense

Fig. 2 shows cell density of *A. tamarense* and the toxicity of *M. edulis* in 1996. In both stations, the toxicity in mussels was high when *A. tamarense* was abundant. The maximum toxicity of the mussels was quite different between the two stations, 22 MU g^{-1} d.g. at Stn M1, and 560 MU g^{-1} d.g at Stn C1 on 10 May 1996. The toxicity in most samples of the mussel at Stn C1 exceeded 4 MU g^{-1}, and in general was much higher than that at Stn M1, while the abundance of *A. tamarense* in May was higher in Stn M1 than in Stn C1.

In 1997, *A. tamarense* occurred from 20 March to 1 May at Stn M2, and from 27 March to 12 April at Stn C2 (Fig. 3). In this study, the toxic dinoflagellate in size-fractionated phytoplankton was identified as *A. tamarense* except for that on 15 May at Stn C2 when the highest toxicity of size-fractionated phytoplankton was detected but *A. tamarense* did not occur that day. The high toxicity in the mussels might have had a relation to the high abundance of *A. tamarense* at both stations, whereas the time lag between the maximum value of the mussel toxicity and the maximum value of *A. tamarense* abundance was about 1-2 weeks. However, the toxicity of the mussels was much higher at Stn C2 (377.3 MU g^{-1} d.g. maximum) than in Stn M2 (13.9 MU g^{-1} d.g. maximum).

Toxin profiles of size-fractionated phytoplankton (A. tamarense) and M. edulis

The toxin profile of the size-fractionated phytoplankton included only C1 and C2 at two stations in 1997. In the mussel, the toxin profile consists of 10 components, C1-2, GTX1-5, dcSTX, STX and neoSTX (Fig. 4). The major toxins were the GTX group, comprising 80 % of the total toxins. Among the GTX group, GTX1+4 comprised a larger proportion than GTX2+3 at both stations.

DISCUSSION

The toxicity of the mussels, *M. edulis* was measured by the mouse assay method in 1996 and 1997. The toxicity of the mussels was in concordance with *A. tamarense* blooms in both years. However, a time lag of 1-2 weeks of between the maximum toxicity of *M. edulis* and maximum abundance of *A. tamarense* was observed; except for that at Stn C1 in 1996, where the maximum toxicity of the mussel and maximum abundance of *A. tamarense* occurred spontaneously. The time lag between toxic dinoflagellate blooms and toxification of mussels has often been reported in previous studies [10,11]. Thus, the results suggest the density of dinoflagellates affected the toxicity of the mussels in Chinhae Bay in 1996 and 1997.

Alexandrium tamarense was the only identified PSP species among size-fractionated phytoplankton in 1997. The toxicity of the fractionated phytoplankton and *A. tamarense* abundance at two stations did not exactly coincide with each other. The toxicity of size-fractionated samples was recorded before or after maximum abundance of *A. tamarense* was observed, and a comparatively high peak of toxicity was shown on 15 May at Stn C2, whereas *A. tamarense* did not occur. Another toxic species of *Alexandrium* possibly occurred on that day, however the taxonomy of the species is beyond the scope of this study (personal observation). The variability in the toxicity of a single isolate is generally attributed to differences in the rate of toxin production or accumulation under different growth conditions, not caused by any differences in toxin composition [8,9].

The toxicity of the mussels was variable between the areas: much higher in the stations near Chilchundo in both years. The toxicity of the mussels in Chilchundo was much higher than that in 1989 (21 MU g^{-1}), whereas that in Masan was almost the same as in 1989 [4]. The maximum value of the toxicity, 560 MUg^{-1} at Chilchundo in 1996 was also higher than those reported in Japan, China, and Indonesia [12]. Generally, the toxicity in a given mussel was reported to vary as a function of dinoflagellate cell density, strain toxicity, and environmental parameters [13]. Greater toxicity of the mussels near Chilchondo in 1996 may be not only due to the higher toxicity of cultured strains, but also may partially reflect a predominance of more potent carbamate toxins compared to the strains at Masan Bay which typically contain higher proportions of the less

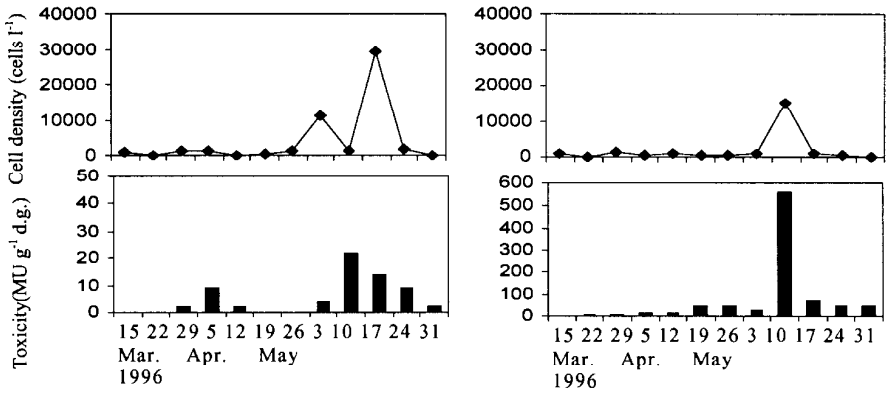

Fig. 2. The number of *Alexandrium tamarense* (cells l^{-1}) and toxicity of the mussel *Mytilus edulis* (MU g^{-1} d.g.) at 2m depth at Stns M1 and C1 in Chinhae Bay, Korea in the spring of 1996.

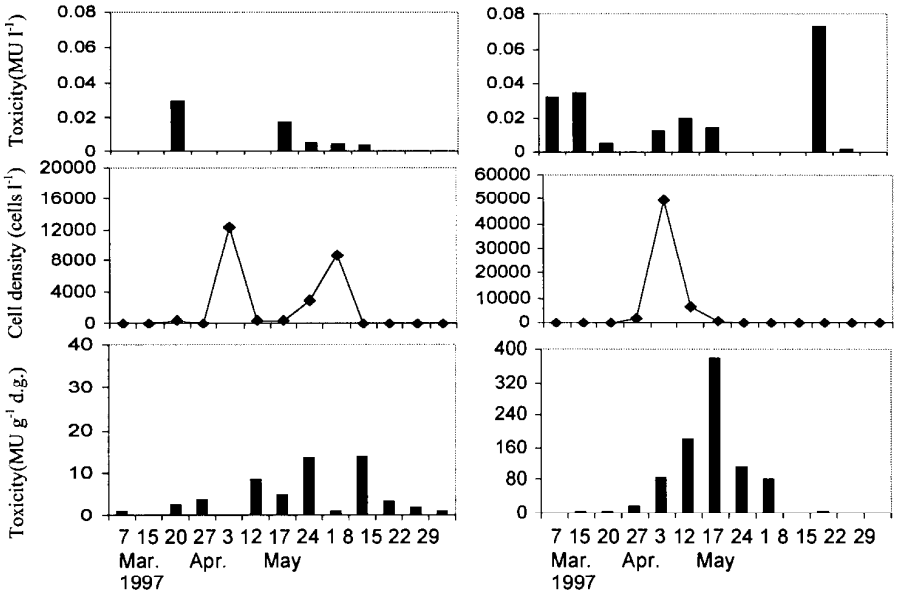

Fig. 3. Toxicity of size-fractionated phytoplankton (MU l^{-1}), the number of *Alexandrium tamarense* (cells l^{-1}) and toxicity of *Mytilus edulis* (MU g^{-1} d.g.) at 2m depth at Stns M2 and Stn C2 in Chinhae Bay, Korea in the spring of 1997.

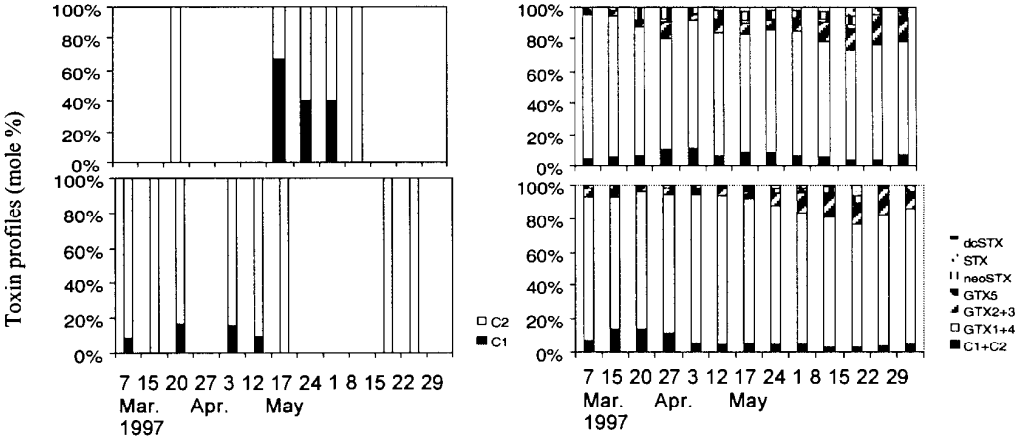

Fig. 4. Toxin profiles (mole %) of size-fractionated phytoplankton and Mytilus edulis at 2m depth at Stns M2 and C2 in Chinhae Bay, Korea in the spring of 1997.

toxic N-sulfocarbamoyl toxins. However, this explanation is still uncertain, because the toxicity of the size-fractionated phytoplankton in 1997 were low or undetectable, while the toxicity of the mussels was different between the two stations. At Stn M1 and M2, the total standing stocks of phytoplankton were much higher than at Stns C1 and C2, thus the portion of A. tamarense was higher at Stns C1 and C2. It is expected that the ingestion rate of the mussels on A. tamarense at Stns C1 and C2 might be higher than Stns M1 and M2, which led to the higher toxicity of the mussels at Stns C1 and C2. However, the influence of the ingestion rate of M. edulis on toxic accumulation is difficult to assess under field conditions [14], and the feeding kinetics of the mussels were beyond the scope of this study. However, the feeding behavior of mussels must be studied for better understanding of the relationship between mussels and toxic dinoflagellate in Chinhae Bay.

The toxin profiles of the mussels in 1997 showed little variation between two stations. The major toxin composition of the mussels, M. edulis was the GTX group, comprising 80 % of the total toxins. The profiles of this study were similar to those of mussels in 1989, while mussels collected in 1990 showed completely different toxin profiles in which neoSTX comprised nearly half of the total toxins [5]. The toxin composition of M. edulis differed from that of the fractionated phytoplankon in which C1 and C2 were the only analyzed toxins. Sullivan et al. [15] suggested that toxin compositions in bivalves might differ from the cells they feed on. The changes in toxin composition in mussels over time may result from enzyme-mediated toxin catabolism, chemical equilibration in the digestive system, and/or selective uptake and elimination of various toxin components. Bioconversion of PSP toxins in mussels contaminated by other dinoflagellate species has also been reported [13]. The relative increases in carbamate toxins, particularly STX in mussels compared to dinoflagellates has been interpreted as evidence of biotransformation. In the present study, the toxin composition of the mussels contains carbamate toxins such as GTX1+4, STX, and neoSTX. It is suggested that the difference in PSP toxins in the mussels M. edulis and that of A. tamarense results from bioconversion.

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PSP TOXIN PRODUCTION OF URUGUYAN ISOLATES OF *GYMNODINIUM CATENATUM* AND *ALEXANDRIUM TAMARENSE*

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ABSTRACT

It has been well documented by the National Monitoring Programme on HAB and Mussel Toxicity in Uruguay that *Alexandrium tamarense* was present during paralytic shellfish poisoning (PSP) episodes along the coast of Uruguay in the late winter/early spring of 1991, '92, '93, '95 and '96 while *Gymnodinium catenatum* was present during PSP events in the late summer/early fall of 1992, '93, '94, '96 and '98. Cultures of these two dinoflagellates, established from the mouth of the Rio de la Plata, have been analyzed for saxitoxin and its derivatives by high performance liquid chromatography (HPLC) for the first time. These toxin profiles have been compared to shellfish extracts taken during four different toxic booms. There is a strong similarity between the toxin profiles of the shellfish collected in September 1991 and August 1993 and cultured *Alexandrium* extracts whereas the best agreement between shellfish extracts, from March of 1993 and 1994 is with cultured *G. catenatum* extracts. These results demonstrate for the unequivocally that PSP in Uruguayan shellfish can arise from two different dinoflagellate species in two different seasons. This has clear implications with respect to the design and operation of an effective monitoring program.

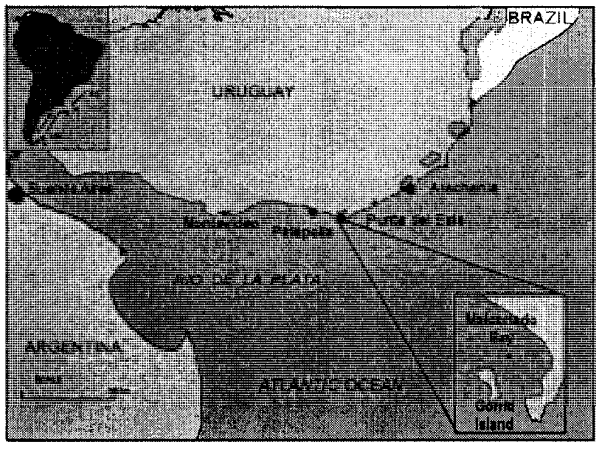

Fig.1. Map of the study area showing sample sites. Shellfish and phytoplankton samples collected at Piriápolis, Punta del Este and Arachania; sediment samples collected at Maldonado Bay (lower insert). The Subtropical Convergence Zone is seen in the upper insert.

INTRODUCTION

The coastal area influenced by the nutrient-rich waters of the Rio de la Plata (Fig. 1) is a very productive habitat [1]. Since the 1980's, impacts caused by frequent harmful algal blooms (HABS) have had a significant effect on the local economy due to decreased fishery and tourist revenues and the public's negative perception of seafood safety [2,3,4]. In 1980 the first toxic outbreak was recorded [5,6] and in the years since then, recurring episodes of PSP in molluscs at times of *Alexandrium tamarense* and *Gymnodinium catenatum* blooms have been recorded. There have, however, not been any detailed toxin analyses from these events.

MATERIAL AND METHODS

Study area

The Rio de la Plata (Fig. 1) is the second largest South American basin, draining an area of approximately 3,170,000 km^2 that extends into five countries. Salinity and temperature of this water body fluctuate dramatically through the year with salinity ranging between 5 and 32 g/L^{-1}, and the annual temperature between 8 and 25 °C. [7]. Seasonal winds, in combination with currents within the Subtropical Convergence Zone (Fig. 1), have a major influence on the Rio and its phytoplankton population dynamics [8,9].

Cultures

Shortly after blooms of *A. tamarense* and *G. catenatum*, sediment samples were collected from the Bay of Maldonado near Punta del Este (Fig. 1). Two clonal cultures of *G. catenatum*, GCURD5 and GCURF11, and three clonal cultures of *A. tamarense*, ATUR01, ATUR01B and ATUR02, were established from germinated cysts isolated from these sediments. Upon reaching mid-exponential growth, duplicate cultures were extracted for PSP toxins with both mild, 0.5 M acetic acid (HOAc) to preserve to original cellular toxin composition, and 0.1 M hydrochloric acid (HCl), heated to convert the N-sulfocarbamoyl toxins to their more potent carbamate derivatives.

Shellfish

Blue mussels, *Mytilus edulis*, were harvested during blooms of *A. tamarense* from the Punta del Este station in the Bay of Maldonado on 4 September 1991 (*Mytilus* 1) and 26 August 1993 (*Mytilus* 3). *Donax hanleyanus*,

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Intergovernmental Oceanographic Commission of UNESCO 2001

a commensal clam, and *M. edulis* were each collected from the Arachania and Piriapois stations on 2 March 1994, (*Donax* 4) and 24 March 1993 (*Mytilus* 2) respectively during blooms of *G. catenatum* (Fig. 1). All shellfish samples were processed according to the standard method of the Association of Official Analytical Chemists (AOAC) [10]. Because there was incomplete hydrolysis of these extracts during the original processing, a subsample was mixed 1:1 with 0.2 N HCl and rehydrolyzed.

All samples were analyzed by HPLC [11] for the saxitoxins using a modified three-step isocratic elution method [12]. External standard solutions, kindly provided by Y. Oshima (Tohoko University, Sendi, Japan), were run prior to the commencement of sample analysis and after every fourth sample. The terms GTX1,4, GTX2,3, C1,2 and C3,4 are used to represent the pooled concentrations of two toxins so as to account for possible epimerization. Toxicities (in STX equivalent $cell^{-1}$) were calculated from molar composition data using individual potencies [11].

RESULTS AND DISCUSSION

Bloom details

Alexandrium tamarense favors the cooler waters (8-18 °C) of the late winter, early spring season with maximum cell densities of over 30,000 cells L^{-1} witnessed in each of the two blooms reported here. Bloom initiation and subsequent enhancement may result from transport of the cells from southern, offshore waters coupled with local *in situ* growth. These same phenomena may also give rise to spring blooms of *A. tamarense* along the coast of Argentina [13, 14].

Gymnodinium catenatum has reached cell densities of over 60,000 cells L^{-1} when warmer water temperatures (19-24 °C) prevail during the late summer and early fall months of the southern hemisphere which are consistent with distributions of this species along the Argentine coast [15, 16]. However, blooms of *G. catenatum* in the Rio de la Plata are considered to be more localized events, as seedbeds of *G. catenatum* cysts, which contain more than 400 cells cc^{-1} sediment, have been discovered in the nearshore area (17, 18).

Toxin content and composition: cultures

HPLC analysis of acetic acid extracts of the two dinoflagellate species reveals that the two *G. catenatum* cultures, on average, had the greatest amount of toxin per cell on a molar basis, (193 vs 70 $fmol/cell^{-1}$) but when the potency of these same extracts is considered, the overwhelming proportion, up to 96 mole percent of the N-sulfocarbamoyl derivative toxins C1-4, GTX5 and GTX6 which is typical for *G. catenatum* strains worldwide [19, 20]. The more potent carbamate toxins such as GTX1, 4 and GTX2, 3 that were found in the *A. tamarense* cultures are only detected in very low levels in the *G. catenatum* extracts (Fig. 2a). While the *Alexandrium* cultures had significant levels of the gonyautoxins, ranging from 24 to as much as 47 mole

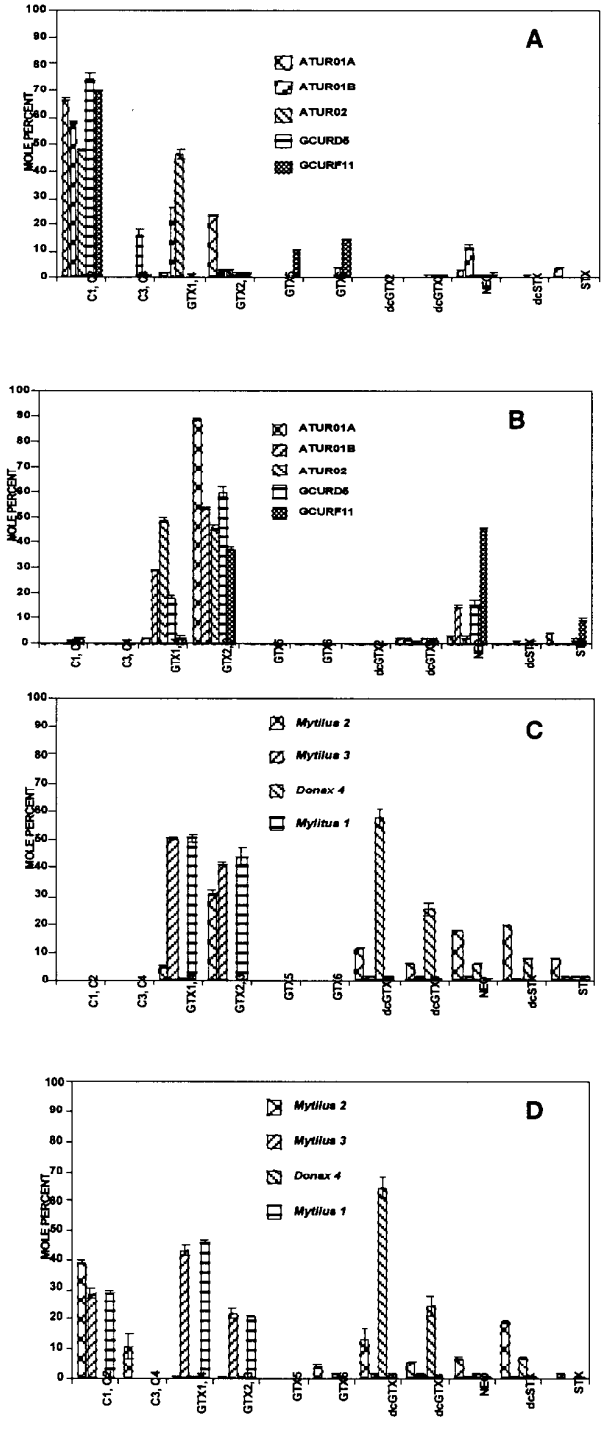

Fig. 2. Mole percent toxin composition of dinoflagellate and shellfish extracts. (A) Culture acetic acid extracts; (B) Culture hydrochloric acid extracts; (C) Original shellfish extracts; (D) Rehydrolyzed shellfish extracts.

Alexandrium isolates are seen to be more toxic (15292 vs. 12200 fgSTX equiv. $cell^{-1}$). The lower toxicity values associated with higher molar concentrations of toxin in the *G. catenatum* cultures result from an percent, they were still dominated by the low potency C1 and C2 toxins, 49–67% (Fig. 2a). Lesser amounts of NEO and STX were contained in two of the three *Alexandrium* strains (<5%) however, ATUR01B was found to have 11% NEO (Fig. 2a).

The toxicity of all these cultures changes if they are subjected to hot acid treatment that converts the less potent N-sulfocarbamoyl derivatives to their highly toxic analogues. Following these preparations, the *G. catenatum* cultures still contained an elevated molar concentration of toxin, but were now much more potent on a cellular basis than the *A. tamarense* cultures, 76,660 vs. 21,221 fgSTX equiv. $cell^{-1}$. This is an increase in *G. catenatum* toxicity of over 600% from the original acetic acid extracts resulting from the creation of high amounts of GTX1,4 and GTX2,3 and NEO following hydrolysis (Fig. 2b). Gonyautoxins 2,3 and 1,4 dominate the *A. tamarense* hot acid extracts with combined values ranging from 83 to 95 mole percent (Fig. 2b).

Toxin composition: shellfish

Prior to rehydrolysis, all of the shellfish extracts, with the exception of sample *Donax* 4, harbored significant levels of the N-sulfocarbamoyl derivative toxins, up to a maximum of 53%, presumably due to incomplete hydrolysis of the sample during the initial processing (Fig 2c). Sample *Donax* 4, collected during a *G. catenatum* bloom, was unique in that it was chiefly composed of decarbamoyl toxins, primarily dcGTX2, prior to, and after rehydrolysis.

The other shellfish sample taken during a *G. catenatum* episode, *Mytilus* 2, was dominated by the low potency N-sulfocarbamoyl derivatives (53%) while also having significant amounts of decarbamoyl toxins GTX2, GTX3 and STX (Fig. 2c). As would be expected, GTX 2,3 and 1,4 as well as NEO mole percents all increased with rehydrolysis of this sample (Fig. 2d).

The two samples, *Mytilus* 1 and *Mytilus* 3, harvested during the *Alexandrium* blooms, had strikingly similar toxin profiles. In the original extract, toxins C1,2, GTX1,4 and GTX2,3 were found to compose over 94 mole percent of the total toxin profile with 29% arising from C1,2 (Fig 2c). After rehydrolysis, toxins C1,2 were converted to GTX2,3 with its concentration increasing from about 21% to 42% of the total molar toxin composition while the percentage of toxins GTX1,4 increased marginally to about 50% of that total (Fig. 2d).

Toxin composition comparisons, cultures vs. shellfish

The fact that the shellfish samples were not completely hydrolyzed by the AOAC extraction procedure is rather disconcerting from a public health and regulatory standpoint, but it proved to be an asset in comparing the toxin profiles of the shellfish and the phytoplankton culture extracts. While the agreement between the toxin profiles of the cultures and the shellfish is not perfect, there is sufficient similarity in the case of shellfish samples *Mytilus* 1, *Mytilus* 2 and *Mytilus* 3 and the cultured dinoflagellate extracts to establish correlations. These are supported by the phytoplankton observations made when the shellfish samples were collected.

The original extracts of shellfish samples *Mytilus* 1 and *Mytilus* 3 had profiles dominated by toxins C1,2, GTX1,4 and GTX2,3. Toxin profiles of *A. tamarense* cultures ATUR01A, ATUR01B and ATUR02 were also dominated by the same epimer groups although the percent composition of each epimer pair in the shellfish and cultured samples were somewhat different (Fig. 2a,c). This discrepancy in mole percent toxin composition can be explained by compositional shifts resulting from metabolic processes (bioconversion) within the shellfish [21, 22, 23, 24] and by the conversion of the low potency N-sulfocarbamoyl derivative toxins to their more potent carbamate forms by hot acid treatment [25]. While there is no way of knowing what the original unhydrolyzed toxin profiles of the dinoflagellates which rendered these mussels toxic was, these data suggest that they are most similar in composition to the cultured *A. tamarense* and not *G. catenatum* from those waters.

The two shellfish samples collected during *G. catenatum* blooms are quite different from one another and only one, *Mytilus* 2, is similar in composition to either of the two *G. catenatum* cultures based on the high proportions of toxins C1,2, C3,4, and GTX6. The toxin profile of shellfish sample *Donax* 4 was dominated by decarbamoyl toxins GTX2, GTX3 and STX and the only indication that *G. catenatum* may have rendered this sample toxic are the low levels of GTX6 (<2 mole percent), that are seen only in the *G. catenatum* extracts, and are found in this sample. These low concentrations of GTX6 are validated by the rehydrolysis data, which reveal an increase in NEO concentrations that coincides with the concentration of GTX6 measured in the original extract. While specific mechanisms for the transformation of toxins in *Donax* have not been investigated, one can postulate that *in vivo* enzymatic decarbamoylation of toxins C1,2 or GTX2,3, described in the other bivalve species occurred resulting in the high percentage of decarbamoyl derivatives [26, 27, 28]

SUMMARY

This paper describes for the first time toxin profiles of two causative organisms responsible for PSP events in Uruguay and links these profiles to those of toxic shellfish harvested during blooms of the two dinoflagellates.

The blooms of *A. tamarense* appear to be restricted to the late winter/early spring seasons and are initiated by the cold current transport of cell populations from southern offshore waters. These cells have toxin profiles rich in the high potency gonyautoxins but contain less

toxin per cell on a molar basis than the *G. catenatum* isolates.

Gymnodinium catenatum is dominated by low potency N-sulfocarbamoyl toxins that are converted to their highly toxic carbamate derivatives by hot acid hydrolysis. Blooms of this species occur during the late summer/early fall season when water temperatures are significantly warmer than those measured during *A. tamarense* blooms. High numbers of *G. catenatum* cysts within the Rio de la Plata suggest that these blooms maybe a result of localized cyst germination events.

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DINOPHYSIS ACUMINATA DISTRIBUTION AND SPECIFIC TOXIN CONTENT IN RELATION TO MUSSEL CONTAMINATION

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ABSTRACT

The lack of correlation between *Dinophysis* cell density and mussel toxin contents is understandable since very often, an instantaneous measure is compared to an integrated value. The dinoflagellate spatial heterogeneity and a possible variability in specific toxin content make the establishment of a quantitative relationship difficult. These different sources of variance have to be estimated and separated.

In situ mussel contaminations were carried out in the Bay of Seine (English Channel) during an oceanographic cruise. Batches of mussels *Mytilus edulis* attached to a rope were immersed at three different dephs (about -2, -5, -8 m tidal average) at 5 stations on both sides of the Seine plume. The area was sampled during the cruise to describe the distribution of *Dinophysis* throughout the bay and in the water column. Five days after immersion, mussels were collected and analyzed for their toxin contents. *D.°acuminata* was mainly present in the upper mixed layer though the maximum cell density was observed at the pycnocline with *D. acuminata* (64,000 cell.l^{-1}) representing 90% of the *Dinophysis*. Toxin contents in mussels showed a significant decrease with depth. This result shows that the toxicity level in mussels mirorred the average distribution of *Dinophysis* in the bay. In parallel, an analysis of *Dinophysis* spp. enriched fractions collected during the cruise revealed variable A.O. cell concentrations of 3 to 50 pg AO.cell^{-1} (cells did not contain DTX1). During the following year, a red-tide *Dinophysis* bloom (up to 1.5.10^5 cell.l^{-1}) occurred in late summer and although the experiment could not be repeated entirely, a number of mussel samples were collected and *Dinophysis* concentrated fractions were analyzed. AO concentrations were very low in all these phytoplankton samples and was undetectable in mussels.

In conclusion, *Dinophysis* spp. distribution in the water and variability of its toxicity must be taken into account in order to improve the precision of toxic phytoplankton monitoring and the management of shellfish surveying.

INTRODUCTION

Besides toxin content in mussels, the French phycotoxin network monitors phytoplankton communities. Every year, *Dinophysis* spp. blooms producing diarrheic shellfish toxins (DST) are followed in relation to DST contamination. However, no clear relation has been found between the presence and density of toxic algae and shellfish toxicity. Mussels may contain toxins when there are few or no *Dinophysis* spp. in the water, and vice versa. This preliminary study reports on the feasibility of a monitoring system involving moorings of mussels in a selected site for early detection of DST events. Two cruises conducted in Seine Bay at times when *Dinophysis* blooms were likely to occur, studied the heterogeneity of the spatial distribution of the algae and the variability in their toxin content. A mussel contamination test was carried out *in situ*. Results are compared to the *Dinophysis* spp. distribution in the zone. Concentrates of *Dinophysis* spp. were analysed to estimate the toxicity of the cells.

MATERIALS AND METHODS

Two cruises (Dinoseine I and II) took place in July 1994 and 1995. Batches of 4 to 5 kg of calibrated mussels (4 to 5 cm length), coming from a DST-free area as checked by control analysis, were immersed at two or three levels, depending on the depth of the water, at four stations on the north and south sides of the mouth of the Seine River. The mussels were removed six days after immersion (Fig.1 a,b, for the position of the stations and Table 1 for the depth of the batches).

An onboard computer system for data and sample acquisition allowed real-time description of the water column according to the following parameters: temperature, salinity, light intensity, fluorescence, particle load and particulate size distribution (30 classes between 0.7 and 400 m). This profiler was used to determine sampling depths. Two liter syringe samples were obtained at desired depths, *e.g.* in relation to the position of the thermocline. Samples were then filtered through a 20 m filter made up to 100 ml and fixed with 1ml Lugol s solution.

Dinophysis concentration: Pumping depth from a hose attached to the profiler, was also determined on the basis of data relative to particle measurements (proximity of the thermocline, fluorescence, particle size distribution). The concentration technique, as described by Maestrini *et al.* [1], used a peristaltic pump which delivered a known volume through two successive screens (100 and 20° m). A precise amount of the enriched fraction was carefully collected from the 20 m screen: 10 ml were removed under gentle stirring for precise evaluation of the *Dinophysis* spp. concentration, and the rest was filtered on a 10 m filter stored in methanol, for further analysis.

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Cell counts of Dinophysis spp.: An aliquot of the sample was counted in a 10 ml sedimentation chamber, after dilution if required. Variability in cell density per liter was estimated at 12% for a mean density ca. 1.000°cell.l^{-1}.

Preparation for analysis: The digestive glands of mussels were dissected and immediately frozen. Extraction was performed according to the protocol of Lee *et al.* [2]. For phytoplankton concentrates, the 10 m filters were washed several times with methanol in an ultrasound bath, and the methanolic fractions were extracted three times with dichloromethane after addition of water. The dry extracts were stored at -18¡C until analysis.

Analytical method: The dry extracts of mussel digestive glands and phytoplankton concentrates were treated for HPLC analysis according to the method of Lee *et al.* [2]. The relative error measured on four replicates extracts of mussel and phytoplankton concentrates was respectively 10% and 13%.

RESULTS

The first field trip in July 1994 coincided with a large bloom of *Dinophysis acuminata.* However the *D.°accuminata* bloom in 1995 occurred in August, so the contamination experiment during the July cruise provided negative results since mussels were not contaminated. However, concentrates of phytoplankton from different depths were performed during the bloom in August and all the mussel samples from the monitoring network were analysed according to the above described procedure.

Distribution of Dinophysis spp. in the zone: The distribution maxima for *Dinophysis* spp. (Fig. 1c), in July 1994, showed a high density point with around 50,000 cells per liter, which is exceptional for this species. The two stations for mussels, III and IV, were in a high concentration zone (from 30,000 to 50,000 cells per liter). An East-West transect through the zone indicated that maximal cell concentrations were close to the surface [between 3 and 6 m (Fig. 1d)] i.e. in the layer influenced by river freshwater input at the interface of the salinity/density gradients (Fig. 1f). During the 1995 cruise, no proliferation of *Dinophysis* spp was observed.

Mussel contamination: Mussel digestive glands contained only okadaic acid (OA). Table 1 presents the concentrations in mussels and *D. acuminata* cell densities in the vicinity of the mussel batches. As might be expected, the comparison of the results of mussel toxicity with the spatial distribution of the maxima for *Dinophysis* spp. in the study zone (Fig.1 c,d,e) shows that maximum mussel contamination (21 to 24 g OA .g^{-1} of digestive gland for two sub-samples in the batch) corresponded to a high *Dinophysis* concentration at station III (35,420 cell.l^{-1}). This value was relatively high. It is noteworthy that a nearly two-fold difference between the contamination of surface and deeper batches was recorded at all stations except at the station I. Water sampling during the five days of contamination in the zone clearly showed a great heterogeneity of the distribution of *Dinophysis* in the water column, with density maxima in sub-surface waters (-2 to -6 m; Table°1). In 1995, results were obtained from the monitoring survey samples. They were all negative except two samples whose concentrations were 0.6 g OA.g^{-1} (the 16th August) and 1.2 g OA.g^{-1} of digestive gland (the 6th September), while the maximum *Dinophysis* concentrations reached 42,000 cells.l^{-1}, the 22th August.

Concentrate toxicity: In all concentrates, the genus *Dinophysis* was largely represented by *D. accuminata. D. skagii* and *D. rotundata* were seldom observed. The concentrates without *Dinophysis* were toxin-free (neither okadaic acid nor DTX1). The results for the 1994 and 1995 concentrates are summarized in Table 2. It may be noted that okadaic acid concentrations were very low in 1995: this observation was consistent with the very low mussel toxicity, observed by the network.

CONCLUSION

These two cruises enabled us to illustrate the patchiness in *Dinophysis* spp. horizontal and vertical distribution. High concentrations in relatively thin layers of water (some tens of centimeters, Fig. 1d). makes the detection of toxic algae by daily samples collected with a standard bottle, difficult. Moreover, due to the great variability in the toxin content of algal species, the presence of cells even at high concentration, may have little relationship to shellfish toxin content. However, the toxicity of mussels is indicative, for a given period, of the density variations in *Dinophysis. D. accuminata* has been shown elsewhere to contain variable okadaic acid concentrations depending on year and season [3], [4], [5]. The reported range of variation is similar to that observed by us: 0 - 57.7 pg OA per cell [3]. The absence of any relation between *Dinophysis* cell density and shellfish toxicity is a concern for network managers [6]. These authors proposed a monitoring scheme based on the mooring of mussels in risk sector. The present study shows that this scheme can provide good results, but that the choice of the site depends on preliminary knowledge about the hydrodynamics of the zone and certain logistic requirements such as feasibility of anchoring the immersed system and the facility of access, that limit its generalized use.

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Table 1: Mussel contamination after five days of immersion and concentration of *Dinophysis* spp. in the vicinity of the mussel batches. Results from both cruises in July 1994 and July 1955 (ND: under the detection limit).

Stations	Depths (m) of mussel batches or water samples		Mussel toxicity g OA.g^{-1} DG		Concentration of *Dinophysis* spp.: Cells.l^{-1} in water samples close to the stations
	July 1994	July 1995	July 1994	July 1995	July 1994
I	-2	-2	1.5	ND	
	-6	-6	2.2	ND	15,764 - 2,399
	-16	-16	0.8	ND	
II	-1.5	-1.5	11.3-14.0 (*)	ND	12,757
	-3.5	-5	8.8	ND	5,286
	-6 to -7	-	5.0	-	950
	-13	-10	-	ND	760
III	-2 to —4	-1	21-24	ND	28,000 - 9,515 - 35,420
	-4 to —6	-4	15.7-19.5	ND	2,950 - 8,140
	-8.8	-8	7.8-9.0	ND	2,560 - 1,260
	-12	-	-	-	1,400
IV	-2 to —3	-1	7.9-9.7	ND	26,730 - 5,730 - 17,380
	-3 to —6	-4	3.8	ND	28,230 - 930 - 950
	-11.4	-	-	-	232
	-16 to -18	-	-	-	460 - 380

Table 2: Toxicity of phytoplankton fractions enriched in *Dinophysis* spp. cells (*D. acuminata*). Results of methanolic fractions (in duplicate). (**) Analyses performed by M. Quilliam (NRC, Halifax) on the same extract.

Dinoseine 1994 (July 15-21)			Dinoseine 1995 (determinations on August 25)	
Stations Depths (m)	Concentration (pg of OA.cell^{-1})		Depth	Concentration (pg of OA.cell^{-1})
DSA 4 (-4.7 m)	3.7-4.4	8.9 (**)	-1	0.20-0.13
DSA 5 (-4.7 m)	6.3-6.9	11.8 (**)	-4	1.38-0.63
			-6	0.52-0.47
DSA 26 (-4m)	43-50.6	53.8 (**)	-9	0.69-0.36
			-12	0-0
			-21	0.24-0.68

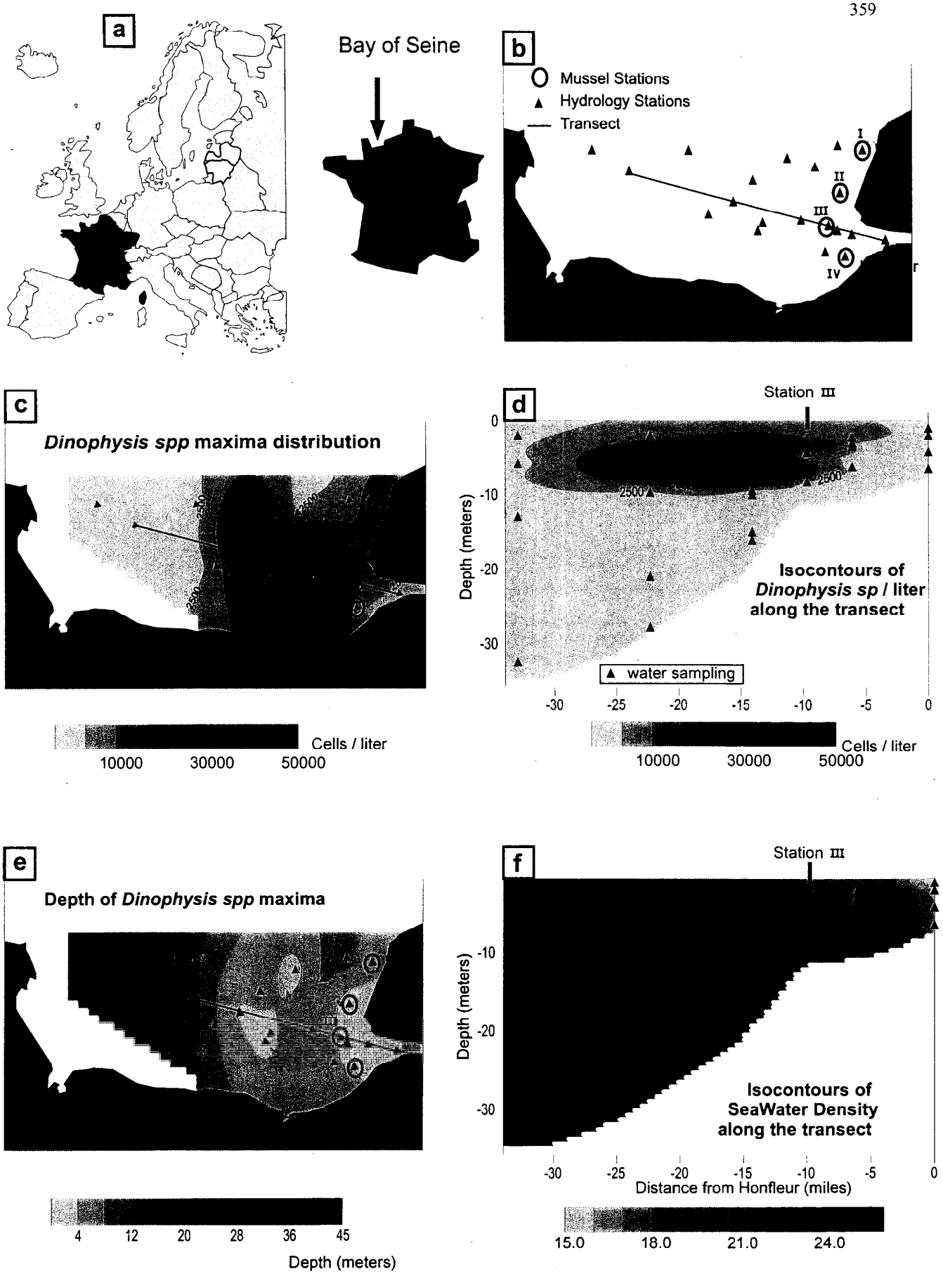

Fig. 1 : DinoSeine I (July 1994) : [a] Map of the study area ; [b] Mussel and hydology stations locations;

[d] [f] : *Dinophysis* spp distribution and density along the transect in Seine Bay;

[c] [e] : *Dinophysis* spp maxima distribution in the area and according to the depth.

TOXIN CONTENT OF *DINOPHYSIS ACUMINATA, D. ACUTA* AND *D. CAUDATA* FROM THE GALICIAN RIAS BAJAS.

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ABSTRACT

Dinophysiw acuminata, D. acuta, and *D. caudata* co-occur during early autumn DSP outbreaks in the Galician Rías Bajas, and their contribution to shellfish toxicity needs to be established. HPLC–FD analyses of single cell isolates during different events confirmed that *D. acuta*, with an OA:DTX2 ratio of 3:2, is the source of DTX2 toxin detected in Galician mussels, and that either *D. acuta* or *D. acuminata* can be the main contributors to autumn toxic outbreaks. Our results suggest that *D. caudata* has trace amounts of OA (<1 pg · cell^{-1}) and confirm the large variability in toxin content in *D. acuta* and *D. acuminata* during different seasonal outbreaks, or even during the same outbreak after natural populations were maintained in the laboratory for 5-9 days. The risk to human health of using a "*Dinophysis* index" as substitute information for the standard mouse bioassay in the early detection of diarrhetic shellfish toxicity is discussed.

INTRODUCTION

Species of *Dinophysis* cause recurrent DSP outbreaks in Galicia and long closures of bivalve marketing in a region with an annual production over $2 \cdot 10^5$ t of mussels [1-3]. *D. acuminata* is very persistent and may cause DSP outbreaks from May to October or even the whole year. *D. acuta* and *D. caudata* are more seasonal and associated with DSP outbreaks from September to November. *D. acuminata* and *D. acuta* can reach concentrations of $1\text{-}2 \cdot 10^4$ cell · l^{-1}, whereas *D. caudata* occurs in moderate concentrations rarely exceeding 10^3 cell · l^{-1}. Large variations in bivalve toxicity related to *Dinophysis* spp concentrations are attributed partly to interspecific and intraspecific variations in toxin content of *Dinophysis* cells (Table I). The main objectives of this study were: i) To obtain the toxin profile and the toxin content per cell in different species of *Dinophysis* causing DSP outbreaks in Galicia; ii) To investigate the presence of conjugated forms of OA (diol-esters and DTX4); iii) To evaluate the contribution of each species during toxic outbreaks associated with multispecific populations of *Dinophysis*; iv) To find possible relations between toxin content per cell and different morphotypes of *D. acuminata* occurring throughout the year.

METHODS

Vertical and oblique plankton net-hauls (20-100-µm fraction) were made during different DSP events in the Galician Rías. Single cell isolation of *D. acuminata, D. acuta* and *D. caudata* was made by microcapillary manipulation under an inverted microscope (25X, 100X). Cells were transfered several times through drops of filter-sterilized seawater (Millipore, 0.22 µm), and finally filtered on preheated (400°C, 6h) Whatman glass fibre filters (1.2 µm, 2cm). For analyses of whole population extracts, net-hauls were filtered on board and inmediately kept in the deep freeze. An aliquot was taken and fixed with neutral formaldehyde for species quantification. Table II shows the number of cells isolated for each analysis. In the case of *D. acuta*, cells were isolated on the day of collection, and 5 and 9 days after being maintained in a culture chamber (L:D 14:10, 15°± 1°C). Most samples were analyzed following the procedure of Lee et al. [4] with slight modifications. A double extraction samples of *D. acuminata* from June'98 the boiling and the freeze/thaw procedures developed by Quilliam et al. [5] for *Prorocentrum lima* cultures were applied: the first method,

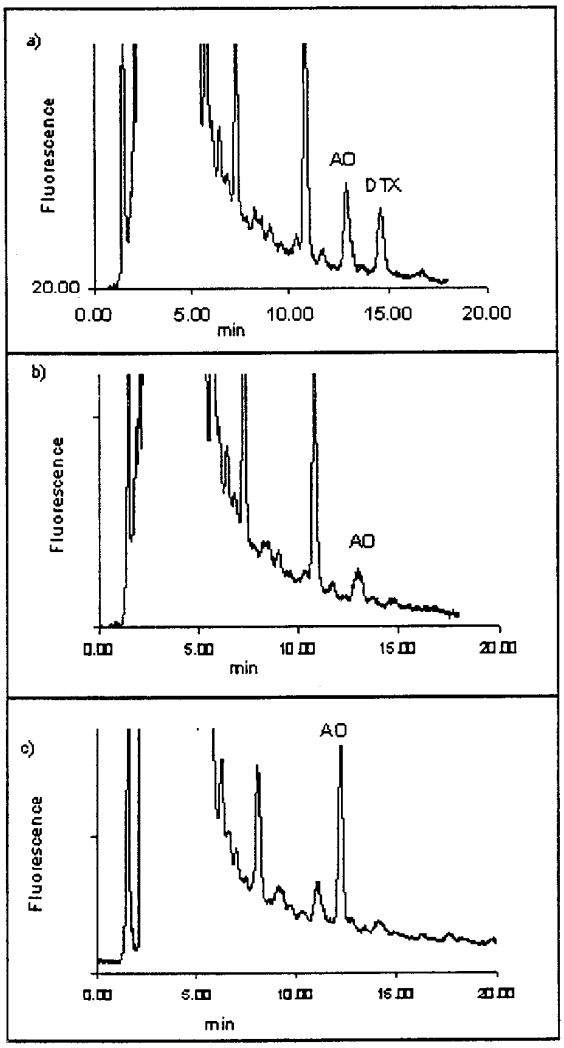

Figure 1 HPLC chromatograms obtained from extracts of a) *D. acuta*; b) *D. acuminata*; and from c) OA standard.

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Table I. Concentration of diarrhetic shellfish toxins (OA and DTXs) in natural populations of *D. acuminata* and *D. acuta* from different parts of the world (nd = not detected).

Species	Locality	Toxin	Toxin content (pg · cell^{-1})	Sampling Procedure	Ref.
D. acuminata	Le Havre, France	OA	1.6	Single cell isolation	[4]
	Bay of Tokyo, Japan	OA	Traces		
	Gulf of St. Lawrence, Canada	OA	25.5	Single cell isolation from net-hauls kept in the laboratory	[13]
	Gulmar Fjord, Sweden	OA DTX1	1.1- 23.2 nd - 0.2		[14]
	Galicia, Spain	OA	1- 37	Average toxicity estimations from multiple regression analyses of net-haul extracts.	[2]
	Denmark	OA	nd - 40	Average toxicity estimations from net- haul extracts	[15]
D. acuta	Galicia , Spain	OA	9.4	Single cell isolation	[4]
	Cork, Ireland	Total OA DTX2	136 58 78		[7]
	Gulmar Fjord, Sweden	Total OA DTX1	4 - 21 nd -14 nd - 7	Single cell isolation from net-hauls kept in the laboratory	[14]
	Galicia, Spain	Total OA DTX2	0.4 – 430 0.6 – 94 0.5 – 169	Average toxicity estimations from multiple regression analyses of net-haul extracts	[2]

Table II. Toxin content (pg · cell^{-1}) in single cell isolates from natural populations of different species of *Dinophysis* from the Galician Southern Rías (total toxin content per cell and OA:DTX2 ratio are in bold).

Species	Sampling Date	Isolation Date	No. of cells isolated	N° of cells injected	Toxin Content (pg · cell^{-1})
D. acuminata	18.06.98	06.98	461	201	**9.9** OA
			491	199	**7.9** OA
	8.09.98	9.09.98	857	246	**21.7** OA
D. acuta	9.10.97	10.10.97	1250	308	**10.7** 6.3 OA 1. DTX2 **OA: DTX2 = 3:2**
	9.10.97	14.10.97	1214	412	**55.1** 33.1 OA 22.0 DTX2 **OA: DTX2 = 3:2**
	9.10.97	16.10.97	534	175	**24.1** 18.4 OA 5.7 DTX2 **OA:DTX2 = 3:1**
D. caudata	9.10.97	10.10.97	1388	342	**0.73** OA

Table III. Estimated toxin content (pg · cell^{-1}) per cell of *Dinophysis* from net-hauls where *D. acuta* was the overwhelmingly dominant *Dinophysis* species

***Dinophysis* cells in the filters**	**Sampling Date**	**Toxin Content (pg · cell^{-1})**
D. acuta (507934); *D. acuminata* (75044); *D. caudata* (5300)	28.08.90	2.6 OA:DTX = 1.6
D. acuta (26928); *D. caudata* (528)	2.10.90	4.0 OA: DTX2 = 1.5
D. acuta (45425); *D. caudata* (7820); *D. rotundata* (115)	22.10.90	0.6 OA: DTX2 = 1.5

to destroy the enzymes of *Dinophysis* cells and prevent hydrolysis of potentially present conjugated forms of OA; the second method to allow the enzymatic hydrolysis of OA conjugated forms. Derivatization was performed with ADAM reagent (SERVA). Characteristics of the system were: Hewlett-Packard 1050, Merck Superspher 100-RP18 (4 mi, 250 x 4 mm); mobile phase MeCN:H2O (80:20); 1.1 ml · min^{-1} flow; column temperature 35°C; fluorescence detector HP 1046 A with excitation and emission waves of 365 and 412 nm respectively. Standards used were OA (NRC, Canada) and DTX2 kindly provided by K.James (Cork, Ireland). The coefficient of variation (C.V) of the analyses is lower than 10%.

RESULTS

Figure 1 shows chromatograms obtained from the extracts of *Dinophysis acuta*, *D. acuminata*, and from the OA standard. Table II shows the main results on toxin content per cell and species from single cell isolations. Table III shows the average toxin content per cell of *Dinophysis* in extracts from net-haul populations where *D. acuta* was the overwhelmingly dominant *Dinophysis* species.

Dinophysis acuminata: OA was found in all samples. Significant differences, indicating the presence of conjugated forms of OA in the cells were not found in the samples from June'98 (9.9 and 7.9 pg OA · cell^{-1} respectively) subjected to the two extraction protocols. A very small peak with retention time corresponding to DTX2 was found. Cells from September showed a toxin content more than double (21.7 pg OA· cell^{-1}) that found in the June cells

Dinophysis acuta: OA and DTX2 were found in the three samples. The OA:DTX2 ratio was 3:2 except in the samples from specimen that were maintained longer in the culture chamber. Important quantitative differences were found in the cells isolated as soon as they were collected at sea (10.7 pg · cell^{-1}) and those which were maintained in the laboratory (55.1 and 24.1 pg · cell^{-1}). In extracts from net-haul populations where *D. acuta* was the overwhelmingly dominant *Dinophysis* species, OA and DTX2 were found in similar proportion, but total toxin content in cells from that year's episode were much lower (0.6 – 4 pg · cell^{-1}) [2].

Dinophysis caudata: The sample chromatogram exhibited a small peak with the same retention time as OA that can be interpreted as traces of OA (0.7 pg OA · cell^{-1}).

DISCUSSION

These results show for the first time that Galician populations of *D. acuta* contain DTX2, and that this is the main species associated with the presence of this toxin in Galician mussels [6]. OA and DTX2 have also been found in *D.acuta* populations in Ireland [7] and in Portuguese mussels associated with the occurrence of *D. acuta* [8]. An OA:DTX2 ratio of 3:2 has been found in plankton net-hauls where *D. acuta* was the dominant species of *Dinophysis*, and in isolated cells, except in those stressed after several days maintained in the laboratory. The lowest toxin content per cell found in 1990, may be explained partly by a dilution effect of the toxicity in populations with a higher growth rate during a year with unsual high concentrations of this species, and partly because of the lower toxin content per cell estimated from net-haul extracts due to the matrix and other effects [9]. Regarding *D. acuminata*, although esterified toxins have not been found in the sample tested in this study by the boiling and the freeze/thaw procedures, the possibility of OA being present in *Dinophysis* cells as diol-esters or DTX4-type compounds should not be discarded, because esterified OA has been found in Galicia in net-haul extracts where *D. acuminata* was the dominant dinoflagellate [10]. For the first time, isolates of *Dinophysis caudata* have been analyzed, showing trace amounts of OA. Blanco et al. [11] obtained a negative correlation coefficient for *D. caudata* from multiple regression of HPLC analyses of net-hauls containing a mixture of *Dinophysis* spp and other microplanktonic organisms [11]. At least in Galicia, *D. caudata* seems to have a minor rôle in autumn DSP toxicity.

Differences in *Dinophysis* cell numbers of at least one order of magnitude have been found in samples collected at the same station within 24 h [12], and toxicity concentrated by the bivalves is largely determined by the proportion that toxic microalgae represent from the total food available [2]. These new data on variability in toxin content per cell of *Dinophysis* together with the problems mentioned above, and the well known sampling problem of planktonic species which are not very abundant and distributed in a patchy fashion, further weaken the value of a "*Dinophysis* index" as a substitute for the compulsory mouse bioassay in the detection of diarrhetic shellfish toxins associated with *Dinophysis* blooms.

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ESTERIFIED OKADAIC ACID IN NEW ZEALAND STRAINS OF *PROROCENTRUM LIMA*

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ABSTRACT

Prorocentrum lima (CAWD33), isolated from Rangaunu Harbour, Northland, New Zealand, produced 6.3 pg okadaic acid (OA) per cell as determined by HPLC (derivitisation with ADAM). Mouse bioassays indicated toxicity in the range of 0.4 – 0.8 MU per 10^6 cells. However the mouse response differed from the expected response to OA, and included neurological symptoms. Analysis by DSP ELISA test kit indicated toxicity x25 greater than was detected by mouse bioassay. HPLC analysis of strains of *P. lima* from different geographic regions in New Zealand was carried out with and without an alkaline hydrolysis step. Results indicated that OA and esterified OA (estOA) were present in strains from Whatawhiwhi and Rangaunu Harbours, but not in strains from Rangiputa, in Northland. No DTX series toxins were detected, whereas a Spanish strain used as a comparison produced OA and DTX-1, but no estOA. There was good correlation between HPLC results and those obtained by protein phosphatase (PP-2A) inhibition assay. The New Zealand strains were similar under scanning electron microscopic investigation, but differed slightly from the Spanish strain. *Perna canaliculus* and *Mytilus galloprovincialis* fed with *P. lima* producing OA and estOA appeared to accumulate estOA preferentially. *Crassostrea gigas* showed minimal uptake of either compound.

INTRODUCTION

Routine monitoring for biotoxin producing micro-algae has been carried out weekly throughout New Zealand for much of the past seven years for both commercial and recreational shellfish harvesters. Phytoplankton monitoring results are, whenever possible, correlated with shellfish biotoxin results so that micro-algal trigger levels (cells l^{-1} seawater) can be set for risk assessments, and the DSP producer *Prorocentrum lima* is one target species.

Analysis of mass cultured cells of *P. lima,* isolated from Northland, NZ (CAWD33), showed that anomalies existed between results generated by the mouse bioassay and those from the DSP ELISA test kit. *P. lima* produced 6.3 pg okadaic acid (OA) $cell^{-1}$ (HPLC: deriv. ADAM), whereas mouse bioassays indicated toxicity in the range of 0.4 - 0.8 MU per 10^6 cells. The mouse response differed from that expected for OA, and included neurological symptoms.

Analysis by DSP ELISA determined toxicity x25 greater than mouse bioassay results (Proceedings New Zealand Marine Biotoxin Science Workshop, No. 6, October 1996, Ministry of Agriculture and Fisheries, Wellington). These results led to the present study, in which analyses of strains of *P. lima* from different geographic regions in New Zealand were carried out, with and without an alkaline hydrolysis step, to determine whether esterified OA (estOA) could be responsible for the anomaly in bioassay results. The protein phosphatase (PP-2A) inhibition assay results indicated that both OA and estOA were present in some strains, and this was supported by HPLC analysis. No DTX1 was present in the NZ strains.

Shellfish contaminated with *P. lima in vitro* were also analysed to determine whether the micro-algal toxin profile was altered during passage through the shellfish gut.

SEM and larval bioassays were carried out for the various *P. lima* clones to determine whether different morphologies or bioassay responses could be detected and correlated with the different toxin profiles.

METHODS

Prorocentrum lima strains were maintained in f2 medium [1] in the Cawthron Micro-algae Culture Collection. Isolates tested (in stationary phase) were from Rangaunu (CAWD33), Whatawhiwhi (CAWD70), Rangiputa a (CAWD69), Rangiputa b (CAWD94), Croissilles (CAWD95) and Spain (CAWD32).

SEM and *Artemia salina* and Pacific oyster (*Crassostrea gigas*) larval bioassays were carried out as described previously [2,3].

Mouse bioassays (AOAC method) and DSP ELISAs were carried out at ESR, Porirua (NZ Marine Biotoxin Management Board). DSP toxins were determined by HPLC (deriv. with ADAM) with an added hydrolysis step to determine the presence of esterified compounds [4]. PP-2A assays were carried out on the same extracts following the method of Mountfort et al. [5,6].

Pacific oysters, Greenshell™ (*Perna canaliculus*) and blue (*Mytilus galloprovincialis*) mussels were held in filtered (0.2 µm), aerated seawater and fed *P. lima* cells (4.8 x10^7 over 8 days). Analysis of shellfish extracts was by HPLC and PP-2A assay.

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RESULTS AND DISCUSSION

The DSP-producing dinoflagellate *Prorocentrum lima* occurs epiphytically on seaweeds, and on oyster racks, mussel lines and in sediments, and is therefore of concern to shellfish harvesters. Clonal *P. lima* cultures isolated from different geographic regions of NZ and analysed by HPLC all produced OA and estOA, but no DTX1. OA and estOA was confirmed by PP-2A, but the assay does not discriminate between OA and DTX1.

The production of estOA varied between the New Zealand strains tested, even in strains from neighbouring sites. A Spanish strain tested produced OA and DTX1 but no esterified toxins (Table 1). The esters could have been cleaved at either 7-O (DTX3) or 1-O (sulphated OA precursors, eg. DTX4,5), and have yet to be characterised. HPLC and PP-2A assay results were comparable, and the latter shows promise for replacing the ethically controversial mouse bioassay.

The morphologies of all the clonal cultures of *P. lima* analysed for DSP were similar to previous SEM descriptions of *P. lima*, irrespective of toxin profile, following the criteria of Faust [7]. Morbidity/mortality results for the *A. salina* and *C. gigas* bioassays were the same for all *P. lima* strains tested, i.e. all larvae exhibited morbidity within 16 h, died in 24 h.

Table 1. DSP toxin content of extracts of *Prorocentrum lima* strains isolated from New Zealand and Spain.

Protein phosphatase inhibition assay (PP-2A) and HPLC results pg cell^{-1}; nt: not tested; hyd: hydrolysis.

Site	OA	OA post hyd.	DTX1	DTX1 post hyd.
Rangaunu				
HPLC	15.1	19.4	0	0
PP-2A	17.4	28.2	-	-
Whatawhiwhi				
HPLC	26.9	35.0	0	0
PP-2A	28.1	44.4	-	-
Rangiputa (a)				
HPLC	14.0	14.0	0	0
PP-2A	22.0	18.0	-	-
Rangiputa (b)				
HPLC	9.9	10.1	0	0
PP-2A	12.0	12.0	-	-
Croissilles				
HPLC	nt	nt	nt	nt
PP-2A	14.8	28.8	-	-
Spain				
HPLC	7.6	6.2	1.3	1.5
PP-2A	12.6	16.6	-	-

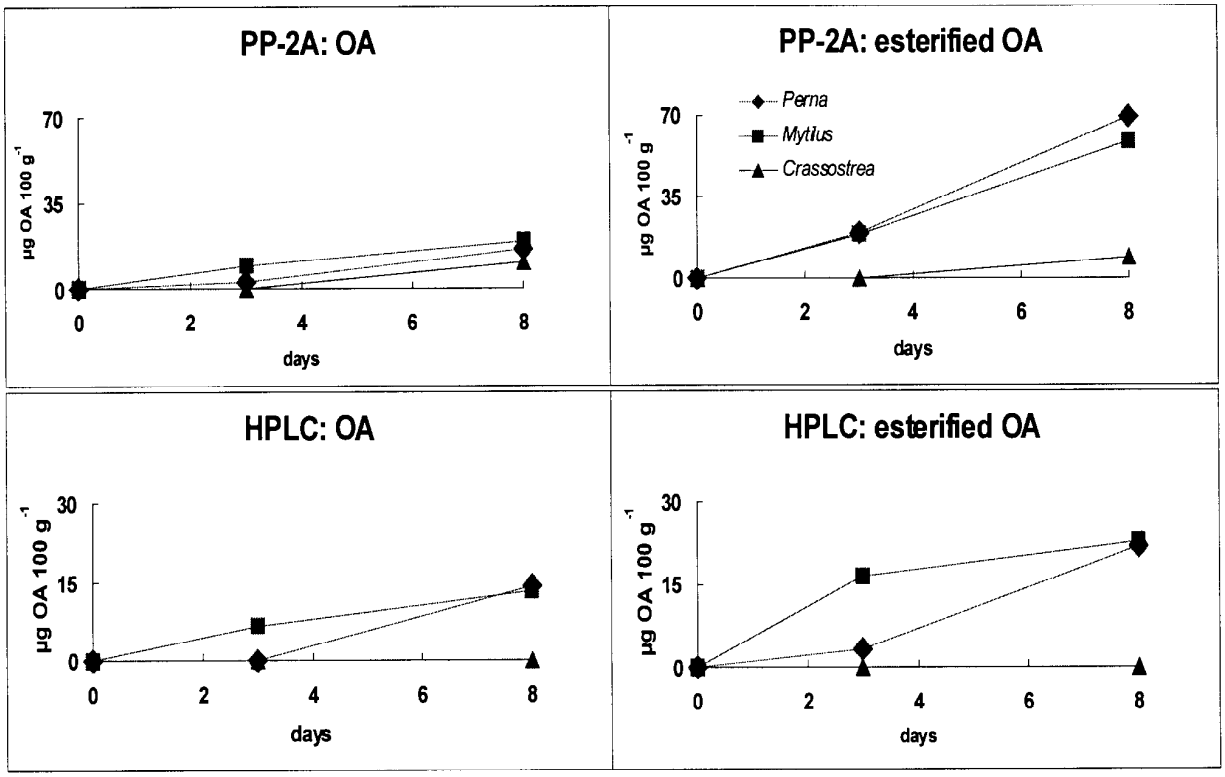

Figure 1. Analysis of shellfish fed *Prorocentrum lima* (strain Whatawhiwhi) by PP-2A and HPLC (left) before and (right) after hydrolysis.

Protein phosphatase inhibition assay (PP-2A) and HPLC results pg cell^{-1}.

Shellfish feeding

Greenshell™ and blue mussels accumulated OA and estOA over the eight-day feeding experiment; the ratio of estOA to OA detected in the mussel hepatopancreas was greater than that determined for the original microalgal culture (Figure 1). It is possible that estOA is selectively accumulated by both mussel species. Previous analyses of blue and Greenshell™ mussels contaminated with OA at Wedge Point, Malborough Sounds, showed a greater accumulation of toxin in the former, suggesting a difference in absorption of the toxin [8]. In this *in vitro* study no real difference in absorption was noted between the two species.

Oysters did not accumulate estOA or OA as determined by HPLC, although the PP-2A assay detected low concentrations at day eight (Figure 1). These results support suggestions that different shellfish species should be subject to different toxic micro-algae cell counts as trigger levels for flesh testing in New Zealand's phytoplankton monitoring programmes.

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DINOPHYSISTOXIN-1 AND ESTERIFIED DINOPHYSISTOXIN-1 IN THE MUSSEL *MYTILUS GALLOPROVINCIALIS* FED ON THE TOXIC DINOFLAGELLATE *DINOPHYSIS FORTII*

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ABSTRACT

Non-toxic mussels (*Mytilus galloprovincialis*) were fed for 5 days with a natural population of the toxic dinoflagellate *Dinophysis fortii*. The total number of *D. fortii* cells ingested by five mussels during the feeding experiment was approximately 2.34 x 10^5. Okadaic acid (OA), dinophysistoxin-1 (DTX1) and esterified toxins of OA and DTX1 in *D. fortii* and mussel extracts were determined by liquid chromatography followed by in-line atmospheric pressure electrospray ionization-mass spectrometric (ESI-MS) detection. DTX1 was the only derivative detected in extracts of the *D. fortii* cells used for the feeding experiment whereas mussels fed on *D. fortii* contained both DTX1 and esterified DTX1. DTX1 was present in significantly higher amounts in the mussels than esterified DTX1. The absorption efficiency of DTX1 in the midgut glands of mussels was approximately 9 % of the total amount of DTX1 in *D. fortii* cells filtered by the mussels. The absorption efficiency is higher than that reported previously in scallops (less than 3 %).

INTRODUCTION

Diarrhetic shellfish poisoning (DSP) toxins, okadaic acid (OA) and its homologues, dinophysistoxin-1 (DTX1), dinophysistoxin-2 (DTX2) are derived from dinoflagellates such as *Dinophysis* spp. and *Prorocentrum* spp. [1,2]. Besides these toxins, the esterified toxin 7-*O*-acyl DTX1 (DTX3) is known as a principle toxin in Japanese scallops [3] (Fig. 1). Recently, we demonstrated the conversion of DTX1 to 7-*O*-acyl DTX1 in Japanese scallops fed on toxic *D. fortii* [4]. This conversion occurred rapidly in Japanese scallops [4]. It is reported that the major toxins in mussels are free toxin such as OA, DTX1, and DTX2 [5-10]. Recently, the presence of 7-*O*-acyl derivatives of OA and DTX2 has been reported in Irish and Spanish mussels [11,12].

The present study was designed to compare the assimilation and transformation of DSP toxins in mussels with observations that have previously been made in scallops [4] by experimentally contaminating mussels with the toxic dinoflagellate *D. fortii*.

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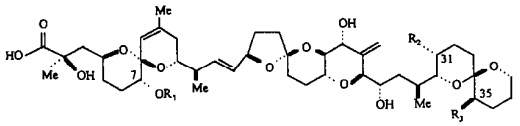
MATERIALS AND METHODS

Reagents

OA, DTX1 and 7-*O*-palmitory DTX1 (7-*O*-16:0 DTX1) standard toxins were supplied by Japan Food Research Laboratories (Tokyo, Japan) [13].

Toxic dinoflagellates and mussels

The feeding experiment was carried out according to the method previously applied to scallops by Suzuki *et al.* [4]. Toxic dinoflagellates *D. fortii* were collected at Tsukahama, Ishinomaki Bay, Japan on 14, 16 and 18 July 1997 when *D. fortii* was observed as the predominant species. *D. fortii* was collected with a sampling pump, and subjected to size fractioning using plankton nets, the 20-50 m fraction which contains *D. fortii* was collected and placed in 200 liters of filtered seawater until used for the feeding experiment. For *D. fortii* toxin analysis, an aliquot of the 20-50 m fraction was suspended in 200 ml of filtered seawater (condensed dinoflagellate sample) as reported previously [14,15]. The concentration of *D. fortii* in the condensed dinoflagellate samples collected on 14, 16 and 18 July 1997 was 95, 252 and 252 cells/ml, respectively.
Non-toxic mussels (*Mytilus galloprovincialis*) were collected at Tsukahama in June 1997. Ten mussels within a size range of 47-58 mm (Mean±SD, 50±3 mm) in shell length were placed in 200 liters of aerated seawater at 19-20...C and acclimated to laboratory conditions one month before the experiment. The seawater supplied to the mussels was changed from natural seawater to filtered seawater one week before the experiment began. Before the feeding experiment with *D. fortii* began five of the mussels (Sample No 1-5) were



	R_1	R_2	R_3
okadaic acid (OA)	H	CH_3	H
dinophysistoxin-1 (DTX1)	H	CH_3	CH_3
dinophysistoxin-2 (DTX2)	H	H	CH_3
dinophysistoxin-3 (DTX3)	Acyl	CH_3	CH_3

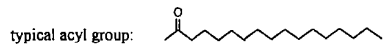


Fig. 1. Chemical structures of okadaic acid homologues.

sacrificed and midgut gland samples subjected to toxin extraction. Other five individual mussels (Sample No. 6-10) were exposed to fresh seawater containing *D. fortii* at a cell density of approximately 160 cells/l twice a day (8:30 AM and 5:30 PM) for 5 days. The number of *D. fortii* cells consumed by the five mussels was determined by the change in tank concentration of cells over each feeding interval. Changes in the number of *D. fortii* cells without mussels were also observed under the same experimental conditions.

Extraction of DSP toxins from toxic dinoflagellates

Extraction of DSP toxins from *D. fortii* was carried out by solid phase extraction (SPE) on a non-polar cartridge column as previously described [14,15]. An aliquot (20 ml) of the condensed toxic dinoflagellate sample was filtered through a 1 m filter paper (No. 5C). The residue was washed with 5 ml of methanol three times (toxic dinoflagellate residue extract). The filtrate of the condensed toxic dinoflagellate sample was transferred to a Sep-Pak plus C18 cartridge column (Waters, Milford, MA, USA) which had been previously conditioned with 10 ml of methanol and distilled water. The Sep-Pak C18 cartridge column was subsequently washed with 10 ml of distilled water and finally washed with 10 ml of methanol. The methanol eluate and the toxic dinoflagellate residue extract were combined and then evaporated. The residue was dissolved in 2.5 ml of 80 % methanol and extracted twice with 2.5 ml of hexane, then 1 ml of 0.2 % acetic acid was added to methanolic solution. The combined hexane extracts were made up to 10 ml with hexane and used for the LC-MS analysis of 7-*O*-acyl DTX1. 7-*O*-acyl derivatives of OA homologues are preferentially solubilized in the hexane fraction in the liquid-liquid partition of DSP toxins in this procedure [12]. The DTX1 and OA in the methanolic solution were extracted twice with 4 ml of chloroform. The combined chloroform extracts were made up to 10 ml with chloroform. An aliquot (1 ml) of the chloroform extract was evaporated and dissolved in 100 l methanol and an aliquot of this solution was analyzed by LC-MS.

Extraction of DSP toxins from the midgut glands of mussels

Extraction of DSP toxins form the midgut glands of mussels was carried out according to the method previously reported [16]. Combined midgut glands of five control mussels (No. 1-5) and individual midgut glands of test mussels (No. 6-10) fed on *D. fortii* were extracted with four times their volume of 80 % methanol. After centrifugation, 2.5 ml of the supernatant was extracted twice with 2.5 ml hexane. The combined hexane extracts were made up to 10 ml with hexane. A 1 ml aliquot of 0.2 % acetic acid was added to the methanolic solution, then the OA and DTX1 were extracted with 4 ml of chloroform. The chloroform extract was made up to 10 ml with chloroform. An aliquot (1 ml) of this extract was evaporated, dissolved in 100 l methanol, and an aliquot analyzed by LC-MS.

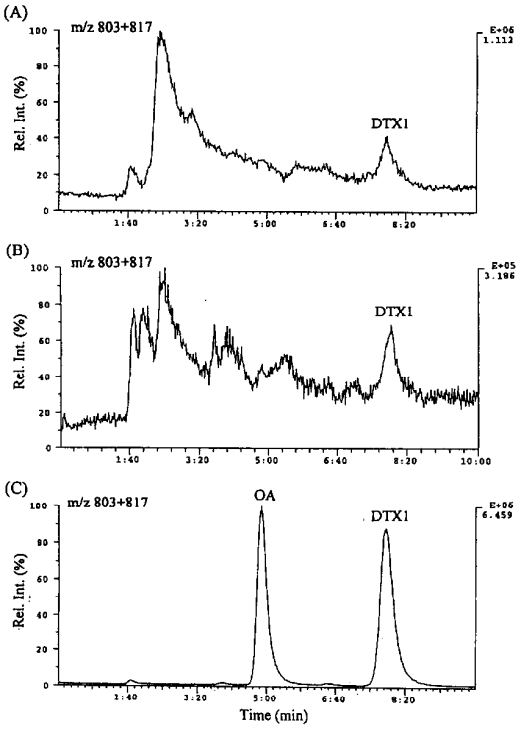


Fig. 2. Selected ion monitoring (SIM) chromatogram $[M-H]^-$ of OA and DTX1 obtained from the toxic dinoflagellate sample collected on 14 July (A), mussels (No. 9) extracts (B), and standard toxin mixture (OA and DTX1) (C).

Hydrolysis of esterified DSP toxins

An aliquot of the hexane extracts (1 ml) was evaporated and 100 l of a 0.5 N NaOH solution in 90 % methanol was added. The solution was kept at 75 _ for 40 min. After evaporating the methanol from the reaction mixture, the aqueous layer was acidified with 300 l of 0.5 N HCl and extracted three times with 300 l diethyl ether. After evaporating the combined ether extracts, the extracts were dissolved in 100 l 80 % methanol and an aliquot directly analyzed by LC-MS.

LC-MS of toxins

LC-MS was carried out identically according to the previous method [4,17]. LC-MS was performed using a Hewlett-Packard Model 1050 Series liquid chromatograph coupled to a Finnigan MAT SSQ-7000 mass spectrometer (San Jose, CA) equipped with an atmospheric pressure ESI interface and an ICIS data system. The LC flow was introduced into the ESI interface without any splitting. Separation of toxins was

achieved on a Mightysil RP-18 column containing an octadecyl phase bonded to 5 m silica gel particles (150 mm x 2 mm i.d.; Kanto Chemical Co. Inc., Tokyo, Japan) at 35 _. Acetonitrile-water (70:30, v/v) containing 0.1% acetic acid was used as the mobile phase with a flow rate of 200 l/min for analysis of OA and DTX1 [17]. 100 % methanol with 1 mM ammonium acetate was used as the mobile phase with a flow rate of 200 l/min for analysis of 7- *O*-16:0-DTX1 [4]. The voltage on the ESI interface was maintained at approximately 4.5 kV. The temperature of the heated capillary was 200 _. High-purity nitrogen gas was used as the sheath gas at an operating pressure of 70 psi and an auxiliary gas at 5 units, respectively. Negatively-charged ions corresponding to $[M-H]^-$ (m/z 803 for OA, 817 for DTX1, 1055 for 7-O-16:0-DTX1) were scanned by selected ion monitoring (SIM).

RESULTS

The total cell number of *D. fortii* filtered by five mussels (No. 6-10) was 2.34 x 10^5 cells. Changes in the cell number in the control container without mussels were insignificant, indicating that reproduction and mortality of *D. fortii* cells did not occur during the feeding experiments.

Fig. 2 (A) shows a SIM chromatogram $[M-H]^-$ of OA and DTX1 obtained from the condensed toxic dinoflagellate sample. OA was not detected in any of the dinoflagellate samples. Table 1 shows the free and esterified DTX1 contents in *D. fortii* used for the feeding experiments determined by LC-MS. The DTX1 contents of *D. fortii* used for the feeding experiment did not change significantly. Although free DTX1 was detected from toxic dinoflagellate extracts, DTX1 resulting from the hydrolysis of hexane extracts was not detected, demonstrating that *D. fortii* did not contain esterified DTX1.

Fig. 2 (B) shows a SIM chromatogram $[M-H]^-$ of OA and DTX1 obtained from a mussel (No. 9) fed with *D. fortii*. As in the condensed *D. fortii* sample, DTX1 was the only free toxin observed.

Fig. 3 (B) shows a SIM chromatogram $[M-H]^-$ of 7-*O*-16:0-DTX1 obtained from the hexane extract of

Table 1. Free and esterified dinopysistoxin-1 detected in *Dinophysis fortii* used in the feeding experiment

Date	Free DTX1*1 (pg/cell)	Esterified DTX1*2 (pg/cell)
14 July	43	ND*3
16 July	40	ND
18 July	33	ND

*1 Free DTX1 obtained from chloroform extracts.
*2 Esterified DTX1 obtained by hydrolysis of hexane extracts.
*3 ND: not detected.

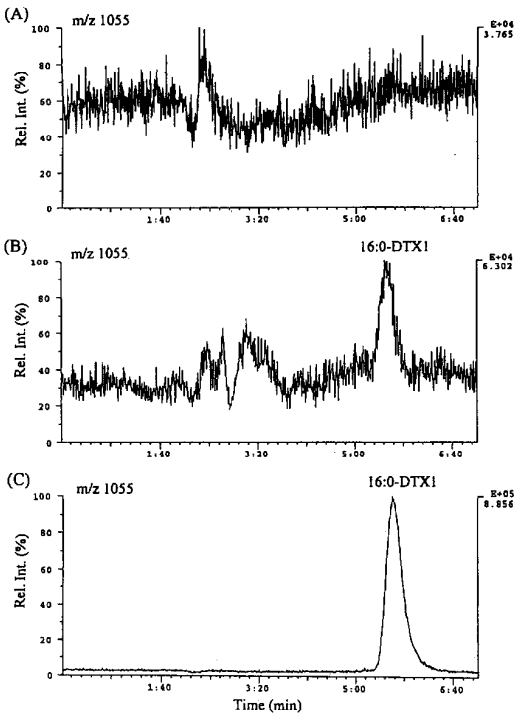


Fig. 3. Selected ion monitoring (SIM) chromatogram $[M-H]^-$ of 7-*O*-16:0 DTX1 obtained from the toxic dinoflagellate sample collected on 14 July (A), mussel (No. 9) extracts (B), and standard toxin 7-*O*-16:0 DTX1 (C).

mussel (No. 9) fed with *D. fortii*. The ion peak which was detected had an identical retention time to the 7-*O*-16:0-DTX1 standard (Fig. 3 C). This ion peaks was not detected from hexane extracts of *D. fortii* (Fig. 3 A) and mussels (No. 1-5) before the feeding experiment.

Table 2 shows the toxin contents in the midgut glands of mussels determined by LC-MS. Mussels fed on *D. fortii* contained both DTX1 and esterified DTX1 though DTX1 was present in significantly higher amounts in the mussels than esterified DTX1. The contents of 7-*O*-16:0-DTX1 were lower than those of esterified DTX1. This indicates the presence of other 7-*O*-acyl-DTX1 in mussels [11].

DISCUSSION

In the present study, mussels were contaminated with DSP toxins by being fed with the toxic dinoflagellate *D. fortii*. Although extracts of *D. fortii* contained only free DTX1 (Table 1), the additional presence of esterified DTX1 in mussels fed on *D. fortii* was confirmed by LC-MS analysis of hydrolysis products of the hexane extracts (Table 2). The presence of 7-*O*-16:0-DTX1 in mussels was also confirmed by LC-MS analysis (Fig. 3).

Table 2. Free and esterified dinophysistoxin-1 detected in the midgut glands of mussels used in the feeding experiment

Mussels DTX1 No.	Free DTX1*1 (g/g)	Esterified DTX1*2 (g/g)	7-*O*-16:0-DTX1 (g/g)
1-5*3	ND*4	ND	ND
6	0.14	ND	ND
7	0.04	0.04	0.01
8	0 13	0.04	0.02
9	0.12	0.09	0.03
10	0.15	0.03	ND
Average (No. 6-10)	0.12	0.04	0.01

*1 Free DTX1 obtained from chloroform extracts.
*2 Esterified DTX1 obtained by hydrolysis of hexane extracts.
*3 Combined extracts of mussels (No. 1 - 5) before the feeding experiment using of *D. fortii*.
*4 ND: not detected.

To our knowledge, this is the first direct evidence of the transformation of DTX1 to 7-*O*-acyl DTX1 in mussels. It is similar to previous observation made by Suzuki *et al.* [4] in Japanese scallops, however in their study, a significantly higher content of esterified DTX1 compared to free DTX1 was observed. It is reported that the major toxins in natural DSP contaminated mussels are free toxin; OA, DTX1, and DTX2 [5-10]. The results obtained in the present experimental study likewise demonstrated that the major toxins in experimentally contaminated mussels are free toxins.

The total cell number of *D. fortii* filtered by the five mussels (No. 6-10) was 2.34 x 10^5. The average DTX1 content in *D. fortii* cells calculated from the data in Table 1 was 39 pg/cell and did not change significantly over the experimental period (Table 1). The total amount of DTX1 in the *D. fortii* cells filtered by the mussels (estimated from the filtered cell number and average toxin content) was approximately 9.1 g. In contrast, the sum of the free and esterified DTX1 contents accumulated in the five mussels was 0.78 g. The absorption efficiency of DTX1 in the midgut glands of mussels under these experimental was therefore approximately 9 % of the total amount of DTX1 in *D. fortii* cells filtered by the mussels. This absorption efficiency was higher than the less than 3% previously reported for scallops [4].

During routine monitoring of DSP toxicity of shellfish in Japan, it is frequently reported that the toxicity of mussels is higher than scallops. This phenomenon may be partly explained by the higher absorption efficiency of toxins in mussels compared to scallops. In addition, because the specific mouse toxicity of DTX1 is higher than that of 7-*O*-acyl-DTX1 (7-*O*-16:0 DTX1) [18], the apparently higher rate of conversion of DTX1 to 7-*O*-acyl-DTX1 in Japanese scallops, in comparison with mussels, may be also be a factor in decreasing the mouse toxicity of scallops.

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ELIMINATION AND DIFFERENTIAL TRANSFORMATION OF YESSOTOXIN BY THE GREENSHELL MUSSEL *PERNA CANALICULUS* AND THE BLUE MUSSEL *MYTILUS GALLOPROVINCIALIS*

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ABSTRACT

Greenshell mussels (*Perna canaliculus*) and blue mussels (*Mytilus galloprovincialis*) were experimentally contaminated with yessotoxin (YTX) by feeding with a culture of the dinoflagellate *Protoceratium reticulatum*. Tissue localisation of YTX and its analogues was measured by HPLC-FD and the changes in toxin composition and concentration monitored over a 10 day depuration period. After feeding ceased YTX was the main toxin analogue present in greenshell mussels and only low levels (<10%) of 45-hydroxy yessotoxin (45-OH-YTX) were detected. In blue mussels on the other hand 45-OH-YTX was the predominant molecular species observed, comprising >90% of the total toxin. Neither analogue could be detected in tissues other than the digestive gland. The relative proportions of these analogues stayed approximately the same in both mussel species throughout the depuration period. The slow rate of disappearance of YTX from greenshell mussel digestive glands over time (0.06 g YTX g^{-1} day^{-1}) was comparable with the rates observed in naturally contaminated shellfish (0.04 g YTX g^{-1} day^{-1}). This experiment demonstrates that different bivalve species may metabolise and eliminate yessotoxin via different physiological/biochemical pathways.

INTRODUCTION

Yessotoxin was first isolated from the Japanese scallop (*Patinopecten yessoensis*) and described as a new ladder form polyether toxin in 1987 [1]. Recently there has been increased interest in YTX contamination of shellfish as its presence has been identified in several new locations throughout the world and a range of new analogues and derivatives have been described [2 to 8]. YTX is nominally included in the diarrhetic shellfish poisoning (DSP) group. By intra-peritoneal administration it is lethal to mice and causes histo-pathological changes in the heart, liver and pancreas [9]. However, recent work on the toxicology of YTX [10] suggests that it has a low, possibly negligible, toxicity by oral administration and may not in fact be a concern as a food poisoning agent. Despite this YTX still remains a concern, firstly because toxicological work sufficiently rigorous to satisfy the requirements of international food quality regulators has yet to be carried out, and secondly because its presence in shellfish may confound the interpretation of the conventional lethal mouse bioassay for DSP and other lipophilic marine biotoxins. YTX contamination of shellfish in New Zealand was first identified in cultivated greenshell mussels from the Marlborough Sounds in 1996 [11] and subsequently in mussels from the Hauraki Gulf in 1998 [12, 13]. A study of the Marlborough Sounds event resulted in the first identification of the planktonic dinoflagellate *Protoceratium reticulatum* (formerly *Gonyaulax grindlyei*) as the causative agent of YTX contamination in New Zealand [14]. Field research into the dynamics of contamination and elimination of the DSP-toxins okadiac acid (OA) and other dinophysis toxins (DTXs) and pectenotoxins (PTXs) by the two edible mussel species (*P. canaliculus* and *M. galloprovincialis*) which occur in New Zealand has shown [11, 15] that they have very different absorption characteristics and rates of elimination of these toxins. The experiment which is reported in this paper was designed to test the hypothesis that these two mussel species might likewise display different characteristics in the sequestering and metabolism of yessotoxin and obtain some preliminary data on the tissue localisation and the rates of elimination of the toxin from the shellfish.

METHODS

Experimental

Forty individual blue and greenshell mussels (65.9 – 4.5mm and 85.3 – 6.8mm mean shell length respectively) were continuously fed over 7 days (for 5 minutes at 30 minute intervals) with early stationary-phase, cultures of a yessotoxin-producing culture of *Protoceratium reticulatum* (Cawthron Institute Culture collection isolate; CAWD40). Microscopic examinations showed that all *P. reticulatum* cells were cleared from the water during the 30 minute feeding interval. During the entire feeding period the 40 mussels in each 35 litre aquarium (at 18^0C) were supplied with approximately 140 litres of culture with a cell density of about (1.0 x10^7 cells l^{-1}). At the conclusion of the feeding phase a sample of 5 mussels was retrieved (Day 0) and the remainder of the mussels held in the aquaria for a further 10 day depuration period during which there was no supplementary feeding. The seawater was changed every two days and 5 replicate mussels removed from the tanks at days 1, 4, 6, 8, and 10. Mussel samples were immediately frozen (-20^0C) and stored prior to dissection, extraction and analysis. Three mussels from each sampling were extracted and analysed.

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Extraction

YTX extraction and clean-up procedures were carried out using a modification [16] of Yasumoto s and Takizawa s method [17]. The hepatopancreas and remaining tissues of the mussels were dissected, homogenates of each of these tissues prepared, and 1g extracted with 9ml 80 % MeOH. Aliquots (0.5 ml) of these extracts were evaporated under N_2, resuspended in 500 l MeOH:20mM phosphate buffer (1:4) and loaded onto a Sep-Pak^R C-18 column (Waters, USA), washed with MeOH:H_2O (2:8) and eluted from the column with MeOH:H_2O (7:3). The eluant was dried under N_2 and derivatized for 2 hrs with 50 l of 0.1% DMEQ-TAD (Wako). The derivatized extract was further purified by re-solution in 500 l MeOH:H_2 O (3:7), loaded onto a Sep-Pak^R C-18 cartridge, washed with 500 l MeOH:H_2O (3:7) and eluted with 500 l MeOH: H $_2O$ (7:3). This eluant was dried, re-suspended in 100 l MeOH and injected (5 l) into the HPLC. The efficiency of YTX recovery from spiked blue and greenshell mussels using this method was 98 – 5% and 104 – 9% respectively [16].

HPLC conditions

Samples were analysed on a Shimadzu LC-10A HPLC system using a Mightysil RP-18 (2.0 mm id x 250 mm) column at 35^{0}C. The mobile phase consisted of 40 mM phosphate buffer (pH. 5.8)/MeOH (3:7) delivered at a flow rate of 0.20 ml/min. The detector excitation and emission wavelengths were set at 370 nm, and 440nm respectively. The YTX analysis was calibrated using a YTX standard solution kindly provided by Professor T. Yasumoto. As we had no standard solution of 45-OH-YTX we also calculated the concentration of this analogue using the YTX standard. Since there are no data on the recovery efficiency of 45-OH-YTX using the clean-up process outlined, the estimates of this analogue reported here must be regarded as approximate.

LC-MS analysis

To confirm peak identities, the extracts of both mussel species sampled at the conclusion of the feeding phase were also analysed by liquid chromatography with mass spectroscopy detection. The apparatus used was a Finnigan MAT SSQ7000 LC-MS system in negative electro-spray ionisation mode using a Mightysil RP-18 column (2.0 mm id x 150mm) and a mobile phase of 80% MeOH with 1mM ammonium acetate at a flow rate of 0.2 ml/min. The ion peaks monitored were for YTX; $[M-Na]^-$, m/z 1163 and $[M-2Na+H]^-$, m/z 1141 and for 45-OH-YTX: $[M-Na]^-$ m/z 1179 and $[M-2Na+H]^-$, m/z 1157.

RESULTS AND DISCUSSION

Early stationary-phase cultures of *P. reticulatum* used in this experiment produced YTX at a level of approximately 6.1 pg YTX per cell with no evidence of the production of significant quantities of any other YTX analogues, including 45-OH YTX (Fig. 1). No trace of YTX or 45-OH-YTX was detectable in blank samples of blue or greenshell mussels taken prior to the commencement of feeding. At the conclusion of the feeding phase (T_0), neither analogue was detectable in any shellfish tissues apart from the digestive gland. At T_0, YTX reached a mean level of 1.09 – 0.71 g g wet wt.$^{-1}$ hepatopanceras in the greenshell mussels (Figs. 2B, 3A), but only very low levels of 45-OH-YTX (0.12 – 0.05 g g $^{-1}$) were observed. In most greenshell mussel samples small peaks with the characteristic form and retention time of 45-OH-YTX were identifiable but at levels to low to quantify accurately (i.e. below the threshold for peak integration), therefore the levels reported here are estimates based on manually measured peak heights relative to the peak height of YTX. In blue mussels at T_0 only low levels of YTX (0.03 – 0.05 g g wet wt.$^{-1}$ hepatopancreas) were detectable though large peaks with the expected form and retention time of 45-OH-YTX were observed with a mean concentration of 0.77 – 0.67 g g wet wt. $^{-1}$ hepatopancreas (Figs. 2C, 3B).

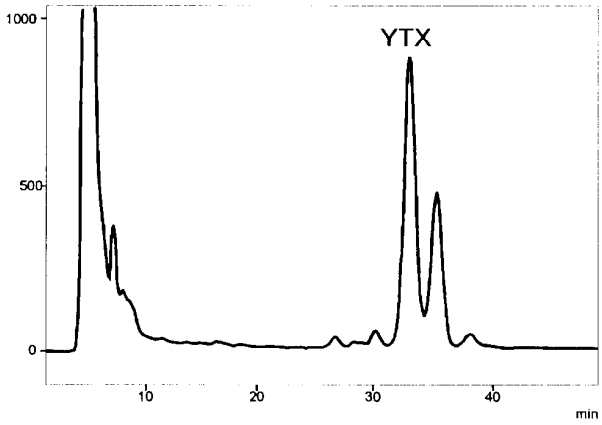


Fig. 1. HPLC chromatogram of yessotoxin (YTX) in early stationary-phase culture of *Protoceratium reticulatum* used in the feeding experiment.

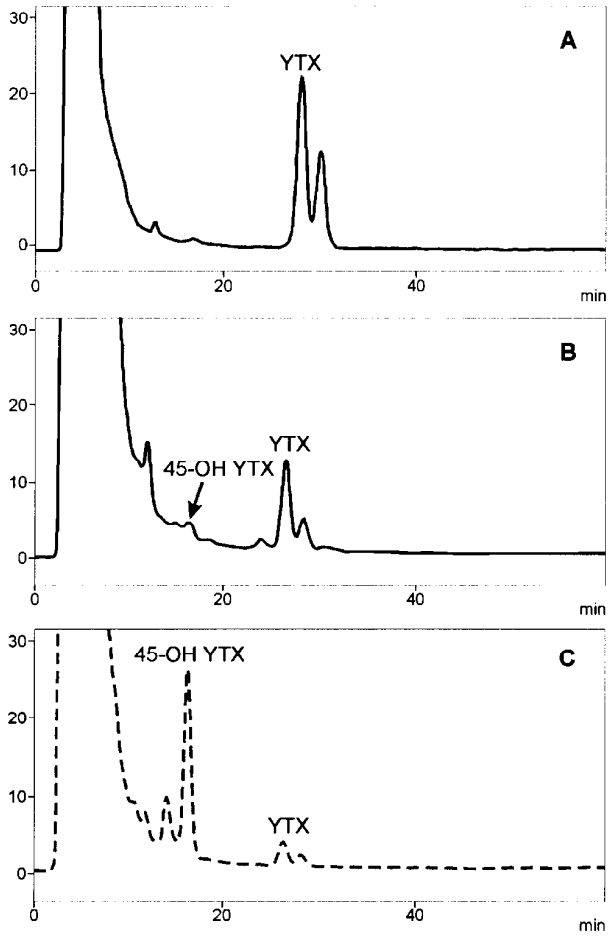
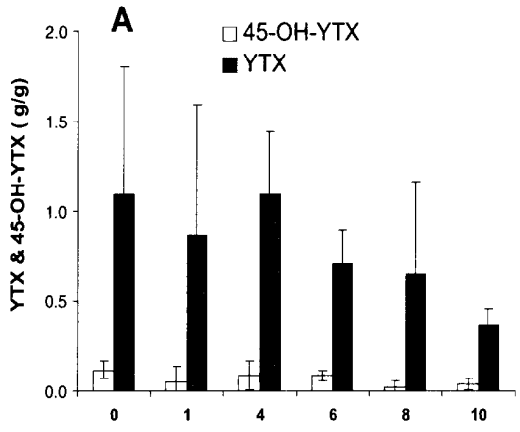


Fig. 2. HPLC chromatograms of the yessotoxin (YTX) standard (A) and yessotoxin and 45 hydroxy yessotoxin (45-OH-YTX) in greenshell mussels (B) and blue mussels (C).



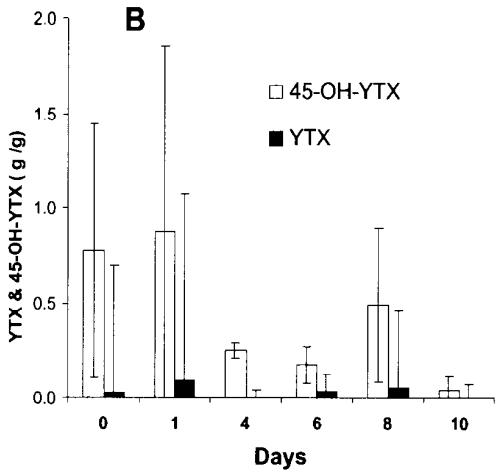


Fig. 3. Yessotoxin (YTX) and 45 hydroxy yessotoxin (45-OH-YTX) concentrations in the hepatopancreas of greenshell mussels (A) and blue mussels (B) through the depuration period (days). The solid bars represent the mean, and the error bars the standard deviation (SD) about the mean, of three replicates analysed on each occasion.

The identity of YTX and 45-OH-YTX in both blue and greenshell mussels was confirmed by the monitoring of two ion peaks ([M- Na]$^-$ & [M-2Na+H]$^-$) in the LC-MS analyses [2]. The LC-MS analysis also confirmed that YTX was the major toxin in greenshell mussels and 45-OH-YTX the major toxin in blue mussels. In the LC-MS chromatograms an interesting and significant difference between the retention times (approximately 30 seconds) of the hydroxy YTX analogues in the different mussel species was observed, suggesting that these were in fact different isomers of 45-OH-YTX.

Although the variation between the 3 replicate mussel samples analysed was rather high, there was a clear decline in the concentration of YTX and 45-OH-YTX in both mussel species over the depuration period (Fig 3). Quite high levels of YTX remained in the greenshell mussels after 10 days (Fig. 3A) and, assuming a linear rate of detoxication, equate to an approximate toxin loss rate of about 0.06 g YTX g wet wt.$^{-1}$ hepatopancreas day^{-1}. This is comparable to a detoxication rate of 0.04 g YTX g wet wt^{-1} hepatopanceras day^{-1} which was observed following a natural YTX contamination event in Queen Charlotte Sound in December 1996 [11]. The elimination of 45-OH-YTX in the blue mussels appears to have been somewhat more rapid (>0.07 g YTX g wet wt.$^{-1}$ hepatopanceras day^{-1}) however, the wide variation between replicates makes this uncertain (Fig. 3B).

The observations reported here show that 45-OH-YTX is a derivative of YTX which is not synthesised *de novo* by the dinoflagellate but is produced within the

hepatopancreas of the shellfish. That it is produced to a lesser extent in the greenshell mussels than in the blue mussels illustrates that there are some fundamental differences in the characteristics of digestion and assimilation of lipophilic compounds like YTX between the two species. It also raises the possibility that other shellfish species have similarly unique metabolic attributes and that other compounds within the newly identified range of YTX analogues and derivatives such as 1-desulfo-yessotoxin, homo-yessotoxin, 45-OH-homo-yessotoxin and Adriatoxin [2, 8] may also be the result of shellfish mediated transformations. This is an important consideration in the context of the design of new highly specific methodologies such as ELISA assays for the detection of YTX contamination which must have broad enough cross reactivity to detect and quantify all these compounds.

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DETECTION OF *N*-ACYL HOMOSERINE LACTONES IN MARINE BACTERIA ASSOCIATED WITH PRODUCTION AND BIOTRANSFORMATION OF SODIUM CHANNEL BLOCKING TOXINS AND THE MICROFLORA OF TOXIN-PRODUCING PHYTOPLANKTON

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ABSTRACT

'Quorum sensing' or 'autoinduction' is a chemical signalling system which enables bacteria to function in a "quasi" multicellular manner by coupling gene expression to a critical population size (quorate) or cell density. Such synchronised behaviour has been shown to play a role in many different metabolic functions including production of exotoxins, virulence determinants and bacteria-plant interactions. *N*-acyl homoserine lactones (*N*-AHLs) are the best known group of chemical signal molecules used in quorum sensing. The aim of this study was to determine if *N*-AHLs are produced by marine bacteria associated with toxic phytoplankton, sodium channel blocking (SCB) toxins and paralytic shellfish toxin (PST) biotransformation. Four different assays were used to determine if homoserine lactones were present in bacteria from the microflora of *Pseudo-nitzschia multiseries* (an AST-producing diatom) and *Alexandrium* spp. (PST-producing dinoflagellates). Bacteria capable of biotransforming PSTs and bacteria reported to produce sodium channel blocking toxins such as PSTs and tetrodotoxin (TTX) were also studied. Results indicated that three of the four bacteria isolated from *P. multiseries*, both TTX-associated strains and four PST-biotransforming isolates, produce *N*-AHLs. However, only one of the seven bacteria isolated from toxic dinoflagellates and associated with PST production gave positive results. This information will be used in future studies to determine if *N*-AHLs play a specific role in amnesic and paralytic shellfish toxins. It also raises the question of a wider involvement of quorum sensing in harmful algal blooms (HABs).

INTRODUCTION

Amnesic shellfish toxins (ASTs) and paralytic shellfish toxins (PSTs) are neurotoxins which accumulate in filter feeding bivalves [1-4]. Both toxin groups are produced by phytoplankton. PSTs are associated with dinoflagellates e.g. *Alexandrium* spp. and ASTs with diatoms e.g. *Pseudo-nitzschia* spp. [1,2]. Tetrodotoxin (TTX) is also a neurotoxin, associated primarily with pufferfish (*Tetraodon* spp.), and blocks sodium channels in mammalian nerve cells in a similar manner to PSTs [5]. Domoic acid (DA), the principle toxin of the AST group, acts as a glutamate agonist in the brain and central nervous system and has been shown to cause neuronal damage in the hippocampal region of the brain [6].

Marine bacteria have been linked to enhanced synthesis of domoic acid [1], production and enhanced synthesis of PSTs [3,4], production of TTX [7-10], and biotransformation of PSTs [11,12]. However, the mechanisms by which bacteria perform these functions is currently unknown. One possibility is *via* production of pheromones. These are small diffusable signal molecules which permit Gram-negative bacteria of the same strain to communicate with one another [13]. Known as 'quorum sensing' or 'autoinduction', this chemical signalling system enables bacteria to function in a "quasi" multicellular manner by coupling gene expression to a critical population size (quorate) or cell density. Such synchronised behaviour is known to play a role in *inter alia*: inducing light emission, regulating plasmid conjugal transfer, swarming, the cessation of cell growth, stationary-phase survival, exoenzyme and capsular polysaccharide synthesis, protein secretion, phenazine antibiotic biosynthesis, the control of secondary metabolism and sporulation, the production of exotoxins, virulence determinants and cytotoxins by human and plant pathogens [14-16]. Bacteria known to use autoinduction associate with higher organisms as pathogenic, mutualistic, commensal or saprophytic symbionts [16].

Several different classes of pheromones have been identified however, the most intensely investigated are the *N*-acyl homoserine lactones (*N*-AHLs) [14]. *N*-AHLs are a group of low molecular weight compounds which have an identical homoserine lactone ring, but vary in length and structure of their *N*-linked acyl side chain. Each side chain, comprising between 4 and 14 carbons, is characteristic of the synthesis of different structural and functional signals. Homoserine lactones are not essential for growth and reproduction hence their classification as secondary metabolites [16,17]. *N*-AHLs have been detected in a variety of marine bacteria, most notably, in several *Vibrio* species [13,15,16]. They have also been identified in the bloom material of the cyanobacteria *Microcystis wesenbergii* [18]. The aim of this study was to determine if homoserine lactones are produced by marine bacteria associated with toxic phytoplankton [1,2], sodium channel blocking (SCB) toxins [5] and PST biotransformation [11,12]. This is the first step in studies on the potential role of *N*-AHLs in bacteria/phytoplankton interactions and neurotoxin production.

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MATERIALS AND METHODS

Bacterial culture

The bacterial strains tested for *N*-AHLs, their origin and function are listed in Table 1. *Vibrio anguillarum* MT 1741 and *Pseudomonas aeruginosa* NCIMB 8295 were used as *N*-AHL positive controls. SCB strains, bacteria demonstrating PST biotransformation and the positive controls were grown for 24 h at 25°C with vigorous shaking in 10 ml of marine broth (Difco). The *P. multiseries* bacterial isolates, which have a longer growth cycle, were grown for 96 h under the same conditions. *Chromobacterium violaceum* CV026 was grown for 24 h at 25°C with vigorous shaking in 10 ml of Lernia-Bertiani (LB) broth (plus 0.5% NaCl). *Escherichia coli* JM107 and JM109 were grown for 24 h at 37°C in 10 ml of LB (0.5% NaCl) supplemented with either 20 μg/ml tetracycline (JM107) or 50 μg/ml ampicillin (JM109) to maintain their plasmids. All strains used in this study were tested for the presence of *N*-AHLs at lag, log and stationary growth phases. Bacterial supernatant was obtained by centrifuging samples at 16,000 *g* for 1 minute.

Table 1: Bacterial strains tested for *N*-AHL production.

Bacterial Strain	Association	Source	Ref.
Cream	diatom microflora	*P. multiseries* CLN-1	[19]
Orange	diatom microflora	*P. multiseries* CLN-1	[19]
Pink	diatom microflora	*P. multiseries* CLN-1	[19]
Yellow	diatom microflora	*P. multiseries* CLN-1	[19]
407-2	PST/SCB dino. microflora	*A. tamarense NEPCC 407*	[3,20]
4αvs1	PST/SCB dino. microflora	*A. tamarense UW4*	[3,20]
4αvs3	PST/SCB dino. microflora	*A. tamarense UW4*	[3,20]
2c3	PST/SCB dino. microflora	*A. tamarense UW2c*	[3,20]
2c6	PST/SCB dino. microflora	*A. tamarense UW2c*	[3,20]
253-13	PST/SCB dino. microflora	*A. lusitanicum NEPCC 253*	[3,20]
667-2	PST/SCB dino. microflora	*A. affine NEPCC 667*	[3,20]
Shewanella alga (OK1)	TTX/SCB	*Jania* sp.	[21,22]
Alteromonas tetraodonis (GFC)	TTX/SCB	puffer fish	[21,22]
M11	PST bio-transformation	mussel	[23]
M12	PST bio-transformation	mussel	[23]
Q05	PST bio-transformation	queen scallop	[23]
R65	PST bio-transformation	razorfish	[23]
S09	PST bio-transformation	king scallop	[23]

PST – paralytic shellfish toxin, SCB – sodium channel blocker, dino. – dinoflagellate, TTX – tetrodotoxin.

Chromobacterium violaceum CV026 bioassay

Chromobacterium violaceum produces a purple pigment known as violacein by means of autoinduction. Strain CV026 is a mutant of the wild type and will only express the purple phenotype when exogenous *N*-AHL is present. The strain thus acts as a biosensor for the detection of *N*-AHL signal molecules with acyl side chains between 4 and 8 carbons long [24]. *C. violaceum* CV026 (200 μl) was spread over an LB agar plate to which supernatant from centrifuged bacterial samples (20 μl) and appropriate controls were spot-inoculated. Plates were left to incubate at room temperature for 48 h prior to analysis for purple pigmentation. *V. anguillarum* was selected as a positive control due to its ability to produce *N*-AHLs with both large and small acyl side chains [24]. Marine broth was used as a negative control.

Reverse Chromobacterium violaceum CV026 bioassay

N-AHLs with acyl side chains between 10 to 14 carbons long can be detected by their ability to inhibit violacein production stimulated by shorter homoserine lactones [24]. An overnight culture of *C. violaceum* CV026 (50 ml) was mixed with 0.6% LB agar (50 ml) and 500 μg of N-3-(oxohexanoyl)-L-homoserine lactone (OHHL, Sigma). This 'sloppy' agar was used to make five 20 ml plates. A sterile cork borer was used to make 5 mm wells in the agar which were then inoculated with supernatant from centrifuged bacterial samples (20 μl) or appropriate controls. Plates were incubated for 24 h at room temperature and checked to ensure they had turned purple. They were incubated for a further 48 h and analysed for white haloes around the sample wells which is indicative of inhibition of violacein production. *P. aeruginosa* NCIMB 8295 which produces *N*-3(oxododecanyoyl)-L-homoserine lactone [15] and marine broth served as controls.

Escherichia coli reporter strain bioassays

Escherichia coli JM107 transformed with plasmid pSB401 and *E. coli* JM109 with plasmid pSB1075 bioluminesce in the presence of *N*-AHLs [25,26]. The plasmids contain the structural genes *lux*CDABE from *Xenorhabdus luminescens* and either the *lux*R gene from *Vibrio fischeri* (pSB401) or the *las*R gene from *Pseudomonas aeruginosa* (pSB1075). Plasmid pSB401 is particularly useful at detecting *N*-AHLs with acyl side chains 6-10 carbons long and pSB1075 for side chains of 8-12 carbons. However, these reporter strains do detect a much wider range of *N*-AHLs with acyl side chains of different lengths and structure [26]. Whereas the *C. violaceum* bioassays rely on the naked eye for determination of results, bioluminescence from these *E. coli* reporter strains can be measured accurately in a luminometer. An overnight

culture (1 ml) of each reporter strain was used to inoculate 100 ml of LB broth containing the appropriate antibiotic. This was incubated with vigorous shaking at 37°C until an OD_{600} of between 0.15 and 0.2 (approximately 2 h later) was obtained. To triplicate luminometer cuvettes (BioOrbit), 500 µl of the *E. coli* reporter strain and 500 µl of bacterial supernatant were added and left to incubate at 37°C for 1 h. Marine broth (500 µl) was used as a negative control and a combination of 490 µl marine broth plus 10 µl of 5 mg/ml synthetic OHHL (Sigma) served as the positive control. The triplicate samples were measured for light output using a luminometer (BioOrbit).

RESULTS AND DISCUSSION

Table 2 lists results from the four different *N*-AHL assays. Only the positive control showed purple coloration using the *C. violaceum* CV026 assay. In the reverse *C. violaceum* bioassay, both TTX-associated strains and three of the PST biotransformers tested positive for *N*-AHLs with side chains between 10 and 14 carbons long. As all of these strains tested negative in the liquid culture assays (Table 2), it is possible they produce homoserine lactones with 14 carbon acyl side chains.

The *E. coli* biosensors gave a positive signal for three of the strains although the agar plate methods did not (Table 2). This may simply reflect the differential sensitivity of the two methods. Alternatively, it has been reported that compounds other than *N*-AHLs activate *E. coli* biosensors [27]. Diketopiperazines (DKPs), such as those found in cell-free supernatant of some *Pseudomonas aeruginosa* cultures, can stimulate *E. coli* biosensors in a concentration-dependent manner, albeit at a much higher concentration than *N*-AHLs. Although the physiological role of DKPs is not fully understood, their activity suggests the existence of 'cross talk' between bacterial signalling systems, therefore they may also play a role in cell communication. We have used well-characterised bioassays to indicate the presence of any *N*-AHLs, however mass spectrometry and NMR spectroscopy are required in order to distinguish homoserine lactones from DKPs [27]. Additionally, thin layer chromatography and high performance liquid chromatography (HPLC) followed by mass spectrometry is required to purify and classify *N*-AHLs [28,29].

N-AHLs were detected in three of the four *P. multiseries* isolates. The orange strain gave a consistently strong signal to both plasmid reporter vectors, although pSB401 gave the best response (Table 2). This may be due to the presence of more than one *N*-AHL between 6-10 carbons long. The yellow strain gave a weak positive signal during the lag phase of growth only. Bacteria have been shown to influence the production of DA in *Pseudo-nitzschia multiseries* [1,2] however, the mechanism for this is currently unknown. We postulate that *N*-AHLs may be a possible route by which bacteria influence diatom toxin production and the data presented here is the first step in investigating this theory. The results also show that *N*-AHL activity was detected in bacteria which produce tetrodotoxin or biotransform PSTs, again this is the first step in ongoing investigations to determine if homoserine lactones are involved in these functions. Only one of the seven PST/SCB toxin producers tested positive in the four assays inferring that homoserine lactones are not involved in the production of sodium channel blocking toxins by these isolates. This does not rule out the possibility that these bacteria utilise other less well understood chemical signalling systems to produce SCB toxins. These bacteria were also part of the microflora of their dinoflagellate of origin, however a higher proportion of isolates require to be examined to determine if bacterial *N*-AHLs are common in dinoflagellate cultures

Table 2: Results of *N*-AHL assays.

Bacterial Strain	*Chromo. violaceum*	Reverse *Chromo.*	*E. coli* 401	*E. coli* 1075
Cream	–	–	+	+
Orange	–	–	+	+
Pink	–	–	–	–
Yellow	–	–	+	+
407-2	–	–	–	–
4αvs1	–	–	–	–
4αvs3	–	–	–	–
2c3	–	–	–	–
2c6	–	–	–	–
253-13	–	–	–	–
667-2	–	–	+	+
S. alga	–	+	–	–
A. tetro.	–	+	–	–
M11	–	–	+	+
M12	–	–	–	–
Q05	–	+	–	–
R65	–	+	–	–
S09	–	+	–	–

(+) indicates positive detection of *N*-AHLs, (–) indicates no detection.

Homoserine lactones have been reported to be involved in bacterial/plant interactions [13,15,16] although to-date there is a dearth of information with regards to these compounds in relation to phytoplankton. Overall we suggest that signalling molecules may have a wider remit in bacteria/phytoplankton interactions which has largely been unexplored. More specifically, they may be involved in the population dynamics of harmful algal blooms (HABs). For example, it has been reported that algal-lysing bacteria must reach a cell density of 10^6 before algal lysis takes place [30,31]. If this activity is shown to be directly related to *N*-AHL production, it will greatly elucidate the mechanisms by which this potential tool for controlling HABs operates. Studies on allelopathic interactions between algae and bacteria would also benefit from investigating the presence and purpose of homoserine lactones.

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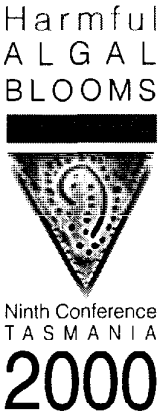
TOXINS : MECHANISMS OF ACTION



NEW AND POTENTIALLY TOXIC BACTERIAL ISOLATES FROM *NOCTILUCA SCINTILLANS* (DINOFLAGELLATA)

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ABSTRACT

Although generally regarded as harmless in European waters, the bloom-forming dinoflagellate *Noctiluca scintillans* has been occasionally associated with fish mortality and reduced shrimp yields in Asia. The reason for this phenomenon is unclear, but it is possible that bacteria may be involved in harmful algal blooms; the bacteria may produce their own toxins or influence the toxic levels of the algae. It is thus noteworthy that up to 1 % of *Noctiluca* occurring in the southern North Sea contain large numbers of intracellular bacteria; these *Noctiluca* cells appear visibly turbid.

Analysis of the diversity and dynamics of bacterial populations associated with *Noctiluca scintillans* by denaturing gradient gel electrophoresis (DGGE) indicates the occurrence of one dominant group of bacteria within *Noctiluca* and different other groups in smaller amounts. In contrast, free-living bacterial populations in the water column consist of several different dominant groups. Up to now 18 bacterial isolates from *Noctiluca* cells have been cultured. The bacteria have been characterized by classic physiological (including antibiotic sensitivity) and molecular biological methods. Phylogenetic analysis of the 16S rDNA of the bacteria revealed a great diversity among the bacterial isolates belonging to different groups of bacteria, i.e. bacteria of the γ-subdivision of the Proteobacteria, and of the Gram positive high G+C% group. Two of the isolates - one belonging to the α-subdivision of the Proteobacteria and *Alteromonas macleodii* - show sodium channel blocking activity. The role and significance of the intracellular bacteria with regard to *Noctiluca* blooms is discussed.

INTRODUCTION

The relationships between bacteria and harmful algal blooms may be very complex. While many algae are able to produce toxins [see reviews by 1, 2] bacteria attached to or associated with algae may also produce toxic substances, or influence the toxicity of the algae [3-7].

Of particular interest are the bacteria living intracellularly in bloom-forming algae, where the conditions for their survival and growth are very different from those in the water column or on the outer cell surfaces. Among the dinoflagellates, intracellular bacteria/cyanobacteria have been found in *Glenodinium foliaceum* Stein and *Gonyaulax diacantha* (Meunier) Schiller [8, 9]; *Amphidinium herdmanii* and *Katodinium glandulum* [10], *Gymnodinium lebourae* Herdman [11], *Gymnodinium splendens* Lebour [9]; *Noctiluca scintillans* Macartney 1810 syn. *miliaris* Suriray 1836 [12, 13]; *Peridinium balticum* [14] and in the genera *Ornithocercus*, *Histioneis* and *Citharistes* [15, 16].

Not all of the above mentioned dinoflagellates form conspicuous or harmful blooms. Although considered as non-toxic in European and American waters, *Noctiluca* has occasionally been implicated in fish mortality and reduced shrimp yields in Asia [17,18]. In many cases, however, the evidence may be circumstantial. Ammonia accumulation and oxygen depletion have also been named as factors in *Noctiluca* toxic phenomena.

In this paper we describe 18 bacterial isolates from *Noctiluca scintillans* cells, all of which have been cultured. These are characterized by classical physiological and molecular biological methods. The role and significance of the intracellular bacteria with regard to *Noctiluca* blooms are discussed.

MATERIALS AND METHODS

The determination of *Noctiluca* abundance in plankton hauls at the Helgoland Roads (German Bight, North Sea), cultivation of *Noctiluca* in the laboratory and experimental conditions are described in [13]. In brief, plankton hauls were examined 5 days a week; clear and turbid cells were counted separately under a stereo microscope. All turbid and clear *Noctiluca* were maintained in the laboratory at 19±1°C in glass vessels containing 50-100 ml of 0.45µm filtered seawater and fed with the unicellular green alga *Dunaliella tertiolecta* Butcher. Cultures were not axenic.

Endocytic bacterial isolates designated as NE1 and NE2 (*Alteromonas macleodii*) were isolated from turbid *Noctiluca* cells [13] and maintained on ZoBell agar slants. Bacterial isolates numbered 1 through 16 originated from both clear and turbid (free living and cultured) *Noctiluca* cells without food vacuoles, treated with cetyl-trimethyl-ammonium bromide (CTAB, 1 µg ml^{-1}), washed 4x with sterile seawater and transferred to 5 ml liquid diluted (10% strength) ZoBell medium (pH range from 3.1 to 7.9). Cells were then pierced and incubated on shakers (90 rpm) at 18°C. After 7 d, 38 µl were plated onto ZoBell agar and incubated further at 18°C.

Isolates NE1 and NE2 were tested by the German Collection of Microorganisms (DSMZ, Braunschweig). Morphological and physiological tests were performed on isolates 1-16 according to [19].

Tests for antibiotic sensitivity of all isolates were based on the method of [20]. Susceptibility discs (Oxoid Ltd., Hampshire, England) included chloramphenicol 10 µg, penicillin G 5 i.u., tetracycline 30 µg, streptomycin 10 µg, penicillin 1 i.u., and nystatin 100 i.u. All isolates were grown on ZoBell agar. Clear zones around

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the discs indicating growth inhibition were measured after 4 days at 20°C. *Escherichia coli* B (Kiel) served as control. Screening for production of antibiotic-like substances was performed by streak tests on ZoBell agar [21], modified. Species tested for growth inhibition by the endocytic bacteria were *E. coli* B, two North Sea isolates and 7 marine bacteria species from the NCIMB (National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland). Laboratory experiments were performed to determine if the endocytic bacteria in turbid *Noctiluca* cells would be visibly reduced by the presence of antibiotics in the culture water. Penicillin G, chloramphenicol and streptomycin were used at a final concentration of 10 mg l^{-1} and also a mixture of all three. Both turbid and clear *Noctiluca* cells were placed in glass vessels (10 cells in 40 ml sterile seawater) with *Dunaliella* food; change of water and food took place every 4 days. Vessels were examined daily and evaluated after 9 days.

The mouse neuroblastome (MNB) assay for the detection of sodium channel blocking (SCB) toxins was performed by the method of [22].

Molecular biological methods were used to describe and classify the culturable as well as non-culturable endocytic bacteria from clear and turbid *Noctiluca* cells, from *Noctiluca* and *Dunaliella* culture water, and from the marine environment.

To characterize the endocytic bacterial population in its entirety within *Noctiluca*, both clear and turbid cells were used. Single *Noctiluca* cells were used for PCR amplification after extensive washing procedure to remove bacteria from the outer cell surface [13]. The ensuing DNA extract was used for PCR amplification and DGGE. Samples of water from the marine environment were taken from 25 June to 6 August 1997. Bacterial biomass was concentrated for nucleic acid extraction using a combination of methods [23]. After prefiltration with 60 µm gauze and 3 µm filters, the water samples were pumped through a 0.22 µm Sterivex filter unit (Millipore Corp.). Filters were stored at -20°C until further processing for PCR and DGGE.

PCR experiments were carried out for *Noctiluca* and bacterial DNA according to [24], briefly described in [13]. DGGE was performed according to [24].

RESULTS

The annual percentages of turbid cells occurring in plankton hauls at Helgoland from 1995 to 1999 were 0.41, 0.46, 0.06, 0.07, and 1.35%, respectively. While the values for 1997-98 are relatively low, there was a large increase in 1999, particularly during the last week in July.

On ZoBell agar all isolates form colonies with a shiny surface; two show yellow pigmentation (1 and 3), the rest are beige. Two are Gram positive, most cells are rod shaped and all but two are motile (Table 1). Isolate no. 1 is oxidase negative; only two do not have the enzyme catalase. Most of the isolates are capable of both oxidative and fermentative hydrolysis of sugars; nos. 1 and 3 are unable to utilize any of the sugars and no. 5 only fermentative.

Table 1. Characterization of bacterial isolates from *Noctiluca* cells (t = turbid *Noctiluca* cell, c = clear; oxid., cat. = presence of cytochrome oxidase and catalase)

Isolate No.	Noctiluca source	Gram stain	Cell form	Motility	Oxid.	Cat.	SCB-blocking activity
NE1	t, free living	-	irreg. rods	+	+	+	+
NE2	t, cultured	-	rods	+	+	+	+
1	t, free living	positive	coccoid	+	-	+	-
2	t, free living	-	curved rods	-	+	-	-
3	c, free living	positive	coccoid	-	+	+	-
4	c, free living	-	spiral rods	+	+	-	-
5	t, free living	-	coccoid	+	+	+	-
6	t, free living	-	spiral rods	+	+	+	-
7	t, free living	-	rods	+	+	+	-
8	t, free living	-	rods	+	+	+	-
9	c, free living	-	rods	+	+	+	-
10	c, cultured	-	rods	+	+	+	-
11	c, cultured	-	rods	+	+	+	-
12	c, cultured	-	rods	+	+	+	-
13	t, cultured	-	rods	+	+	+	-
14	t, cultured	-	rods	+	+	+	-
15	t, cultured	-	coccoid	+	+	+	-
16	c, cultured	-	rods	+	+	+	-

Production of antibiotic-like substances: in streak tests none of the isolates inhibited the growth of ten species tested (data not shown).

Antibiotic sensitivity of endocytic bacteria: clear zones around the antibiotic discs indicating growth inhibition were measured. Results show that all isolates are sensitive to chloramphenicol and streptomycin, and only 3 to nystatin. Isolates nos. 2 and 4 were inhibited by all antibiotics tested.

The addition of antibiotics to the culture water either singly or combined did not visibly reduce the turbidity of *Noctiluca* cells as observed over a 9-day period under a dissecting microscope. The growth rates were determined after 4 days before some cells ceased to divide and formed swarmers. The growth rates µ of turbid *Noctiluca* with antibiotics present ranged from 0.08 to 0.13; the number of cells in the control remained unchanged. Clear cells with antibiotics had µ values from 0.83 to 0.99 (0.95 for the control). Higher growth rates for clear cells compared to turbid have been previously reported [13].

Isolates NE1 and NE2 demonstrate sodium channel blocking activity. The remaining isolates do not.

Molecular biological analyses of endocytic bacteria in their entirety within free-living turbid *Noctiluca* cells by DGGE showed a single band of high intensity, and above this, several weaker bands (Fig. 1). The same pattern was found for endocytic bacteria from laboratory cultured turbid *Noctiluca* cells. This indicates the presence of one dominant bacterial group and several less abundant groups. There was no change in band patterns corresponding to length of *Noctiluca* cultivation, whether the original turbid cells were free-living or longer in laboratory culture, at the time of analysis (data not shown).

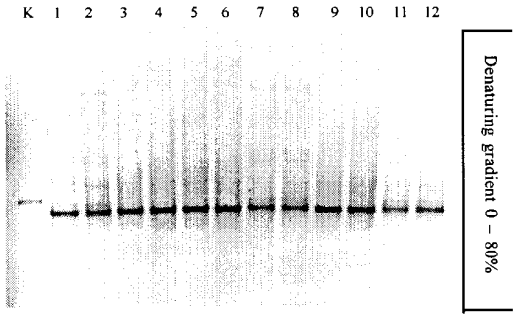

Figure 1. DGGE gel band patterns of endocytic bacteria from free living turbid *Noctiluca* cells. Lanes 1-12 represent samples taken 7 to 11 July 1997.
K : reference strain *E. Coli* J53.

Free-living marine bacteria showed a variable gel pattern with at least 10 bands of different intensities. Thus as many as 10 dominant bacterial groups may occur; no single main band was observed. Bands produced from bacteria from *Dunaliella* and *Noctiluca* culture water did not show any agreement with those from free-living and cultured *Noctiluca* cells (data not shown).

DISCUSSION

Visibly turbid *Noctiluca* cells occur regularly in plankton hauls at Helgoland. Unusually high numbers were recorded at the end of July 1999, at the end of a *Noctiluca* bloom ("red tide"). While endocytic bacteria are transferred to daughter cells during cell division [13] it is not yet known how the bacteria were initially able to infect *Noctiluca* and enter its cytoplasm. Possibly "sloppy feeding" may be involved, whereby bacteria entering the cytostome with other food particles bypass a vacuole membrane and actively invade the host cytoplasm [12]; indeed, most of the endocytic bacterial isolates from *Noctiluca* cells are motile. The bacteria are not enclosed within a membrane as reported for other dinoflagellates with endocytic bacteria [11, 14]. Ingested diatoms with sharp edges could rupture the food vacuole, allowing bacteria to escape. Destruction of the vacuole membrane by bacterial enzymes is also conceivable.

An estimated number of 10^5 - 10^7 bacteria may be found within a single turbid *Noctiluca* cell [13]. The turbidity was not reduced when antibiotics were added to the culture water. Turbid cells in culture have lower growth rates than clear cells, and turbid stock cultures are more prone to collapse than clear cells under the same conditions. Large numbers of intracellular bacteria may therefore be detrimental to the growth of *Noctiluca.*

In the marine environment, free living or attached bacteria may influence the growth of harmful algal species, whereby the effect tends to be more inhibitory than stimulatory [2]. Algicidal activity of bacteria has already been described for various phytoplankton species including dinoflagellates, diatoms and raphidiophytes [25-29]. It is possible that endocytic bacteria in *Noctiluca*, without being directly algicidal, may bring about a decline during a bloom. This would not be without precedent: algicidal marine bacteria (free living, not endocytic) may be involved in the termination of an algal bloom in Hiroshima Bay, Japan [30].

Endocytic bacteria have been found in many species of algae [10-15]. In most of these cases, the algae cells were cultured. The bacteria were described, if at all, as rods or cocci, but were not further identified. With molecular biological methods, it could be shown that endocytic bacteria from turbid and clear *Noctiluca* differ from one another in quality as well as quantity. Turbid *Noctiluca* cells, whether free-living or cultured over different periods of time, harbor similar groups of bacteria consisting of one dominant and several less abundant groups. A cultivation effect may thus be excluded. Bacterial DNA from clear *Noctiluca* cells shows a different pattern from that of turbid cells [13, 31]. Endocytic bacteria produce DGGE gel patterns which are different from those of free-living marine bacteria and again from those present in *Noctiluca* and *Dunaliella* culture water. This indicates that the endocytic bacteria in *Noctiluca* consist of populations which are especially adapted to the environment in the host cytoplasm.

Characterization of 18 isolates from both turbid and clear *Noctiluca* cells shows that the majority belong to the γ-subgroup of Proteobacteria, and one to the α-subgroup. The phylogenetic diversity of these isolates compared with bacteria from other biotopes is discussed in detail elsewhere [31]. Two isolates show sodium channel blocking activity: NE1 and NE2. Isolates 10-13 and 16 show a similarity to isolate PCOB-2 which also shows sodium channel blocking activity, and was isolated from the toxic alga *Protogonyaulax* (= *Alexandrium*) *cohorticula* [32]. Four (nos. 6-9) belong to the *Pseudoalteromonas* group, species of which are generally found in association with marine eukaryotes and show e.g. antibacterial and algicidal activity; several in the group produce toxic substances [29, 33]. Members of the genera *Aeromonas, Alteromonas/Pseudomonas* and *Vibrio* were frequently isolated from dinoflagellate bloom as well as non-bloom waters [3, 7].

Noctiluca is regarded as a non-toxic species in European waters, despite occasional reports of toxic phenomena in connection with *Noctiluca* blooms in other areas of the world [34]. However, *Noctiluca* cells may contain large numbers of endocytic bacteria including strains or species which may be involved in the production of harmful substances. These turbid *Noctiluca* cells demonstrate a reduced growth rate compared to clear cells. Large numbers of endocytic bacteria may conceivably be involved in the decline of a *Noctiluca* bloom in the North Sea, as e.g. in July 1999 when the proportion of turbid cells was relatively high. The bacteria which had become concentrated within the cells may be released into the water column after a bloom breakdown.

It is known that attached bacteria may alter the toxicity of an algal species [7] or themselves be involved in the production of toxic substances [4, 32, 6,]. With the exception of a *Moraxella* sp. isolated from an apparently axenic culture of *Protogonyaulax* (= *Alexandrium*) *tamarensis*, which possibly was inside the

algal cells [4], other toxin-producing bacteria reported appear to be externally associated with the algae and present in the environmental water. There are no reports of bacterial enrichment within the algal host cells.

In conclusion, it is recommended to examine *Noctiluca* cells obtained from other parts of the world, especially where toxic phenomena occur, for turbid cells with endocytic bacteria.

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INVESTIGATIONS INTO THE TOXICOLOGY AND PHARMACOLOGY OF SPIROLIDES, A NOVEL GROUP OF SHELLFISH TOXINS

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ABSTRACT

The mammalian toxicity of spirolides, a novel group of macrocyclic imines, was first revealed in routine monitoring for diarrhetic shellfish poisoning (DSP) toxins from shellfish aquaculture sites in Nova Scotia, Canada. Spirolides elicit a novel and highly potent toxic response in mice after intraperitoneal injections. In toxicological studies, the oral and intraperitoneal toxicities of spirolides in mice were determined to be approximately 1 mg kg^{-1} and 40 μg kg^{-1}, respectively. The pharmacological effects of spirolides were also characterized by subjecting mice to various drugs (e.g., atropine, physostigmine, propanolol and epinephrine), followed by a challenge with a spirolide-rich extract of *A. ostenfeldii* cultures or purified spirolides. Some therapeutants were capable of enhancing survivability, whereas others produced faster death times. After administration of "antidotes" to these therapeutants, and the observation of reversal or enhancement of spirolide effects, at least one mode of action was indicated. Spirolides appear to affect the muscarinic acetylcholine receptors in mammalian systems. The potential implications for human health, if any, from consuming spirolide-contaminated shellfish have not yet been determined.

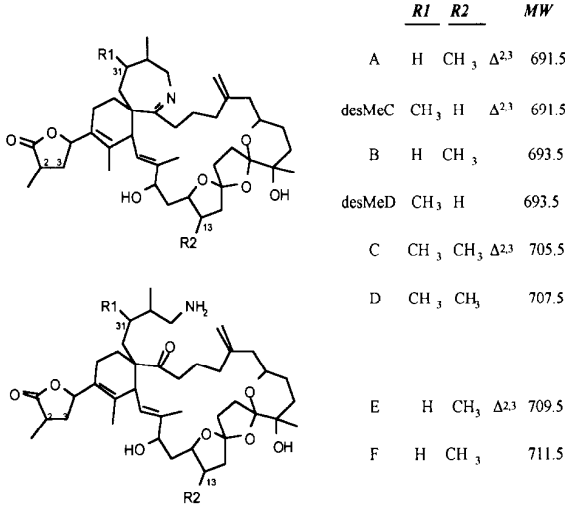
INTRODUCTION

In 1991, routine biotoxin monitoring of bivalve molluscs at aquaculture sites along the eastern shore of Nova Scotia, Canada, first revealed a novel and highly potent toxic response in mice after intraperitoneal (IP) injections of lipophilic extracts. The symptoms, which included rapid deaths preceded by neurological symptoms, were very different from those associated with known shellfish toxins, including those responsible for DSP or PSP intoxication. Such toxic episodes are now annually recurrent at multiple sites in Nova Scotia, but have not been categorically linked to any human intoxication.

The symptoms of this novel "fast acting toxin" in mice after IP administration of contaminated shellfish extracts, included piloerection, abdominal muscle spasms, hyperextensions of the back, and arching of the tail to the point of touching the nose. Rapid death was observed within 3-20 min, preceded by neurological symptoms, including convulsions. If a mouse survives past 20 min while demonstrating symptoms, it will recover fully and quickly.

The original episodes (1991), and most of the subsequent annual toxic events, were confined to the southeastern shore of Nova Scotia, specifically sites at Ship Harbour and Mahone Bay. Although these events have not been categorically linked to any human intoxication, there have been anecdotal consumer reports of vague symptoms (gastric distress, tachycardia) after shellfish consumption from these sites during periods (May to July) when so-called "fast acting toxicity" was apparent in mouse bioassays of lipophilic extracts of shellfish. As a precautionary measure, in most years, the local aquaculturists voluntarily withheld shellfish from the market until the unusual mouse toxicity abated.

The episodic seasonal nature of the toxic symptoms in mice, and rapid shifts (within days) in toxicity, as inferred from mouse death times, made it difficult to collect enough toxic shellfish to permit isolation and identification of the causative agent(s). In 1994, 3.9 kg of toxic scallop digestive glands were collected and subjected to a purification scheme. The results revealed the presence of a family of novel macrocycles, named spirolides, consisting of a spiro-linked tricyclic ether ring system and an unusual seven-membered spiro-linked cyclic iminium moiety [1]. The structures of spirolides belonging to groups A, B, C, and D are shown in Figure 1. Also shown are two other spirolides, E and F, isolated from shellfish [2]; these have been shown to be biologically inactive, presumably due the opening of the cyclic imine function. They appear to be shellfish metabolites or degradation products.



	R1	R2		MW
A	H	CH_3	$\Delta^{2,3}$	691.5
desMeC	CH_3	H	$\Delta^{2,3}$	691.5
B	H	CH_3		693.5
desMeD	CH_3	H		693.5
C	CH_3	CH_3	$\Delta^{2,3}$	705.5
D	CH_3	CH_3		707.5
E	H	CH_3	$\Delta^{2,3}$	709.5
F	H	CH_3		711.5

Fig. 1. Chemical structures of spirolides that have been identified in Nova Scotian shellfish and *A. ostenfeldii*. Compounds indicated with a $\Delta^{2,3}$ have a double bond between carbons 2 and 3.

The biological origin of spirolides was unknown until recently, although the evidence (geographical extent, seasonality, and occurrence in multiple shellfish species)

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strongly suggested a planktonic source [3]. This hypothesis was also supported by the high degree of structural homology between spirolides and other macrolides of marine dinoflagellate origin, including gymnodimine (from *Gymnodinium mikimotoi*) and prorocentrolides (found in *Prorocentrum lima*) [4]. Using liquid chromatography-mass spectrometry (LC-MS) analyses, various spirolides of the A, B, C and D groups were detected in size-fractions of planktonic material from Nova Scotian aquaculture sites. The dinoflagellate *A. ostenfeldii* was determined to be the causative organism [5]. A spirolide-producing culture of *A. ostenfeldii* was eventually obtained from Ship Harbour, NS, and used to generate the biomass required for spirolide isolation and purification, as well as for toxicity trials using crude cell-free extracts [6].

The mode of action of spirolides, resulting in the "fast acting" toxic response in the mouse bioassay, has so far been elusive. To foster further research into the potentially toxic effects on humans, we report on initial investigations using the laboratory mouse, to confirm and validate the toxic effects of spirolides in a whole animal model. Previous researchers have used various therapeutants and whole animal bioassays as a means of identifying general modes of actions for various bioactive compounds. For example, Schantz experimented with whole animal assays (mice and rabbits) in his attempts at characterizing the mode of action of saxitoxin [7]. The use of whole animals as a means of describing toxic effects has also been reported for tetrodotoxin and saxitoxin [8,9].

METHODS

Materials

Toxicity tests were performed on either small quantities of spirolides B and D isolated from toxic shellfish [1] or crude extracts of the more readily available *A. ostenfeldii* culture (strain AOSH1) isolated from Ship Harbour, Nova Scotia. The *A. ostenfeldii* extract, hereafter named AOE, was prepared from cellular biomass collected by centrifuging samples of cultures in exponential growth phase, followed by sonication of the pellet in a small amount of water. The residue was then suspended in 1% Tween 80R in physiological saline (0.85% NaCl) and centrifuged again. A typical preparation involved extraction of a 0.34-g pellet into a 20-mL volume, thus resulting in 17 mg wet plankton equivalents per mL. The total spirolide content of this AOE was found to be 129 $\mu g\ mL^{-1}$ according to LC-MS analyses.

Toxicity assays

Mice (female, CD-1 strain) were purchased from Charles River (Canada) and the husbandry was regulated according to the Canadian Council on Animal Care guidelines. For intraperitoneal (IP) toxicity measurements, mice of uniform size were injected with 1 mL volumes of spirolide standard solutions or dilutions of AOE. The intragastric (IG) toxicity was assessed using 0.5-mL doses of AOE at four dilutions: 17, 8.5, 6.4, and 4.3 mg wet plankton per mL.

Pharmacological investigations

In an attempt to characterise the pharmacological effects of spirolides, mice were subjected to various drugs followed with a challenge of AOE. The initial screening evaluation involved four drugs: epinephrine, propanolol, physostigmine and atropine. Each drug was prepared at concentrations that would provide a sub-lethal, but therapeutic, dose. Pre-screening was used to confirm the lethality of the doses. Each of four mice (per drug) was then inoculated with 0.5 mL of the therapeutant and the time recorded. After 5 to 7 minutes, a challenge consisting of 0.5 mL of AOE was administered by IP injection and the time recorded once more. Subsequent experiments were performed to determine specific sub-receptor effects by administering the drugs tropicamide, gallamine, pirenzepine, and methacholine, and observing the resulting times to death.

RESULTS AND DISCUSSION

Intraperitoneal toxicity

Due to the short supply of pure spirolides, a crude aqueous extract of *A. ostenfeldii* (AOE) was used for most of the experiments. Preliminary IP assays revealed that the toxicity of pure spirolides and AOE were essentially equal when death times were normalized to the total amount of spirolide injected, based upon LC-MS analyses. This gave us confidence that dose-response data based on AOE was legitimate, at least for IP work. It should be noted that the principal spirolide present in the *A. ostenfeldii* culture of strain AOSH1 is desmethyl-C (>90% on a molar basis), along with analogues C, C3, D, D3 and desmethyl-D [3]. Another important point is that all spirolides from both the A/B and C/D classes isolated to date have shown similar IP toxicities.

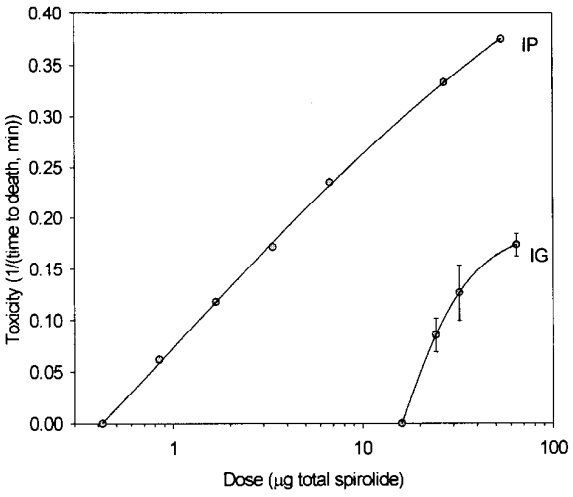

Fig. 2. Toxicity of spirolides in mice through intraperitoneal (IP) and oral (IG) dosing. The curves are logistic dose response curve fits generated by SigmaPlot. The IP data points are means of duplicate tests, whereas those for the IG tests are means of 6-8 replicate tests, with standard deviations shown as error bars.

IP injection of AOE into CD-1 female mice revealed an almost linear dose-response relationship, as shown in Figure 2. These data indicate an LD_{50} toxicity value of approximately 40 μg kg^{-1}. It is interesting to note that, on a molar basis, spirolides (MW 691-707) have a toxicity about half that of the potent paralytic shellfish poison, saxitoxin dihydrochloride (MW 372, LD_{50} = 10 μg kg^{-1}).

Oral toxicity

Having demonstrated toxicity of spirolides via the IP route of administration, it was important to test the oral potency, which is particularly critical for evaluating human health implications. Although spirolides and the related dinoflagellate toxin, gymnodimine, have not been associated with any significant incidents of human illness, some other structurally similar cyclic imines, the pinnatoxins (isolated from the bivalve *Pinna muricata*), were implicated in major human toxicity incidents in Japan and China [10,11]. It is possible that the difference could be due to the doses to which humans were exposed, but it is also possible that gymnodimine or spirolides could be destroyed in the digestive tract by acid or enzymes, or that they are not even absorbed by the intestine into the blood. Interestingly, it had been observed previously that when spirolides A and B are subjected to acid hydrolysis (oxalic acid, 60°C), the imine ring opens and non-toxic spirolides E and F are produced (see Figure 1) [2]. It was also shown that spirolides C and D do not decompose under the same conditions. This was attributed to the steric effects of two methyl groups on the imine ring.

A quick test was perfomed to see if spirolides would survive stomach acid conditions by measuring the relative rates of hydrolysis of spirolides B and D in pH 3 aqueous hydrochloric acid at 37°C. Surprisingly, no ring opening was observed for either spirolide, even after 24 h. This suggested that acid degradation in the stomach is not a significant factor. Despite this, it is still important to determine if there is difference between the toxicities of the A/B and the C/D types, as steric effects can be significant in metabolic degradations as well.

The intragastric (IG) toxicity of spirolides was assessed with CD-1 female mice using 0.5-mL doses of AOE at four dilutions: 17, 8.5, 6.4, and 4.3 mg mL^{-1}. The percent mortality at the four concentrations was 100%, 100%, 75% and 0%, respectively. The toxic response as a function of total spirolide dose is summarized in Figure 2. It indicates that the minimum lethal dose results in a death time of about 15-18 min. By extrapolation, the oral LD_{50} was estimated to be 20 μg $mouse^{-1}$ or 1 mg kg^{-1}.

To confirm that the oral toxicity was due to the spirolides in the extract and not due to other components in the plankton matrix, some previously isolated and purified spirolides A and D were also tested for oral toxicity. IG administration of 50 μg of spirolide D into each of three mice resulted in three deaths (two at 4 to 5 min, and a third at 8 min; there was some leakage from the syringe upon injection into the latter subject). There was only enough spirolide A for administration of 40 μg into each of two mice: one mouse survived whereas the other died in 19 min. These results suggest that spirolide A might be less toxic than D, but this needs to be confirmed with further testing when more spirolide A has been isolated. These specific toxin tests support the observations made with *A. ostenfeldii* culture extract, which consists of C/D class spirolides.

Pharmacological investigations

In an attempt to characterise the pharmacological effects of spirolides, mice were subjected to sub-lethal, but therapeutic, doses of various drugs followed with a challenge of AOE. The working hypothesis was that decreases or increases in death times could be used to classify the actions of AOE, and to infer the possible mode of action of spirolides.

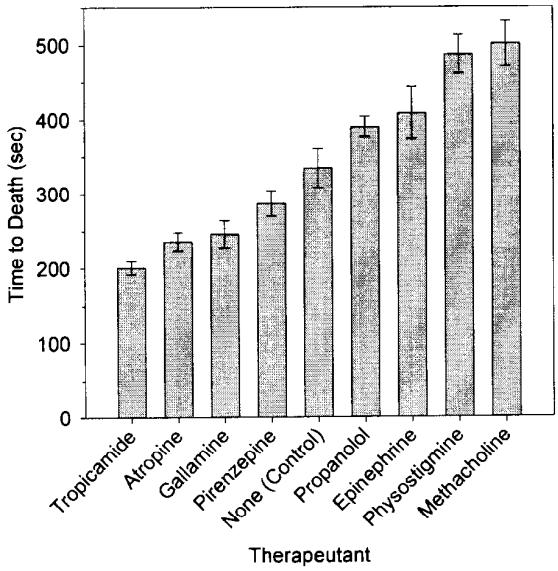

Fig. 3. Results of testing the effect of various agonists and antagonists on the mouse death time. The results shown are means of four replicates and the error bars indicate the standard deviations.

The initial screening evaluation focused on the effect of four drugs: epinephrine, propanolol, physostigmine and atropine. As seen in Figure 3, the control raw extract resulted in a death time of approximately 5 min (334 ± 26 s). Any deviation in death times would then represent the apparent effect of the therapeutant. Initial screening demonstrated little effect due to epinephrine (adrenaline) and propanolol at the doses tested. However, the effects of the physostigmine pre-treatment resulted in reduced time to death (487 ± 26 s). This indicates that the effect of the putative toxic agent is blocked by physostigmine, a cholinesterase inhibitor. On the other hand, mice pre-treated with atropine died more quickly (236 ± 12 s). These results could indicate similar effects of atropine and the putative agent (spirolides). It is significant that atropine is the drug of choice to counteract the effects of physostigmine poisoning, and vice versa. The effects of atropine (and by inference spirolides, if the mechanism is similar) in humans include the following: (a)

anticholinergic activity though a blocking of the muscarinic acetylcholine receptor; (b) smooth muscle relaxation; (c) reduced salivary and bronchial secretions, dryness of mouth; (d) increased heart rates, possibly inducing a sinus tachycardia; (e) reduced gastrointestinal motility, abdominal cramps; (f) pupillary dilation; (g) blurred vision; (h) urinary retention; (i) hyperthermia, possibly due to decreased sweating; (j) bitter taste; (k) vomiting; and (l) headache, dizziness, drowsiness.

These preliminary results indicated that spirolides may affect the muscarinic acetylcholine receptor. As such, this avenue was pursued further for evidence of specific sub-receptor effects by administering the drugs tropicamide, gallamine, pirenzepine, and methacholine, and observing the resulting effects. The results, also summarised in Figure 3, strongly suggest that spirolides mimic the actions of muscarinic acetylcholine antagonists. The acetylcholine agonists offer protection, as expected, which confirms at least one mode of biological effect by spirolides. In summary, although we cannot exclude other biological effects, spirolides have demonstrated a strong affinity to the muscarinic acetylcholine receptor.

CONCLUSIONS

1. Spirolides are produced by *Alexandrium ostenfeldii*, a common marine dinoflagellate in natural plankton populations in temperate coastal zones.
2. Spirolides concentrate in shellfish tissues to levels that can cause rapid death of mice when using a "DSP-type" lipophilic extract.
3. The symptomology and toxic effects in mice administered (IP and IG) with aqueous extracts of cultured *A. ostenfeldii* is consistent with that of purified spirolides, i.e., biological activity in plankton is accounted for by the spirolides alone.
4. Spirolides are highly toxic to mice via both intraperitoneal (IP) and intragastric (IG) administration, with estimated LD_{50} values of 40 $\mu g\ kg^{-1}$ IP and 1 $mg\ kg^{-1}$ IG.
5. Although all spirolides appear to be toxic via IP administration, there may differences between the oral toxicities of spirolide types A/B and C/D.
6. Spirolides have been shown to produce an antagonistic effect at the muscarinic acetylcholine receptor, although it is possible that they might produce effects at other biological receptors as well.

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PARALYTIC SHELLFISH TOXINS AND GLUTATHIONE *S*-TRANSFERASES IN ARTIFICIALLY INTOXICATED MARINE ORGANISMS

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ABSTRACT

The metabolism of algal toxins by marine organisms is a field of emerging interest. In spite of numerous studies on the depuration of paralytic shellfish toxins (PSTs) from marine bivalves, little work has been conducted on the mechanisms of PST detoxification. Exposure experiments show PSTs to cause induction in the activity of the phase II detoxification enzyme, glutathione *S*-transferase (GST) in salmon (*Salmo salar*) livers and mussel (*Mytilus edulis*) digestive glands. Liver samples from salmon periodically injected ip over 21 days with saxitoxin and a toxic dinoflagellate extract showed elevated GST activity relative to controls. Immunodetection of GST isoenzymes by western and dot blotting with mammalian antibodies suggests that increased expression of GST protein may be the primary cause of the activity induction observed.

Mussels were also exposed to doses of saxitoxin by intra-muscular injection. A small but significant elevation of GST activity was noted in the digestive glands of exposed groups relative to controls. This activity induction may be due to increased GST expression or enzyme activation. This work suggests that there may be a role for detoxification enzymes such as glutathione *S*-transferase in the detoxification and elimination of PSTs from these fish and shellfish models.

INTRODUCTION

Paralytic shellfish toxins (PSTs) accumulate in filter feeding bivalves and pose serious health risks to human consumers. Exposure of fish to such toxins can result in fish kills [1] and other deleterious effects in marine food webs [2]. A greater understanding of the precise fate of PSTs in marine organisms is therefore required. Studies in this field to date have focussed on the dynamics of PST depuration from bivalve molluscs [3], yet little is known of the mechanisms responsible for eliminating these toxins from fish and shellfish. It has been suggested that drug metabolising enzymes (DMEs) may be involved in the metabolism of algal toxins [4]. Intra-peritoneal (ip) exposure of salmon (*Salmo salar*) to saxitoxin (STX) has been shown to effect the induction of cytochrome P-4501A, a phase I DME, as measured by 7-ethoxyresorufin *O*-deethylase activity [5].

Investigated here is the potential role of the phase II DME glutathione *S*-transferase (GST) in PST metabolism. GSTs catalyse the conjugation of reduced glutathione (GSH) to electrophilic centres on substrates. This activity is inducible on exposure of the organism to the substrate. The objective of this study was to determine the induction response of GST activity in marine organisms exposed to PSTs by injection. Two species of commercial importance to Scottish aquaculture were chosen to represent model fish and shellfish respectively: Atlantic salmon (*Salmo salar*) and blue mussels (*Mytilus edulis*).

MATERIALS AND METHODS

Salmon

Four groups of 10 post-smolt Atlantic salmon (single sex, average weight 400 g) were acclimated for 2 weeks in 1 m diameter, circular tanks supplied (through-flow) with fresh, aerated sea water. Toxins were administered ip at 10-day intervals to fish under anaesthesia (MS-222™) and all fish were sampled 21 days after initial exposure. Fish were exposed to:

a) Physiological saline (control)
b) Saxitoxin diacetate (Calbiochem, 3 injections of 2 µg/kg live fish in physiological saline)
c) Saline extract of *Alexandrium fundyense*, CCMP 1719 (3 injections of 1.51 µg STXeq/kg live fish [2.26 µg PSTs/kg live fish] of which: NEO 41.67%, GTX4 28.11%, C2 22.52%, GTX3 2.60%, GTX1 2.21%, STX 1.55% and C1 1.33%)
d) A saline extract of the non-toxic dinoflagellate *Scrippsiella trochoidea*, NEPCC 15 (control, toxins not detected).

Injections were administered at a fixed value of 125 µl/100 g live fish. Dinoflagellate extracts were prepared in physiological saline using equal numbers of toxic and non-toxic cells from stationary phase cultures in Guillard's f/2 (without silicates) media (Sigma, UK). The toxicity of extracts was determined by HPLC according to Franco & Fernández-Vila [6].

On sampling, livers were rapid-dissected from fish and snap frozen in liquid nitrogen for storage at -70 °C. Thawed samples (200 ± 10 mg) were homogenised on ice using a Potter-Elvehjem glass / teflon homogeniser (1000 rpm, 7 strokes) in 1 ml of 0.2 M potassium phosphate buffer containing 1 mM DTT and 1 mM EDTA adjusted to pH 7.4. Homogenates were centrifuged (10,000 g, 20 minutes, 4 °C) and the protein fraction collected for analysis. GST activity of protein fractions was determined at 20 °C using 1-chloro 2,4-dinitrobenzene as a substrate according to Habig *et al.* [7]. Data were normalised to the protein content of the sample as determined by BioRad DC™ protein assay against bovine serum albumin standards.

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Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J. (eds)
Intergovernmental Oceanographic Commission of UNESCO 2001

Analysis of GST protein was achieved by probing western and dot blots using anti-rat π class GST antibodies. SDS PAGE was performed by the method of Laemlli [8] using BioRad tris-HCl ready gels in a BioRad mini protean II cell. Salmon samples (40 μg protein) were run alongside rat liver protein fractions (positive control, 40 and 80 μg protein) and BioRad pre-stained low range protein standards. Immediately after electrophoresis, proteins were transferred to a nitrocellulose membrane (Hybond C-extra, Amersham Pharmacia Biotech) using a BioRad mini trans-blot electrophoretic transfer cell.

Vacuum dot blots were performed using a Life Technologies / GibcoBRL manifold to filter salmon samples (200 μg protein) onto nitrocellulose membranes. Membranes were probed with rabbit anti-rat π class GST primary antibodies. Detection was by immuno-peroxidase staining: donkey anti-rabbit IgG (secondary antibody), rabbit peroxidase-anti-peroxidase complex (tertiary antibody) and incubation with 4-chloro 1-naphthol to achieve staining. All antibodies were used at 1:1000 dilution. Secondary and tertiary antibodies were obtained from the Scottish Antibody Production Unit. Stained membranes were scanned and analysed by Phoretix 1D software (Phoretix International).

Results were checked for normality and homogeneity of variance prior to analysis by one-way ANOVA followed by Tukey's multiple comparison of means.

Mussels

Farmed *Mytilus edulis* from the West Coast of Scotland were acclimated (1 week) in 50 L glass aquaria supplied (through-flow) with fresh, aerated sea water at 12 °C. Prior to exposure, mussels were free of PSTs as determined by mouse bioassay [9] and HPLC [6]. All individuals were attached and gaping with mantle edges exposed.

Five groups of 10 individuals were exposed to various doses of saxitoxin diacetate (Calbiochem) in physiological saline [10] by injection into the posterior adductor muscle [11]. Exposure was achieved at doses of 10, 33, 100 and 333 μg/100 g. Control mussels were injected with physiological saline alone. A positive control group was included where mussels were injected with a known inducer of GST activity, 3-methylcholanthrene (3-MC, administered in squalene, 200 mg/100 g soft tissue).

Two days after exposure, digestive glands were dissected and stored as described for salmon. Samples (200 ± 10 mg) were homogenised in 1 ml of 20 mM tris-HCl buffer containing: 0.5 M sucrose, 0.15 M KCl, 1mM EDTA, 1mM DTT, 100 μM PMSF and adjusted to pH 7.6 [12]. Protein fractions were prepared and assayed for GST activity and protein as above.

Results were analysed by one-way ANOVA followed by Dunnett's multiple comparisons test.

RESULTS AND DISCUSSION

Salmon

Hepatic GST activities were found to differ significantly between treatment groups ($P<0.001$). Fish exposed to both saxitoxin and a toxic dinoflagellate extract containing a number of PST analogues demonstrate nearly two-fold induction of activity over controls (Fig. 1). This level of induction is comparable to the 'modest two-fold' induction caused by ip exposure of fish to known inducers of GST activity such as polycyclic aromatic hydrocarbons [15] No significant differences in hepatic activity were apparent between saline and non-toxic *S. trochoidea* extract injected fish. This suggests that the presence of PSTs in toxic *A. fundyense* extract is responsible for the induction of activity observed in fish exposed to this extract.

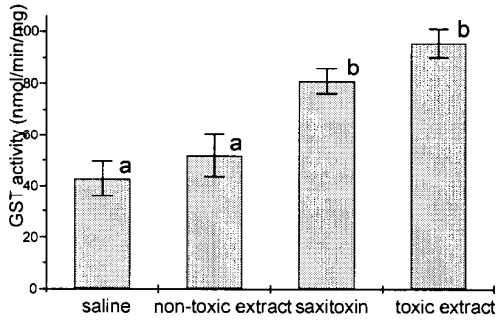

Fig. 1. Hepatic glutathione *S*-transferase activity of Atlantic salmon exposed over 21 days to multiple ip injections of: physiological saline, saxitoxin and extracts of toxic (*Alexandrium fundyense*, CCMP 1719) and non-toxic (*Scrippsiella trochoidea*, NEPCC) cultured dinoflagellates. Data are means ± SE, n = 10. ab Groups with different notation are significantly different ($P<0.05$).

The major GST isoform in salmon livers has been shown to be homologous to mammalian π class GST [13]. Antibodies raised against rat π GST detect a single protein band in protein fractions from both rat (positive control) and salmon livers (Fig. 2). Rat π class GST was estimated at 26.8 kDa in size, while the 'π class GST-like' protein detected in salmon samples was 27.6 kDa.

Variation in salmon 'π class GST-like' protein was inferred from immuno-peroxidase stained dot blots by quantifying band intensities on scanned blots. This analysis showed that fish injected with an extract from toxic *A. fundyense* not only demonstrate the highest levels of hepatic GST activity (Fig. 1), but also contain significantly elevated levels of 'π class GST-like' protein (Fig. 3). However, the lower level of GST activity induction caused by STX exposure is not reflected by a significant increase in the level of 'π class GST-like' protein in the same samples. Nevertheless, data from

these dot blots suggest that elevated levels of the major GST protein in salmon livers may be responsible for inducing hepatic GST activity in salmon exposed to PSTs extracted from cultured *A. fundyense*.

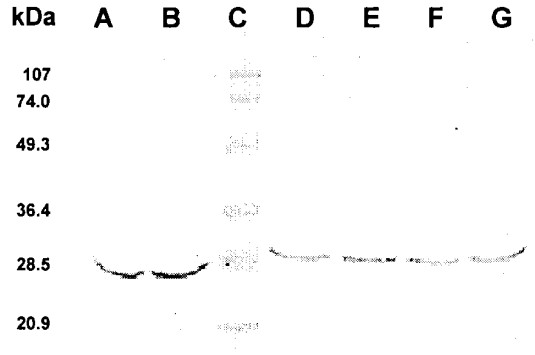

Fig. 2. Western blot probed with anti-rat π class glutathione *S*-transferase antibodies and visualised by immuno-peroxidase staining. A-B) protein fractions from rat liver run as a positive control at 40 and 80 μg protein loadings respectively. C) pre-stained BioRad low range size marker. D-G) protein fractions from salmon livers (40 μg protein).

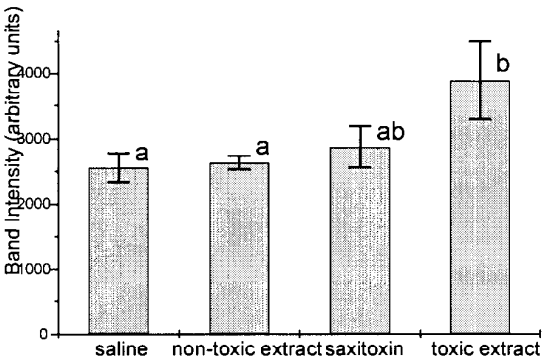

Fig. 3. Hepatic salmon protein homologous to rat π class glutathione *S*-transferase. Samples (200 μg protein) are from fish exposed over 21 days to multiple ip injections of either: physiological saline, saxitoxin or extracts from toxic and non-toxic cultured dinoflagellates. Variation in protein levels is inferred from band intensity of immuno-peroxidase stained dot blots. Data are means ± SE, n = 10. ab Groups with different notation are significantly different ($P<0.05$).

Mussels

Two days after exposure of mussels to an intra-muscular (im) injection of STX, significant differences in digestive gland GST activity were apparent between treatment groups ($P<0.005$). Mussels exposed to STX all have mean digestive gland GST activities higher than controls. Groups injected with higher doses of STX (100 - 333 μg/100 g) demonstrated a significant induction (approximately 1.5-fold) of digestive gland GST activity over controls (Fig. 4.). Mussels exposed to 3-MC (a known inducer of GST activity) also demonstrate a significant induction of digestive gland GST activity (Fig. 4). The level of induction shown by this positive control group is almost 2-fold, slightly higher than mussels exposed to STX, but still comparable.

This induction of GST activity in response to STX exposure may be caused by increased GST protein expression as seen in salmon exposed to PSTs from a cultured dinoflagellate. Alternatively, induction may be via indirect post-transcriptional activation of the enzyme by toxins. Without quantification of GST protein in these mussel samples neither mechanism can be inferred.

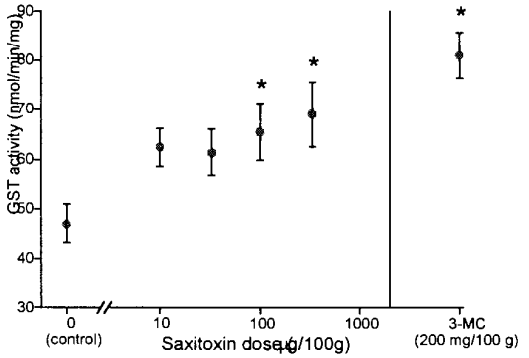

Fig. 4. Glutathione *S*-transferase activity in digestive glands of mussels two days after exposure to single injections (im) of either physiological saline (control) or various doses of saxitoxin diacetate in saline (10, 33, 100 & 333 μg/100 g). Data are means ± SE, n = 10.
* Indicates significant difference from control ($P<0.05$).

Conclusions

In the fish and shellfish models chosen, artificial intoxication with PSTs (saxitoxin or extracts from toxic dinoflagellates) results in induction of GST activity in the livers of salmon and equivalent organ (digestive glands) of mussels. Such induction suggests that this enzyme system may play a role in the metabolism of this group of algal toxins. Exposure of marine organisms to PSTs from toxic dinoflagellate blooms may therefore result in a greater capacity of the liver or digestive gland to conjugate GSH to absorbed toxins.

GST catalysed conjugation of GSH to PSTs could result in reduced sodium channel blocking activity of the toxin. Altered chemical properties of the conjugated toxin may also render it more excretable [14]. Such effects could aid in the elimination of PSTs from marine organisms and impact the residence time of toxins in fish and shellfish.

Future studies will aim to confirm the precise effect of this enzyme system on PSTs by determining the structure of any glutathione conjugates produced. Efforts will also be made to elucidate the mechanism by which these toxins induce GST in marine invertebrates. The potential role of other DMEs will also be addressed.

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CELLMEMBRANE P-GLYCOPROTEIN ACTIVITY (MULTIDRUG RESISTANCE) DOES NOT CONTRIBUTE TO THE RESISTANCE OF MUSSEL *(MYTILUS EDULIS)* HEMOCYTES TO THE CYTOTOXIC EFFECTS OF OKADAIC ACID

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ABSTRACT

The viability of mussel hemocytes incubated in 10 nM-1 µM okadaic acid (OA) was studied. After 72 hours of exposure, viability was reduced to 54% in 1 µM OA compared to 88% in control cells. This yielded a LC_{50} of >1 µM for OA which is 30-100 times higher compared to other cell systems. To test if P-glycoprotein (P-gly) activity (multidrug resistance) contributed to the resistance against OA in hemocytes, we used a documented substrate (vincristine) and two inhibitors (verapamil and staurosporine) of P-gly. In contrast to the predicted increase, intracellular substrate accumulation was reduced in the presence of both inhibitors in repeated experiments. Various concentrations of OA did not change the dynamics of the substrate. Our results indicated that mussel hemocytes do not express functional P-gly in the plasma membranes which would hence confer to the resistance against the cytotoxic effects of OA.

INTRODUCTION

Okadaic acid (OA), the main causative agent of diarrhetic shellfish poisoning (DSP), is a specific inhibitor of protein phosphatase 1 (PP1) and protein phosphatase 2A (PP2A) [1]. By blocking the activity of PP1 and PP2A, OA inhibits dephosphorylation of numerous enzymes which in turn causes major effects on signal transduction pathways and hence essential cellular functions [2]. Low concentrations of OA are cytotoxic in a vast range of cell culture systems and induce apoptotic cell death [3, 4].

Although intensively studied in various mammalian systems, the impact of OA in shellfish, the main vectors for DSP, has been given little attention. Svensson & Förlin [5] identified both PP1 and PP2A in the digestive gland of blue mussels and showed that mussel phosphatases were just as sensitive to *in vitro* inhibition by OA as other studied organisms. Although highly sensitive to OA on a molecular level, no effects of OA *in vivo* on a cytosolic enzyme, regulated by PP1 and PP2A, were detected. It was suggested that mussels may have mechanisms which prevent OA from accumulating within the cytosolic environment [5].

The apparent lack of intracellular effects of OA could be due to so-called multidrug resistance (MDR), or P-glycoprotein (P-gly) activity, in mussel cells [6, 7]. MDR cells have been found to be resistant to the cytotoxic effects of OA [8]. Also, cell clones selected for resistance to OA show a MDR phenotype [9, 10, 11]. One mechanism involved in the resistance is probably due to an increased extrusion of OA, since resistance can be reversed by the addition of verapamil, a competetive inhibitor of P-gly activity [9, 10, 12]. The MDR phenotype is not restricted to mammalian cells [13]. In *Mytilus* sp., P-gly expression and activity has been found in gill and mantle tissue [14, 15], in isolated membrane vesicles of the digestive gland [16] and in hemocytes [17].

In this paper, mussel hemocytes were used as a model cell system to study the cytotoxicity of OA. We found that these cells were highly resistant to the cytotoxic effects of OA. As a protective mechanism, we tested if mussel hemocytes contain functional P-gly in the plasma membranes which are capable of reducing intracellular [OA] and thus the cytotoxicity of this compound.

MATERIALS AND METHODS

Animals

Adult *Mytilus edulis* (7-10 cm shell height, mean dry weight 6 g) were collected in 1998 from Inre Vattenholmen, off Tjärnö Marine Biological Laboratory, Sweden, during a period of non-detectable levels of OA in the mussels. They were returned to the laboratory and maintained in flow-through tanks with the addition of *Isochrysis galbani.*

Extraction of mussel hemolymph

Hemolymph was withdrawn from the posterior adductor muscle into a sterile 5 ml hypodermic syringe. The blood was immediately diluted with an equal volume of physiological saline (PS) (20 mM HEPES, 436 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 53 mM $MgSO_4$, pH 7.3) and put on ice. Cell concentration was determined by staining cells 1:4 with Gentiana violet and counted in a hemocytometer. In all experiments, the following protocol was used: hemolymph suspensions from three individuals were pooled, cell concentration was determined and the suspension was further diluted in PS to a final concentration of $2*10^5$ cells ml^{-1}. 10^5 cells (0.5 ml) were seeded into wells of a sterile 24-well tissue culture plate. The cells were left to attach to the bottom surface for 30 min and the overlying solution was removed by a Pasteur pipette. 0.5 ml of fresh PS was added with our without various drugs as described below.

Cytotoxicity assay

OA (1 mM stock solution in DMSO, Sigma) was diluted in PS supplemented with 2% (w/v) glucose and 1% (v/v) antibiotics (gentamicin, 10 mg ml^{-1}, Sigma). The cells were exposed to various concentrations of OA

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(10 nM to 1 µM) in 0.5 ml of supplemented PS for 24 to 72 hours. As controls, cells were incubated in the OA vehicle (DMSO). The microplates were incubated in 10° C in a light-proof humidity chamber. After each incubation period, the cells were detached from the surface by adding 0.25% (w/v) trypsin. Cell viability vas determined using the red dye Eosin Y (Sigma). 20 µl of the cell suspension was mixed with an equal volume of 0.05% Eosin Y (w/v, dissolved in PS) and transferred to a hemocytometer. Viable (unstained) and non-viable (stained) cells were counted immediately and the viability was calculated as % viable cells of total cell count (minimum 200 cells counted).

Assay for P-gly activity

The activity of P-gly was estimated by measuring the intracellular uptake of the *Vinca* alkaloid vincristine (VCR), a well-documented substrate for the P-gly transporter. Radiolabelled G-^{3}H-vincristine sulphate (0.75 µCi/ml, Amersham) and unlabelled vincristine sulphate (Sigma) was mixed to a final concentration of 0.5 µM VCR in PS. As inhibitors of P-gly activity, 10-20 µM verapamil (VP) and 0.5 µM staurosporine (ST) were used. The cells were incubated in 0.5 µM VCR for various time periods in 15° C in the dark in the absence (controls) or presence of the inhibitors. Also, the effects of OA (10 nM to 1 µM) on the accumulation of VCR was tested. At the end of each incubation period, the radioactive solution was removed and the cells were washed in 0.5 ml PS for one minute which was repeated three times.The cells were lyzed by adding 0.5 ml of a solution containing 1 M NaOH and 5% (v/v) Triton 100-X for 2 hours. The solution was transferred to vials containing 4.5 ml scintillation liquid (Opti-phase 'HiSafe' 3, Wallac OY) and the radioactivity was counted.

Statistical tests

All data was analysed using one factor or two factor analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) aposteriori test for differences among means.

RESULTS

Cytotoxicity of OA

The viability of hemocytes incubated for 24, 48 and 72 hours in different concentrations of OA are shown in fig. 1. Cells incubated in 1 µM OA showed a significant reduction in viability in time compared to the other treatments (treatment*time, $p<0.01$). After 24 h, there was no difference in viability among treatments. However, viability was significantly reduced to 72% after 48 hours in 1 µM OA compared to controls (90%) and a further reduction to 54% was observed after 72 hours (control cells, 88% viability). Since the viability for hemocytes incubated in 1 µM OA were >50% after 72 h, the LC_{50} value (concentration that reduces the number of viable cells to 50%) for OA was >1 µM in this system.

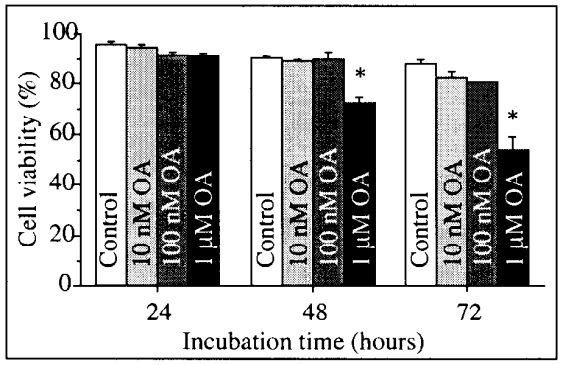

Fig. 1. Viability of mussel hemocytes incubated in various concentrations of okadaic acid (OA) for 24 to 72 hours. Values are means ± SE (n=2).

Activity of P-gly

Hemocytes were exposed to 0.5 µM VCR in the absence and presence of 20 µM VP or 0.5 µM ST. The intracellular accumulation of VCR was measured in intervals of 15 minutes up to 120 minutes of drug exposure (fig. 2). There was a highly significant difference among treatments ($p<0.01$) and among times ($p<0.01$). In all treatments, uptake of VCR increased in time. Cells treated with VP had a lower content of VCR at all times compared to the control (only VCR added). In the ST treatments, the content of VCR was lower compared to contols at 45 minutes and thereafter. These results are in strong contrast to the predicted effects of VP and ST on the accumulation of VCR. A 'classical' MDR response to P-gly inhibitors is an increased accumulation of VCR.

To estimate the rate of efflux of VCR, cells were first exposed to 0.5 µM VCR in the absence (control) and presence of 20 µM VP for 60 minutes. The drug solution was removed and the cells were washed with PS three times. The cells were then incubated in 0.5 ml of PS and the efflux of VCR was measured after 0 (max uptake), 30 and 60 minutes. Cells exposed to VP had accumulated 33 % less VCR compared to the control after 60 minutes of exposure to the drugs, (fig. 3, 0 minutes). The intracellular content of VCR was then significantly reduced for both treatments in time ($p<0.05$), however, the rate of reduction was similar for both. The intracellular content of VCR after 60 minutes in drug-free medium was reduced to 60% compared to the max uptake in VP-treated cells. The same value for control cells was 68%. Thus, exposing cells to VP for 60 minutes reduced the uptake of VCR, however, the rate of efflux was not affected by pre-exposure to VP.

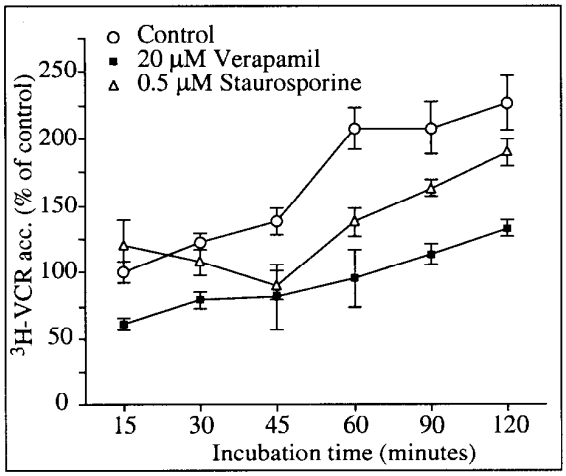

Fig. 2. Accumulation of ^{3}H-vincristine (^{3}H-VCR) in hemocytes incubated for 15 to 120 minutes in the absence (control) and presence of 20 µM verapamil or 0.5 µM staurosporine. The intracellular ^{3}H-VCR content in control cells after 15 minutes of incubation was set at 100%. Values are means ± SE (n=2).

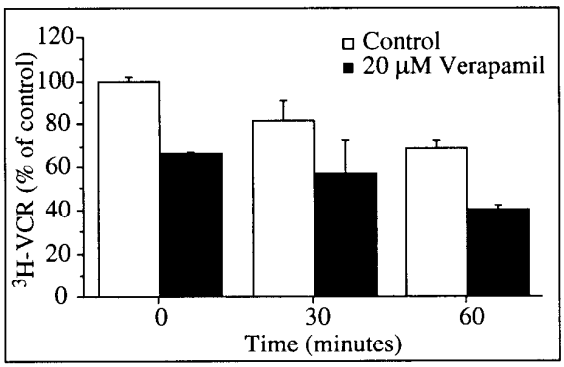

Fig. 3. Efflux of ^{3}H-vincristine (^{3}H-VCR) in hemocytes incubated for 60 minutes in the absence (control) and presence of 20 µM verapamil. After the exposure, the drug-containing medium was replaced by physiological saline and intracellular content of ^{3}H-VCR was measured immediately (0 minutes, max uptake) and then after 30 and 60 minutes. The max uptake of ^{3}H-VCR in control cells at was set at 100%. Values are means ± SE (n=2).

To test if OA and VCR are competing substrates for the P-gly transporter, hemocytes were incubated in 0.5 µM VCR for 45 minutes in the absence (control) and presence of various combinations of OA (10, 100 nM and 1 µM), VP (20 µM) and ST (0.5 µM), (fig. 4). The uptake of VCR was not affected by the addition of OA alone (fig. 4, control bars, $p>0.05$). Also, OA in combination with VP or ST did not cause any significant change in the accumulation of VCR compared to VP or ST alone. The accumulation was significantly reduced by both inhibitors ($p<0.01$) in the same manner as seen in previous experiments. Thus, in mussel hemocytes, OA does not appear to affect the dynamics of VCR.

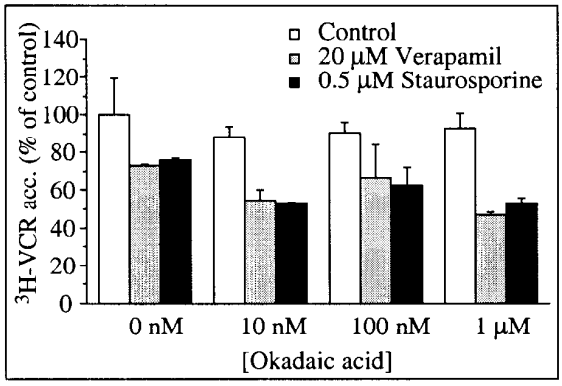

Fig. 4. Accumulation of ^{3}H-vincristine (^{3}H-VCR) in hemocytes incubated for 45 minutes in the absence (control) and presence of various combinations of OA (10, 100 nM and 1 µM), verapamil (20 µM) and staurosporine (0.5 µM). The intracellular ^{3}H-VCR content in control cells (no OA) was set at 100%. Values are means ± SE (n=2).

DISCUSSION

The cytotoxicity of OA has been studied in various cell culture systems where it is generally regarded as a highly toxic substance. LC_{50} values reported for OA range from 1.5 nM in human epidermoid carcinoma cells (KB) cells [18], 13 nM in chinese hamster ovary cells (CHO-K1) [10], 30 nM in rat pituitary GH_3 cells [9] and 31 nM in mouse neuroblastoma cells (N1E-115) [19]. In this study, mussel hemocytes exposed to 1 µM OA showed a reduction in viability compared to our control treatments. However, >50% of the cells were still viable after 3 days of exposure to 1 µM OA, yielding a LC_{50} >1µM in this system. Considering the low LC_{50} values previously reported for OA, mussel blood cells seemed to be highly resistant to the cytotoxic effects of OA, surviving 30 to 100 times greater concentrations compared to other cell types.

Using radiolabelled VCR as a model substrate of P-gly activity and the P-gly inhibitors VP and ST, we initially aimed to characterize P-gly activity in hemocytes. Both VP, a calcium channel blocker, and ST, an inhibitor of protein kinase C, have been shown to interact directly with the P-gly molecule, thereby inhibiting the efflux of VCR [20, 21]. Thus, our findings that the accumulation of VCR was reduced in the presence of both inhibitors was contrary to the predicted effects of VP and S. Additionally, only the uptake and not the efflux of VCR seemed to be affected by VP.

Our results strongly indicated that mussel hemocytes did not express 'classical' P-gly activity in the plasma membranes. Instead, there was a transport of VCR into or within the cells which could be reduced by VP and ST. A possible explanation to these results is that the main site for P-gly-like activity in mussel blood cells is within subcellular membranes, such as the endosomes or lysosomes.

The possibility for an intracellular MDR-mediated transport is discussed in Moore & Willows [22]. In this model, cell surface membranes and its associated proteins are incorporated into endosomal and lysosomal membranes and toxic compounds accumulate through P-gly-mediated transport from the cytosol into these compartments. Also, human blood cells show P-gly expression which is not connected to the plasma membrane [23].

When hemocytes were exposed to various concentrations of OA, this did not affect the dynamics of VCR, suggesting that OA is not a competing substrate for the VCR-transporting system. Similar results were found by Wielinga et al. [24] who treated MDR and wildtype cells with 200 nM OA. This did not change the accumulation of three different substrates of P-gly which indicated that OA may not be a substrate for P-gly. They also concluded that PP1/PP2A do not regulate the drug transport activity of P-gly. It has previously been suggested that P-gly activity is regulated by reversible phosphorylation via protein kinase C [25] but recent evidence indicate that phosphorylation does not play a significant role in the function of P-gly [24, 26].

In conclusion, we found no support for the hypothesis that mussel hemocytes contain active P-gly in the plasma membranes which would contribute to the resistance against the cytotoxic effects of OA. In future experiments, the role of the lysosomal system in protecting cells from cytotoxic effects of okadaic acid will be investigated.

ACKNOWLEDGEMENTS

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SMALL INTESTINAL INJURIES IN MICE CAUSED BY A NEW TOXIN, AZASPIRACID, ISOLATED FROM IRISH MUSSELS

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ABSTRACT

Pathological changes of the small intestine caused by a new toxin, azaspiracid, from Irish mussels were studied. Human poisoning cases included both diarrhetic shellfish and paralitic shellfish poisoning symptoms. The present paper focused on the former. Injuries were observed in the upper part of the small intestine, where lamina propria in the villi became atrophied at the initial stage, followed by desquamation of epithelial cells and shortening of villi. The injuries were different from the DSP toxin okadaic acid; 1) they developed very slowly after a lag time of about 3 hr, 2) recovery was very late, 3) initial target and process were different.

INTRODUCTION

Occurrence of a new type of food poisoning resulting from ingestion of the mussel *Mytilus edulis* was first reported in the Netherlands in 1995. These mussels were cultivated in Killary Harbour in Ireland [1; 2]. The mussel toxicity was again observed in 1997 at Arranmore Island region in Ireland [3]. Symptoms observed in patients were nausea, vomitting, diarrhea and stomach cramps, and thus resembled those of diarrhetic shellfish poisoning (DSP). However, in bioassay tests with mice, other than DSP symptoms, neurological symptoms were also observed. In the mussel farming area, there were no DSP or PSP (paralytic shelfish poisoning) producing plankton. The new toxin, azaspiracid (Fig. 1) was isolated from the mussels [4].

Pathological changes in orally administered mice were fatty changes in the liver, decreased number of lymphocytes, and small intestinal erosion. The injuries of the villi in the small intestine by azaspiracid were different from those by the DSP toxin okadaic acid, that is, the former had a time lag of about 3~4 hr before destruction and recovery took a long time, whereas the latter had transient changes [5].

In the present report, the changes in the small intestine according to dose and time-course by orally administered azaspiracid were studied in *in vivo* experiments in mice.

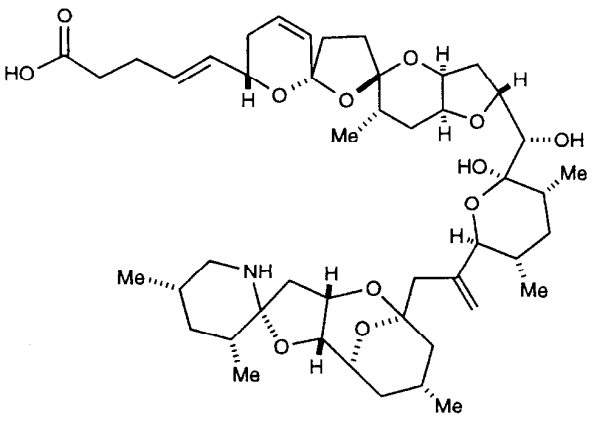

Fig.1 Structure of azaspiracid

MATERIALS AND METHODS

Azaspiracid was extracted from mussels *Mytilus edulis* collected at Killary Harbor in Ireland in 1996 and purified as described previously [4]. Stock solution of azaspiracid was prepared by dissolving 100μg of the toxin in 1 ml of aqueous 50% ethanol (w/v). ICR male mice were used for the experiments. For administration by gastric intubation, aliquots of the stock solution were diluted in 0.2 ml of saline. After administration at 300μg/kg, the mice were killed at 30, 60 min, 2, 3, 4, 8, 16 and 24 hr. With lethal doses of 500 and 600 μg/kg, mice were killed at 16 and 24 hr, and mice given 700 and 900 μg/kg were killed at very weakened stages. Internal organs from all mice were fixed in 20% neutral formalin for light microscopy and in Karnovsky's solution (4% paraformaldehyde, 5% glutaraldehyde in 0.2M cacodylate buffer, pH 7.4) for scanning electron microscopy. For observation, the specimens were treated as described previously [6].

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RESULTS

General observation

With the lethal dose of 500 μg/kg, mice showed neither diarrhea nor body weight loss within 24 hr. Even at the higher dose of 900 μg/kg, mice did not show any behavioral changes during 4 hr before sudden death. At autopsy, characteristic changes were swelling and fatty changes of the liver and accumulation of fluid in the small intestine.

Changes in the small intestine

Tissue changes caused by azaspiracid were observed in the upper part of the small intestine, and moderate volume of fluid was seen; these were similar to those caused by okadaic acid.

With 600 μg/kg, epithelial cells were eroded, followed by exposure of the lamina propria in the lumen and intestinal glands of Lieberkühn after 8 hr (Fig. 2a). With the higher dose of 900 μg/kg, desquamation of epithelial cells occurred, resulting in exposure of the lamina propria and empty crypts of Lieberkühn after 4 hr (Fig. 2b). Thus, in fatal cases, azaspiracid injured epithelial cells in a short period.

Fig. 2a

Fig. 2 Small intestinal injuries of mice treated p.o. at higher doses.
a After 8 hr with azaspiracid at 600 μg/kg, epithelial cells of villi became desquamated. LP: lamina propria; arrows: intestinal glands of Lieberkühn. SEM.

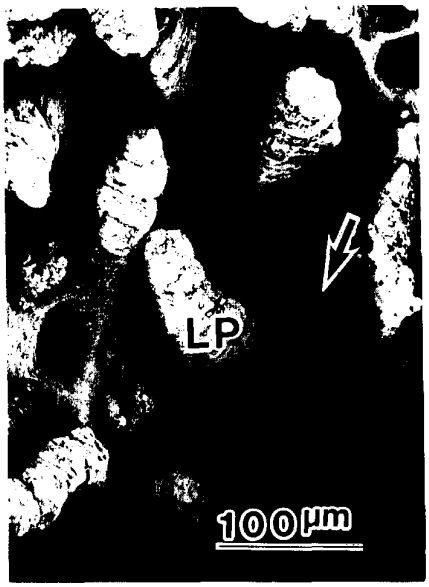


Fig. 2b. At 4hr with azaspiracid at 900 μg/kg. Epithelial cells of villi desquamated together with crypt cells. LP: lamina propria; arrow: hole of crypt of Lieberkühn. SEM

On the contrary, with a lower dose the injury started in the lamina propria. With 300 μg/kg, the onset of sporadic necrosis caused by nuclear pyknosis in the lamina propria of the villi was confirmed after 3 hr (Fig.3a). The atrophied lamina propria was spatially separated from epithelial cells, and vacuolization of epithelial cells was prominent after 4hr (Fig. 3b). Then villi became shorter by losing their upper parts after 8 hr, and the degenerating cells were still separating from the top (Fig. 3C), but neither the lamina propria nor crypts of Lieberkühn were exposed.

After 24 hr, epithelial cells showed signs of recovery but lamina propria lagged in recovery. Some villi were still empty inside or contained necrotized lamina propria (Fig. 4a), but on the surface, villi did not reflect the inside condition and looked as if they were undergoing a normal recovery process (Fig. 4b).

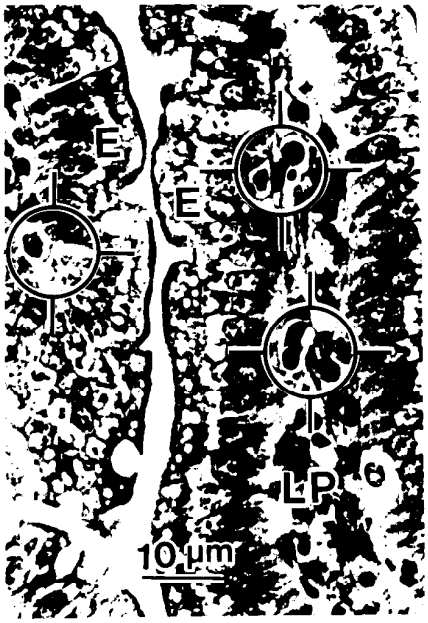
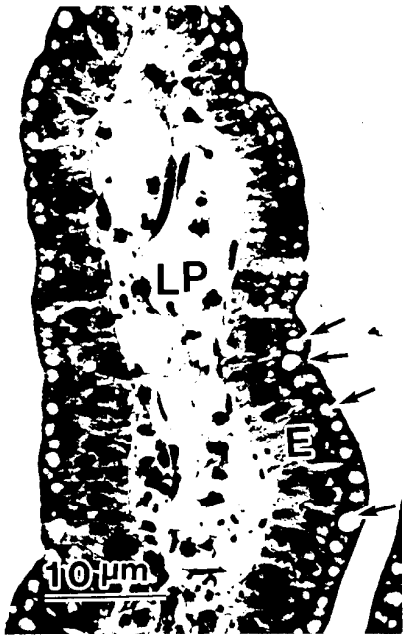


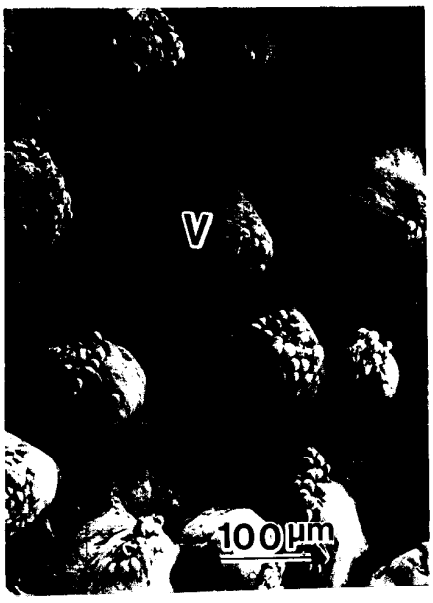
Fig. 3a

Fig. 3 The progression of small intestinal injuries.

a. Changes initiated at the lamina propria after 3 hr with 300 μg/kg, and sporadic necrosis with nuclear pyknosis is seen.
E: epithelial cells of the villi; LP: lamina propria circles: necrosis with nuclear pyknosis.LM.



3b. After 4 hr with 300 μg/kg. Atrophyc lamina propria was spacially separated from epithelial cells and the inside of villi became almost empty LP: lamina propria; arrows: vacuolar degeneration of epithelial cells (E). LM.

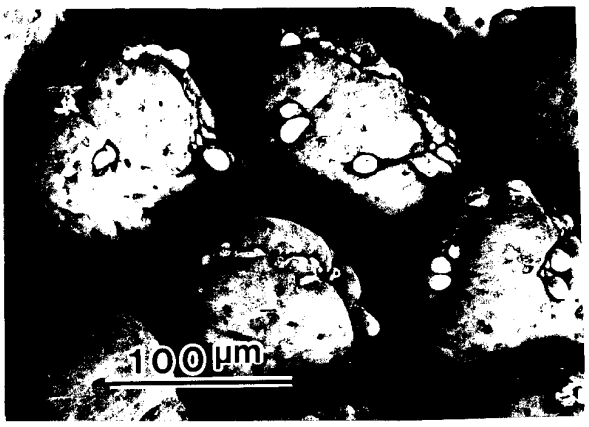


3c. After 8 hr, villi (V) became shorter, losing the upper parts. Necrotizing cells still cover the tops. SEM.

Fig. 4a

Fig. 4 Recovery process 24hr after administration of 300 μg/kg.

4a. Lamina propria lagged in recovery, and some villi were still empty inside (*) or contained necrotized lamina propria. LP: lamina propria; E:epithelial cells. LM.



4b In the lumen, the surface arrangement of villi was irregular, but normal recovery seemed underway. V: villi. SEM.

DISCUSSION

In the small intestine, severe changes by azaspiracid at 600~900 μg/kg caused desquamation of the epithelial cells during 8 hr. With a sublethal dose of 300 μg/kg, the onset of the degeneration of villi was characterized
by necrotic atrophy of the lamina propria by nuclear pyknosis within 3 hr.

There are some types of pathological changes of villi; 1) hypersecretion of mucus from goblet cells, such as cholera toxin and ciguatoxin [7], 2) edema of epithelial cells resulting in desquamation, such as okadaic acid [8], 3) degeneration of blood vessels (a rise in permeability or breaking) such as palytoxin [9], lyngbyatoxin [unpublished] and aplysiatoxin [6; 10], 4) atrophy of lamina propria, and 5) others.

With sublethal doses of okadaic acid or azaspiracid, although the appearance of villi looked to have similar changes at the developed stage, the representative DSP of okadaic acid was type 2) and its injuries were not so severe and the recovery was rapid, meaning that the process was transitional [5; 8]. On the other hand azaspiracid was type 4), because the target in the villi was the base of its structure, and therefore the recovery was extremely late. The partially purified toxin fraction KT3 caused almost the same injuries in each organ as azaspiracid, such as fatty changes in the liver and necrosis in lymphoid tissues, but it caused much more fluid accumulation in the small intestine [11] than this pure toxin. Thus the azaspiracid fraction should be explored in the future to explain the differences in severity of diarrhea.

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IN VIVO PROTEIN PHOSPHATASE 2A INHIBITION AND GLUTATHIONE REDUCTION BY MICROCYSTIN-LR IN GRASS CARP (*CTENOPHARYNGODON IDELLUS*)

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ABSTRACT

Blooming of algae in eutrophic waters can result in the production and accumulation of secondary metabolites e.g. okadaic acid class compounds including microcystins. Microcystins occur worldwide, predominantly in freshwater blooms, but have also been reported in brackish and coastal marine waters. In mammalian toxicological studies, the intoxication of rodents exposed to microcystins is characterized by an inhibition of protein phosphatases. In this study, *in vivo* inhibitory effects of microcystin-LR (MC-LR) on protein phosphatase type 2A (PP-2A) in the liver of grass carp (*Ctenopharyngodon idellus*) were investigated. Results showed that MC-LR had specific and potent inhibitory activity against liver PP-2A in fish. PP-2A activity was significantly inhibited in fish one hour after they have been injected (ip) with 100μg MC-LR/kg, or higher, and this was accompanied by a corresponding decrease in the glutathione (GSH) content in the fish liver. When 8 mgGSH/kg was injected into the fish two hours before 900 μgMC-LR/kg was ip administered, the ultrastructure of the fish liver was unaffected by MC-LR as compared to fish that were not pre-treated with GSH. Hepatocytes from fish ip injected with MC-LR, but with no pre-treatment with GSH, showed complete dissociation and severe ultrastructural damage, suggesting that GSH could offer some protection to the fish liver against the toxin.

INTRODUCTION

The blooming of cyanobacteria and dinoflagellates in eutrophic waters often result in the production of toxic secondary metabolites such as okadaic acid, nodularins and microcystins [1]. Microcystins, produced by cyanobacteria of genera such as *Microcystis, Anabaena, Oscillatoria* and *Nostoc*, constitute one of the most well studied groups of toxins, many of which are potent hepatotoxins. Microcystins, have a worldwide distribution, and have been implicated in numerous intoxication cases involving humans and domestic animals [1]. Although microcystins are found predominantly in freshwater blooms, recent studies have revealed their occurrence in brackish and coastal marine waters [2].

Microcystins are a group of cyclic peptides consisting of seven amino acids. The general structure of microcystins is cyclo-*D*-alanine-*L*-R_1-*erythro*-β-methyl- *D*-isoaspartic acid-*L*-R_2-Adda-*D*-isoglutamic acid-*N*- methyl-dehydro-alanine (Mdha), where R_1 and R_2 represent two variable *L*-amino acids. Adda is the amino acid 3-amino-9-methoxy-2,6,8-trimethyl-10-phenyl- deca-4,6-dienoic acid. Over 60 structural variants of microcystins corresponding to various combinations of R_1 and R_2 have been identified to date [3].

Once absorbed into a vertebrate, the toxins are transported through the ileum to the liver where they are preferentially taken up by hepatocytes via the bile acid transport system [4]. Some microcystin variants have been shown to be an inhibitor of serine/threonine protein phosphatases type 1 and type 2A [5]. This inhibition leads to hyperphosphorylation of proteins associated with the structural components of cells causing cytoskeletal disintegration [6]. The shrinking of hepatocytes and collapse of sinusoidal architecture can lead to a concentration of blood in the liver and hypovolaemic shock [7]. Through their strong inhibitory effects on protein phosphatases [8], microcystins disturb the regulation of phosphorylation of subunit proteins on the disassembly of intermediate filaments in normal cell mitosis. Thus, apart from their acute toxicity, microcystins are also notorious for their tumour-promoting characteristics [9]. Microcystins have also been shown to cause liver cell necrosis, and a recent study suggests that the mechanism of acute toxicity in liver cells could be different from that of liver tumour promotion [10]. Recent epidemiological studies have suggested that these compounds may be linked to the high rate of primary liver cancer in certain parts of China [11].

Previous studies on microcystins have focussed primarily on mammalian models, and there have been relatively few studies on the effect of microcystins on fish. Laboratory exposure experiments have indicated that microcystins can cause histopathological damage to fish [12].

Microcystins have been shown to exhibit a highly specific, potent *in vitro* inhibitory effect on protein phosphatases isolated from grass carp liver [13]. Recent *in vitro* studies further suggested that microcystins may be detoxified via the glutathione *S*-transferase mediated formation of a microcystin-glutathione conjugate in a range of aquatic organisms, including a plant, a cladoceran, a freshwater bivalve and zebrafish [14]. Our study aims to (1) examine the *in vivo* inhibitory effect of one common structural variant of microcystin (microcystin-LR) on liver protein phosphatase type 2A of the grass carp (*Ctenopharyngodon idellus*); and (2) investigate the possible role of glutathione in moderating the toxic effect of microcystin-LR in fish.

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MATERIALS AND METHODS

Chemicals and Reagents

Microcystin-LR (MC-LR) was isolated and purified form *Microcystis aeruginosa* TAC95 according to the method described in Harada *et al.* [15]. Glycogen phosphorylase b and phosphorylase kinase are products of GIBCO. All reagents are of analytical grade.

In vivo intoxication

Grass carp, *Ctenopharyngodon idellus* Val., (about 25 g) were obtained from a local fish farm, and acclimated for one week at 20 ± 1_C in the laboratory. The fish were intra-peritoneally (ip) injected with 0.1 mL of 0.9 % NaCl solution containing various amounts of MC-LR to achieve final doses of 0, l0, 50, 100 and 400 μgMC-LR/kg. There were 4 replicates in each treatment group. The animals were sacrificed 1 hour after injection. Livers were dissected from individual fish, and the activities of protein phosphatase type 2A (PP-2A) and glutathione *S*-transferase (GST), as well as concentrations of glutathione (GSH) were determined.

Preparation of protein phosphatase 2A from fish liver

Protein phosphatase type 2A (PP-2A) was purified from the liver dissected from the sacrificed grass carp following the procedure described in Xu *et al.* [13]. A buffer containing 50 mM Tris-HCl at pH 7.4, 2mM EDTA, 2mM EGTA, 0.2 mM PMSF, 2mM 2-mercaptoethanol, 10% glycerol and 0.25M sucrose at pH 7.4 was used as the buffer for homogenization. The homogenate was centrifuged at 6,000g for 30 minutes and then 100,000g for 60 minutes. The supernatant was applied to a DEAE-cellulose column pre-conditioned with a buffer similar to the homogenization buffer, but without sucrose. The column was first eluted with the above buffer containing 0.1 M NaCl to remove other protein phosphatases (including protein phosphastase type 1), and PP-2A was then obtained by eluting the column with the same buffer containing 0.2 M NaCl. PP-2A was concentrated by ultrafiltration, partitioned and stored in small vials at -20_C.

Determination of PP-2A activity

^{32}P-labelled glycogen phosphorylase a was prepared by the phosphorylation of glycogen phosphorylase b using phosphorylase kinase. A typical reaction mixture contained 30_l buffer (167mM Tris-HCl at pH 7, 0.3mM EGTA, 0.3% 2-mercaptoethanol, 16.7mM caffeine, 2mgml^{-1} bovine serum albumin), 10 _l protein phosphatase 2A, 10 _l ^{32}P-labelled glycogen phosphorylase a, and 50 _l distilled water. The reaction was initiated by the addition of ^{32}P-labelled glycogen phosphorylase a. The reaction mixture was then incubated at 30_C for 10 minutes. The reaction was terminated by an addition of 30% TCA. The mixture was placed on ice for 10 minutes, and then centrifuged at 12,000 rpm for 5 minutes, 150 _l supernatant was added to a scintillation cocktail and counted by a liquid scintillation counter. PP-2A activities of individual samples were measured according to Wong *et al.* [16].

Measurement of GST activity, and GSH content

200 mg of liver tissue was homogenized in a solution containing 100mM phosphate, 5mM EDTA and l.0 ml 10% TCA on ice using a glass homogenizer. The homogenate was centrifuged at 10,000g for 30 min, and the supernatant retained for GSH determination following the fluorometric procedure of Hissin & Hilf [17]. The supernatant of homogenate of 100 mg liver tissue in a 0.1 M phosphate buffer (pH 7) was used for GST measurement using 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate according to Habig *et al.* [18].

Protein determination

The protein contents of individual enzyme preparations were estimated using the Bio-Rad™ microassay kit with Bovine Serum Albumin (BSA) as a standard.

Effect of GSH on toxicity of MC-LR in fish

Experimental fish were divided into 4 groups (A, B, C, and D). Each group comprised 15 fish. Group A, B, C were given ip injections of 0.9 % NaCl, 0.9% NaCl containing 8 mgGSH/kg, 0.9% NaCl containing 900 μgMC-LR/kg, respectively. Fish in group D were ip injected with 0.9% NaCl containing 8 mgGSH/kg (i.e. same treatment as group B), and then with 0.9% NaCl containing 900μgMC-LR/kg (same treatment as group C) two hours later. Three fish were sampled from each group 6 hours, 24 hours, 48 hours, and 72 hours post injection. Livers were dissected from the fish, fixed, embedded, sectioned and stained, and then examined under both optical and transmission electron microscopes.

Statistical Analysis

An analysis of variance was used to test the null hypothesis that the different treatments had no effect on PP-2A and GST activities, nor GSH contents. In the cases where the null hypotheses were rejected, pair-wise comparisons between the control and individual treatments were performed using the Dunnett's test [19].

RESULTS

In vivo inhibition of fish liver PP-2A by MC-LR

Fish in all experimental groups showed no abnormal behaviour. Liver removed from fish injected with MC-LR had an intact contour and showed slight congestion. PP-2A activities of liver homogenates collected from fish injected with 10 or 50 μgMC-LR/kg showed no significant difference from the control fish (Dunnett's test: $q < 2.2$, $P > 0.05$). Liver PP-2A activities of fish dosed with 100 μgMC-LR/kg were reduced to about

10% of the control ($q > 14.0$, $P < 0.01$), while PP-2A activities at 400 μgMC-LR/kg were completely inhibited (Fig. 1). The above results showed that ip injection of 100 μgMC-LR/kg or higher resulted in a rapid (within 1 hour) inhibition of PP-2A activities in fish. GSH contents of fish dosed with 10 μgMC-LR/kg or higher were significantly lower than that of the control fish ($q > 7.0$, $P < 0.01$; Fig. 2). There were, however, no significant differences in GST activities between the control and all the treatment groups.

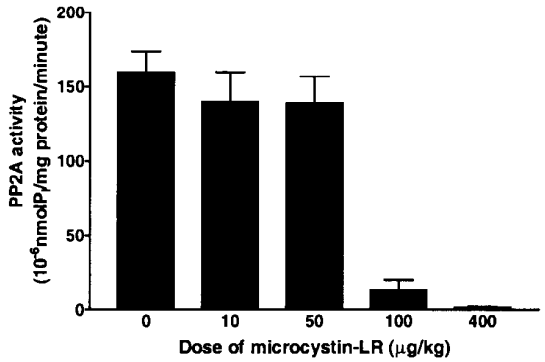

Fig. 1 *In vivo* inhibition of protein phosphatase 2A (PP-2A) activity by microcystin-LR in grass carp liver. The vertical lines are 1 S.E.

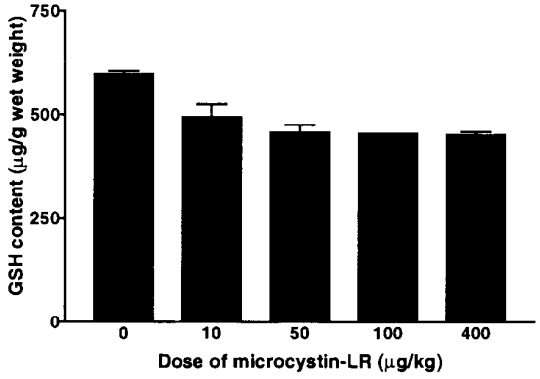

Fig. 2 Glutathione (GSH) contents in the liver of grass carp ip injected with various doses of microcystin-LR. The vertical lines are 1 S.E.

Effect of GSH on toxicity of MC-LR in fish

Six hours after injection, marked histopathological alterations (including hepatocyte dissociation and microfilament separation) were observed in the livers of fish injected with 900 μgMC-LR/kg only (group C) when compared to both fish dosed with 0.9% NaCl (group A) and fish dosed with 8 mgGSH/kg only (group B). After 72 hours, there were massive necroses of hepatocytes, infiltration of erythrocytes into, and accumulation of blood cells in areas of dissociated hepatocytes in fish dosed with MC-LR alone (group C). Fish pre-treated with 8 mgGSH/kg (group D), however, showed no such histopathological change in the liver ultrastructure, even when followed with an ip injection of 900 μgMC-LR/kg two hours later.

DISCUSSION

A recent study has shown that MC-LR, -YR, and -RR have potent *in vitro* inhibitory effect on protein phosphatases type 1 (PP-1) and type 2A (PP-2A) isolated from grass carp liver and kidney [13]. Also, the patterns of inhibition were similar irrespective of whether pure enzyme extracts or crude tissue homogenates were used, indicating that the inhibition of PP-1 and PP-2A by microcystins is highly specific. In the present *in vivo* investigation, activity of protein phosphatase 2A was completely inhibited just 1 hour following an ip injection of 400 μgMC-LR/kg. This finding revealed that many important biochemical processes regulated by protein phosphatases, PP-2A in particular, could be significantly disrupted by microcystins within a period much shorter (1 hour following toxin administration) than that generally employed in toxicological studies (> 24 hours). Indeed, studies on the molecular interactions between protein phosphatases and microcystins using reverse-phase liquid chromatography [20] and biosensor technique [21] have revealed a very rapid binding of these molecules, leading to an inactivation of the protein phosphatase catalytic subunits within minutes.

Common carp (*Cyprinus carpio*) ip injected with the median lethal dose of 550 μgMC-LR/kg showed a total loss of the liver parenchymal architecture and degeneration of kidney tubuli, while those receiving sublethal doses of the toxin still suffered severe liver damage with dissociation of hepatocytes and hydropic degeneration [12]. 26-hour exposure to MC-LR at a dose of 400 μg/kg caused heptatocellular swelling, lysis of hepatocyte plasma membrane, and liquifactive necrosis in rainbow trout, *Oncorhynchus mykiss* [22]. Andersen *et al.* [23] found that ip injection of MC-LR into healthy Atlantic salmon (*Salmo salar*) induced similar pathological alterations as the "netpen liver disease", including diffuse necrosis and hepatic megalocytosis. In this study, similar histopathological changes were observed in grass carp liver six hours after the fish were ip injected with 900 μgMC-LR/kg.

Glutathione (GSH) is a tripeptide widely present in animals, most plants, and bacteria. This compound is involved in many aspects of cellular metabolism, particularly in the protection of cells against two types of metabolic stress. First, it can non-enzymatically reduce a number of chemical species, such as peroxides or free radicals, which may cause oxidative stress. Second, it can participate in the detoxification of many xenobiotics (including toxins) by serving as a substrate for glutathione *S*-transferase (GST) [24]. This process converts, through conjugation with metabolites of endogenous compounds, a toxic substance into a more soluble, less toxic derivative of the original compound which can readily be eliminated. In this study, fish ip injected with 10 μgMC-LR/kg or higher had

significantly lower GSH contents in the liver as compared to the control fish. A study on primary rat hepatocytes also revealed that exposure to cylindrospermopsin, a hepatotoxic alkaloid, caused a reduction in cellular GSH [24]. Furthermore, Takenaka & Otsu [10] suggest that GSH may play an important role in the metabolic pathway leading to detoxification as well as increasing clearance. Conceivably, the reduction in GSH contents, following microcystin challenge, may be due to increased utilization in detoxification processes, or decreased GSH synthesis, or both. Further investigations are required to distinguish between these two possibilities.

Hermansky *et al.* [25] reported that treatment of mice with 4 mgGSH/kg two hours before MC-LR administration provided complete protection against microcystin lethality. Similarly in this study, no histopathological damage was observed in the liver of fish that have received a dose of 8 mgGSH/kg two hours prior to MC-LR administration. These findings suggest that GSH treatment may offer effective protection against the toxin in number of animal taxa. In vitro experiments have demonstrated that GST extracted from different organisms (plants, invertebrates and fish) can metabolize MC-LR, resulting in the formation of GSH conjugates of MC-LR within an hour [14]. GSH-microcystin conjugates, with reduced toxicity compared to microcystins, have also been detected in toxin-treated mice [26]. There is evidence to suggest that the conjugation reaction to GSH catalyzed by GST may represent the first step of microcystin detoxification in a wide range of organisms. It would be instructive to undertake further studies to test the generality of this assertion.

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INSECTICIDAL ACTIVITY OF HARMFUL CYANOBACTERIA: THE ROLE OF TERPENE SUBSTANCES

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ABSTRACT

Biocidal characteristics of terpene fractions and their components were revealed in laboratory culture and natural populations of the cyanobacterium *Microcystis aeruginosa* Kütz. emend. Elenk. Toxicity, deterrent, and the other inhibitory phenomena were observed in fruit fly and fall webworm. The presence of citronellol, nerol, geraniol, β-phenylethyl alcohol and eugenol in laboratory culture and linalool, linalyl acetate, terpineol, geraniol and β-phenylethyl alcohol in the natural populations was ascertained. The composition of terpene fractions varied depending on the method of raw biomass treatment (lyophil and thermal drying, Folch and hexane extractions). Studies of the composition and activities of terpene compounds produced by laboratory culture resulted in the discovery of their toxicity and statistical dependence between insect mortality, terpene content and concentration of β-phenylethyl alcohol. Terpene fractions isolated from natural populations were toxic for fall webworm larvae and inhibited their nutrition and metamorphosis. Mainly larvae were killed over 10-20 days exposition regardless of treatment of raw biomass. Linalool and linalyl acetate mixture (1:1) had the highest insecticidal effect and caused total mortality on the 5-th day. The results demonstrate the role of terpene substances in plant protection and pest control, especially in the regions of intensive silkworm breeding.

INTRODUCTION

Biocidal activity of 23 cyanobacteria and microalgae, including water bloom species in Ukrainian waters and Black Sea coastal region, was revealed in some injurious organisms (bacteria, helminths, insects). Cyanobacteria and microalgae have displayed inhibitory action on vital functions (nutrition, growth, metamorphosis and reproduction) of herbivorous insects - Colorado potato beetle *Leptinotarsa decemlineata* Say, fall webworm *Hyphantria cunea* Drury, gypsy moth *Lymantria dispar* L., ermine moth *Hyponomeuta padella* L., brown-tail moth *Euproctis chrysorrhae* L. and lackey moth *Malacosoma neustria* L. Now it is necessary to pay attention to research and isolation of active biocidal substances produced by cyanobacteria and microalgae, in particular terpene complexes [1-5]. The terpene compounds are very important in relationships between different species of microalgae, seaweeds and bacteria, insects and plants, parasites, predators and prey in ecosystems. They can be found as a part of alarm pheromones, defensive secretions, sexual and trophic attractants of insects or secondary metabolites of plants. In the last case they can act as a repellent and deterrent for some species, or toxin for others, or even as an attractant. The use of terpene compounds in pest control may be very interesting, but this requires the stable sources to obtain terpenes. Insect metabolites have a very short-time action, and some of them reveal bifunctional activity. Terpene concentration in plants is variable and depends on a number of factors. Terpene produced by organic synthesis can contaminate the environment. Meanwhile it is possible to get terpene compounds by microbial synthesis. In particular, cyanobacteria can be used, because many terpene classes (hydrocarbons, alcohols, ethers, aldehydes etc) have been described among their metabolites [6-8]. The main objective of this paper is research of insecticidal activity of cyanobacterial terpene complexes and their compounds.

MATERIALS AND METHODS

Terpene fractions and their components were isolated from cultures and natural populations of the cyanobacterium (blue-green alga) *Microcystis aeruginosa* Kütz. emend. Elenk. The laboratory culture was obtained from collection of Institute of Hydrobiology of National Academy of Sciences of Ukraine. The Fitzgerald medium in Zehnder and Gorham modification was used for its cultivation [9]. A series of investigations was conducted to determine relationship between biocidal activity, content of terpene compounds in algal culture and composition of its medium (cultures of different composition of nitrogen and phosphorus were used for this aim). These experiments took place over ten-days duration with a two-month period. The natural material was collected in the Dnieper (Krementchug reservoir) during an August-September "water bloom". It consisted mainly of *M. aeruginosa* (96.0-98.0%). This biomass was exposed to thermal (37°C) and lyophil drying; then the experimental samples were prepared in laboratory mills: diameter of powder fractions after grinding was less than 0,063-0,1mm. The terpene fraction was isolated by water-steam distillation by Ginzberg and Dalmatov, but in some cases we used hexane extraction in Soxlet apparatus and extraction of lipid and pigment complex by Folch [9]. The quantitative and qualitative composition of the terpene fraction was examined by gas-liquid chromatography; the pure culture was examined every 10 days. The concentration of terpene

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compounds was very small in water-steam distillate (0,01%), so we carried out an additional step to concentrate the sample: terpene extraction with diethyl ether and subsequent waterbath vaporization. Several columns with different polarity and selectivity [10] were used for individual identification of components.

Widespread species of insects were chosen as the test objects. To determine the terpene activity in pure culture we added cyanobacteria to trophic medium for cultivation of experimental fruit flies *Drosophila melanogaster* Meigen (wild type). This species was selected for test because of its small feeding requirements in comparison with the species tested by extracts from natural populations. After the preparation of a test medium, insects - four males and four females - were placed on its surface. Each treatment consisted of three repetitions. Insect viability (specimens) was observed daily.

The second instar larvae of herbivorous insect - the fall webworm - were used for the study of deterrent and insecticidal activities of natural cyanobacterial material. The insect larvae were collected from their natural populations in the agricultural area near Simferopol in Central Crimea. During the experiments larvae were kept in vessels, 10-15 specimens in each. Each experimental treatment consisted of three or five repetitions. The leaves of the most preferred host-plant of fall webworm - ash-leaved maple *Acer negundo* L. - were used for feeding. They were treated with extracted substances from cyanobacterial biomass before placing the insects on the substratum. Leaf area consumed by fall webworm larvae was registered (%) during 7 days after the treatment. Dynamics of insect mortality (%) was studied during 10-20 days.

RESULTS AND DISCUSSION

The experiments demonstrated biocidal activities of terpene compounds produced by the pure culture of *M. aeruginosa*. The accumulation level of terpene compounds in cyanobacterial cultures was closely connected with the mortality of the experimental drosophila fruit flies. The suppression of insect vital functions took place in 5-7 days. The increase of terpene content from the 10th till the 30th day of cyanobacteria cultures was accompanied by the increase of fly mortality (Table 1). The gas-liquid chromatographic analysis ascertained the presence of citronellol, nerol, geraniol, β-phenylethyl alcohol and eugenol in the terpene fraction (Fig. 1, 2). Their ratio varied depending on the age of cultures. Concentration of terpene fraction increased during 10-30 day period in both cultures. The increase of insecticidal effect was observed simultaneously. This time the content of β-phenylethyl alcohol became higher in all samples. This process correlated with the reduction of eugenol content. We can assume some connection between the β-phenylethyl alcohol presence and insecticidal action (Table 1).

The terpene fraction from the natural populations of *M. aeruginosa* contained a number of components, including linalool, linalyl acetate, terpineol, geraniol and β-phenylethyl alcohol. But the different methods of raw biomass treatment affected some differences in terpene composition:

- the water-steam distillate obtained from a lyophil dried cyanobacterial biomass contained β-phenylethyl alcohol (the main component), and also linalool, linalyl acetate and geraniol (Fig. 3).
- the fraction from thermally dried biomass contained β-phenylethyl alcohol and linalool in approximately

Table 1. Biologically active substances and insect mortality during 7 days (24 fruit flies in every assay, 8 specimens x3 replicates)

Names of fractions and compounds	*Microcystis aeruginosa* (standard culture), age in days				*Microcystis aeruginosa* (culture with double nitrogen content and half phosphorus content), age in days			
	10	20	30	40	10	20	30	40
Terpenes in total, %	$2.1 \cdot 10^{-4}$	$2.1 \cdot 10^{-4}$	$2.4 \cdot 10^{-4}$	$1.6 \cdot 10^{-4}$	$1.8 \cdot 10^{-4}$	$1.8 \cdot 10^{-4}$	$3.9 \cdot 10^{-4}$	$3.0 \cdot 10^{-4}$
Citronellol, % in fraction	8.59	9.35	-	-	7.58	7.58	-	-
Nerol, % in fraction	4.79	3.77	3.8	10.7	7.69	7.69	7.3	7.3
Geraniol, % in fraction	3.64	-	--	3.58	9.9	9.9	--	--
β-phenylethyl alcohol, % in fraction	10.3	8.16	10.2	34.9	8.89	8.89	41.9	41.9
Eugenol, % in fraction	64.42	78.72	71.7	50.81	58.91	58.91	50.78	50.78
Unidentified components	8.26	--	14.3	--	7.03	7.03	--	--
Mortality of insects (specimens), in total	14±1.0.2	17±1.7	21±0.68	19±0.34	16±.0.3	16±.0.3	21±0.7	19±0.7
Mortality of insects: control nutrient medium without cyanobacteria	2±0.3	0	3	3	2±0.3	0	3	3
Least significant difference, 0.05 level	3.11	4.76	1.9	0.95	1.19	0.25	1.96	1.96

equal proportions, and also linalyl acetate, terpineol and geraniol (Fig. 4).
- filtrate from raw natural cyanobacterial material contained β-phenylethyl alcohol and terpineol.
- the alcoholic extract from cyanobacteria lipid and pigment complex contained geraniol, linalool, linalyl acetate, β-phenylethyl alcohol, terpineol and the number of non-identified components (Fig. 5).

Reanalysis after two years confirmed the presence of the same components. It manifested the stability of terpene fraction. But linalool and linalyl acetate content in thermally dried biomass became significantly less, because the evaporation and disintegration had occurred in the sample.

The comparative investigation of deterrent and insecticidal effects revealed high biological activity of terpene compounds. The isolated fraction was toxic for the tested insects in the different series of experiments and regardless of the method of cyanobacterial biomass treatment. Terpene compounds inhibited the process of larval nutrition. Mainly mortality of larvae took place during 10-20 days. So terpene substances not only suppressed nutrition and metamorphosis of insects, but also killed the majority of larvae (Table 2). The fraction isolated from thermally dried biomass was more toxic than from the lyophilised one. But the terpene fraction isolated by Soxlet apparatus caused the elimination of the largest number of insects; this was connected with the most complete extraction of active compounds.

The special comparative tests determined the different biocidal activity of some pure terpenes in 0.002% concentration. The linalool-linalyl acetate mixture (1:1) had the highest effect and caused the total mortality of fall webworm larvae on the 5th day (Table 2). However, the other compounds were toxic for caterpillars during a longer period. We observed metatoxic effect: survived caterpillars were turned into pupae more rarely, but no adults were produced from pupae formed from such larvae. So no specimens completed the metamorphosis as imago.

The obtained data founded evidence on toxicity of

Table 2. The deterrent and insecticidal activity of terpene compounds isolated from the natural populations of *Microcystis aeruginosa* (test objects: fall webworm larvae, 2nd instar)

Variant (terpene fractions isolated by different experimental methods and some of their compounds)	Method of cyanobacterial biomass drying	Number of insects	Nutrition during 7 days, % of leaf surface	Mortality of larvae, %		
				10 day	15 day	20 day
Terpene fraction obtained according to Ginzberg method	Lyophil	60	43.6±6.5	51.7±6.4	68.3±4.8	81.7±4.8
	Thermal	60	15.6±2.6	63.3±6.4	85.0±3.2	90.0±1.6
Terpene fraction obtained according to Dalmatov method	Lyophil	60	21.7±3.5	53.3±4.8	71.7±8.1	85.0±3.2
	Thermal	60	18.8±3.5	70.0±6.4	85.0±6.4	86.7±6.4
Terpene fraction obtained in Soxlet apparatus	Lyophil	105	33.4±4.9	76.0± 3.8	91.3±3.8	96.0±1.3
Linalool & linalyl acetate mixture	-	135	25.9±3.1	100.0		
Terpineol	-	75	68.0±1.9	13.3±3.8	42.7±3.8	42.7±3.8
β-phenylethyl alcohol	-	75	60.0	22.7±5.1	53.3±3.8	53.3±3.8
Control: :water	-	135	100.0	0	0	0
Least significant difference, 0.05 level	-		21.0	28.3	27.1	20.7

terpene compounds of cyanobacteria in relation to some herbivorous insects.

It is interesting to discuss the specific effects of isolated terpene compounds for different insects. So linalool was discovered in defensive secretions and alarm pheromones of Hymenoptera and Coleoptera and in repellent mint species [11]. Citronella oil containing geraniol and citronellol repels *Tribolium castaneum* Hbst., *Callosobruchus chinensis* L. and *Periplaneta americana* (L.) [12]. Citronellol is known as the main part of ant *Lasius umbratus* Nyl. defensive secretion [13] and as the repellent for some mosquito species. But _-terpineol and _-phenylethyl alcohol were described as the attractants for silkworm *Bombyx mori* L. [14] as well as geraniol for honeybee *Apis mellifera* L. [15]. Therefore, these cyanobacterial substances are not harmful for non-target insects and can be used for plant protection in regions of the silkworm breeding where application of chemical and bacterial insecticides is undesirable. In the other cases it is possible to use a pest control system based on the complex of bacterial insecticides and terpene compounds [16].

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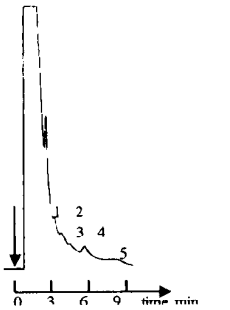


Fig.1. Laboratory culture with increased nitrogen content and limiting in phosphorus, 20th day of laboratory cultivation.
1. Citronellol 3. Geraniol 5. Eugenol
2. Nerol 4. β-phenylethyl alcohol

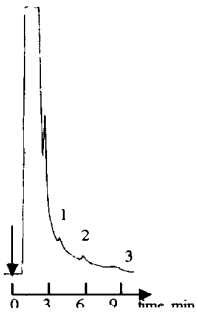


Fig. 2. Laboratory standard culture, 20th day of laboratory cultivation.
1. Nerol 2. β-phenylethyl alcohol 3. Eugenol

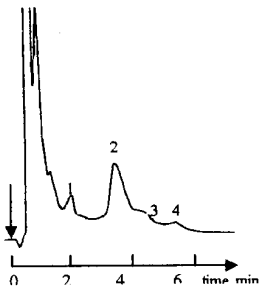


Fig.3. The natural populations (lyophilization and water-steam distillation)
1. Linalool 2. β-phenylethyl alcohol 3. Linalyl acetate 4. Geraniol

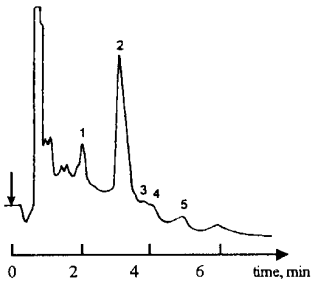


Fig. 4. The natural communities (thermal drying and water-steam distillation)
1.Linalool 2.β-phenylethyl alcohol 3.Terpineol
4.Linalyl acetate 5.Geraniol

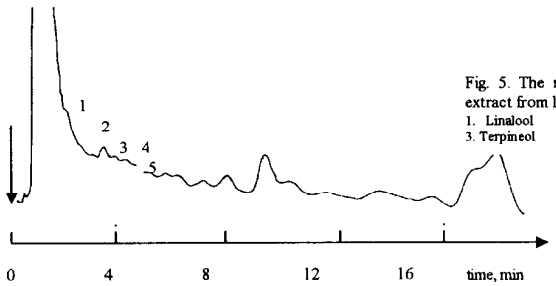
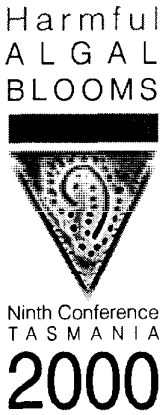


Fig. 5. The natural populations (alcoholic extract from lipid-pigment complex)
1. Linalool 2. β-phenylethyl alcohol
3. Terpineol 4. Linalyl acetate 5. Geraniol

INTERACTIONS BETWEEN HARMFUL MICROALGAE AND MARINE INVERTEBRATES



FEEDING BEHAVIOR OF INDIVIDUALS AND GROUPS OF KING SCALLOPS (*PECTEN MAXIMUS*) CONTAMINATED EXPERIMENTALLY WITH PSP TOXINS AND DETOXIFIED

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ABSTRACT

An experimental recirculating flume was used to study PSP contamination and detoxification patterns in scallops fed *A. minutum* and then non-toxic flagellates. Experiments were performed simultaneously with i.) a set of five isolated individuals compared to a control and ii.) two groups of 30 animals placed in a 100-L raceway (biodeposits method).
Isolated scallops showed considerable interindividual variation in Feeding Time Activity (FTA) values despite identical feeding conditions. Toxin absorption rates varied with time from one individual to another, but were always distributed in tissues in the following order: digestive gland > kidneys > other tissues. Mean bioaccumulation efficiency (Be) in tissues reached 19% for an absorption efficiency (Ae) of 42%.
For both groups, feeding behavior at the time of the shift to a non-toxic diet changed drastically depending on the algal species used to detoxify the scallops. A diet based on *Tetraselmis suecica* appeared to stimulate feeding processes, whereas one based on *Isochrysis galbana* had the opposite effect. During detoxification periods following initial toxicity of either 150 or 350 µg STX.eq./100 g of whole flesh, an average of more than two weeks was needed for toxin levels in scallops to reach the quarantine threshold. Toxin analysis in tissues also showed obvious STX neoformation in kidneys.

INTRODUCTION

Several areas of Northern Brittany are almost every year subjected to intense blooms of *Alexandrium minutum* Halim, a PSP toxins producer. Even if only oysters and mussels have been contaminated so far, some risk of *A.minutum* cysts or vegetative forms transfer along Northern Brittany coasts cannot be discarded and makes necessary further studies on the physiology of contamination/detoxification patterns in scallops.
A particular aspect of scallops contamination by phycotoxins is the fact that generally only adductor muscle and gonad are eaten by consumers. As a result, some national monitoring networks for phycotoxins are based on assessment of the toxicity in digestive gland and not n in the whole flesh or in each separate tissue. A toxin threshold in digestive gland, different from the international safety threshold used for the whole flesh (80 µg.STX eq.100 g-1), can then be used, provided the relative toxin ratio between digestive gland and muscle is known. Predicting gonad toxicity from digestive glands toxic level is rather rash [1] since no correlation, for instance, was found between toxicity of the gonad and that of viscera in wild populations of sea scallops, *Placopecten magellanicus*. Furthermore, it seems that once PSP toxins are accumulated by scallops, they are very slowly eliminated [2].
Previous studies [3,4,5] involving experimental contamination of King Scallops by toxic *Alexandrium* species showed high levels of contamination and detoxification as well as marked differences in the toxin profiles of different organs. They showed also an increase in gonad toxicity during detoxification due to conversion of gonyautoxins to saxitoxin.
Regarding toxin uptake the following questions were therefore addressed : i.) what proportion of toxin from ingested algal food is durably assimilated in scallop tissues ii.) is toxin body burden correlated to filtration rate and iii.) are GTX2/GTX3 (major toxin analogs in the French strain of *A.minutum*) hydrolysed or transformed through enzymatic pathways ?

MATERIAL AND METHODS

Scallops (*Pecten maximus*) were obtained in February 1998, from fishermen catches in the Bay of Brest and with no previous history of toxic algal blooms. The individuals were in sexual resting phase and had a mean shell height of 77.4 ± 3.3 mm (n=52). Before transfer into the experimental unit, they were acclimated for 5 to 6 days at 16 ± 0.5°C, and fed daily *Scrippsiella trochoidea*.
Algae were cultured at 16 ± 1°C with a light intensity of 50 ± 4 $\mu E.m^{-2}.s^{-1}$ and a 12 h light/12 h dark photoperiod. The AM89BM strain of *A. minutum*, isolated in Morlaix Bay in 1989, had a mean toxicity evaluated during experimental time-course at 2.4 ± 0.1 pg.eq. STX per cell (n=12). The non-toxic strains *S. trochoidea* Paulsen, *Tetraselmis suecica* Kylin, and *Isochrysis galbana* Parke, were cultured, like *A. minutum*, in Provasoli's nutrient medium [6]. The cultures were used at the end of exponential growth phase.
A recirculated flume identical to the one previously used for long-term experiment on oysters [7] was used. The exposure period to the toxic algal strain was of 9 days, while the subsequent non-toxic diet was applied during 14 days.
A 30-L "buffer" tank placed within the circuit just after the flume outlet contained the heat exchanger and the pumps ensuring circulation of the water and continuous measurement of fluorescence. Microalgae were supplied with a peristaltic micropump started and stopped as a function of the threshold set for in vivo fluorescence. The continuous measurements provided by the fluorometer were integrated via an acquisition and control card (AD Clone interface) connected to a PC, in order to keep constant fluorescence values, i.e cell concentrations (.in vivo fluorescence was itself calibrated by cell counts performed with a microscope or

a Coultronics particle counter). Cell concentrations during contamination and detoxification phases were equivalent to 0.5 mg.L^{-1} TPM, i.e. the quantity of *A. minutum* required to induce a toxic concentration in the bivalves greater than the salubrity threshold (80 µg.eq.STX, 100 g^{-1} of meat) at the end of the exposure period. This corresponded to 120 cells.L^{-1} for *A. minutum* [8], 12,000 cells.mL^{-1} for *I. galbana* and 2,000 cells.mL^{-1} for *T. suecica.*

Scallops biodeposits (feces and pseudofeces) were collected twice a day and total (130 L) renewal of the water circuit was performed every day in order to avoid high concentrations of ammonia. The overall feeding behavior of scallops (30 animals) was monitored using the biodeposit method [9].

The impact of a change of food in a raceway for given experimental conditions was assessed using a general linear model (Draper and Smith, 1981).

Another experimental device was used to investigate individual responses of scallops to *A.minutum* feeding. For that purpose, 6 1L boxes (5 live scallops and a control with an empty shell) were continuously supplied (input) with seawater directly pumped in the 100 L raceway. Residual seawater (output) was then recirculated in the buffer tank. Each experimental box was connected to an automatic sampler so that an aliquot of sea water + algae was pumped through a Turner spectrofluorometer and analysed for fluorescence level. As a result, sea water of each experimental box was analysed during 1 mn every 6 mnd. The difference between fluorescence time-series continuously recorded for the 5 scallops and that of the control allowed a non-stop evaluation of phytoplankton particles uptake versus time for each animal. . The Feeding Time Activity (FTA) could then be defined as the percentage of time where filtration activity was recorded.

The purpose of the 'individual' experiment was to make certain of individual variations in feeding activity and thus have a better approach of the relationship between FTA and toxin uptake. It was also an interesting way to evaluate Toxin Bioaccumulation Efficiency (Tbe) such as TBe = [Tox]/TAR, with [Tox] = PSP toxin concentration detected in scallop tissues and TAR = toxin absorption rate. TAR is calculated from : OAR (organic absorption rate), PSP toxin concentration per algal cell and the relationship between POM and cell concentrations.

For PSP toxin analysis, four scallops were randomly collected during the detoxification period. Digestive gland, kidneys and the remaining part of each individual were dissected and ground with 0.1 N CH_3COOH at 4°C on a v/w basis. After centrifugation (3,000 x g, 15 min, 4°C), the pH of extracts was adjusted to 3.0-3.5 using glacial acetic acid to prevent excessive dilution. After half-dilution, supernatants were ultrafiltrated and then kept at 4°C until analysis. Ten ml of *A. minutum* culture were pipetted at the end of the exponential phase, and cells were centrifugated (3,000 x g, 15 mn, 4°C). Once supernatant removed, 1 mL of 0,03N Acetic Acid was added. Samples were frozen (-80°C).and a freeze-thaw method was then used to extract the toxins

Analysis was performed by reverse-phase ion-pairing high-performance liquid chromatography (IP-HPLC) according to the method of Oshima [10]. Diluted solutions (1:200) of each component of PSP1-B standard (MACSP, NRC-Halifax, Canada) were used as external standard for quantitative detection.In consideration of the dilution factors, molar concentration was then converted into µg STXeq.100 g^{-1} scallop meat using the conversion factors of Oshima, i.e. 297 and 168 µg STXeq.µM for GTX3 and GTX2, respectively.

Comparison of regression line slopes (ANCOVA) was used to determine significant differences, if any, between detoxification curves, i.e. *Isochrysis* versus *Tetraselmis*. For a P-value greater than or equal to 0.1, differences between slopes were considered not statistically significant from observed values at a ≥90% confidence level. For a P-value less than 0.05, differences between slopes were considered statistically significant at a 95% confidence level.

RESULTS

Individual experiment : Feeding Time Activities (FTA) showed wide variations among all 5 animals (Fig 1) during the 9d contamination phase. Two out of five had mean FTAs comprised between 90 and 98 %, one had 66 % and the two other bivalves showed low activities of 37 and 43 %. In term of PSP toxins absorbed, cumulated curves (Fig 2) obviously showed highest toxin amount in scallop which reached highest FTA value (C4) whereas scallops with low or mean FTA (C1, C2, C3) did not reach more than 63 µg STX.eq at the end of the contamination. Curiously, C5 scallop reached the same score, despite high FTA.

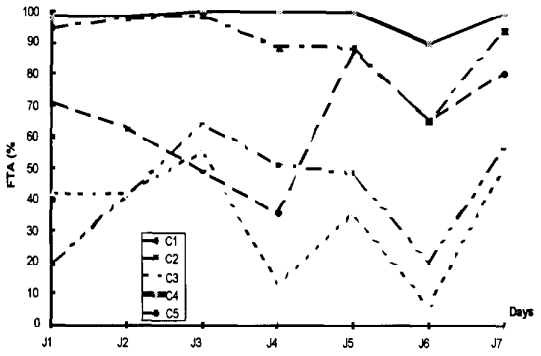

Fig 1 : Feeding Time Activities recorded for each experimented scallops of the 'individual' system during the contamination phase.

When comparing mean toxin body burden per tissue (or bulk of tissues) reported to total wet weight of flesh (100 %) a classical distribution was observed, i.e : digestive gland>>kidneys>other tissues. It was nevertheless noteworthy that kidneys participation to total toxin body burden reached 20 %.

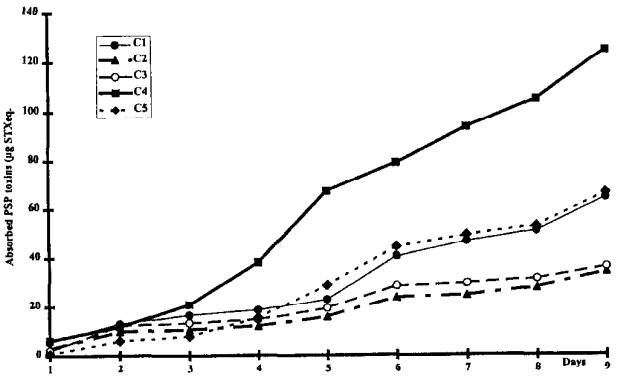

Fig 2 : Cumulated amounts of PSP toxins in each scallop whole flesh during the contamination phase

Mean Absorption efficiency (Ae) calculated as Ae = OAR/OFR (with OAR : Organic Absorption Rate and OFR : Organic Filtration Rate), was 42 % and mean Toxic Bioaccumulation Efficiency (TBe) was 19 %, which means that only 42 % of organic matter from filtered algal food was absorbed, out of which PSP toxins absorbed was 19 %. It was noticed that about 8 % of filtered toxins was accumulated in scallop tissues.

Population experiment : Different feeding processes were observed during *Tetraselmis* or *Isochrysis* diet-based detoxification periods .When *Tetraselmis* non-toxic diet is applied to scallop population previously fed *A.minutum* there is no detectable 'food effect' at the moment of the shift in diets, and feeding processes are continuously increasing up to a roughly constant level. At the opposite; *Isochrysis*-based non-toxic diet, when applied to scallop population actively feeding on *A.minutum*, produces a sharp decrease of feeding processes just at the moment of the switch of diets.

General linear model results were expressed as significant or not in term of 'food effect' and positive or negative trend in the slope of the curve during detoxification phase. A 'food effect' was observed only when switching from *A.minutum* to *Isochrysis* diet, with a sharp decrease of each of the three ecophysiological parameters. No special trend was detected during detoxification with this algae except a slight positive trend in BR. At the opposite, results were varying with *Tetraselmis* according to the observed parameter : negative and positive trends during detoxification respectively for CR and BR and significant food effect only for CR and FR, with an increase of each of these parameters.

Detoxification pathways : Detoxification kinetics for each of the two non-toxic diets (Fig 3) depict wide individual variations within each of the 5 scallops samples, especially at day 9 of contamination, thus corroborating what was observed in the 'individual' experiment. The safety threshold is not reached, even after 14 days detoxification in each case. A typical exponential function such as $y = y_0 e^{-kt}$ [11,1] was tentavilely applied to the '*Isochrysis*' curve to explain [Tox]/time relationship but unsuccessfully. Considering that individual variations at day 9 of contamination did not reflect the correct toxin status of the population just before being exposed to *Tetraselmis* diet, detoxification kinetics were therefore compared between days 4 and 14 of detoxification.

No difference between each kinetic (P-value : 0.12) was demonstrated. A significant slope was found only for '*Isochrysis*' detoxification curve although the most appropriate mathematical model describing the detoxification pathway was a linear relationship and not an exponential function.

The relative ratio of STX and GTX2/GTX3 gonyautoxins was evaluated in kidneys during detoxification time-course to estimate bioconversion of toxins in the excretory system. This ratio continuously increased between day 0 and day 8 of detoxification, to reach a steady level of 25 % the rest of time.

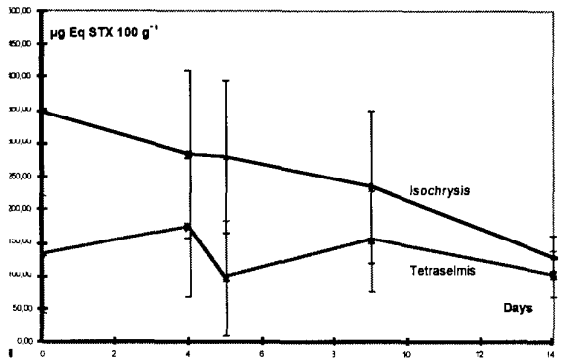

Fig 3 : Detoxification kinetics of PSP toxin concentrations in whole shellfish flesh and for *Isochrysis*-based diet (blue) and *Tetraselmis*-based diet (red).

DISCUSSION AND CONCLUSIONS

Wide individual variations in FTA, daily toxin uptake and total toxin body burdens at the end of the contamination phase were observed. This is in agreement with what was stated by Shumway and Cembella [12].One of the most surprising results obtained here was the variation in PSP toxin daily uptake which seems unrelated to daily FTA pattern. Yet, in at least one specimen, high FTA values were consistently associated with high PSP toxin content in shellfish flesh.

PSP toxins distribution in tissues follows a classical pattern, with the highest amounts in digestive gland and the lowest in other tissues, except kidneys, which represented 20 % of total toxin body burden, i.e definitely more than in a previous study (11 %) using static system and performed on larger size adult King scallops [5] with a more toxic *Alexandrium* species.

The efficiency of toxin bioaccumulation in tissues seems relatively poor, i.e only 8 % of toxin contained in ingested algal food, but this is enough to pass the safety threshold after 6 days of continuous feeding on

A.minutum diet at a mean cell concentration level of 150 cells.ml-1, at least for actively feeding animals.

Results on population experiments were more difficult to analyse. A 'food effect', or a sharp change in the level of at least two physiological parameters : both clearance and filtration seemed to be enhanced with *Tetraselmis*, and, at the opposite, decreased with *Isochrysis*. In previous experiments using Pacific oyster and the same experimental system [7], *Isochrysis*-based diet showed no detectable effects on feeding parameters whereas *Tetraselmis*-based diet showed an increase, particularly marked with biodeposition rate.

Albeit *Tetraselmis*-based diet seems the most appropriate non-toxic algal food to restore a healthy feeding activity, further experiments are needed, especially with diatoms-based diets. As to detoxification pathways, it is noteworthy that STX toxin, absent from the algal food used as toxin vector, was detected in shellfish flesh, particularly in kidneys, with an increased ratio during detoxification time-course. This result is in agreement with previous findings [5] showing a very slow decreasing trend of PSP toxins concentrations in King scallop kidneys as well as an increase in neoformed STX during detoxification.

Similar results are mentioned [12] on another scallop species : *Patinopecten yessoensis*, for which, once PSP contaminated, a 6 months detoxification resulted in a parallel decrease in GTX1/4 and increase in NeoSTX and STX in the kidneys.

The fact that *Isochrysis*-based detoxification kinetic was fairly described by a linear relationship rather than by an exponential function and that safety threshold was not met after 14 d detoxification would suggest King scallops might be assimilated to slow detoxifiers rather than to 'fast detoxifiers', likely to what was stated [1] for *Placopecten magellanicus* and *Patinopecten yessoensis*. If confirmed, these observations could lead to consider a possible physiological homogeneity of the group

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EFFECTS OF HARMFUL ALGAE ON THE EARLY PLANKTONIC LARVAE OF THE OYSTER, *CRASSOSTREA GIGAS*

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ABSTRACT

Short-term effects of the harmful algae, *Alexandrium tamarense*, *A. taylori*, *Chattonella antiqua*, *Cochlodinium polykrikoides*, *Gymnodinium catenatum*, *G.mikimotoi*, *Heterocapsa circularisquama*, *Heterosigma akashiwo*, and *Scrippsiella trochoidea* on the larvae (trochophore stage) of the Pacific oyster, *Crassostrea gigas* were examined in laboratory in order to determine the cytotoxicity of the nine algae on early life stages of the oyster. Lethal effects were found in larvae exposed to *A. tamarense*, *A. taylori*, *G. mikimotoi*, and *H. circularisquama* at cell densities of 10^5-10^7 cells^{-1}. In the case of exposure to *C. polykrikoides*, the trochophore larvae showed an extremely retarded metamorphosis to the D-shaped larval stage. In contrast, exposure to *C. antiqua*, *G. catenatum*, *H. akashiwo*, and *S. trochoidea* did not affect the survival rate and development of oyster larvae at the cell densities tested. Based on the results, the effects of harmful algae on the oyster larvae are concluded to vary greatly among species.

INTRODUCTION

Harmful algal blooms can cause a public health hazard resulting from the consumption of phycotoxins accumulated in marine organisms [1-3]. Harmful algal blooms can also affect the physiological processes and survival of marine animals [2,4]. In general, the latter is concerned with economic losses in the aquaculture industry [2,4]. Blooms of several harmful algae are known to kill marine finfish, molluscs, crustaceans, micro- and macroalgae. It is necessary to elucidate the mechanisms responsible for these effects in order to future develop damage prevention strategies. However, the effects of harmful algae on marine animals are species-specific and complex.

Oyster farming is the most successful aquaculture industry worldwide but some farming locations suffer economic loss from harmful algal blooms [5]. Oyster farming largely depends on the collection of naturally occurring larvae. Oyster larvae are planktonic during their early stages in the coastal water column that is they are exposed to harmful algal bloom directly. Although larval bivalves are considered to be strongly influenced by harmful algal blooms, information on the effects of harmful algae on an early larval development remains unclear. In this study, we preliminaly examined the effects of seven harmful algae, on the larvae of the commercially important oyster, *Crassostrea gigas* in order to verify the toxicity of harmful algae on the early developing stage of oyster larvae.

METHODS

Algal culture

Isolation records of nine microalgae used in the present study are listed in Table 1. Clonal cultures of these species were obtained by repeated washings using capillary pipettes.

Table 1 Isolation record of nine algae

Species	Strain name	Locations
Alexandrium tamarense	AT97	Hiroshima Bay, Japan
Alexandrium taylori	ATY-IN	Gondol coast, Indonesia
Chattonella antiqua	OC-B5	Osaka Bay, Japan
Cochlodiniumu porikrikoides	CP-EHI	Uwa Sea, Japan
Gymnodinium catenatum	GC97-HI	Hiroshima Bay, Japan
Gymnodinium mikimotoi	GM92-HI	Hiroshima Bay, Japan
Heterocapsa circularisquama	HA94-2	Ago Bay, Japan
Heterosigma akashiwo	HS92	Hiroshima Bay, Japan
Scrippsiella trochoidea	SCP2	Hiroshima Bay, Japan

The algae were grown in 300 ml flasks filled with 125 ml of f/2-Si medium without $CuCl_2$ because copper contamination to the assay procedure may interfere the development of larvae. The medium was autoclaved in polycarbonate bottles and transferred to the glass flasks when the temperature decreased to 70-80°C. All algae grew at a normal rate without $CuCl_2$ conditioning. Algal were cultured at 22°C (except for *A. tamarense*; 15°C) on a 12h light: 12h dark cycle under illumination at 100μmol photons $m^{-2}s^{-1}$ provided by cool-white fluorescent lamps. Cultures in the late exponential to early stationary phase were used for the experiments. In *C. polykrikoides* and *G. catenatum*, cells were concentrated using a plankton net (10μm mesh) because the maximum yield did not reach 10^7 cells^{-1}. Cultures were diluted with filtered and oxygenated seawater (GF/F pore size ca. 0.7μm Whatman Co., 31 psu) to the required concentrations. Cell counts were conducted on 0.5-1ml aliquot of the culture media more than three times under a microscope.

Larval oyster rearing

Adults of the pacific oysters, *Crassostrea gigas* were collected from Hiroshima Bay in the Seto Inland Sea, Japan. Water temperature and salinity in the natural habitat were 24°C and 31 psu, respectively. The sperms and eggs were sampled from a male and female individual, and then distributed to the 900 ml of oxygenated filtered seawater immediately. The mixture of sperms and eggs were incubated about two hours at 25°C. Fertilized eggs were gently washed with filtered

seawater on the 30μm opening of nylon mesh in order to remove the excess sperm, then distributed in oxygenated filtered seawater. In order to minimize bacterial growth, 25 mg Penicillin G potassium l^{-1} and 20 mg Streptomycin l^{-1} were introduced in rearing media. The nonshelled larva (trochophore stages, 17 h after the fertilization) were subjected to exposure experiment described below.

Five ml of larval suspensions was transferred to 6-well culture plate (ca. 130 trochophores/well), added the cultured harmful algae at several step of concentrations (0, 10^5-10^8 cells^{-1}). Controls were conducted with aditioning 15% (v/v) f/2 medium. Exposure experiments was conducted at temperature of 24°C, in a low light intensity (10-30μmol photons $m^{-2}s^{-1}$) for 8-10 h. Observations were conducted on sub-sample by a microscope. Death individuals were judged from the lost of motility and marked morphological disintegration (probably internal cell discharge due to cytotoxiciy). Mortality was estimated from the sum of disappeared and dead individuals. All experiments were conducted at duplicates.

RESULTS

Mortalities of trochophore exposed to the various concentrations of the nine harmful algae after 8-10 h are given in Fig. 1 and Table 2. Mortality was 5% in the control [15%(v/v) of the f/2 medium] and 82% of larvae changed to the straight-hinge stage (D-shaped larvae) within 27 h after fertilization. Notable mortality was observed for *A. taylori* and *H. circularisquama*. Both dinoflagellates killed >90% of the oyster larvae within three hours at cell densities of 10^5-10^6 cells^{-1}. In the 10^7 *H. circularisquama* cells^{-1} exposure, affected individuals showed marked visible changes (Fig. 1-3) within 30 min, and 86% died within 2 h after the exposure. Further, *G. mikimotoi* and *A. tamarense* also killed >92% of oyster larvae within 8 hours in cell density of 10^7 cells^{-1}. However, a marked mortality of larvae was not found in 10^5-10^6 cells^{-1} of *G. mikimotoi* and *A. tamarense* and larvae succeeded to develop to the D-shaped larvae within 10 h after the exposure. In the *C. polykrikoides* exposure, marked mortality and visible damage was not observed in larvae at 3 x 10^7 *C. polykrikoides* cells^{-1} exposure. However, larvae showed a retardant of metamorphosis to the D-shaped larvae (Table 2). No marked increase of mortality (1-7%) was observed with *C. antiqua*, *H. akashiwo*, *G. catenatum*, *S. trochoidea* at all the cell densitis examined.

DISCUSSION

Effects of harmful algal blooms on bivalves have been revealed in many previous studies [2]. However, quantitative relationships between various harmful algae and the effects on development during the early life stage of bivalves remain unclear.

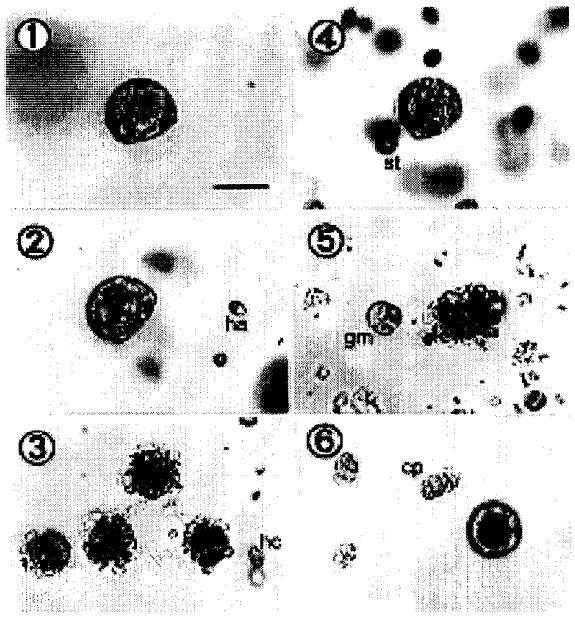

Fig. 1. 1-6 Photomicrographs of oyster larvae exposed to various harmful algae for 8 h. 1.Control. A nonshelled trochophore started to possess a straight-hinge. 2. *H. akashiwo* (ha); 1 x 10^8 cells^{-1}. No effect. 3. *H. circularisquama* (hc); 1 x 10^7 cells^{-1}. Affected individuals produce a mass mucus and aggregate together. Cilia of the trochophores were crashed and a number of cells discharged to surface. 4. *S. trochoidea* (st); 5 x 10^7 cells^{-1}. No effect. 5. *G. mikimotoi* (gm); 3 x 10^7 cells^{-1}. Trochophores completely lost motility and number of cells discharged to surface. 6. *C. polykrikoides* (cp); 3 x 10^7 cells^{-1}. No visible damage or significant mortality were observed but metamorphosis to the straight-hinge stage was clearly arrested.

Effects of the nine harmful algae on oyster larvae are summarized into three types

Type-I Lethal to oyster larvae at visible bloom density (red tide)

H. circularisquama > *A. taylori* > *G. mikimotoi* > *A. tamarense*,

Type-II Non-lethal effects but induce a delay in metamorphosis

C. polykrikoides

Type-III No effect

C. antiqua, *H. akashiwo*, *G. catenatum*, *S. trochoidea*

Some previous studis have indicated that *A. tamarense* bloom is not only cases PSP outbreak in oyster but also brought about considerable negative effect on oyster themselves [6].

A. tamarense killed the oyster larvae at 10^7 cells^{-1} but this is a magnitude higher than natural bloom densities. However, plankton cells sometimes accumulate at a hundred to thousand fold level by physical events such as tidal fronts and eddies, oyster aquaculturist should be

Table 2 Effects of harmful algae on the survival of Pacific oyster *Crassostrea gigas* larvae. A. healthy individuals, B. unusual individuals, C. mortality, D. Metamorphosed individuals, E. mortality.

Species name	cells l^{-1}	conditions of trochophore (% of initial density) 8h A	8h B	8h C	10h D	10h E
Alexandrium tamarense	1×10^7	0	8	92	1	96
	1×10^6	95	3	2	69	11
	1×10^5	91	6	3	77	7
Alexandrium taylori	2×10^7	0	0	100	0	100
	2×10^6	0	0	100	0	100
	4×10^5	15	23	62	3	87
Chattonella antiqua	1×10^7	95	2	3	78	5
Cochlodiniumu porikrikoides	3×10^7	95	2	2	25	5
Gymnodinium catenatum	1×10^7	94	3	3	82	3
Gymnodinium mikimotoi	3×10^7	0	1	99	0	100
	3×10^6	91	5	4	71	6
	3×10^5	94	3	3	80	2
Heterocapsa circularisquama	1×10^7	0	14	86	2	93
	2×10^6	17	28	55	6	83
	5×10^5	45	19	35	27	55
Heterosigma akashiwo	1×10^8	96	3	1	80	4
Scrippsiella trochoidea	5×10^7	93	5	2	77	2
15%(v/v) f/2 medium	-	96	2	2	82	5
Filtered seawater (GF/F)	-	93	3	4	82	7

aware of the potential cytotoxic effects of *A. tamarense*. No data are available on the potential impact of *A. taylori* on marine animals. The present strain was established from a red tide (1.6×10^7 *A. taylori* $cells^{-1}$) which occurred in Indonesia, Gondol coast, Bari Island in November, 1996. The red tide was not associated with fish-killing and shellfish poisoning. HPLC analysis on cultured cells of *A. taylori* detected no PSP toxins (S. Sakamoto, per. comm.). However, *A. taylori* had an extreme lethal effect on the oyster larvae. This dinoflagellate recently formed a red tide in Wakayama Prefecture, central Japan and laboratory tests showed that the red tide led to acute toxicity on juveniles of the abalone *Haliotis discus* (T. Takeuchi, per. comm..).

G. mikimotoi and *C. polykrikoides* are known to cause massive finfish kills [4] but little is known on their effects on molluscs and crustaceans. *G. mikimotoi* (type I) kills not only oysters but also finfish, other molluscs, and crustaceans [7]. Particular, some gastropods such as the abalone *Haliotis discus* and *Sulculus diversicolor* frequently are killed by *G. mikimotoi* [8]. Therefore, this dinoflagellate is clearly toxic to molluscs. A previous study indicated that *G. mikimotoi* is closely related to *Gyrodinium* cf. *aureolum* [9,10]. Exposure of a *G. aureolum* bloom water to embryos of the oyster *C. gigas* interfere with the normal embryonic development and affect survival [11]. This dinoflagellate also shows a cytotoxic effect on adult mussels, *Mytilus edulis* [12,13]. Bloom water of the dinoflagellate *C. heterolobatum* that is closely related to *C. polykrikoides* induced a significant mortality and depression of calcium uptake in larval eastern oyster *C. virginica* [14]. According to our study, *C. polykrikoides* did not cause any visible damage (loss of motility, cell discharge, or mucus production) or lethal effects on oyster larvae but did cause a considerable retardation of metamorphosis.

The present study revealed that the dinoflagellate *H. circularisquama* has an extreme lethal effect on oyster larvae which is associated with observable damages using *H. circularisquama* has caused mass mortalities of various bivalves in western Japan since 1988 [15]. Juvenile pearl oysters (*Pinctada fucata*) exposed to 5-10 $\times 10^6$ *H. circularisquama* $cells^{-1}$ frequently resulted in death within 48 hr in association with vigorous clapping, contraction of their mantle and gills, and cardiac arrest [16]. A significant inhibition of the filtration rate during feeding experiments on the blue mussel, *Mytilus galloprovincialis* was observed in 5-10 $\times 10^4$ *H. circularisquama* $cells^{-1}$ [17].

Humans and some marine mammals are affected by phycotoxins such as PSP, DSP, and ASP etc. when the toxin is accumulated in food through the food web. However, it seems that the relationships between harmful algae and other marine animals are different from those of mammals. In the present study, both *A. tamarense* and *A. taylori* belonging to type-I but the latter used at present study does not produce PSP toxins. Another PSP producer *G. catenatum* showed no harmful effects on oyster larvae even at 10^7 *G. catenatum* $cells^{-1}$. It seems that the cytotoxic effects of *Alexandrium* spp. on oyster larvae are not primarily caused by the PSP toxins. Therefore, acute toxic effects of *Alexandrium* spp. on oyster larvae are probably mediated by another toxic nature. Cell-free filtrate of *A. tamarense* and *A. taylori* have less cytotoxicity on the oyster (data not shown). The direct contact of larvae with toxic cells of *Alexandrium* is important process causing cytotoxicity, therefore, it is highly likely that toxic nature would localized on cell surface of *Alexandrium*.

Furthermore, it is interesting that the ichthyotoxic raphidophyte species *C. antiqua* and *H. akashiwo* did not affect the oyster larvae. *H. akashiwo* did not affect the shell-growth responses of mussels [18] and cells were readily taken up by pearl oyster spat at 10^8 $cells^{-1}$ [16]. The present study indicates that mechanism of damage occurrence in bivalve physiology due to harmful algae is different from those of finfish and mammals. Such differences require further examination..

It should be noted that some physical and chemical factors such as excess particle density and oxygen depletion may have affected the results in the present study. However, mortality and metamorphosis rates of trochophores exposed to the non-toxic dinoflagellate *S. trochoidea* (5×10^7 $cells^{-1}$) were not different from the

control. Thus, we believe that the effects obtained in present study were brought about by the specific toxicity of the harmful algae.

In the early developing phase, larval bivalves show rapid growth because of their relatively high metabolic activity. Therefore, the species composition and abundance of phytoplankton as a food strongly affect the development and survival of bivalve larvae. Generally, planktonic bivalve larvae constantly swim and stay in the surface and subsurface layer where their food of phytoplankton, bacteria, protozoa, and detritus are commonly abundant. Hence, swimming is the most important strategy for the development, survival, and dispersal of larval bivalve from an ecological point of view [19]. However, these habitats concurrently have a high potential of being directly affected by harmful algal blooms. These facts indicate that planktonic larval bivalves are affected by certain harmful algal species when exposed to a bloom density in nature. Further work is necessary to elucidate the potential impact of harmful algae on the early development of commercially important molluscs.

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AN ADVANCED, *IN VIVO* METHOD TO INVESTIGATE THE DISTRIBUTION OF PARTICLES IN THE BLUE MUSSEL, *MYTILUS EDULIS*, USING A GAMMA CAMERA TECHNIQUE

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ABSTRACT

This paper presents an imaging tool for *in vivo* studies on bivalve shellfish using advanced gamma camera technique. The distribution of different particles, labelled with gamma emitting radionuclides, in the digestive tract of blue mussels, *Mytilus edulis*, was investigated. The stomach was identified from the image and outlined as a "Region of Interest" (ROI) and the temporal changes of particle concentration within the ROI were analysed. The results indicated that the uptake rate correlated to the size of the particles. It was concluded that this method is useful for studies on particle processing, such as uptake and elimination, in bivalves.

INTRODUCTION

Accumulation of harmful substances, such as marine biotoxins and bacterial or viral pathogens by shellfish is a major problem for the aquaculture industry and requires extensive and reliable quality control. In order to improve present monitoring programs, more knowledge is desired about how individual mussels digest different particles and how these processes can influence the retention and degradation of harmful compounds.

The aim of this study was to evaluate the usefulness of gamma-camera technique to study the particle processing in *M. edulis*.

METHODS

The gamma camera was developed by Hal Anger (2) and has become the main imaging tool in nuclear medicine. The principle of the method is to use particles labelled with a gamma-emitter such as ^{57}Co, ^{51}Cr, ^{113}Sn or $^{99}Tc^{m}$ from which the gamma rays pass through a collimator which projects the direction to a sensitive crystal, made of thallium-activated sodium-iodide [(NaI(Tl))], for detection. The pulses from the gamma camera are converted to digital signals and displayed as an image on a computer-screen (HERMES-system (NuD) Nuclear Diagnostic AB, Hägersten, Sweden). The radioactivity is recorded as a function of time.

To evaluate the method, eight mussels were used of which four sets of two were given different particles. The particles used were 1) non-degradable microspheres, 15 µm, made from styrene-divinyl benzene resins, labelled with ^{57}Co (half-life 256 days) (NEN-TRAC Microspheres, Du Pont), 2) stannous colloids (90% 1-10 µm), labelled with $^{99}Tc^{m}$ (half-life 6 hours) (ISOPHARMA, IFE Tinn-kolloid TC.32S), 3) macro aggregate human serum albumin (90% ca 40 µm), labelled with $^{99}Tc^{m}$ (TechniScan®LyoMAA, MALLINCKRODT MEDICAL, Petten, Holland, B.V.) and 4) micro colloids preparated from human serum albumin (0.2-1) µm, labelled with $^{99}Tc^{m}$ (SOLCO®ALBU-RES).

Each mussel was placed in front of the camera in a beaker with temperature-controlled and oxygenated seawater. A magnetic stirrer was used to obtain a homogeneous distribution of the particles in the water, and to avoid disturbance the mussel was placed in a basket and hung above the bottom. The magnetic stirrer was turned off when the activity in the water was visually reduced to an undetectable level, in order to avoid re-ingestion of radioactive faeces. The distribution of the radioactive particles was registered during 360 minutes and was followed from the image, as is illustrated in fig. 1. The area where the particles accumulated after passing the gills was identified as the stomach and was outlined as a "Region of Interest" (ROI). The activity from this area was converted to numbers and presented as a graph. The background correction of activity was done by drawing a ROI outside the beaker. To minimise the influences of differences in the used amount of activity and variability in geometry of the mussel body as well as the distance between the mussel and the camera, all values were normalised to the amount of radioactivity offered to the mussel. The following parameters were extracted from the graphs: 1) maximum activity, given as the maximum value of activity registered in the ROI (%); 2) uptake rate, as the accumulation of activity per minute, until 50 % of the maximum activity was reached (% min^{-1}); and 3) retention, as the amount of activity (%) measured in the ROI after 360 min. All the values were corrected for background activity and for the half-lives of the radionuclides.

Harmful Algal Blooms 2000
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Intergovernmental Oceanographic Commission of UNESCO 2001

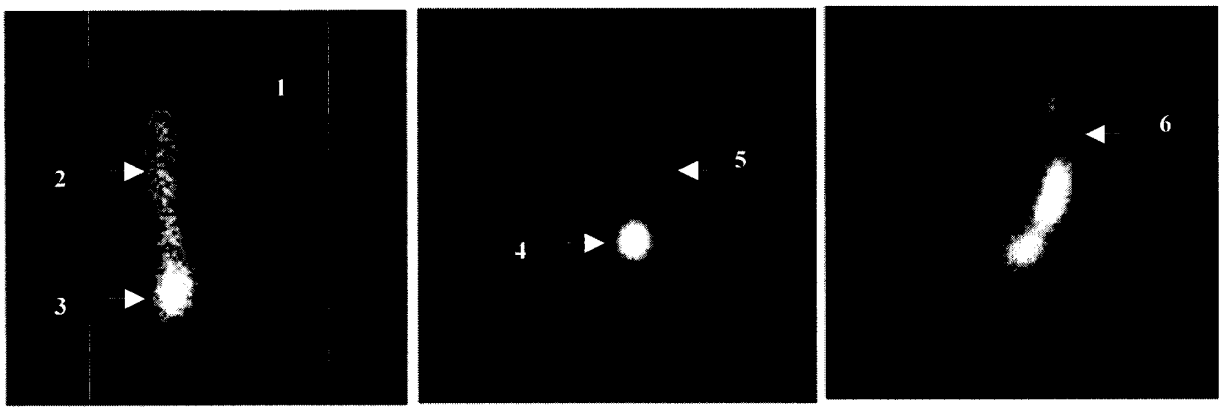

Fig. 1. Gamma camera images (scale 1:1.5) from a mussel fed macro aggregate human serum albumin, LyoMAA. Image **A)** illustrates the distribution of activity two minutes after the particles were offered to the mussel. The activity is shown in the beaker (1) and a pronounced accumulation has taken place on the gills (2) and the oesophagus (3). Image **B)** shows that after 10 min, no activity is visible in the beaker or on the gills but in the digestive gland (4). In addition, some activity is shown in the first part of the gut (5). Image **C)** shows that after 120 min there is still activity in the digestive gland but a major part has been transported to the terminal part of the gut (6).

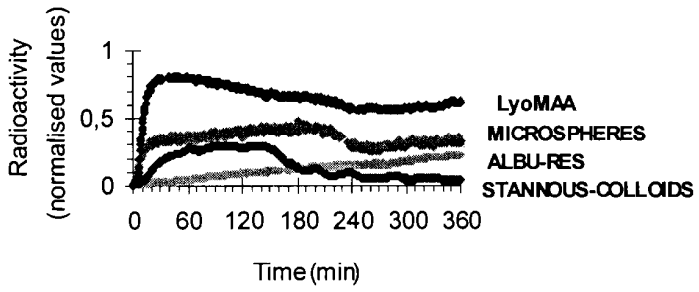

Fig. 2. Chart lines showing the amount of radioactivity in the digestive glands in four mussels fed microspheres (15 μm), stannous-colloids (1-10 μm), LyoMAA (40 μm) and ALBU-RES (0.2-1 μm), respectively. The radioactivity is normalised to the amount of activity offered to the mussel.

Table1. Results obtained from the gamma camera measurements of radioactivity in the stomach of eight individual mussels. Four sets of two mussels were given different radiolabelled particles. The parameters are calculated as described in text.

Particle given to the mussel	Max.value (%)	Uptake rate (% min^{-1})	Retention (%)
Microspheres	42	1.7	76
(15 μm)	35	0.4	86
Sn-colloids	26	0.5	19
(<10 μm)	36	1.8	50
LyoMAA	80	4.0	75
(ca 40 μm)	66	0.8	100
ALBU-RES	20	0.1	100
(0.2-1 μm)	32	0.1	100

RESULTS

Figure 2 shows the temporal changes in the radioactivity of the different particles in the stomach (ROI). The largest particle, LyoMAA was the most rapidly and efficiently ingested particle whereas the slowest ingestion was observed for the smallest, micro colloidal material, ALBU-RES. These small particles constantly increased in this ROI during the observation time. The mean maximum
activity for the two individuals fed LyoMAA was 73 (SD 10)% and for ALBU-RES it was 26 (SD 8)%. The mean uptake rate for LyoMAA was 2.4 (SD 2.3)% min^{-1} and for ALBU-RES it was 0.1% min^{-1}. The maximum activities for the microspheres and the stannous-colloids were comparable [39 (SD 5)% and 31 (SD 7)%, respectively]. Stannous-colloids were eliminated faster than the other particles and had the lowest retention [35 (SD 22)%] (Table 1)

DISCUSSION

With this investigation, we were able to present a gamma camera method, which can be used to study individual behaviour in processing of various particles in blue mussels. A previous study on *M. edulis,* by Møhlenberg and Riisgård (3), showed a marked decline in the uptake of particles smaller than 7 μm. In our study there was a large variability between individuals, feeding the same particle. Nevertheless, the results from the limited numbers of individuals we used, indicated that the uptake correlated to particle size. The retention of particles observed in our study could partly be dependent on the uptake rate. The mussels cleared the water from particles when fed macro aggregate serum albumin, microspheres and stannous colloids and we could follow their subsequent transport from the digestive gland into the gut as a reduction in the digestive gland. However, when mussels were fed the smallest particles (ALBU-RES), they were not able to clear the water and the uptake continued during the whole observation time. This effect must be considered and can be avoided by limiting the time spent feeding and thereafter transferring the mussels to clean water to continue the observations. It is also possible to use several outlined regions like the gills, the gut and the whole mussel body to supplement the evaluation. Likewise, increasing the observation time gives the possibility to estimate and compare the elimination rates of particles.

This method can be used for several applications. The camera has two channel facilities, which allow simultaneous recording of two gamma emitters with different energy levels, such as ^{51}Cr and $^{99}Tc^{m}$. This can for example be used to study discrimination or preference in uptake, absorption and elimination of different particles. The gamma camera method can also be used to study the uptake and elimination of radiolabelled micro-organisms such as bacteria and algae. It has been shown that it is possible to label Gram-negative bacteria with $^{99}Tc^{m}$ (4, 5) and it might also be possible to label heterotrophic dinoflagellates through ingestion of labelled bacteria or other labelled compounds, like amino acids. The conclusion from this study is that the gamma camera method can be a useful tool when studying the distribution of various particles in individual bivalves.

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ACCUMULATION OF DOMOIC ACID ACTIVITY IN COPEPODS

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ABSTRACT

Herbivorous copepods are key intermediates for the transfer of algal toxins into marine food webs. Domoic acid activity was detected by receptor binding assay in copepods fed on the diatom *Pseudo-nitzschia multiseries* (Hasle) Hasle within 3 h. Results of receptor binding assays indicated a range of 3 to 7 ng domoic acid equivalents per copepod within that period. A 1000-fold accumulation of domoic acid activity above ambient cellular concentrations of the toxin was documented in *Acartia tonsa* Dana after 20.5 h of exposure and grazing rates were as high as 1,600 cells copepod^{-1} hr^{-1} during that time. There was no significant difference in copepod grazing rates on non-toxic *Pseudo-nitzschia pungens* (Grunow) Hasle and toxic *P. multiseries*.

INTRODUCTION

The first incidence of domoic acid entering marine food webs was documented in 1987 on Prince Edward Island in eastern Canada when 107 human cases of encephalopathy [1], dubbed amnesic shellfish poisoning [2], were reported. All the victims had eaten cultured mussels contaminated with domoic acid, a potent neurotoxin produced by the diatom *Pseudo-nitzschia multiseries* (Hasle) Hasle [3]. In September 1991, dead pelicans and cormorants in Monterey Bay tested positive for domoic acid [4] and reports of tainted razor clams and dungeness crabs in Oregon and Washington followed in October. Evidence was mounting for widespread food web contamination by domoic acid associated with *Pseudo-nitzschia* blooms [5, 6]. Marine mammal mortalities from as early as 1972 through 1992 were suspect [7] and Scholin and colleagues [8] have now conclusively demonstrated direct food chain links from *Pseudo-nitzschia australis*
Frenguelli, another domoic acid-producing diatom, through planktivorous fish to sea lions.

Another major point of entry for domoic acid into marine food webs is through zooplankton. Crustacean zooplankton are key links in the transfer of phytoplankton carbon to higher trophic levels and can serve as vectors for algal toxins as well [9-12]. In this effort we used a receptor binding assay to demonstrate the ability of copepods (*Acartia tonsa* Dana) to graze toxic *Pseudo-nitzschia* cells and rapidly accumulate domoic acid activity. Comparative grazing experiments using non-toxic *Pseudo-nitzschia* showed no significant differences in grazing rates between toxic and non-toxic cells.

METHODS

Nonaxenic clonal batch cultures of *P. multiseries* (MU1) and *P. pungens* (Grunow) Hasle (CL20; gift of S. Bates) were grown in T1 medium [13] at 20 ± 1°C under 100 µmol photons m^{-2} s^{-1} cool white fluorescent light in Charleston, South Carolina, USA. Early stationary phase cultures (5 to 15 x 10^4 cells ml^{-1}) were shipped overnight to Beaufort, North Carolina, USA for the grazing experiments.

Copepods were collected on 15 December 1998 and 29 March 1999 with a 333 µm-mesh plankton net towed at the surface in Beaufort Inlet, NC, (34° 41.8'N, 76° 40.3'W) (salinity = 34 psu, temperature ~10-15°C). Zooplankton were returned to the laboratory within one hour and sorted immediately. Actively-swimming adult female *A. tonsa* were selected with a micropipette under a dissecting microscope and were acclimated on non-toxic *P. pungens* (7.5x10^3 cells ml^{-1}) in 30 psu seawater for 24 hours. Replicate samples of *A. tonsa* were frozen to serve as controls for toxin exposure. The initial cell concentration for the *P. multiseries* culture, prior to dilution for use in grazing experiments, was 50x10^3 cells ml^{-1}.

For the grazing experiments, 16 copepods each were added to triplicate samples of *P. multiseries* concentrations at both 1,225 and 4,812 cells ml^{-1} and to *P. pungens* concentrations of 503, 973 and 10,785 cells ml^{-1}. Initial aliquots of *the P. multiseries* and *P. pungens* cultures were filtered (2 ml) and filters and filtrate were analyzed for domoic acid. At each *Pseudo-nitzschia* con-centration, *A. tonsa* were allowed to graze in 240 ml suspensions for 16.5-20.5 h. Copepods were counted at the end of the grazing periods and < 3% mortality was noted. The grazing samples were preserved in 5% Utermohl's solution and the *Pseudo-nitzschia* cells were counted in a settling chamber at 400x on an inverted Wild microscope [14]. Grazing and ingestion rates were calculated according to Frost [15].

For the toxin accumulation experiments, the copepods were held in 5 L containers at the same cell concentrations for the same time periods as the grazing experiments. Copepods were removed using a 210 µm-mesh sieve and rinsed in filtered seawater three times before being micropipetted on to GF/F filters (Whatman) (31, 50 or 100 copepods each) and stored at -80°C until analyzed. Filtered cells, filtrate, and copepod samples were extracted in 10% aqueous methanol and tested using a domoic acid receptor binding assay [16]. All toxin values represent DA activity relative to a domoic

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acid reference standard (DACS-1C; NRC Inst. for Marine Bioscience, Halifax, NS, Canada) and are expressed as DA equivalents.

RESULTS

Preliminary experiments with *P. multiseries* cultures relieved concerns about the efficacy of shipping cultures overnight. Even with periodic shaking of the culture flasks over 24 h the cells retained >80% of their original toxin. Initial observations of copepods fed *Pseudo-nitzschia* cells during the 24 h acclimation period indicated they were healthy and had high grazing rates and high egg and fecal pellet production rates. From two grazing experiments it appeared that food saturation was reached between 1,000 and 5,000 *Pseudo-nitzschia* cells ml^{-1} and the highest rate of 1,600 cells $copepod^{-1}$ hr^{-1} was reached at food levels of 4,812 cells ml^{-1} (Fig. 1).

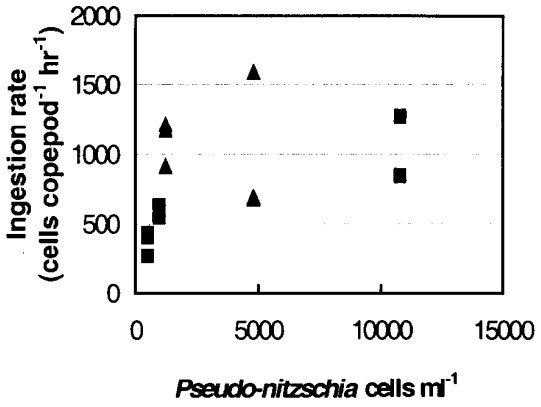

Fig 1. Ingestion rates of copepods (*Acartia tonsa*) grazing on (▲) toxic *Pseudo-nitzschia multiseries*) and (■) non-toxic (*Pseudo-nitzschia pungens*) cells. N=14 (672 copepods)

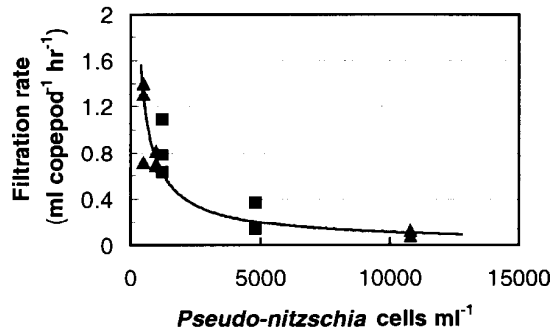

Fig 2. Filtration rates of copepods (*Acartia tonsa*) grazing on toxic (▲) (*Pseudo-nitzschia multiseries*) and non-toxic (■) (*Pseudo-nitzschia pungens*) cells. N=14 (672 copepods)

Food levels above this concentration did not increase the grazing rate (cells consumed per copepod per hour). This can be seen more clearly in the ingestion rate data with an exponential decrease in ingestion (volume of culture swept clear of cells per copepod per hour) with increasing cell concentrations (Fig. 2).

Detection of domoic acid activity in *A. tonsa* was possible within 3 h of the onset of grazing on *P. multiseries*, but toxin levels varied between 3.1 and 7.0 ng DA equiv. $copepod^{-1}$ (Fig. 3).

This reflects the variable grazing rates during the early part of the experiment. Higher and less variable domoic acid contents (6.3-7.3 ng DA equiv. $copepod^{-1}$) were noted at 20.5 h. After this time the copepods were screened, rinsed, and moved into filtered seawater to allow the domoic acid to depurate for 4.3 h. After depuration the domoic acid content was reduced (5.0-5.6 ng DA equiv. $copepod^{-1}$), yet toxicity clearly remained present in these animals.

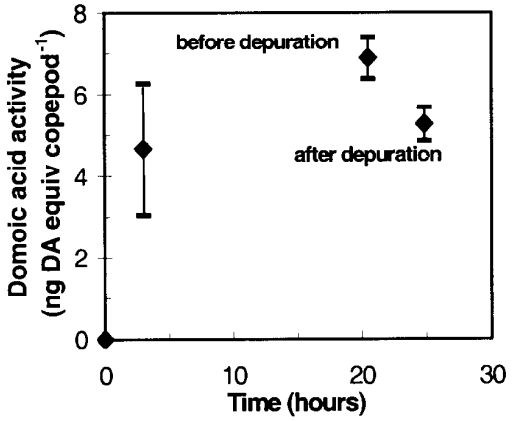

Fig. 3. Accumulation of domoic acid activity in copepods (*Acartia tonsa*) grazing on *Pseudo-nitzschia multiseries* (MU1) after 3 and 20.5 hours. Copepods were moved to filtered water after 20.5 h and allowed to depurate. N=10 (781 copepods)

The domoic acid content of *P. multiseries* cells at the outset of the experiment (0 h) was between 1.3 and 2.3 pg DA equiv. $cell^{-1}$, while the cell-free filtrate concentrations ranged from undetectable (1,225 cells ml^{-1}) to between 66.3 and 72.3 pg DA equiv. ml^{-1} (4,812 cells ml^{-1}). The dissolved DA levels did not change appreciably from the start of the experiment through the 3 h and 20.5 h time points, remaining between 60.1 and 78.0 pg DA equiv. ml^{-1}. These data indicate that copepods ate the *Pseudo-nitzschia* cells while causing little breakage and thus minimal release of DA. Therefore, exposure of the copepods to dissolved DA was relatively uniform throughout the >20 h experiment.

DISCUSSION

The rapid accumulation of domoic acid activity by zooplankton grazing on *P. multiseries* observed herein is not surprising given the high turn over rate of *A. tonsa* reported by Guerrero & Rodriguez [17]. The highest concentration of domoic acid activity recorded in copepods (7.43 ng DA equiv. copepod^{-1}) in this study could have been accumulated in <3 h, assuming the cells retained 80% of their domoic acid (~1.6 pg DA equiv. cell^{-1}) and were consumed at the maximum grazing rate of nearly 1,600 cells copepod^{-1} h^{-1}. With such rapid toxin transfer from phytoplankton to primary consumers, it is understandable why Scholin et al. [8] noted that even a short 'pulse' of domoic acid (days to weeks) may be sufficient to accumulate in the food web to kill marine fauna. During the 1998 Monterey bloom of *P. australis,* receptor assay based domoic acid concentrations ranged over an order of magnitude from 7.22 to 74.6 pg DA equiv. cell^{-1} [8]. The lowest domoic acid level from those field samples was approximately the highest cellular toxicity measured in this study (7.6 pg DA equiv. cell^{-1}). It is reasonable that with cellular domoic acid levels 10 fold higher, the copepods in this study would have accumulated a proportionally higher concentration of domoic acid as well.

Results of our depuration experiment indicate there may be some retention of toxicity after toxic cells are no longer available as food. The gut retention time of *A. tonsa,* while temperature and food dependent, is approximately 30 minutes [18]. If all the domoic acid activity was contained in the copepod gut and was not incorporated in tissue, complete depuration would be expected in <1 h. This was not the case. After >4 h in filtered seawater the domoic acid level was still ~5-5.6 ng DA equiv. copepod^{-1}. Rapid accumulation of domoic acid, and perhaps slower release or depuration than might be predicted, certainly suggests the potential for crustacean zooplankton to be a major source of toxicity in *Pseudo- nitzschia* bloom events.

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GYMNODINIUM BREVE TOXINS WITHOUT CELLS: INTRA-CELLULAR AND EXTRA-CELLULAR TOXINS

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ABSTRACT

The dinoflagellate, *Gymnodinium breve*, produces several neurotoxins causing neurotoxic shellfish poisoning (NSP), massive fish kills and respiratory irritation in marine mammals and humans. The common method for public health advisories is enumeration of live cells in the water. Evidence for stability of the neurotoxins outside of cells indicates that contamination could result from water masses carrying suspended/dissolved neurotoxins in the absence of viable *G. breve* cells. Therefore, reliance on cell counts only for public health protection may be insufficient. A stirred ultra-filtration cell concentrator was used for separating viable cells from water collected during natural *G. breve* blooms. The results showed that early stages of *G. breve* blooms contained primarily intra-cellular toxins and that the relative amount of extra-cellular toxins increased as the bloom progressed.

INTRODUCTION

These studies were undertaken to assess the relative amount of intra-cellular (inside cells) and extra-cellular (outside cells) toxins produced during a natural bloom and in cultures of the toxic dinoflagellate, *G. breve*, the most prevalent species of harmful alga affecting the Gulf of Mexico [1,2,3,4]. This dinoflagellate produces as many as nine potent neurotoxins (brevetoxins) that contaminate shellfish resulting in neurotoxic shellfish poisoning (NSP), cause massive fish kills and produce severe respiratory irritation in marine mammals and humans that are exposed to air-borne neurotoxins in association with marine aerosol [1,5,6,7,8,9]. Although naturally occurring in the Gulf of Mexico in background concentrations <1000 cells/L, harmful bloom conditions result when concentrations reach thousands to millions of cells/liter producing toxic quantities of the neurotoxins [4].

The neurotoxic chemicals produced by *G. breve* are characterized as polycyclic ethers with molecular weights averaging 900 [5,6,10]. The neurotoxins are contained within the cell until the cell ruptures (lysis) [11]. The most common method for discerning toxic levels of the neurotoxins for public health advisories is enumeration of the *G. breve* cells in a given water mass [4]. A previous study found brevetoxins to persist for 21 days in samples of water taken from a natural bloom [12] indicating that reliance on cell counts may be insufficient for public health protection. This study investigated the amounts of intra-cellular and extra-cellular toxins during red tide blooms from two locations along the Florida (USA) Gulf coast.

MATERIALS AND METHODS

Intra- and Extra-Cellular Toxin Method Assessment:

Verification of the intra- and extra-cellular toxin analysis method was established by analysis of laboratory cultures of *G. breve* with known concentrations of cells and toxins. The method was tested by analyzing the distribution of toxins in the original culture, in culture with all cells lysed (all extra-cellular toxins) and in a 50/50 mixture of the original culture and the lysed culture.

Gymnodinium breve culture used for these studies was grown in f/2 enriched seawater [13] using 12/12 h light/dark at 24 ± 2° C at the Mote Marine Laboratory phytoplankton culture facility. A culture of approximately 30 x 10^6 cells/L was diluted to 5 x 10^6 cells/L for the experiment. Total brevetoxins in the whole culture was established by extraction and recovery according to the method of Pierce et al. [14], using a C18 bonded-phase extraction disc (Metachem, Torrance, CA) for toxin recovery. The 5-L of culture was filtered through a glass-fiber filter GF/D (Whatman, Clifton, NJ), the cells were lysed by the addition of distilled water and the free brevetoxins were collected in the filtrate. Methanol was then added to the filtrate (10% by volume) and the entire sample filtered through a C18 extraction disc to recover brevetoxins. The brevetoxins were eluted from the disc with 20 ml of methanol and reduced under vacuum to 3 ml volume in preparation for analysis by high performance liquid chromatography (HPLC). Standard brevetoxins PbTx-2 and PbTx-3 were obtained from Chiral Corp. Miami, FL. Verification of PbTx-1 and PbTx-5 was accomplished by secondary standards recovered from *G. breve* culture and identified by HPLC retention values.

The PbTx nomenclature follows the protocol established by Poli et al. [5]. HPLC analyses were performed using a Shimadzu LC-600 HPLC containing a B&J,OD5 C-18 reverse-phase, 25 cm x 0.46 cm column interfaced with a SM6A photo-diode array uv-vis detector and mobile phase 85/15 methanol/water at 1 ml/min.

The amounts of intra- and extra-cellular toxins were determined in a 5-L sample of 5 x 10^6 cells/L processed through a stirred ultra-filtration cell concentrator (Amicon, Beverly MA). The sample was filtered through a 0.8 micron polycarbonate membrane (Osmonics, Westborough, MA) in the stirred cell at 5 psi to gently collect the viable *G. breve* cells (intra-cell toxins) on the filter and to allow extra-cellular toxins to pass through with the filtrate. The extra-cellular toxins were recovered by passing the 4.8 L of filtrate (with 10% methanol added) through a C18 bonded-phase extraction

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disc as above. The Intra-cell toxins were extracted from approximately 200ml of solution remaining above the membrane (containing viable *G. breve* cells) by rinsing the cell and membrane with distilled water and recovering toxins on a C18 extraction disc as above. Methanol extracts were reduced in volume to 3 ml in preparation for HPLC analysis as above.

The procedure was repeated using a 5-L sample of the above culture with the cells lysed (ruptured) using an ultra-sonic processor (Sonics and Materials, Danbury, CT) releasing all toxins into the solution as extra-cellular toxins. A mixture of 2.5 L of the original culture plus 2.5 L of the lysed culture was also tested. The samples were processed through the cell concentrator and the intra- and extra-cellular toxin fractions were collected for HPLC analysis as described above.

1998 ECOHAB-FL November 1998 Process:

Surface and bottom water samples were collected from five sites during the ECOHAB-FL November 1998 Process Cruise D in Gulf of Mexico coastal waters adjacent to Charlotte Harbor, Florida in the vicinity of 26.5^{0}N and -82.3^{0}W. Samples collected for brevetoxin analysis exhibited low to moderate *G. breve* cell counts (range from 1×10^{4} to 2.3×10^{6} cells/L) representing a developing bloom. Samples were processed on board ship through the cell concentrator unit and the intra- and extra-cellular toxins were recovered for HPLC analysis as described above.

1999 ECOHAB-FL September 1999 Process Cruise:

Surface and bottom samples were collected from ten sites during the ECOHAB-FL September 1999 Process Cruise B and C in the vicinity of 30.3^{0} N and -87.2^{0} W. The samples were processed for intra- and extra-cellular toxins through the cell concentrator as described above. *G. breve* cell counts ranged from moderate, during cruise Leg-B (5.5×10^{4} to 9.5×10^{5} cells/L) to high concentrations during cruise Leg-C (1.5×10^{5} to 3.9×10^{6} cells/L).

RESULTS AND DISCUSSION

Intra- and Extra-Cellular Toxin Methods Verification:

Results of the recovery of intra- and extra-cellular brevetoxins from *G. breve* culture are given in Fig. 1, showing the four most abundant brevetoxin molecules in decreasing order of abundance were PbTx-2, -1, -3, and -5. Toxin analysis of the original culture showed the intra-and extra-cellular toxins to be about 40% and 60% respectively. Upon lysing the cells with ultra sound the cell count dropped to $<1 \times 10^{3}$ cells/L, the intra-cell brevetoxin concentration dropped to 3% and the extra-cellular concentration increased to 97 %. The mixed lysed and original culture cell count was intermediate between (2.4×10^{6} cells/L) with 23% intra-cellular toxin and 77% extra-cellular toxin content. It is important to note that the definition of "intra- and extra-cellular" brevetoxins is an operational definition based on toxins passing through or being retained on a 0.8 Fm membrane filter.

ECOHAB-FL November 1998 Process Cruise:

The 1998 ECOHAB-FL Process Cruise encompassed a low to moderate *G. breve* bloom (1×10^{4} to 2.3×10^{6} cells/L) within 10 miles of shore. Concentrations of intra- and extra-cellular toxins are given in Fig. 2 for a series of five sets of surface and bottom water samples collected from 20:00 hours on 17 November to 16:30 hours on 18 November, 1998. This sequence of samples shows that as the first day ended most of the cells were on the bottom and the majority of toxins were intra-cellular. As the second day progressed, the intra-cellular concentrations diminished as the extra-cellular brevetoxins became more abundant. This occurred as the *G. breve* cell counts diminished, suggesting cell lysis and release of toxins into the water column as the bloom aged.

ECOHAB-FL September 1999 Process Cruise:

Results of the November, 1999 ECOHAB-FL cruise legs B and C are shown in Fig. 3a and 3b. Leg B exhibits low toxin concentrations in conjunction with the low *G. breve* cell counts with both bottom and surface samples exhibiting greater amounts of intra-cellular than extra-cellular toxins (Fig. 3a). Cruise Leg-C began in the midst of high *G. breve* cell counts with the intra-cellular toxins initially much higher than extra-cellular, however, as the cruise continued the amount of extra-cellular toxins increased as the cell count diminished (Fig. 3b). The amount of brevetoxins as pg/cell was calculated for both surface and bottom samples showing that surface intra-cell toxins ranged from 12 to 40 pg/cell where as in bottom water the intra-cell toxins were much lower ranging about 4 to 6 pg/cell.

Brevetoxin concentrations have been reported in the range of 12 to 30 pg/cell depending on the growth phase of the majority of cells in the sample [15,16]. During the initial portion of the 1999 *G. breve* bloom, when cell counts were below 5.0×10^{5} cells/L, surface water samples exhibited 8 to 40 pg/cell brevetoxin, where-as cells in bottom water contained from 3 to 14 pg/cell, suggesting different growth phases in surface and bottom waters [17]. As the sampling progressed through higher concentrations of *G. breve* cells, the intra-cellular toxin concentrations in surface water diminished while intra-cellular toxins increased in bottom water indicating a change in the growth phase of cells in surface and bottom water. The increase in extra-cellular toxins with progression of the bloom is indicative of a change in bloom dynamics with many of the cells lysing and releasing toxins into the water.

Bioaccumulation of brevetoxins has been shown to occur through trophic transfer from copepods to fish and by filter-feeding molluscs by ingestion of intra-cellular toxins in live *G. breve* cells [11,16]. The Extra-Cellular toxins also may cause NSP in filter-feeding bivalves through ingestion in association with suspended

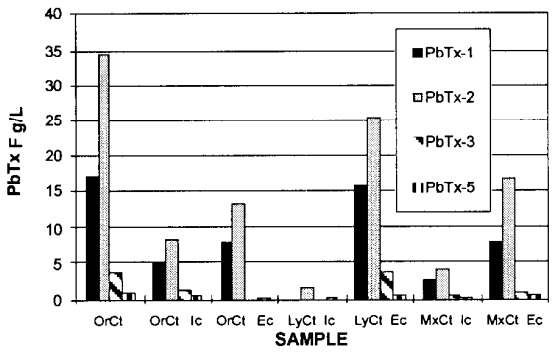

Figure 1. Intra- and extra-cellular brevetoxin method verification: Original culture (OrCt) diluted to 5 x 10^6 cells/L (intra-cellular toxins); Lysed cell culture (LyCt) (extra-cellular toxins); Mixed culture (MxCt) of 50/50 OrCt/LyCt. PbTx nomenclature after Poli et al. [5].

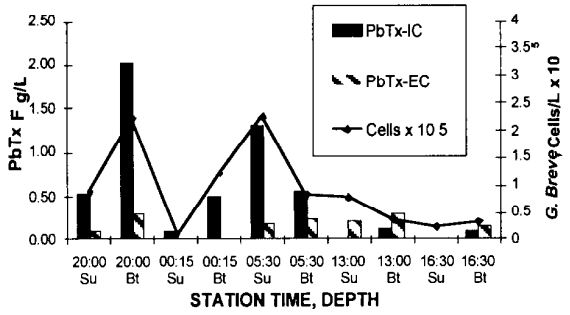

Figure 2. Intra- and extra-cellular brevetoxins in surface (Su) and bottom (Bt) water samples, with surface *G. breve* cells/L: ECOHAB-FL Cruise-D, November 17 & 18, 1998.

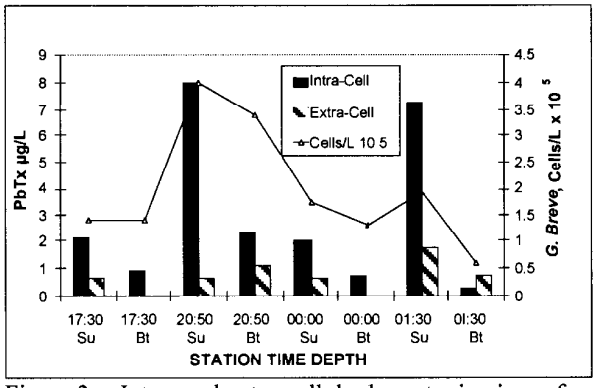

Figure 3a. Intra- and extra-cellular brevetoxins in surface (Su) and bottom (Bt) water samples, with surface *G. breve* cells/L: ECOHAB-FL Cruise-B September 21 & 22, 1999.

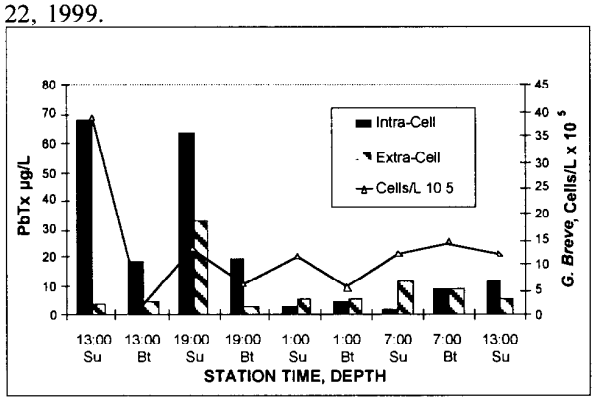

Figure 3b. Intra- and extra-cellular breve toxins in surface (Su) and bottom (Bt) water samples, with *G. breve* cells/L: ECOHAB-FL Cruise-C September 27-29, 1999.

particulate matter. The Extra-Cellular toxins also enter directly into fish through gill filaments providing rapid distribution through their circulatory system.

Bubble-mediated transport of extra-cellular toxins to the sea surface results in subsequent incorporation of brevetoxins into marine aerosol [7]. Thus the extra-cellular toxin concentration must be considered an important component for assessing the potential for adverse respiratory impacts on marine mammals and public health. Monitoring toxins in a water mass over a longer period of time would be required to ascertain if the extra-cellular toxins remain, posing a threat to environmental and public health after the *G. breve* bloom has diminished.

CONCLUSIONS

The results of this study support the hypothesis that that the amount of extra-cellular toxins in the water increases as the *G. breve* bloom progresses due to cell lysis releasing toxins to the water. This conclusion has significance for understanding toxin fate and transport and suggests that monitoring the number of HAB cells in the water column may not be sufficient for protection of public health and natural resources, indicating the importance of monitoring the actual toxin content of suspect water masses.

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SHELLFISH TOXICITY IN NORWAY – EXPERIENCES FROM REGULAR MONITORING, 1992-1999

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ABSTRACT

Shellfish toxicity and the presence of toxin producing phytoplankton have been monitored along the Norwegian coast since 1992 in nearly the same way of frequency (weekly) and methods for sampling, microscopy, and toxin extraction and analysis. The number of sampling sites has increased from 17 in 1992 to 24-27 after 1994, now scattered along the whole Norwegian coastline between Sweden in the south and Russia in the north. The cumulated information provides a substantial knowledge of the variations in shellfish toxicity and the occurrence of toxin producing algae along the Norwegian coast and

The combined phytoplankton and toxicity data demonstrate that both DST and PST may occur all along the coast from 58°N to 70°N; however, with large inter-annual and regional variations. In general the risk of DSP is highest along the south coast and in the inner parts of the large fjords along the west coast in the autumn/winter period (September-February). PSTs have been detected all along the coast, with some hot spots at the northwest coast. The risk of PSP is highest in late spring and early summer (April-July) in southern Norway and a couple of months later in northern Norway. Although several blooms of potentially AST producing diatoms have been observed, domoic acid has so far not been documented in bivalves from the Norwegian coast.

With few exceptions, periods with toxic shellfish were recorded every year at all sampling sites. Up to 15-16 of the 27 stations could be closed for shellfish harvesting due to the risk of intoxications. During the period 1992-99 the northern border of documented presence of DST in mussels moved northwards from station 22 (66°N, 13°E) to station 26 (70°N, 23°E), and for PST from station 23 (63°N, 12°E) before 1992 to station 27 (70°N, 30°E) in 1997. The observation period is probably too short to make conclusions concerning trends in spreading of toxic phytoplankton northwards. Our data do not support an obvious linkage between eutrophication and the risk for PSP or DSP. On the other hand, they also cannot exclude a connection between the nutrient conditions in the sea and occurrence of PSP and DSP.

INTRODUCTION

In this century, up to the sixties, single episodes of mussel toxicity were recorded along the Norwegian coast; however, without leading to a holistic managing policy. From the sixties a regional monitoring of mussel toxicity was performed, usually in connection with high-risk situations for PSP outbreaks [see 1]. After a massive outbreak of DSP in the autumn 1984 [2], additional and the presence of toxin producing phytoplankton was organized and partly funded by the Norwegian Food Control Authority (SNT). Results from this monitoring program provide valuable information concerning distribution of the shellfish toxicity along our coast.

Monitoring and communication to the public

The number of sampling sites in the national monitoring program has increased from 17 in 1992 to 24-27 after 1994, now scattered along the whole Norwegian coastline between Sweden in the south and Russia in the north. The location of the monitoring stations in 1999 is shown in Fig. 1. Algae samples are collected weekly in the

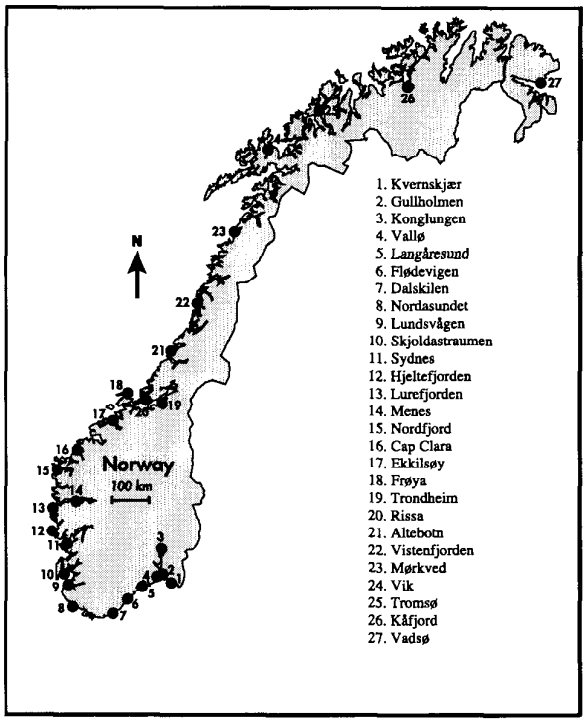

Fig.1. National monitoring stations for shellfish toxicity in Norway in 1999.

period March-October. The sampling procedure includes a net-haul (20 micron mesh size) and a water sample from 0-3m (by a hose), which are used for identification and enumeration of potentially toxic phytoplankton species. The net hauls are fixed with neutralized formaldehyde and the water samples with neutralized iodine. Furthermore, upon the start of the yearly program blue mussel (*Mytilus edulis*)

shellfish along the coast at the beginning of the monitoring season. In order to reduce the use of animals in toxicity testing our monitoring is mainly based on analysis of phytoplankton in net hauls and water samples. The harmful genera included in the program are *Alexandrium* associated with PSP (Paralytic Shellfish Poisoning), *Dinophysis* associated with DSP (Diarrhetic Shellfish Poisoning) and *Pseudo-nitzschia* associated with ASP (Amnesic Shellfish Poisoning). Based on experience gained from the monitoring, supplied by literature studies [see 4] we have established algal levels of warning (Table 1). If these levels are exceeded, it triggers the closure of an area, i.e. the public is warned against consuming wild mussels. Analysis of phytoplankton is performed at five laboratories, which apply slightly different methods, but use the same detection limit, 40 cells·l^{-1}. Parallel testing at the laboratories are done. At station 6 we have established "normal occurrence" (Fig. 2) for some toxin producing species as a result of about 10 years of regular monitoring [5].

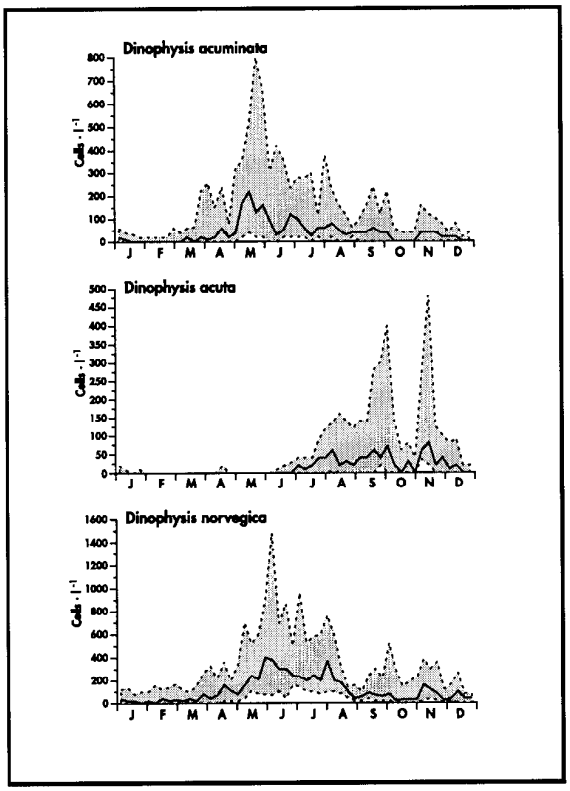

Fig. 2. The "normal occurrence" of *Dinophysis acuminata, D. acuta* and *D. norvegica* at station 6 (The Flødevigen Bay). The bold curve is medians for every week based on all data for the period 1989-1998. Dotted lines are first and third quartiles.

During the monitoring season samples of shellfish are only collected and analyzed for toxicity when the level of toxin producing algae had decreased and remained low for several weeks after a period of closure. However, at three stations (6, 16 and 20 in Fig. 1) shellfish are tested for toxicity every other week throughout the monitoring and 1 500 phytoplankton analyses (750 net hauls and 750 waters samples) within the monitoring program.

Table 1

Levels of warning of selected toxin producing phytoplankton 1999

Species	Levels of warning (cells ·l^{-1})
Dinophysis acuminata	900
Dinophysis acuta	900
Dinophysis norvegica	1 200
Dinophysis spp.(in total)	1 200
Alexandrium spp.	more cells recorded in net-haul
Pseudo-nitzschia	1 000 000*

Explanation: * - leads to hygienic evaluation

The weekly information on the algae situation along the coast is communicated on a web-side http://algeinfo.imr.no/. The information consists of a map with symbols indicating the amounts and kinds of algae, and a short descriptive text with links to useful additional information. If acute situations should occur, more frequent updating of the web-side is possible as well as direct contact with management authorities and aquaculture industry in threatened areas. The advice to the public about the wholesomeness of the shellfish is issued by SNT. Information is available to the public via a web-side (http://www.snt.no/nytt/blaskjell/), a "mussel-phone" and text-TV (Norwegian Broadcasting).

RESULTS AND DISCUSSION

The advice issued by SNT concerning wholesomeness of shellfish in 1999 is shown in Table 2. Roughly the data from this year only show that PST (Paralytic Shellfish Toxins) and DST (Diarrhetic Shellfish Toxins) may occur in shellfish along the entire Norwegian coast. However, both the toxin levels in mussels, the duration of periods with toxins in mussels and the number of stations where toxicity is recorded vary greatly during a monitoring season. Some areas are more likely to have toxic mussels than others.

In general the risk of DSP is highest along the south coast and in the inner parts of the large fjords along the west coast. The season with highest DSP problems is autumn/winter (September-February). The dominating DST-producing organisms are *Dinophysis* spp. with *D. acuta* the most potent [6]. *D. acuta* has its main season in late summer and autumn (Fig. 2). There are, however, many situations with *D. norvegica* and/or *D. acuminata* at concentrations of 3000-6000 cells·l^{-1}, especially in the period March-July, which apparently are not associated with toxicity of shellfish. This corresponds with the large variability in toxicity per *Dinophysis*-cell reported [7, 8]. The large variability in toxicity per cell makes the enumeration of *Dinophysis* spp. and application of levels of warning a rather un-precise management tool, causing many unnecessary closures. However, in several cases when *D. acuta* occur in the autumn the opposite has been recorded; DST exceeding the acceptance level in shellfish

levels present in the shellfish late in the autumn tend to remain in the shellfish until the spring bloom of diatoms in March the following year. The northernmost registration of DST in shellfish so far in Norway was recorded in 1999, at station 26 (Fig. 3).

PST may occur all along the coast, but in recent years hot spots are located at the northwest coast (station 14, 16 and 18). The high-risk season for PSP is late spring and early summer (April-July) in southern Norway and a couple of months later in northern Norway. When our northernmost county Finnmark was included in the monitoring for the first time in 1997, PSTs were recorded and have recurred every year since. Without having any hard evidence, due to little parallel data on the occurrence of *Alexandrium* and PST in mussels in northern Norway, we believe the PST-content per *Alexandrium* cell may be rather high. It would not be unexpected, because very high PST-levels have previously been recorded. The highest values so far are about 20 000 µg saxitoxin per 100g mussel meat recorded near station 19 (Fig. 1) in 1992.

Although several blooms of the potential domoic acid producing diatoms (*Pseudo-nitzschia* spp.) have been observed, domoic acid has so far not been documented in bivalves from the Norwegian coast. However, for precautionary reasons, warnings against collection of wild shellfish have been issued due to high concentrations of *Pseudo-nitzschia* present, see Table 2 (station 5 in week 43). Presence of yessotoxin (YTX) [9] and probably some unknown toxins [10] may occasionally trigger positive response in the mouse bioassay for DST (refer symbol D! in Table 3). This seems to be most common in some large fjords along the west coast, e.g. the Sognefjord (station 14). We have not identified the source organisms of these toxins, but *Gonyaulax grindleyi* which has been associated with YTX in other countries, is common along the coast although normally in low numbers. There is an urgent need to reach international agreement on which toxins should be included in the DST complex. In our opinion YTX should be regulated separately.

After some incidents with PST in shellfish in the Oslofjord and other eutrophicated locations in the sixties and seventies (1, 11, 12] it was speculated that the risk of PSP was associated with eutrophication. This idea may have been due to a limited sampling at that time with focus on some more densely populated areas. Our data, from nearly ten years of regular monitoring, do not support an obvious linkage between eutrophication and the risk for PSP or DSP. On the other hand, they cannot exclude a connection between the nutrient conditions in the sea and occurrence of PSP and DSP.

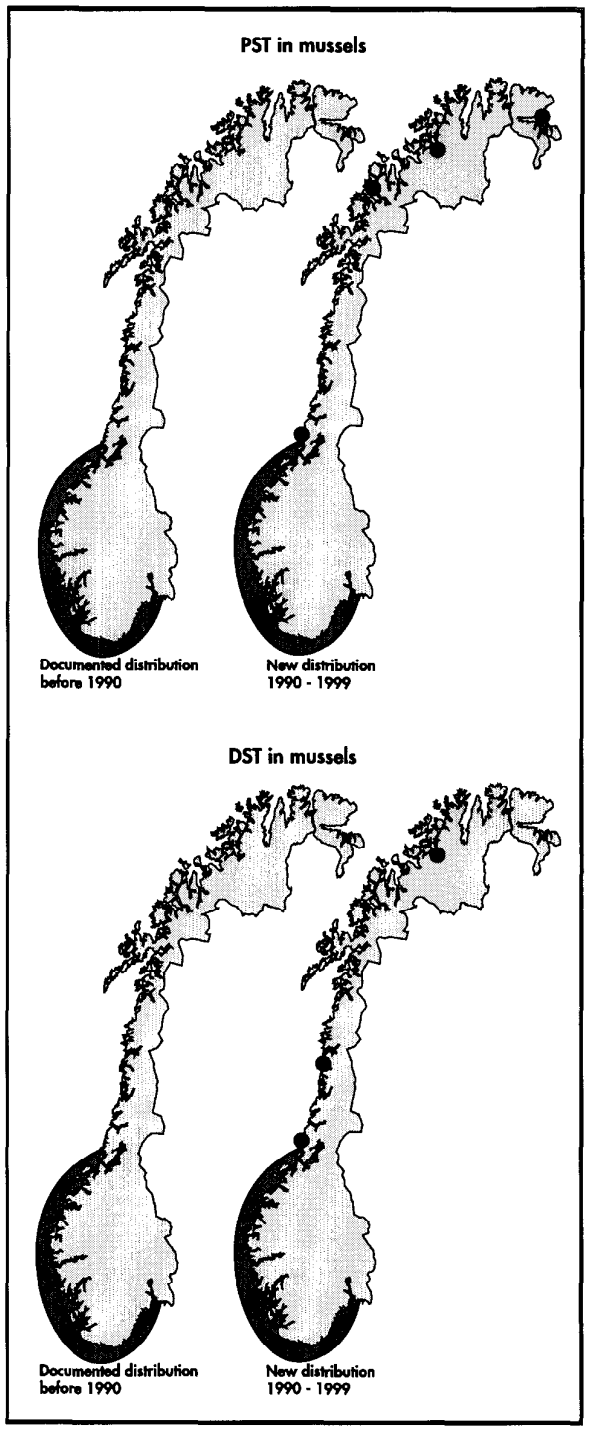

Fig. 3. Documented distribution of PST and DST in mussels along the Norwegian coast before and after 1990.

ACKNOWLEDGEMENTS

All colleagues participating in the monitoring program are acknowledged for fruitful co-operation. The monitoring is partly funded by the Norwegian Food Control Authority (SNT).

Table 2.
Weekly advices issued by SNT concerning wholesomeness of shellfish along the Norwegian coast in 1999.
The basis for the advices is shown by symbols, see explanation.

St.no	St.name\ Week no.	APR 11	12	14	15	16	17	MAY 18	19	20	21	22	JUN 23	24	JUL 25	26	27	28	29	30	31	AUG 32	33	34	35	SEP 36	37	38	39	40	OCT 41	42	43
1	Kvernskjær	•					•							D	D	•									D	D	D	•			D	D
2	Gullholmen	•																														
3	Konglung	?	•	D	D	D	D	•			D	D	D*	D	D!	D!	•								D	D	D	D	•		•	
4	Valle	•												D	D	•																
5	Langåresund	?	A*	A	A*	A	A	•						D	D	D	•			D	D	•			D	D	D	D*	D	D	•	P-N
6	Flødevigen	D	D*	A*	A	D*	D	•	D	•		A*	AD	•		•	D	•		D!	D	•	A	•	•	D	D!	D	D*	D	D*	D
7	Dalskilen	A*	A	A	A*	A	A	•	A	A	•	A	AD	AD	AD	•																
8	Nordasundet	•												AD	AD	AD	D!	D!	•													
9	Lundsvågen	•		D	D	D	•			D	D	•	A	AD	AD	AD	AD	AD	D*	D	D	D!	D!	D!	D!	D*	D!	D!	•				
10	Skjoldastraumen	•		A	A	A	•	A	A	A	•				•		D	D	D	•												
11	Sydnes	A*	A	A	•			A	A	A	AD	AD	•	D	D	D	•			?	?	?	D	D	•	?						
12	Hjelteffjorden	•	?	?	?	•								D	D	D	D	•					D	D	D	•						
13	Lurefjorden	•	?	A	A	A	•							A	A	•																
14	Menes	?	A*	A	A*	A	A	A	A	A	?	D	D*	D	D	D	D	D!	D!	D!	D!	D*	D!	D!	D!	D!	D!	D!	D!	D!	D!	D!	D!
15	Nordfjord	A*	A	A	A*	A	A	A	A	•	A	A	A	A	A*	A	•									•		A	A	•		
16	Csp Clara	?	•	A*	A	A*	A	A*	A	A	A	A*	AD	•		AD*	AD	•		D!	D!	•		•		•		•		•		•
17	Ekkilsøy	?	A*	A	A*	A	A	•		?	?	?	?	•								?	•									
18	Frøya	•	?		?	•																								?		
19	Trondheim	?	•																				•						D	D	D	•
20	Rissa		•	•		•		•		•		•	•		D?	•	•			D!	D!	D!	•	•		•		•	D	D!		•
21	Alteboth	?	•												D	D	D	•					•									
22	Vistenfjord	?	•				A	A	A*	A	A	A*	A	A	•			A	A	•													
23	Mørkved	•												?	?	•				D	D	D	•									
24	Vik i Kvæfjord	?	?	?	•			A	A	A	•																					
25	Tromsø	?	•																													
26	Kåfjord	?	•	?	?	?	•				?	?	?	A*	A	A*	A	AD	AD	•		D	D	D	D*	D	D	D*	D	D	•		
27	Vadsø	?	?	?	?	?	•				?	?	A	A	A*	A	A	A	A*	A	A	A	A*	A	A	A	A*	A	•		?	?	?

Explanation:
A - Alexandrium
D - Dinophysis
P-N - Pseudo-nitzschia
* - mouse bioassay performed
l.w. - levels of warning
? - No data

(blank) can eat, algae below l.w
• can eat, algae below l.w., negative tox. test
A* do not eat, A above l.w., negative tox. test
D* do not eat, D above l.w., negative tox. test
D! do not eat, YTX?, no negative tox. test

A do not eat, A above l.w.
D do not eat, D above l.w.
P-N do not eat, P-N. above l.w.
A* do not eat, A above l.w., positive tox. test
D* do not eat, D above l.w., positive tox. test
D! do not eat, YTX?, positive tox. test

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THE INTEGRATION OF DNA PROBES INTO NEW ZEALAND'S ROUTINE PHYTOPLANKTON MONITORING PROGRAMMES

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ABSTRACT

New Zealand has established two-tier shellfish biotoxin monitoring programmes for industry and Public Health, with the first tier providing risk assessments for harvesters based on analyses of seawater for toxic microalgae, and the second determining whether there is biotoxin contamination of shellfish flesh. A drawback to phytoplankton monitoring based on light microscopy has been the morphological similarity of some microalgae which exhibit different toxin content. The rapid identification of species by ribosomal RNA-targeted DNA whole cell format probes has proved invaluable for differentiating these species and the probes are now used routinely. Sandwich hybridisation DNA probe assays (SHA) have also been used successfully to detect *Pseudo-nitzschia* species and raphidophytes in seawater. SHA detection of the ichthyotoxic *Heterosigma akashiwo* offers finfish farmers a cost- effective management tool for monitoring bloom development.

INTRODUCTION

Phytoplankton monitoring is carried out routinely in New Zealand as a reliable tool for assessing the risk of biotoxin contamination of shellfish, and the risk of fish deaths due to ichthyotoxins. A two-tier programme is in operation, with phytoplankton monitoring being the first tier for the 70 commercial and non-commercial sites from Parengarenga Harbour in the far north to Stewart Island in the south. Shellfish flesh testing, by mouse bioassay, HPLC for domoic acid (DA), or ELISA for diarrhetic shellfish toxins, is the second tier, although in recreational shellfish gathering areas phytoplankton monitoring has largely replaced shellfish flesh testing.

Pseudo-nitzschia species are common bloom formers in New Zealand waters, with the trigger level for voluntary closures being 50,000 cells l^{-1} (based on correlations between cell numbers of *P. australis* and DA contamination of shellfish). However, millions of cells l^{-1} are required for some species to cause toxicity, and some species are non-toxic. Species differentiation is therefore vital, so that harvesters can determine the true risk of re-call of product due to positive flesh tests [1]. Harvesters also require identification of *Alexandrium* to species level, as the paralytic shellfish toxin producing *A. catenella* and *A. tamarense* commonly occur in northern waters with the morphologically similar but non-toxic *A. fraterculus*.

Whole cell format ribosomal RNA (rRNA)-targeted oligonucleotide probes [2,3,4] have proved invaluable for identification of species of both genera in the New Zealand biotoxin monitoring context [5]. The New Zealand government has funded an evaluation of rRNA probes in both whole cell and sandwich hybidisation assay formats. The results to date of these trials, and the integration of probes into New Zealand's monitoring programmes, are presented and discussed in this study.

METHODS

DNA probe-based assays

Whole cell (*in situ*) hybridisation with species-specific large-subunit ribosomal RNA (rRNA)-targeted oligonucleotide probes were tested against *Pseudo-nitzschia* and *Alexandrium* isolates as described [2,6], using the filtration method [3,4,7].

Pseudo-nitzschia targeted probes were auD1, puD1, muD1, muD2, heD2-2, frD1, amD1 and deD1. *Alexandrium* probes were min4 (which binds to an Australian *A. minutum* strain as well as New Zealand strains) and min6 (designed for New Zealand strains of *A. minutum*), and TA4F and cat/tam for *A. catenella* and *A. tamarense*. The controls were UniC (universal positive) and UniR (negative). Sea water samples collected during routine phytoplankton monitoring and containing the target species, as determined by light microscopy, were fixed for 1 h and, following washing and probe addition, were hybridised for 1.25 h or 0.3 h for *Pseudo-nitzschia* and *Alexandrium* respectively. The probe response was observed by viewing the filters under epifluorescence microscopy (excitation, 490 nm; emission, 520 nm).

Sandwich hybridisation DNA probe assays (SHA) were carried out as described [2,8]. This was done using commercially prepared multi-well format assay plates pre-dispensed with oligonucleotide probes for the detection of *P. australis, P. multiseries* and *P. pungens*, or for *Heterosigma akashiwo* and *Fibrocapsa japonica* (developed at Monterey Bay Aquarium Research Institute and produced by Epoch Pharmaceuticals, Saigene Corp., U.S.). Probes for the *A. catenella/ tamarense* complex were dispensed as required. Cells were collected on filters, treated with hybridisation buffer, and the filtered lysate dispensed into plates (195 µl per well). The assay was run on an automated analyser (Saigene Corp., U.S.). The final enzyme-driven colour reaction was enhanced by the addition of 10%

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H_2SO_4 and the resultant colour reaction quantified on a micro-plate reader (450 nm; BioRad 3550).

RESULTS AND DISCUSSION

Whole cell format DNA probes

Pseudo-nitzschia: The results of 200 assays of sea water samples, collected from around New Zealand for research and regulatory purposes over the last 2 years, were analysed. Of the industry requested assays 16% were dominated by the most toxic species, *P. australis* (unpublished data). Non-toxic species comprised 40% and the balance were low level toxin producers, which require >1.0 x10^6 cells l^{-1} for blooms to constitute a risk. The percentage dominance of species for all probe assays carried out during 1997-99 is presented in Figure 1.

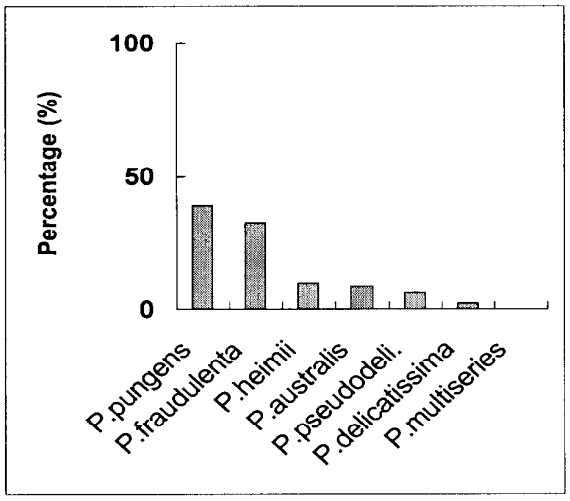

Fig. 1. Percentage dominance of *Pseudo-nitzschia* species throughout 1997-99, determined by whole cell format DNA probes. (Analysis of Min. of Health and industry data.)

In the course of carrying out regular DNA probe assay trials the non-toxic sigmoid species, *P. multistriata*, was found to cross-react with the *P. australis* specific probe (auD1) [7,9]. This species is in the "delicatissima" complex and closely resembles *P. delicatissima*. Seawater samples from Marsden Point (Northland), Tamaki Strait (Hauraki Gulf) and Matakana (Bay of Plenty; February-March 1998), fluoresced with the auD1 probe and there was also pale fluorescence with the probes for *P. multiseries, P. delicatissima* and *P. pseudodelicatissima* (muD1, deD1 and muD2 respectively). The distinctive morphology of *P. multistriata*, when observed under epifluorescence microscopy, has meant that the auD1 probe can continue to be used until a new *P. australis* probe is developed.

Alexandrium: The *Alexandrium* probes were successful in differentiating the major toxic and non-toxic species. The min4 and min6 probes reacted to the cultured New Zealand strains of *A. minutum* tested (CAWD11, 12 and 13), and detected *A. minutum* in field samples from Wellington Harbour (1100 cells l^{-1}; results for Ministry of Health, Nov. 1999). Results of mouse testing indicated shellfish toxin contamination at that site, but results (31 STX equivalents) were received >2 days later than probe derived data, accentuating the fast turnaround time of probes in generating risk assessment information. Assays to determine whether the probes exhibited cross-reactivity showed fluorescence with *A. ostenfeldii* with hybridisation times of >30 min (Table 1). However, at 20 min no cross-reactivity is detected.

The *A. catenella/tamarense* probes were specific for New Zealand strains of these two species (CAWD20, 44, 45, 46, 47, 49 and 50), which mainly produce C toxins. All species tested for cross-reactivity were negative, except *A. concavum*, which was negative with the TA4F probe but positive with the cat/tam probe (Table 1). The TA4F probe will be used preferentially in future. *A. pseudogonyaulax* (syn: *Goniodoma pseudogonyaulax*) [10] was negative with the *Alexandrium* genus probe, which raises questions as to the classification of this species. *Fragilidium subglobosum* was positive with this probe and also warrants further investigation. The speed of application of the *Alexandrium* probes, and the circumvention of the taxonomic expertise required in plate structure interpretation for the genus, mean that with further refinement these probes will be useful risk assessment tools in the future. Raphidophyte cells lost their morphological integrity during the assay and were more suited to the SHA format.

Shellfish and health regulators in New Zealand have shown confidence in the use of whole cell DNA probe assays for underpinning phytoplankton risk assessments by requesting their use on numerous occasions over the last 2 years, particularly for the genus *Pseudo-nitzschia*.

Sandwich hybridisation assays

Heterosigma/Fibrocapsa: Inter-laboratory tests indicate that the SHA system being trialled in New Zealand is running optimally, and that the raphidophyte probes elicit similar results with strains from different geographic regions [8,11]. In field trials, both *Heterosigma* and *Fibrocapsa* were detected with a sensitivity of less than 3.0 x10^3 cells l^{-1}. Due to cell lysis (the SHA detects free rRNA as well as rRNA liberated from cells) it was difficult to draw a standard curve from the field data, and so results were based on phytoplankton monitoring counts. The incremental increases in OD correlated with incremental increases in cell counts, and it is clear that this assay is now ready to be integrated into the suite of monitoring tools for both fish farm managers and shellfish industry regulators.

Table 1. Species specificity of whole cell format DNA probes, targeted to *Alexandrium catenella, A. tamarense* and *A. minutum* rRNA.

Micro-algal species	Culture collection code[a]	Probe[b]	Fluor.[c]	Probe[b]	Fluor.[c]
Alexandrium catenella	CAWD50	TAF4	+	cat/tam	+
		min4	-	min6	-
A. concavum	CAWD52	TAF4	-	cat/tam	+
A. fraterculus	CAWD97	TAF4	-	cat/tam	-
A. margalefii	CAWD10	TAF4	-	cat/tam	-
A. minutum	CAWD13	TAF4	-	cat/tam	-
		min4	+	min6	+
A. ostenfeldii	CAWD16	TAF4	-	cat/tam	-
		min4	-	min6	-
A. pseudogonyaulax	CAWD54	TAF4	-	cat/tam	-
A. tamarense	CAWD20	TAF4	+	cat/tam	+
Coolia monotis	CAWD77	TAF4	-	cat/tam	-
Fragilidium subglobosum	nd	TAF4	-	cat/tam	-
Gymnodinium spp.	CAD66, 90	min4	-	min6	-
Heterocapsa triquetra	CAWD36	min4	-	min6	-
Lingulodinium polyedrum	CAWD27	TAF4	-	cat/tam	-
Prorocentrum balticum	CAWD38	min4	-	min6	-
Protoceratium reticulatum	CAWD40	TAF4	-	cat/tam	-
Scrippsiella trochoidea	CAWD67	min4	-	min6	-

[a] Cultures tested are held in Cawthron Micro-algae Culture Collection. [b] TA4F and cat/tam: probes for *A. catenella/tamarense*; min4 and min6 probes for *A. minutum*. [c] Fluor. = fluorescence (indicating rRNA binding); nd = now dead.

Alexandrium and *Pseudo-nitzschia*: Eight sites monitored during New Zealand's routine phytoplankton programmes were also monitored using the SHA for *P. australis, P. multiseries* and *P. pungens*. When *Alexandrium* was reported in seawater samples, assays were also run for *A. minutum* and *A. catenella/tamarense*.

The SHA resulted in a cross-reaction of *P. multistriata* with the *P. australis* specific probe, but not with the *P. multiseries* probe. The sigmoid isolate required 0.9 x10^6 cells l^{-1} to elicit an OD of 0.13, whereas *P. australis* required 18 x10^3 cells l^{-1} to give an OD of 0.16. A new SHA probe for *P. australis* will be developed but is not considered a high priority, as the millions of cells of the non-toxic *P. multistriata* required to produce a positive response would rarely occur in field samples. Trials of the *Pseudo-nitzschia* SHA continue as the probes are being refined to suit "regional" species characteristics.

An advantage of the *Pseudo-nitzschia* SHA is that rRNA that is free in seawater is also detected, even when no cells are observed under the light microscope. As DA may be produced only as blooms collapse in some cases, this could be a good indicator of shellfish toxicity and warrants further research.

The SHA assays for *A. catenella* and *A. tamarense* have been successful in detecting both species. Negative SHA results when the non-toxic *A. fraterculus* has been reported during phytoplankton monitoring have given harvesters confidence in deciding whether to continue harvesting (with the risk of having to dump contaminated stock) or whether to impose voluntary closures. This work is ongoing as improvements are made to the levels of detection.

In conclusion, *Pseudo-nitzschia* and *Alexandrium* whole cell format DNA probes are now used routinely in New Zealand's phytoplankton monitoring programmes as an aid to risk management decision-making, and the monitoring laboratory (Cawthron Institute) is approved by International Accreditation New Zealand (recognised under ISO-IEC Guide 25).

Sandwich hybridisation format assays are available for the ichthyotoxic raphidophytes, *Heterosigma akashiwo* and *Fibrocapsa japonica*, and SHA trials for several *Pseudo-nitzschia* and *Alexandrium* species are progressing. The SHA may prove more suitable than the whole cell probes for fragile microalgae such as raphidophytes and *Gymnodinium* species.

More micro-algal species are being targeted, and there is the potential for DNA probes to be used as regulatory tools in New Zealand in the not too distant future.

ACKNOWLEDGMENTS

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BETTER CONSUMER PROTECTION AGAINST DSP TOXINS

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ABSTRACT

DSP human toxication outbreaks with detection in causative bivalves of very low concentrations of toxins, have called attention to possible deficiencies in total toxin extraction.
The presence of acyl esters of okadaic acid and DTX-2 has been confirmed in several species of Portuguese shellfish by HPLC with fluorometric detection. In certain instances, the content of esters has surpassed 50% of total DSP toxins found. The fluorometric method of Lee, currently used by our laboratory to confirm the presence of DSP toxins, does not detect these toxins. The need to introduce a hydrolysis step in sample preparation for HPLC is thus emphasized.
Experiments were also carried out on sample preparation for mouse bioassay with results showing an increased toxicity.

INTRODUCTION

In bivalve molluscs other toxins derived from OA, DTX-1 and DTX-2, are also found and globally designated as "dinophysistoxin-3" (DTX-3). Originally DTX-3 referred to the complex mixture of 7-*O*-acyl derivatives of dinophysistoxin-1 (DTX-1) and it was the main diarrhetic toxin found in scallops in Japan, while DTX-1 was the main toxin detected in mussels [1]. Later it was demonstrated that either OA, DTX-1 or DTX-2 may be acylated to produce "DTX-3" [2].
Research of these toxins has led to their identification in Portuguese mussels (*Mytilus edulis* Linné) back in 1995 [3]. Later we found they made a major contribution to a human outbreak of diarrhoea in early 1998 [4], leading to the necessity of studing them for monitoring purposes.
As chemical analysis with the alkaline hydrolyse step have shown a marked increase in total okadaic acid content it was interesting to examine the method of sample preparation for mouse bioassay. The introduction of this step in preparation for mouse bioassay provides new data on total toxicity and increases the related effects in the bioassay.

MATERIAL AND METHODS

Mouse bioassays

A modified version of the method Lee *et al*; 1987 [5] was used. Briefly: 100g edible parts were extracted with acetone, filtered and solvent removed under reduced pressure. The dried residue was re-suspended with aqueous 80% methanol, washed with hexane, and partitioned into dichloromethane. The latter layer was removed under reduced pressure, re-dissolved with 4 ml 1% Tween-60. Three 18-20 g albino mice were injected i.p. with 1 ml of extract.
Alkaline hydrolyses were also carried out on methanol and dried hexane layers, for 1 hour at ambient temperature with 1M NaOH, acidified with 2M HCl, and extracted into dichloromethane. The dried residue was injected in mice as above.

HPLC

Okadaic acid and analogues analyses were carried out according to Lee *et al*; 1987 with minor modifications. Alkaline hydrolysis was used for transformation of okadaic acid esters into the parent toxin and determined indirectly as the parent toxin (details in [4]).

RESULTS

The chromatograms of an extract of mussel digestive glands harvested back in 1995, before and after alkaline hydrolysis, demonstrated clearly the presence of esters of okadaic acid and dinophysistoxin-2, as shown in Fig.1.
Chromatograms of extracts of whole meat of donax clams (*Donax trunculus* Linné) harvested in 1998 and 1999, respectively, before and after alkaline hydrolysis, are depicted in Fig. 2 and Fig. 3, and provide evidence of the presence of okadaic acid and dinophysistoxin-2 esters.
The results obtained by mouse bioassay, before and after an alkaline hydrolysis treatment, both on methanol and dried hexane layers are summarised in Table 1. The results show an increase in toxicity of the hexane fraction after alkaline treatment. This increase was expected, since the parent toxins have a higher i.p. toxicity.

DISCUSSION

The presence of esters has been a recurrent event since their first detection in Portuguese bivalves. Their presence is widespread throughout the Portuguese coast and is not confined to a single species or a localised geographical area (Fig. 4.).
The concentration of esters is many times higher than that of the respective parent toxins.
Toxicity by mouse bioassay with hydrolysed fractions was even higher than expected, taking into account HPLC results; showing how importante is the introduction of this step, to prevent human toxications.
From the results obtained, we strongly recommend that analysis specifically for okadaic acid and analogues (by HPLC-FLU, ELISA, LC-MS and/or PP2A assays) always be carried out with an alkaline hydrolysis step. Recently, this has been implemented successfully with preparation of samples screened with an ELISA test [6].

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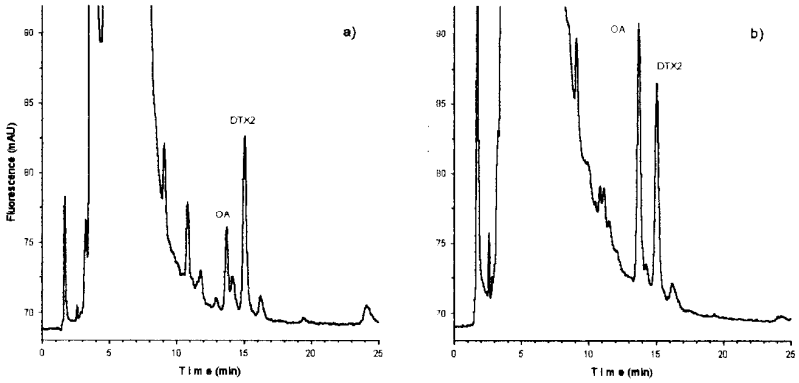

Fig. 1. Chromatograms of the BAP esters of DSP toxins from *Mytilus edulis* contaminated during a *Dinophysis acuta* Ehrenberg bloom (Aveiro, 25 October 1995): a) Sample extracted according to the conditions of Lee's method (OA: 1.7 µg/g; DTX2 4.3 µg/g); b) Total methanolic fraction after NaOH treatment (total OA: 5.5 µg/g; total DTX2: 4.9 µg/g).

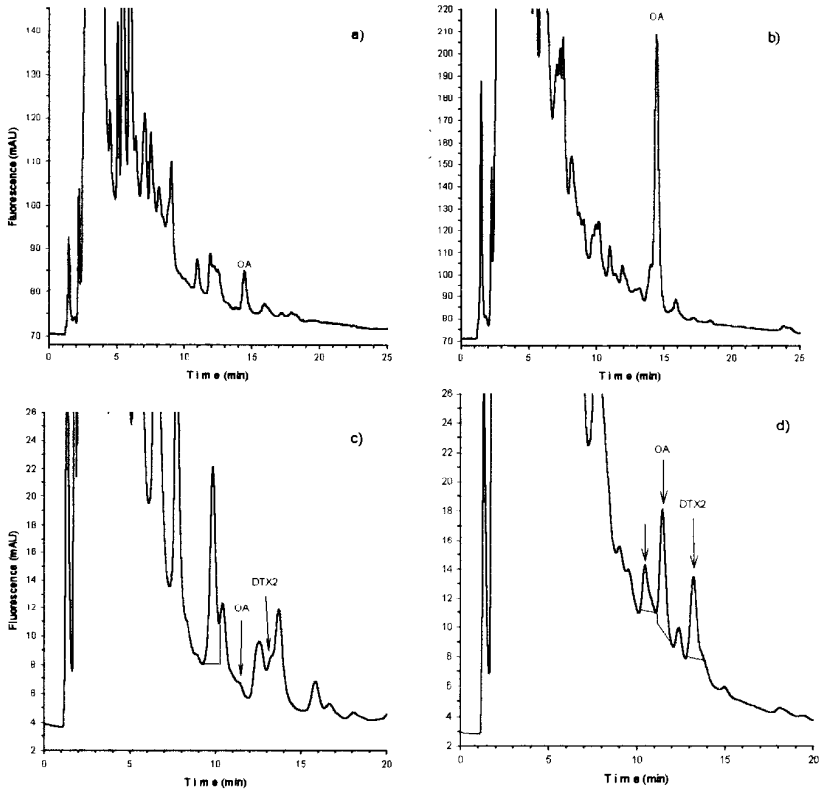

Fig. 2. Chromatograms of the ADAM esters of DSP toxins from *Donax trunculus* contaminated during blooms.
Figs 2a-2b. A *Dinophysis acuminata* Claparède & Lachmann bloom (Olhão, 12 February 1998), responsible for registered human toxications in Loulé: a) Sample extracted according to the conditions of Lee's method (OA: 9 µg/100g); b) Total methanolic fraction after NaOH treatment (total OA: 130 µg/100g).
Figs 2c-2d. A *Dinophysis acuta* bloom (Olhão, 12 February 1998): c) Sample extracted according to the conditions of Lee's method; d) Total methanolic fraction after NaOH treatment (total OA: 64 µg/100g; total DTX2: 49 µg/100g).

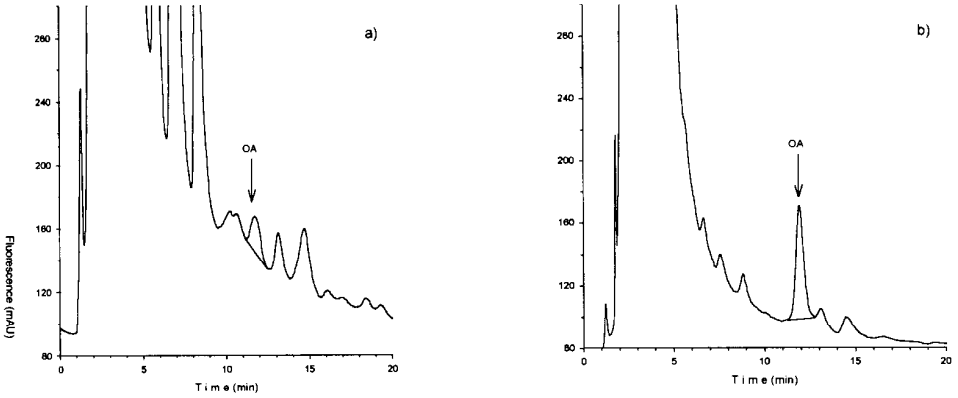

Fig. 3. Chromatograms of the ADAM esters of DSP toxins from *Donax trunculus* contaminated during a bloom of *Dinophysis acuminata* Claparède & Lachmann at Costa da Caparica (15 July 1999).
a) Sample extracted according to the conditions of Lee's method (OA: 20 µg/100g).
b) Hexanic fraction after NaOH treatment (total OA: 92 µg/100g).

Table 1. Comparison between results of donax clams analysed by mouse bioassay, before and after an alkaline hydrolysis treatment, with chemical analysis results.

Sample Identification*	Analysis Method		MeOH	MeOH + NaOH	Hexane	Hexane + NaOH
493/99	MBA	Median survival time (hrs)	7	2	24	2
	HPLC	OA (µg/100g)	10	12	nd**	10
546/99	MBA	Median survival time (hrs)	48	alive	alive	6
	HPLC	OA (µg/100g)	0	--	nd**	4

* 493/99 and 549/99 - Donax clams harvested in July 1999, at Costa da Caparica.
** nd = non determinable by Lee's method.

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TWO CONFIRMED CASES OF HUMAN INTOXICATION BY MARINE BIOTOXINS IN PORTUGAL

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ABSTRACT

In 1994 several cases of PSP occurred at Ericeira, and DSP was reported in Loulé in 1998.
The PSP toxication occurred on 18 October 1994 after eating blue mussels (*Mytilus edulis* L.) picked up at Ericeira beach. Several people went to the local hospital and nine of them were so ill, with neurological symptoms, that they had to be transported to Santa Maria Hospital in Lisbon.
For the DSP syndrome, which occurred on 12 February 1998, the responsible bivalves were donax clams (*Donax trunculus* L.) harvested at Fuzeta/Olhão (Algarve litoral) and eaten by 19 Loulé Health Care Unit employees and relatives. All affected people had abdominal cramps, headaches, vomits and diarrhoea after 6 hrs of ingesting the clams. In both cases IPIMAR, the National Reference Laboratory for Marine Biotoxins, was contacted and samples from the leftovers of the respective bivalves were retrieved for analysis.
Data on toxicity, responsible phytoplankton species and toxications are presented. Measures to prevent other cases are pointed out.

INTRODUCTION

Mollusc bivalves are filtering organisms, which concentrate marine biotoxins, without being visibly affected and, in this way they can become a menace to human consumers. To prevent human illness, monitoring of exploited areas is mandatory both for potentially toxic microalgae and accumulation of toxins by bivalves.
In Portugal this monitoring is carried out along the coast by IPIMAR, the National Inst. for Fisheries Research [1].
Though we consider that bivalve producers benefit from the quality and safety guarantee strict monitoring program can provide. We try to avoid unnecessary closures and make bans as short as possible, so cautions closures seldom take place. Nevertheless tourists or private consumers, may harvest bivalves, even when the area is closed, risking their health. This happened in the present cases, again calling attention to the necessity for a better alert, when suspicion of contamination arises, in order to prevent health hazards.
The first case, paralytic shellfish poisoning (PSP), occurred on 19th October 1994, when the whole Portuguese coast was preventively closed, due to the detection of relative abundant *Gymnodinium catenatum* Graham, since the beginning of October. A red tide of *G. catenatum* was recorded in November, with a maximum of 6.1×10^5 cells L^{-1} [2]. At Ericeira, a beach 35 km north from Lisbon, several people after eating blue mussels (*Mytilus edulis*) collected by themselves, became ill with neurological symptoms dominating other manifestations. Nine of them were so ill that they had to be transferred from the local hospital to Santa Maria hospital in Lisbon.
The second case presented, diarrhetic shellfish poisoning (DSP), occurred 12th February 1998, with donax clams from Fuzeta/Olhão, Algarve coast [3], just after a storm that prevented monitoring sampling. *Dinophysis acuminata* Claparède & Lachmann, the responsible species, had been found at the end of January, but without toxin detection in bivalves.

MATERIAL AND METHODS

PSP 1994
The leftovers of the responsible mussels were frozen and sent to IPIMAR for biotoxin analysis. For PSP detection and quantification, in blue mussels, the mouse bioassay [4] was used both during the monitoring [1] and on the shellfish leftovers. *G. catenatum* quantification was made with a Palmer-Maloney chamber after water concentration through centrifugation. The affected people's symptomatology were kindly provided by Dr Mamede Carvalho and his team from Santa Maria Hospital.

DSP 1998
Regular monitoring of ASP, DSP and PSP as well as phytoplankton is carried out for the Portuguese coast all the year around [3]. Water and bivalve samples are sent weekly to IPIMAR from all shellfish culturing areas, except when weather conditions do not allow for the sampling. DSP analysis are done both by mouse bioassay and HPLC [5,6].
On 17th February Dr Tavares de Sousa, Director of Loulé Health Care Centre, informed us that 18 employees and relatives from the Centre, who had eaten donax clams picked by one of them at Fuzeta/Olhão, were ill with vomiting and diarrhoea. The leftovers of the donax clams were sent to IPIMAR for toxin analysis.

RESULTS

PSP 1994
Data on PSP concentrations in the leftovers of blue mussels confirmed the presence of PSP toxins (364 μg sxt eq/100g). This was not as high as we expected from the described symptomatology, which could have been due to loss of toxins during defrosting. The highest value obtained for the area was in a sample arriving directly to IPIMAR during monitoring (3677 μg sxt eq/100g) in November (Fig. 1). Data on the occurrence of *G. catenatum* in the area are also shown in Fig. 1.
The symptomatology of the nine patients which had to be transferred to the Lisbon Hospital includes paresthesia (sensation of itching or prickling of the skin) on tips, orofacially and scattered, unsteadiness to total walking

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disability, but no digestive complains. The neurological examination showed: walking ataxia in all, dizziness in 3, defective articulation of sounds in 3, distal hypostasis in 6. Hospital internment varied between one and three days, with one patient having to stay one whole week. Total recuperation took 2-3 days for the less affected and 3 months for the most affected, which was the eldest patient.

We think that deficient information on the ban and health possible hazards associated with PSP were the main reasons for this case.

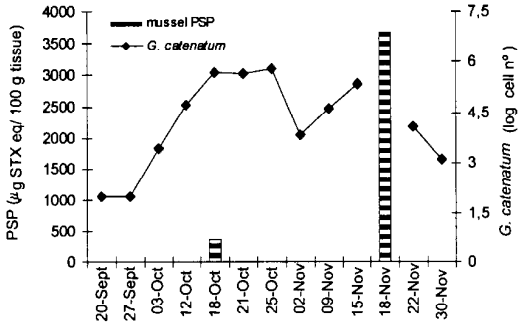

Fig. 1. Development of a *Gymnodinium catenatum* bloom at Ericeira coast, 1994, and resultant blue mussel toxicity data.

DSP 1998

Data of DSP in donax clams from the area and occurrence of *Dinophysis acuminata* show that the dinoflagellate started to be detected on 29th January and at that time okadaic acid (OA) was not detected (Fig.2). As monitoring is normally weekly we expected to have samples on the 5th and 12th February, but bad weather prevented sampling and new monitoring samples were only received on the 17th.

The donax clams responsible for the toxication were collected from the beach on the 12th and they contained 130 µg/100g, the highest detected value of OA in the area (Fig. 2).

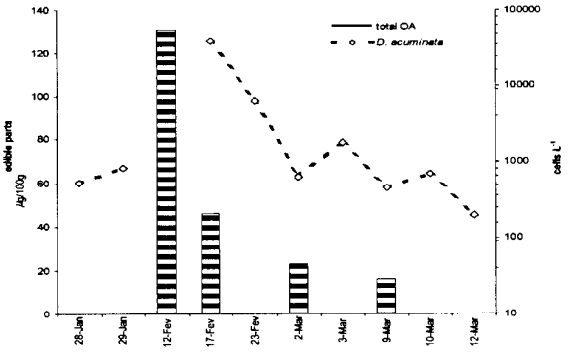

Fig. 2. Development of a *Dinophysis acuminata* bloom at Olhão coast 1998, and total Okadaic acid concentration in donax clams.

All affected people had eaten donax clams collected in Fuzeta/Olhão on the 12th February. Dr Tavares de Sousa described symptomatology, as including diarrhoea, vomiting, epigastric pains, muscular weakness and headaches. The symptoms started 6 hours after ingestion. Severity was proportional to the amount ingested and not related to the cooking procedure, illustrating the temperature stability of DSP toxins.

These symptoms developed over 4 days and only one person had to go to hospital emergency service.

From Fig. 2 we can see that the highest detected number of *D. acuminata* (39.3×10^3 cells L^{-1}) was on the 17th February. The highest detected value for total OA [3] was on the 12th, meaning that, with the lack of sampling due to bad weather, we may have missed the responsible species peak, and that we should have closed the area cautiously on the 29th January.

CONCLUSIONS

In both cases (PSP-1994 and DSP-1998) the described symptomatology accords with the literature [7,8].

In order to prevent health hazards, these two confirmed cases of human toxication with marine biotoxins have called attention to:

- importance of a strict monitoring.
- sampling frequency, which must be weekly, at least at our latitude.
- necessity for an alert that reaches all potential shellfish harvesters and consumers (via TV, Internet, radio and newspapers).
- preventive closures based on potentially toxic microalgae must be made often, especially when sampling is prevented by bad weather.

ACKNOWLEDGMENTS

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THE USE OF NON-ANIMAL ASSAYS WITHIN A BIOTOXIN MONITORING PROGRAMME

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ABSTRACT

Two alternative methods to the mouse bioassay for biotoxin detection in shellfish, the RIDASCREEN Saxitoxin ELISA for PSP detection and the KB cytotoxicity assay for DSP detection, were incorporated into a monitoring programme undertaken in England and Wales. This was to establish the reliability and feasibility of carrying out these alternatives as a prescreen to the mouse bioassay within an active monitoring programme.

There was good agreement between the KB assay and DSP mouse bioassay in all samples tested with no false positives or negatives. Results from this study suggest that the KB assay would be a good potential candidate for incorporating into a routine biotoxin monitoring programme as a prescreen and could help reduce dependence on the DSP mouse bioassay.

The predominance of GTX's and lack of STX in shellfish samples resulted in the underestimation of toxin content by the ELISA kit by comparison to the PSP mouse bioassay. Although the kit was easy and quick to use, in areas where STX is not the dominant toxin in shellfish the implementation of this test kit as a replacement to the mouse bioassay is not recommended.

INTRODUCTION

Consumption of bivalves presents a health risk if shellfish originate from areas with toxic algae problems. During periods of toxic algae occurrence bivalves can bioaccumulate toxins from the algae which can present serious, possibly fatal, health risks to the consumer. The UK is required to undertake a biotoxin monitoring programme under the EC Shellfish Hygiene Directive (91/492/EEC). The biotoxin monitoring programme currently in place in England/Wales involves the monitoring of water for toxic algae and shellfish flesh for biotoxins. The flesh monitoring programme relies on the EC approved AOAC mouse bioassay for detection of PSP toxins (1) and Yasumoto's mouse bioassay for detection of DSP in shellfish flesh (2).

The use of animal bioassays presents ethical considerations, especially within England and Wales where the occurrence of biotoxins is sporadic and with the occasional exception low. Usually less than 10% of mouse bioassays performed within the biotoxin monitoring programme are found to contain biotoxins. The implementation of a non-animal prescreen assay to remove shellfish samples which are negative for toxins within the biotoxin monitoring programme, would greatly reduce the number of mouse bioassays performed. With such an aim in mind two alternative methods for Paralytic Shellfish Poisons (PSP) and Diarrhetic Shellfish Poisons (DSP) detection were incorporated into a shadow monitoring programme at areas which had a previous history of biotoxins. This was to establish the reliability and feasibility of carrying out these assays within an active monitoring programme.

Several methods were examined for rapid easy inclusion into the biotoxin monitoring programme and the KB cytotoxicity assay for DSP detection and RIDASCREEN Saxitoxin ELISA for PSP were decided upon. The cytotoxicity assay uses oral epidermoid carcinoma cells (KB) which when exposed to medium polarity protein phosphatase inhibitors (PPI) such as DSP toxins - okadaic acid (OA), dinophysistoxin-1 (DTX-1) and dinophysistoxin-2 (DTX-2), undergo morphological changes which cause rounding of the cells (3). By visually estimating the proportion of viable cells in the presence of these toxins it is possible to calculate a minimal active concentration (MAC) at which >30% of the cells are affected. This MAC can then be used to determine toxin concentration in shellfish samples. The RIDASCREEN Saxitoxin ELISA is a competitive enzyme immunoassay which is specific to saxitoxin (STX) but has cross reactivity of between 25-55% for gonyautoxin (GTX) 2, 3, C1, C2 and neosaxitoxin (NEOSTX).

METHODS

Shellfish were collected from 5 sample areas (Figure 1) involved in the 1999 England/Wales biotoxin monitoring programme which had histories of PSP and/or DSP episodes. Samples were collected throughout the year by local authority officers and sent to the CEFAS, Weymouth Laboratory where they were immediately extracted for PSP and DSP testing.

DSP monitoring methods

Mussel samples (*Mytilus edulis*) were dissected and digestive glands (DG) extracted and analysed by mouse bioassay in accordance with Yasumoto's procedure (2). A total of 33 shellfish samples were analysed by mouse bioassay and simultaneously tested by KB assay. Extraction for the KB assay was as per the mouse bioassay extraction with the exception that the residue was resuspended in ethanol. Aliquots of digestive gland homogenate were frozen (-20°C) for liquid chromatography-mass spectrometry (LC-MS) testing which was performed throughout the sampling programme. The extraction method for the LC-MS was

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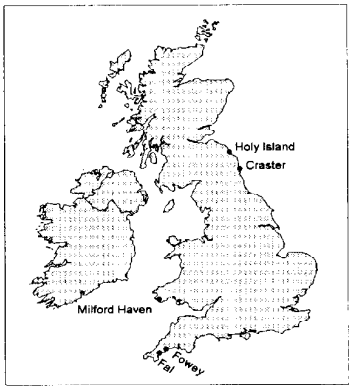
based on that for the DSP mouse bioassay (2). All techniques were calibrated with certified OA and DTX-1 solutions obtained from the National Research Council (NRC), Canada and DTX-2 was provided by Dr K. James of the Cork Institute.

Figure 1. Samples locations 1999



The semi-quantitative KB assay was based on Amzil (4) with the following amendments: 100µl of media containing 10,000 cells was dispensed into each well of a 96 well test plate and left for at least 24 hours to attach. 100µl of OA standard or test solution were added to wells and left for 48 hours with cell examination at 24 hours and 48 hours to determine MAC when >30% cell rounding occurred. The plates consisted of 6 test solutions with dilutions ranging from 1/400 to 1/4000, 6 OA concentrations were used ranging from 25ng/ml-2.5ng/ml. OA standards and test samples were run in duplicate on 2 test plates for each run. Tissue culture conditions are as per Amzil (4) but with a reduction in fetal calf serum concentration to 5%.

PSP monitoring methods

Shellfish samples were extracted and analysed by mouse bioassay in accordance with the AOAC procedure (1). A total of 33 shellfish samples were analysed by mouse bioassay and simultaneously tested by ELISA kit (Ridascreen, R-Biopharm Gmbh, Germany). Aliquots of shellfish homogenate were frozen (-20°C) for high performance liquid chromatography (HPLC) testing which was performed during November 1999. The HPLC method was based on that developed by Lawrence (5) using acetic acid extraction and pre-column derivatisation. All standards were certified calibrated PSP solutions obtained from the National Research Council (NRC), Canada.

The ELISA was performed in accordance with the manufacturers instructions except that shellfish samples were diluted 1/2000 with buffer and STX was prepared from NRC standard.

RESULTS

DSP KB cell assay method comparison

The 3 sites involved in DSP testing were Craster and Holy Island in England and Milford Haven in Wales, all had previous histories of DSP. During the trial no identifiable DSP toxins were detected at Milford Haven by either LC-MS or mouse bioassay. However, at this site an isomeric form of OA was detected in one sample taken on the 7 September. DSP was detected at both Craster and Holy Island during the sampling period (Figures 2 and 3). DSP toxins were detected from May until the end of sampling in September in both areas by LC-MS, levels did not rise sufficiently to produce positives in the mouse bioassay until late July/early August. Only 2 DSP toxins were found, OA and DTX-2. The latter was the predominant toxin and comprised 71% of toxin encountered.

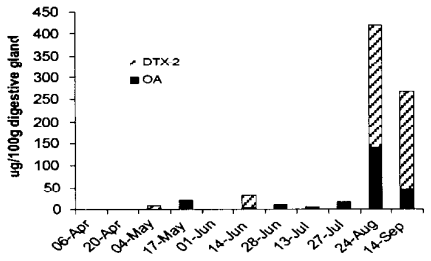


Figure 2. DSP occurrence at Craster by LC-MS during 1999

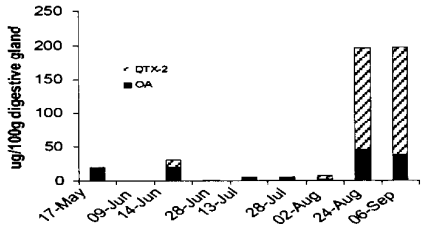


Figure 3. DSP occurrence at Holy Island measured by LC-MS during 1999

The MAC for OA, DTX-1 and DTX-2 are listed in Table 1. The MAC for OA was used to calculate the toxin concentration in shellfish digestive glands as OA is commercially available and has a similar activity to DTX-2. OA and DTX-2 were the dominant toxins found in England/Wales DSP incidents. By performing a range of sample dilutions and using the MAC it was possible to detect a range of toxin concentrations in test samples (Table 2). The minimum detectable level was 4-8µg/100g and maximum detectable level was 40-80µg/100g depending on the test duration time.

Table 1.
DSP toxin minimal active concentration obtained during the KB assay

Toxin	MAC 24hr (ng/ml)	Limit of detection (ng)	MAC 48hr (ng/ml)	Limit of detection (ng)
OA	5	1	2.5	0.5
DTX-1	2.5	0.5	1.25	0.25
DTX-2	3.25	0.75	≥2.5	≥0.5

Table 2
Dilution factors employed within the KB assay and corresponding detection level of DSP toxin in 100g of mussel digestive gland

Dilution Factor	24hr OA equiv µg/100g	48 hr OA equiv µg/100g
1/400	8	4
1/500	10	5
1/670	13	8
1/1000	20	10
1/2000	40	20
1/4000	80	40

The KB assay showed good agreement with the mouse bioassay, in all samples where a positive mouse bioassay resulted the KB assay showed levels sufficiently high to cause mouse death (Table 3). Samples taken from Craster and Holy Island tested by LC-MS and KB assay were mostly within the semi-quantitative parameters of the KB assay. At lower concentrations of OA and DTX-2 some variation occurred within the KB assay. Overestimation was found in 4 instances, however, in 2 of these instances the mouse bioassay also showed the presence of toxins at levels sufficient to cause DSP mouse symptoms or death. The overestimation could therefore indicate the presence of compounds other than those searched for, which affected both the cells and mouse bioassay. Underestimation occurred in 3 samples, in these instances levels were insufficient to cause mouse death. There were no false negatives by KB assay on comparison to the mouse bioassay during the trial.

At Milford Haven toxin was detected at low levels on 2 occasions by the KB cells (Table 3). One of the occurrences coincided with an LC-MS peak of an unknown lipid soluble toxin which could have caused cytotoxic activity.

There was little variation between results obtained over the 24 hour and 48 hour exposure times for the KB assay, nearly all results conformed within the ranges expected within each test examination time.

Table 3
DSP content in mussel digestive gland by KB assay, LC-MS and mouse bioassay

Sample Location	Sample Date 1999	KB (ug/100g) 24 hr	KB (ug/100g) 48 hr	LC-MS OA	LC-MS DTX-2	24hr Mouse Bioassay
Craster	06-Apr	ND	ND	0	0	NG
	20-Apr	ND	10	0	0	NG
	04-May	8	8	0	7	NG
	17-May	8	5	20	0	NG
	01-Jun	ND	ND	0	0	NG
	14-Jun	20	20	3	30	NG
	28-Jun	13	20	12	0.3	NG
	13-Jul	13	10	3	2	NG*
	27-Jul	40	40	15	3	PS
	24-Aug	>80	>40	140	280	PS
	14-Sep	>80	>40	47	220	PS
Holy island	17-May	8	8	20	0	NG*
	09-Jun	ND	ND	0	0	NG
	14-Jun	8	8	20	10	NG
	28-Jun	10	5	1	0.7	NG*
	13-Jul	13	10	6	0.2	NG
	28-Jul	13	10	4	2	NG
	02-Aug	40	40	2	5	PS
	24-Aug	>80	>40	46	150	PS
	06-Sep	>80	>40	37	160	PS
Milford Haven	12-Apr	ND	ND	ND	ND	NG
	27-Apr	ND	ND	ND	ND	NG
	11-May	ND	ND	ND	ND	NG
	26-May	ND	ND	ND	ND	NG
	11-Jun	ND	ND/10	ND	ND	NG
	23-Jun	ND	ND	ND	ND	NG
	12-Jul	ND	ND	ND	ND	NG
	28-Jul	ND	ND	ND	ND	NG
	16-Aug	ND	ND	ND	ND	NG
	25-Aug	10	5	ND	ND	NG
	07-Sep	20	7.5	ND/	ND	NG
	22-Sep	ND	ND	ND	ND	NG
	21-Oct	ND	ND	ND	ND	NG

ND- DSP toxins not detectable NG- negative mouse bioassay
*- some DSP symptoms PS- positive mouse bioassay
/- unknown DSP isomer detected

PSP ELISA method comparison

During the study the levels of PSP found in shellfish from the 5 areas never exceeded the EC action level of 80µg STX equiv./100g shellfish flesh. The mousebioassay only detected PSP toxins in 3 samples (Table 4). The ELISA in comparison with the mouse bioassay underestimated these values by up to 81%.

Table 4
PSP levels detected by mouse bioassay and ELISA

Sample	Mouse Bioassay (µg STX equiv./100g)	ELISA (µg STX equiv./100g)
Craster 1/6	33	8
Fal 22/7	33	6
Holy Island 9/6	36	9

The ELISA found no PSP toxins or levels well below the detectable limit of the mouse bioassay in all other tests. No false positives were obtained using the ELISA. HPLC examination of samples showed the dominance of GTX 1/4 and GTX 2/3 in samples, no STX was detected (Figure 4).

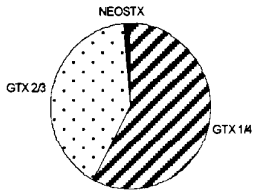


Figure 4. The PSP profile of mussels during the study

DISCUSSION

DSP KB assay method

The current study aimed to evaluate the reliability and practicality of the KB assay as a negative screen to the mouse bioassay. The method employed in this study was extended 48 hours with cell examination at 24 and 48 hour intervals rather than 4 hours as used by Amzil (4). This allowed a large dilution of mussel DG extract which increased ease of reading plates whilst maintaining a minimum detection limit considerably higher than the mouse bioassay. Determination of DSP in shellfish extracts by KB assay would appear to be a valid method. The KB cells showed clear morphological changes on exposure to OA, DTX-1 and DTX-2 which could easily be discerned by light microscope without staining. Reproducibility was good with 98% concurrence between duplicate wells and plates examined during the same time durations. The agreement between the 24 and 48 hour time intervals for OA concentration was also good with 96% of results falling within expected ranges. There was also agreement between the KB assay and mouse bioassay in all samples tested with no false positives or negatives.

Incorporation of the KB assay into an active monitoring programme did not present any difficulties. The KB cell line has good temporal stability (6) and was easy to maintain. The KB plates could be prepared and stored for up to 3 days before use, a constant supply of plates for testing could therefore be provided throughout the week with minimal time commitment. Each plate can be used for 8 samples or 4 if samples are run in duplicate. The time involved in preparing and running this assay over 24 hours is comparable to that of the mouse bioassay. In addition the KB assay format could be adjusted to be comparative in sensitivity to the mouse bioassay, which would allow a reduction in the time required for the assay run.

The KB assay has only been developed for the detection of protein phosphatase inhibitors and toxins such as yessotoxin and azaspiracid which can be detected by the DSP mouse bioassay may not be detected by this method. However, during this study only OA and DTX-2 were found to be responsible for toxicity occurrences in mussels in England and Wales, the major toxins currently associated with DSP would therefore be detected by this method. Results from this study suggest that the KB assay would be a good potential candidate for incorporation into a routine biotoxin monitoring programme as a prescreen to the DSP mouse bioassay and could help reduce dependence on this bioassay.

PSP ELISA method

In the 3 samples in which toxin was detected by mouse bioassay the ELISA underestimated total toxin concentration. The PSP profile of toxins in the mussels showed the predominance of GTX 1/4 and GTX 2/3 but no STX. Kasuga (7) found that cross-reactivity to GTX-1 and GTX-4 was less than 0.1% and the RIDASCREEN Saxitoxin ELISA instruction manual describes the relative cross-reactivity to GTX-2 and GTX-3 as 30% and 55% respectively. The lack of STX in samples taken during this study resulted in the underestimation of toxin content by the ELISA kit. Kasuga (7) found a similar problem with kits exhibiting unpredictable cross-reactivities to a mixture of GTX's, and underestimation of the toxicity of some naturally contaminated shellfish samples. The variation in cross-reactivity against PSP toxins with this kit is clearly very important when applied to real samples. Although the kit was easy and quick to use, in areas in which STX is not the dominant toxin in shellfish the implementation of this test kit as a replacement to the mouse bioassay is not recommended. However, there may be scope for inclusion of this method in areas which have a more mixed PSP toxicity profile providing trigger levels were set sufficiently low.

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A RATIONAL STRATEGY TOWARD THE MANAGEMENT OF SEAFOOD POISONING IN THE WESTERN INDIAN OCEAN REGION

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ABSTRACT

Numerous seafood poisonings have been identified in the Western Indian Ocean region, involving reef fishes (ciguatera), sharks (carchatoxism), turtles (chelonitoxism), sardines (clupeotoxism), puffer fishes (tetrodotoxism). Carchatoxism is an endemic and severe problem specific of Madagascar. Ecotoxicological processes involved are poorly known (sources and natures of toxins, species involved, etc.). Given the regional seafood safety and external trade, the Regional Environment Program of the Indian Ocean Commission/European Union (REP-IOC) established in March 1998 a "Marine Ecotoxicology" component which has focused its activities in the following areas:

- Establishment of a regional network based on the existing national facilities of the IOC countries,
- Capacity building with organisation of training workshops,
- Survey of risk factors following the 1998 bleaching event,

A practical manual for monitoring epidemiological, toxicological and environmental data produced by member of the network has been developed.

SEAFOOD POISONING IN THE WESTERN INDIAN OCEAN REGION

In the Indian Ocean region, focus is almost exclusively on the phenomena of marine ecotoxicology by phycotoxins due to their impact on the health of local populations and on fishing as part of the regional economy. After a long period of being overlooked by researchers, the Indian Ocean is today the object of particular interest due to the multiplicity of existing phenomena and the innovative nature of some of them.
Historically recognised last century in Rodrigues, Mauritius and Reunion, cases of seafood poisoning have proved to be present in the whole of the lower Indian Ocean Region. Specific diseases appear to be characteristic of certain countries, as shown by the available epidemiological data, which remains fragmented and recently acquired.

Harmful Algal Blooms 2000
Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J. (eds)
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Studies undertaken over the last few years, using a pluri-thematic approach have helped to establish the incidence and geographical distribution of diverse forms of seafood poisoning in the IOC countries (Comoros, Madagascar, Mauritius, Reunion, Seychelles).
Eight different types of seafood poisoning are documented in the region (Table 1).

Table 1: Seafood poisoning in the Western Indian Ocean.

Seafood poisoning	causative
Carchatoxism	Sharks
Chelonitoxism	Sea turtle
Ciguatera	Coral reef fish
Clupeotoxism	Sardines
Tetrodotoxism	Pufferfish
Ichtyoallyenotoxism (hallucinatory)	Herbivorous fish
Scombrotoxism	Pelagic fish
Xanthitoxism	Crab

Overall, the geographical location of seafood poisoning seems to be marked by two local areas:

• Ciguatera, which develops endemically in warm seas around the world, seems to affect mainly the Mascareignes plateau (Reunion, Mauritius, Rodrigues extending up to the North banks) and may be sustained by the regular passage of cyclonic conditions.

• Food poisoning from sharks (presently specific to Madagascar), sardines and turtles affect countries near the Mozambique Channel (Madagascar and Comoros).
The other forms of seafood poisoning have less impact on health and socio-economic levels. Accidental cases of tetrodotoxism are no longer recorded in the region. Hallucinatory fishes types are often deliberately consumed and anecdotal. As for histamine food poisoning (scombrotoxism) often associated with poor handling of storage, this concerns mainly pelagic fish.

REGIONAL ACTION

The appearance of certain forms of seafood poisoning is not new in the region (reports date from 1775 in

Mauritius). However, awareness of damage to the marine environment affecting countries of the region, coupled with legitimate objectives to guarantee public health and economic development (traditional fishing, tourism) has, since 1992, drawn the Indian Ocean Commission's attention to the region's toxic marine animals. This drive, which has developed considerably within the framework of the REP-IOC (supported by the European Union) between 1996 and 1999, is directly in keeping with the concerns of the international community and the noting of a significant increase in problems that the algal blooms may cause for economic activities (traditional fishing, trading, tourism, aquaculture, and the health of local consumers.

PAREM (Regional Action Plan for Marine Ecotoxicology) was identified as an unifying theme during the Tuléar workshop in April 1998. It aims to overcome the problems of marine ecotoxicology due to natural toxic compounds (phycotoxins) in the region by understanding their eco-environmental causes, forecast their manifestations and reduce their effects on health and socio-economic factors. In this way, by the implementation of regional and national action plans, which supports the ICZM (Integrated Coastal Zone Management) strategy in the IOC countries, it is possible to fulfil: (i) the double objective of monitoring the quality of marine environments and resources; (ii) the need to protect public health through the alert, monitoring and control services of network set up to lead PAREM.

REGIONAL ACTION PLAN

Establishing networks

The forming of national networks with the necessary skills in each country of the IOC has been one of the REP-IOC/EU's chief activities. Co-ordinated around a focal point, the latter negotiates with the regional level.

Once the member states accept the Action Plan they have to ensure continuity of its activities.

Training programs

Two training programs have been carried out:

The first one concerned the epidemiological level. The second one dealt with the observation of benthic microalgae in relation to the establishing of monitoring processes in the area.

These two training programs have (i) facilitated a sharing of experience and information, (ii) and the understanding that it is necessary to make use of common epidemiological survey forms, drawn up jointly.

Common methods for gathering information

Several common methods have been worked out as part of this program. By April 2000 they will constitute the basis of a practical manual on monitoring epidemiological, toxicological and environmental data [1]. They will form the basis for data exchange on a regional level.

Action in response to regional alerts

A survey of ciguatera risk factors following the 1998 coral bleaching event was undertaken by studying benthic dinoflagellate and herbivorous fish toxicity. In addition to the operational wing, this undertaking has resulted in (i) raising awareness in a way suited to each country and (ii) transferring methodologies for the collecting of Dinophyceae samples.

The second reaction to an alert concerned a source of food poisoning from a sea turtle on the island of Anjouan in November 1999 (90 taken ill, 2 dead). This provided the opportunity to compile medical information vital for a better knowledge of this type of food poisoning, especially from the observation of patients.

CONCLUSION

The reality of an epidemiology of marine eco-toxicology phenomena associated from seafood poisonning, has only been recognised recently in the Indian Ocean region, compared to other intertropical regions. The body of work undertaken over the last few years indicates this.

The reduction of risk factors inherent in seafood products lies in the establishment of a regional integrated strategy. PAREM (Regional Action Plan for Marine Toxicology) provides a relevant, appropriate response operating from several levels of action:

- the application of appropriate regulations (avoidance of dangerous species, hygiene control, etc.)
- improving care for patients following a positive, differential diagnosis,
- control of fishing zones or seafood products,
- monitoring environmental parameters,
- research is essential in our region as knowledge of most of the bio-ecological and toxicological processes is still insufficient.

Only an approach simultaneously involving these different elements can reconcile the interests of consumers, professional fishermen and institutions all at once.

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HARMFUL ALGAL BLOOMS OCCUPATIONAL SCREENING PILOT STUDY

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ABSTRACT

In 1997, fish kills in North Carolina estuaries were attributed to a new organism, *Pfiesteria piscicida*. Guidelines were developed to diagnose "Estuarine Associated Syndrome (EAS)" as a surveillance tool for persons with reported symptoms and exposure to estuaries. Although *Pfiesteria* had not been reported in Florida, a cryptoperidiniopsoid dinoflagellate (a *Pfiesteria*-like organisms (PLO)) had been associated with fish lesions and kills in Florida.

A cross-sectional study was performed to determine the association between occupational exposure to estuarine harmful algal blooms (HABs) and potential human health effects. Florida environmental workers, with estuarine exposure, served as the study population, creating 3 exposure groups: 1) cryptoperidiniopsis exposure, 2) *only* fish kill and/or fish lesion exposure, and 3) a control group. Phone interviews were conducted using a revised EAS Questionnaire to inquire about reported exposure and symptoms.

The study population was a homogeneous group of 53 workers (2 refusals); 41 (80.4%) *ever* exposed to fish events, with 13 (25%) specifically exposed to cryptoperidiniopsis. No participant met the criteria for EAS. The subgroup (n=28) exposed *only* to fish kills/lesions reported more health effects (61%) than those exposed to cryptoperidiniopsis (23%) or the control group (50%) (p=0.08), always associated with Florida Red Tide exposure.

Occupationally exposed workers exposed to HABs in Florida only reported symptoms with Florida Red Tide exposure, not with exposure to a PLO, and there were no cases of EAS. These workers represent an excellent population to follow for future investigations of occupational HAB exposure.

INTRODUCTION

In addition to their impact on other creatures and the environment, harmful algal blooms (HABs) can produce toxins that may be harmful to humans. HABs appear to be increasing worldwide (1-5). Over the past several years, HABs in the mid Atlantic states have been associated with extensive fish kills and reports of variable human health effects associated with exposure to reportedly HAB-contaminated water (6-13). In February 1998, a new marine organism, a *cryptoperidiniopsoid* dinoflagellate resembling *Pfiesteria piscicida* morphologically and genetically, was identified in the estuarine waters of the St. Lucie River (Martin County, FL) in conjunction with fish lesions. No definitive human health effects were reported associated with estuarine water exposure, although there was considerable community and public health concern (10).

A surveillance system of Estuarine Associated Syndrome (EAS) was set up by the Centers for Disease Control and Prevention (CDC) and several states affected by these organisms (ie. *Pfiesteria* and the *Pfiesteria*-like organisms) in 1997, including disease and exposure definitions (11-13).

METHODS

This Pilot Study was a cross-sectional study of persons with extensive occupational estuarine water exposure to investigate self-reported exposure and health effects. The study population consisted of Florida Department of Environmental Protection (DEP) employees whose work involves extensive environmental and laboratory exposure to estuarine waters throughout the State of Florida. Their actual work includes collection of water and marine animal samples, with laboratory processing of these samples.

The questionnaire, adapted from the surveillance of Estuarine Associated Syndrome (EAS) by the CDC with the States (11-13), was administered by telephone. The questionnaire explored self-reported symptoms, geographic estuarine exposure and demographic data, collected anonymously. The protocol for this study was submitted to and approved by the internal review board of the University of Miami Human Subjects Committee.

Florida DEP environmental workers were selected because of their occupational estuarine exposure during normal times, as well as during fish events. Fish events were defined as the presence of the *cryptoperidiniopsoid* dinoflagellate, fish kills and/or fish lesions. Individuals from the 6 major field stations throughout Florida were chosen to participate. Since some individuals from

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different stations joined the crews in the affected areas during the fish events, the unit of analysis in this study was the individual.

A database was created in Epi-Info Version 6 and analyzed using the statistical software SPSS version 8.0 to conduct chi-square analyses and Fischer's exact tests, and ANOVA and t-tests. Using Query 4.0 software to calculatea sample size for G (groups) times 2; the groups were the three discussed below. With an alpha level of 0.05, the power of this study to detect a difference between the 3 groups was 95%.

RESULTS

The six DEP field stations were composed of 53 individuals; 51 (96.2%) participated with 2 refusals. There were 10 (19.3%) women with 49 (96.1%) Whites and 3 (5.9%) Hispanics. The average age of the study population was 34.00±7.19 years. All participants had received college degrees with 24 (47.1%) finishing graduate school.

With regards to exposure, 41 (80.4%) were "ever exposed to fish events"; 13 (25.5%) individuals specifically reported exposure to the *cryptoperidiniopsoid* dinoflagellate during the peak fish event time of February-April of 1998. The majority of the participants were exposed to estuarine organisms in the DEP laboratories.

Overall Symptoms

Twenty-six (51.0%) of the 51 participants experienced no reported health effects; no individual reported any symptom lasting for more than 2 weeks. None of the participants suffered from memory loss or confusion, nausea, vomiting, or muscle cramps. Respiratory symptoms were relatively common, occurring in individuals who reported concomitant exposure to Florida Red Tide. Of the 13 people exposed to the *cryptoperidiniopsoid* dinoflagellate in St. Lucie River, 10 (76.9%) reportedly experienced no health effects throughout the study time periods.

Ever exposed vs. Never exposed to fish events

There were 41 (80.4%) individuals who had *ever* been exposed to fish events, including those present at the St. Lucie River events; only 10 (19.6%) individuals had *never* been exposed to fish events (controls). There were no significant differences between these groups for demographics or reported symptoms.

Table 1. Three Exposure Groups

		Ever Exposed to fish events		
Variable	***Never* exposed (N=10)**	***Only* exposed to fish kills (N=28)**	**Exposed to Dinoflagelle (N=13)**	***P value**
Age (mean)	33.87±8.52	35.06±7.77	31.82± 4.17	0.41
Gender	3F (30.0%)	6F (21.4%)	1F (7.7%)	0.35
Race White	10 (100%)	26 (92.9%)	13 (100%)	1.00
Hispanic	1 (10.0%)	2 (7.1%)	0	0.76
Graduate School	7 (70.%)	14 (50.0%)	3 (23.1%)	0.09
Mean yrs employed	4.40±2.72	5.61±4.59	4.15±3.72	0.50
Ever Symptom	**5 (50.0%)**	**17 (60.7%)**	**3 (23.1%)**	**0.08**

* Fishers Exact Test and ANOVA

Cryptoperidiniopsoid dinoflagellate vs. *fish kills* vs. *controls (Table 1)*

A comparison was performed between 1) the 13 (25.5%) individuals exposed to the *cryptoperidiniopsoid* dinoflagellate, 2) the 28 (54.9%) individuals *only* exposed to fish kills/lesions, and 3) the 10 (19.6%) unexposed controls. There were no significant differences for the demographic variables.

When comparing all three groups with regards to reported experience of any symptoms, no statistically significant difference was found (p=0.08). Although not statistically significant, the group *only* exposed to fish kills (n=17; 60.7%) and even the unexposed controls (n=10; 50.0%) had substantially more people with symptoms than those individuals exposed to the *cryptoperidiniopsoid* dinoflagellate (n=3; 23.1%).

DISCUSSION

This was a cross sectional study of persons occupationally exposed to estuarine waters during and after a major fish event with a new dinoflagellate. None of the study participants met the CDC/State criteria for "Estuarine Associated Syndrome" (EAS) (11-13). An increased prevalence of respiratory symptoms was associated with exposure to known Florida Red Tides; respiratory symptoms have been associated with the aerosolized Florida Red Tide toxins and studies are ongoing (5, 10). The majority of the individuals reporting exposure to the *cryptoperidiniopsoid* dinoflagellate did not report health effects associated with estuarine exposure. Analysis of people *ever* exposed to fish kills compared to people *never*

exposed to fish kills showed no significant difference in the report of symptoms. The individuals exposed to the *cryptoperidiniopsoid* dinoflagellate did not have a higher risk of health effects than those with exposure to *only* fish kills or the unexposed control group.

Although there was sufficient statistical power to detect a difference between the 3 groups, multiple comparisons were not taken into account given the relatively small study population. Furthermore, the study information was cross sectional and by self-report; there were no objective measures of health effects, and only limited objective data on exposure. The possibility of biased reporting is present since these workers could either over-report symptoms (to receive workers compensation) or under-report (due to fear of work reprisals). However, the significant response of reported upper respiratory symptoms associated with exposure to Florida Red Tide, consistent with other reports, would indicate that reporting bias was not an issue in this study (3, 5).

This Pilot Study has a number of advantages not seen in the previously published *Pfiesteria*/EAS literature on possible human health effects (6-13). In addition to the high participation rate, these environmental workers are a uniform group with similar educational and socio-economic backgrounds in a stable job situation. The participants are not necessarily representative of the general population, yet they are an important group of occupationally exposed individuals. They are very highly exposed to estuarine events due to the nature of their work. Therefore, if a dose response relationship is postulated for the health effects possibly associated with exposure to these organisms, then health effects should be seen in this occupational population first and in greatest quantity. Finally, although the study population is small, this study is the largest to date that epidemiologically evaluates individuals with occupational HAB exposure.

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A PILOT STUDY TO EXPLORE THE RELATIONSHIP OF OCCUPATIONAL EXPOSURE TO *GYMNODINIUM BREVE* (DINOPHYCEAE) TOXIN AND PULMONARY FUNCTION

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Occupational Exposure to *Gymnodinium Breve*
Keywords: spirometry, pulmonary function, marine aerosol, Florida red tide, *Gymnodinium Breve*, respiratory effects of red tide

ABSTRACT

The Western Coast of Florida frequently experiences a harmful algal bloom caused by the dinoflagellate, *Gymnodinium breve*, Davis. *G. breve* releases a toxin when the cells lyse, and this toxin becomes part of the marine aerosol. When humans are exposed to *G. breve* toxin in marine aerosol, upper respiratory symptoms such as runny nose, nasal congestion, cough, and sore throat are commonly reported. Since the estimated size of the particle is 7 - 10 μm most toxin should be filtered by the upper airway. If the red tide toxin impacts the lower airway, bronchoconstriction of the smooth muscle may occur. The common method to detect bronchoconstriction is the measurement of the Forced Vital Capacity (FVC) and the forced vital capacity exhaled in 1 second (FeV_1). The purpose of this pilot study was to see if occupational exposure to red tide toxin during a scientific research cruise could be detected through spirometry. A spirometer providing hard copy of the flow volume loop was placed on a research cruise in September 1999. The primary purpose of this cruise was to conduct experiments in a red tide bloom in the Gulf of Mexico. Seventeen volunteer scientists were instructed on the correct method to perform the FVC maneuver. They were also asked to complete a Health History Questionnaire (©Hollister Inc, 1980). The volunteers then performed forced vital capacity maneuver at varying times of the cruises and also documented any respiratory symptoms. Variation in FVC and FeV_1 in correlation to surface cell counts, wind speed, and scientist subjective symptoms were analyzed. Findings revealed that most scientists did not have an abnormal variation in spirometry when occupationally exposed to *G. breve*. However, two scientists did have change in their spirometry beyond normal circadian variation and reported symptoms during those changes. Further investigation in occupational exposure to *G. breve* aerosol is indicated.

INTRODUCTION

The Florida red tide, *Gymnodinium breve* is a toxic phytoplankton that can cause both gastrointestinal and respiratory symptoms in humans. The gastrointestinal effects are due consumption of contaminated shellfish. Human respiratory symptoms appear to be unique to *G. breve*. The respiratory effects appear to be most pronounced when a strong marine aerosol is present [6]. Common reported symptoms are runny or stuffy nose, tickle in the back of the throat, cough, and headaches.

These respiratory symptoms appear to be primarily focused in the upper airway. The majority of the particle size of the *G. breve* aerosol has been determined to be greater than 7 μm [5]. The average particle size filtered by the upper respiratory system (from nose to the larynx) is greater than 5 μm [3]. Therefore, most of the aerosol brevetoxin particles should be filtered by the upper respiratory system and have little impact on the lower respiratory system (below the larynx to alveoli). To reach the lower airways, particle size should be in the .8-to 2-μm range [3]. Numerous anecdotal reports from the Sarasota, Florida area during a significant red tide bloom do not support the concept that brevetoxin does not reach the lower airway. Reports of asthmatic type episodes, tightness in the chest, and shortness of breath are frequently reported. People with pre-existing lung disease appear to have more lower airway symptoms than people with no prior history of lung dysfunction. However, the local medical community reports cases of asthma type symptoms in people with no prior history of reactive lung disease. Whether the lung reactions are directly due to the inhalation of brevetoxin in the lower airway or if it is a systemic reaction to brevetoxin from upper airway impaction and absorption has not been established. When brevetoxin was administered via intravenous route in cats, both cardiovascular and respiratory effects were documented [4]. The respiratory effects were apnea followed by hyperpnoea. In another study, guinea pigs exposed to aerosolized brevetoxin had a bronchospastic response [3].

The purpose of this pilot study is to measure forced vital capacities (FVC) and also the forced expiratory vital capacity in one second (FeV_1) in volunteer scientists working in the natural environment in a large, dense *G. breve* bloom. The FeV_1/FVC is the indices most sensitive to detect borderline and/or mild airway obstruction [1]. Therefore, if *G. breve* toxin is penetrating the lower airway and causing asthma-like reactions, the spirometry data may reflect an acute change in lung function.

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MATERIALS AND METHODS

The Ecology and Oceanography of Harmful Algal Blooms: Florida (ECOHAB: FL) process cruise in September, 1999, studied a significant red tide bloom off the coast of Panama City, Florida. A spirometer was placed onboard ship, in an air-conditioned laboratory.

The scientists participating in the ECOHAB: FL process cruise were asked to voluntarily participate in the study. No stipend or other type of incentive was offered. All scientists agreed to volunteer for the study. The only modification in their work schedule was to perform the vital capacity maneuvers and complete a symptom score sheet. The score sheet was a Likert scale of 1-5 assessing cough, nasal congestion, tightness in chest, and shortness of breath. A section was provided for scientist comments. Participants also completed a health history questionnaire (©Hollister Incorporated) for the study.

Scientists were asked to perform each forced vital capacity maneuver three times in each sampling session [1]. In addition, they were asked to perform the FVC in the beginning of their shift and at the end of their shift. Scientists were also requested to complete a symptom score sheet at each measurement.

A Creative Biomedics DX_1 portable spirometer was used for all pulmonary function screening in this study. A Creative Biomedics 3 liter syringe was used for calibration. All calibrations met American Thoracic Society (ATS) guidelines [1]. Flow/volume loops were hard copied after each exam. All predicted values were provided through the spirometer software using an Enright nomogram. The ECOHAB: FL scientists provided cell counts. Wind speed data was provided through the ship's, the R/V Pelican, weather station.

One volunteer scientist was instructed in the calibration of the spirometer and the technique for the FVC maneuver. Detailed written instructions of the FVC maneuver were also provided. The spirometry scientist instructed participants in the FVC maneuver.

RESULTS

The scientists performed a total of 494 spirograms. After analysis of the flow/volume loop and reproducibility [1], 112 FeV_1's were acceptable exams. Data was separated into the "B" and "C" legs of the cruise.

All volunteers were nonsmokers. No one reported any known lung conditions such as asthma or chronic obstructive pulmonary disease. No prescription medications for cardiopulmonary disorders were reported.

On the "B" leg of the cruise, four women and five men participated. The average age was 37 and the age range was 22 to 46 years. The "B" leg participants had variations in the FeV_1 consistent with normal circadian changes. Although red tide was found on this leg of the cruise, no scientists reported symptoms beyond nasal stuffiness and mild cough.

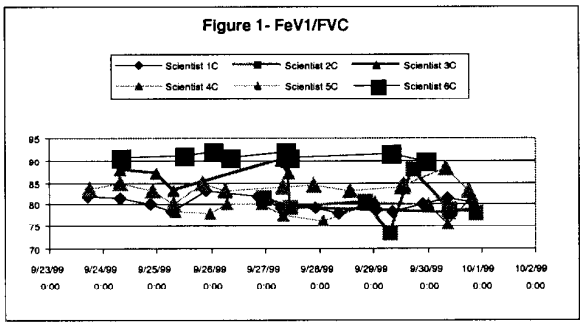
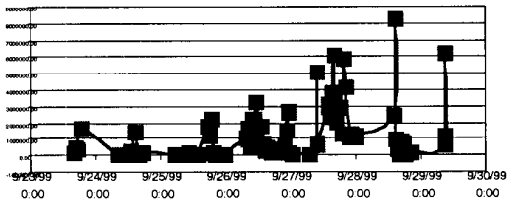
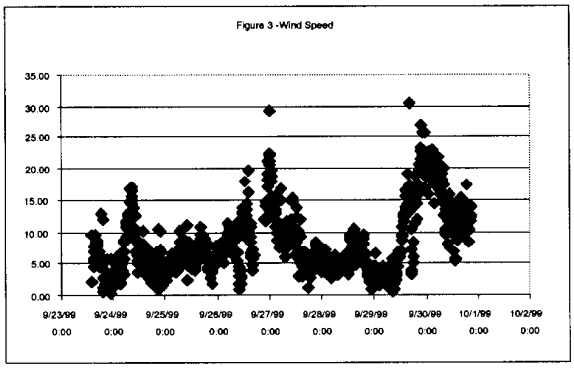


Figure 2 - SURFACE CELL COUNTS





On leg "C" of the cruise, eight scientists volunteered to participate in the study, three women and five men. The average age of the group was 32.5 and the age range was from 29 to 47 years. One participant reported a history of an abnormal breathing test. However, none of this scientist's spirograms met ATS Guidelines and were not evaluated with the data in the study. Two scientists reported a history of childhood asthma but adult asthma was not reported. Shown above are the FeV_1/FVC ratios (Figure 1) for all acceptable spirograms on the "C" leg. The wind speed (Figure 3) and surface cell counts (Figure 2) for Leg "C" are also shown.

Table 1 details the wind speed, cell counts, and respiratory symptoms on the days two of the scientists had spirometry changes beyond normal circadian variances.

Table 1

Date/Time	Wind speed (knots)		Cell Counts	FeV1/FVC Actual		Symptoms	
	Avg.	Max.		Scientist 3	Scientist 2	Scientist 3	Scientist 2
27 Sep 0700	8.7	10.5	5 million	90%	No data	+1 NC	No data
27 Sep 1030- 1145	10.4	15.2	2-6 million	79%	79%	+1 cough	+1 cough
28 Sep 2030	4.3	6.9	190,000	78%	80.5%	+1 NC	+1 cough +1 TC
29 Sep 0730	2.9	5.6	660,000	No data	73%	No data	+2 TC +1 SOB
29 Sep 1700-30	17.5	26.9	1-5 million	78%	87%,78%	No data	+1 TC
30 Sep 2200	No data	No data	No data	No data	78%	No data	None

TC = tightness in chest,
SOB = shortness of breath,
NC = Nasal congestion

DISCUSSION

During the "C" leg, the data from two of the scientists merits some discussion. Scientist 3's FeV_1/FVC decreased from 90% at 0700 on 27 September 1999, to 87% at 1030, and then further decreased to 79% at 1100 on the same day (Figure 1). The corresponding symptom log reflected '+1' nasal congestion at 0700, a '+1' tightness in chest at 1030 and '+2' tightness in the chest at 1100 (Table 1). It should be noted that the scientists performed spirometry in the middle of the day on 27 September 1999 as conditions were perceived to be favorable for brevetoxin in the marine aerosol, (wave action and wind). The next acceptable spirometry maneuver was 1000 on 30 September 1999, with symptoms of '+1' nasal congestion.

Scientist 2 had a decrease in FeV_1/FVC from 80% on 28 September 1999 at 2030 to 73% on 29 September 1999 at 0730. Although this certainly could be due to normal variability, when combined with the symptom log sheet completed by the scientist, there could be a detected change in lung function. On 28
September 1999 at 2030, the scientist reported a '+1' for both cough and tightness in chest. Prior to that time, only a '+1' cough had been reported. At 0700 on 29 September 1999, the scientist reported both '+1' tightness in chest and '+2' shortness of breath. On 29 September 1999 at 1730, the FeV_1/FVC improved to 87% with a "+1" tight chest symptom reported.

All other participant symptoms logs reported only nasal congestion and periodic cough. Although the spirometry data from the two scientists may be effort and or individual variability, it is interesting that they also reported symptoms of tightness in the chest and shortness of breath at that time. Lung variations due to circadian rhythms are reported as only an increase of 0.15 L in FeV_1 by noon and a decrease in FeV_1 of 0.05 L in the afternoon [1].

Collection of brevetoxin aerosol was attempted during Leg "C" and positive data was reported for 27 September. However, data was also collected on 24 September when no pulmonary function changes or symptoms were found. The collection of brevetoxin aerosol may require further investigation.

By definition, occupational exposure is a 15% change in pulmonary function from the start of a workday to the end [3]. No scientists in the study had a change meeting this definition.

A limitation of this pilot study was the scientist self-supervision in the forced vital capacity maneuver. As stated earlier, all spirograms of three of the scientists were unable to be used due to the lack poor quality of the flow-volume loop. The spirograms used in this report met ATS flow volume loop criteria. Further studies would be enhanced by the addition of a respiratory therapist to coach participants in their FVC maneuvers.

CONCLUSION

The results from this pilot study indicate that most of the scientists working onboard ship in a dense bloom of *G. breve* do not have acute changes in pulmonary function. Some variation in pulmonary function was detected in two scientists and the decrease correlated with their self reporting of lower airway symptoms. In addition, the two scientists were young, nonsmokers, with no known history of pulmonary disease. When combined with anecdotal reports from the community during a *G. Breve* bloom, further investigation appears to be appropriate.

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PHYSICIAN DIAGNOSIS AND REPORTING OF CIGUATERA FISH POISONING IN AN ENDEMIC AREA

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ABSTRACT

Ciguatera fish poisoning is the most common marine seafood toxin disease worldwide. It is associated with the consumption of large reef fish contaminated with extremely potent natural marine toxins elaborated by micro-algae known as dinoflagellates. Although a reportable disease with a proven acute therapy, Ciguatera is largely under-diagnosed and under-reported in the United States and elsewhere. This study presented a classic case of Ciguatera to family medicine physicians in the endemic area of Dade County, Florida (USA) to evaluate their knowledge of diagnosis, treatment and reporting of Ciguatera.

Of the 78 eligible participants, 36 (46%) participated. The majority of the participants were male, born in the US, and attended medical school in the US. Although 25(68%) of the participants diagnosed Ciguatera, only 6 (17%) correctly recommended intravenous mannitol therapy as the acute treatment of choice. Almost all the participants (97%) had heard of Ciguatera, but only (64%) had ever diagnosed a case with an average of 0.14 ± 0.42 cases in the past year. Furthermore, only 17 (47%) of the participants knew that Ciguatera was a reportable disease. Foreign-born physicians were significantly more likely to know that Ciguatera was a reportable disease ($p=0.02$); foreign-trained physicians were also more likely to know, although not significantly ($p=0.08$).

This study illustrates that even in an endemic area, Ciguatera is an under-diagnosed, inadequately treated and under-reported disease, especially among US born and US trained physicians.

INTRODUCTION

Ciguatera fish poisoning is the most common marine seafood toxin disease worldwide (1-6). It is associated with the consumption of large reef fish contaminated with extremely potent natural marine toxins elaborated by the dinoflagellate, *Gambierdiscus toxicus*. This dinoflagellate produces a variety of potent neurotoxins, including ciguatoxin and maitotoxin, which are heat and acid stable. Very small amounts of these toxins (probably picogram doses) cause an acute gastrointestinal and neurologic illness with subsequent development of a debilitating chronic neurologic disease lasting weeks to months (1,3,4-6).

Ciguatera has been reported since the 1500s, first by European explorers as they ventured into the tropical and subtropical regions of the world. Until recently, Ciguatera was primarily an endemic disease of island peoples in the tropics. However, with increasing international tourism, seafood trade and consumption, Ciguatera has been reported in non-endemic areas, including North America and Europe (7). Of note, at this time there are no official monitoring programs for Ciguatera in the US seafood industry, although new Food Safety Legislation may make it mandatory in the future.

Although a reportable disease, Ciguatera is highly under-reported in the United States and elsewhere (2, 4-6, 8,9). The US Centers for Disease Control and Prevention (CDC) estimated that Ciguatera is only reported 2-10% of the time (2,3). Part of this under-reporting is due to under-diagnosis by physicians and other healthcare workers, even in endemic areas. Under-diagnosis is an important problem not only because it contributes significantly to subsequent under-reporting, but also because persons with undiagnosed Ciguatera will receive inadequate acute treatment. If Ciguatera is not correctly diagnosed within the first days from exposure, then the appropriate acute therapy, intravenous mannitol, will not be given during its most efficacious period; persons who do not receive intravenous mannitol within the first 2-3 days from exposure are much more likely to experience subsequent chronic Ciguatera, a debilitating neurologic disease, for which there is no definitive treatment (4-6, 10-13).

The objective of this study was to explore the extent of knowledge of Ciguatera, its treatment and reporting requirements among family medicine physicians in the endemic area of Miami-Dade County, Florida with expected incidence rate of 5/10,000/yr (14).

METHODOLOGY

This study was a cross sectional study of the knowledge and behavior of Ciguatera among family physicians in an endemic area. Approval for this study was obtained from the University of Miami Human Subjects Committee. All family medicine physicians listed in the annual Registry of the American Academy of Family Physicians (AAFP) in Miami-Dade County (Florida) were contacted by phone at their workplace by the first author, as a 2nd year medical student. Once verbal consent to participate in a study was obtained, the family

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physician subject was presented with the following classic case of Ciguatera and asked a series of brief questions concerning diagnosis, treatment, reporting issues, and demographics.

"*We have a 50 year old man who presented with nausea, vomiting, and diarrhea, followed within 24 hours by itchy skin without a rash and a burning sensation when touching cold objects. He and his wife returned from the Bahamas 2 days ago where they had caught and eaten a large grouper (which was delicious) within hours prior to the onset of symptoms. His wife is complaining of similar symptoms.*"

All physician subjects approached were ultimately offered a Ciguatera Information Packet through the mail regardless of participation.

The data were entered into EpiInfo 6 software and analyzed by SAS version 7 statistical software. After reviewing frequencies and means, the data were evaluated by ttests and Fishers exact test for possible associations.

RESULTS

From the annual Registry of the American Academy of Family Physicians (AAFP) in Miami-Dade County, 78 eligible participants were identified. Of those eligible, 36 (46%) agreed to participate, 2 refused, and 40 (51%) were lost to follow up. Those lost to follow up did not respond after at least 3 phone calls/eligible participant.
Of the 36 (46%) participants, the majority were male (27 (75%)). Their mean age was 51.3±10.9 years and the mean years in practice were 20.6±11.7. The majority were both born in the US (19 (53%)) and attended US medical schools (23 (64%)).

Although 35 (97%) reportedly had heard of Ciguatera, only 23 (64%) had ever diagnosed a case, with a mean of 0.14±0.42 cases/year of practice. The majority, 25 (68%), were able to make a correct diagnosis of Ciguatera in response to the classic case presentation; there was no significant difference by age, gender, years of practice, birthplace, or medical school location with respect to making this diagnosis. However, only 6 (17%) of the physician subjects recommended the correct treatment of IV mannitol; again, there was no significant difference by age, gender, years of practice, birthplace, or medical school location with respect to this prescribing treatment.

Furthermore, only 17 (47%) knew that Ciguatera was a reportable disease to the public health authorities. Foreign born physicians ($p=0.02$) and foreign trained physicians ($p=0.08$) were significantly more likely to know that Ciguatera was an officially reportable disease; there were no significant differences in knowledge by age, gender, or years of practice. The majority of the foreign-born and foreign-trained physicians were from the Caribbean, a highly endemic Ciguatera area.

CONCLUSIONS

This study is a small cross sectional study of primary care physicians in an endemic Ciguatera area. The study is limited not only by the small numbers of participants but also by the relatively low participation rate. Nevertheless, this is the only study of its kind in the marine toxin disease literature.

In conclusion, family medicine physicians in an endemic area, have relatively little experience recognizing and diagnosing Ciguatera when presented a classic case to evaluate. Furthermore, these physicians do not know the correct treatment, intravenous mannitol. Therefore, even if they were able to diagnosis a classic acute case, they would not be able to prevent the subsequent occurrence of chronic Ciguatera. The majority (53%) of these family medicine physicians did not know that Ciguatera is a reportable disease to health authorities. Therefore, there is no possibility of primary prevention by removing contaminated fish from the home, eating establishments and fish stores before it is shared with others, leading to the possibility of additional cases of Ciguatera. Furthermore, this finding helps to explain the significant public health under-reporting of this marine toxin associated disease, even in an endemic area.

In order to ensure timely diagnosis, treatment and reporting of Ciguatera, physician education is essential (2, 4-6). Given its acute presentation, education should be targeted not only at primary care providers, but also at emergency rooms. Official reporting to health authorities must be made as simple and efficient as possible given the time constraints on busy healthcare providers.

In Florida, for example, the Florida Poison Information Centers in collaboration with the Florida Department of Health and the National Institute of Environmental Health Sciences (NIEHS) Marine Center at the University of Miami have established a toll free, 24 hour/day, 365 days/year Marine Hotline (888-232-8635) in English, Spanish and Haitian Creole (with access to other languages if necessary). Not only does this Marine Hotline provide diagnosis and treatment information concerning all the marine toxin related diseases for all callers including healthcare providers, official reporting of Ciguatera and other marine toxin disease cases are made to the Florida Dept of Health by the Florida Poison Information Center- Miami on behalf of the healthcare provider.

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HARMFUL ALGAL BLOOMS IN HONG KONG WATERS - WHERE ARE THEY FROM?

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ABSTRACT

Hong Kong is one of the sites with most frequent Harmful Algal Blooms (HABs) in the world. There have been over 500 algal bloom incidents recorded in Hong Kong waters during the past 25 years, with 62 causative species responsible for these events. Why are there so many HABs in Hong Kong waters, and where are they from? Guangdong and Hong Kong share the same coastal waters in Southern China, and each one's HABs affect the other as shown by the example of the historical record of the 1998 bloom. Based on the available data, literature and our own studies, we compared the HAB events and causative species recorded in both Hong Kong and Guangdong waters. The results reveal that of the 67 records for HABs from January 1980 to May 1998 in southern Chinese waters, 24 blooms occurred in the same time period in both Guangdong and Hong Kong waters. Of these, 16 included *Noctiluca scintillans*, which is the most frequently recorded HAB species in both waters. Of the remainder, six were *Gymnodinium/Gyrodinium* species, and the other two were *Prorocentrum sigmoides* and *Chattonella marina*. This overlap of bloom occurrences underlines the need to consider HABs in Hong Kong in the context of a contiguous water mass, with flow moving in both directions between Hong Kong and Guangdong waters. Dapeng Bay (called Mirs Bay in Hong Kong) is where the most frequent HABs have taken place.

INTRODUCTION

In March and April of 1998, the worst harmful algal bloom ever recorded occurred in south China waters[1], affecting about two-thirds of the fish farms so that almost all of the cage cultured fishes died in the affected waters. The causative species was identified as a combination of two new species of *Gyrodinium*, together with *Gymnodinium mikimotoi*.

Hong Kong and nearby south China waters can be considered one of the most active places in the world for harmful algal bloom occurrences. In Tolo Harbour, Hong Kong, there were many harmful algal bloom incidents recorded every year, with the maximum in 1988[2,3]. In nearby Guangdong waters, harmful algal blooms were also frequently recorded in Dapeng Bay, Daya Bay, and Shenzhen Bay[4].

The historical 1998 bloom affected almost all of the east coast of Guangdong (including Hong Kong) at the same time. Why did it occurred on such a large scale, and where was it from. Based on our own case study and historical harmful algal bloom records for the area, an attempt is made to provide answers to these questions.

MATERIALS AND METHODS

The historical records of harmful algal blooms in Hong Kong come from the Red Tide Database of the Agriculture, Fisheries and Conservation Department (AFCD), Hong Kong Special Administrative Region Government. Other sources are referred to in the text, and the remaining data are from our own studies carried out in Junk Bay, Hong Kong, and Daya Bay, Guangdong. The sampling techniques and analytical methodologies followed those of the Intergovernmental Oceanographic Commission[5].

RESULTS

Harmful Algal Blooms (HABs) in Hong Kong Waters

The HAB phenomena in Hong Kong began as early as the 1970's. The first recorded HAB incident (which was caused by *Noctiluca scintillans*) was in the southern bays of Hong Kong[6]. The weather conditions associated with the passage of a typhoon were thought to be one of the factors causing this bloom.

HABs remained a rare event until the later seventies, but the number of outbreaks increased rapidly in the eighties with a peak of 89 total incidents recorded in 1988[7]. Tolo Harbour experienced the majority of HAB incidents which occurred in Hong Kong waters[8-10].

From 1980-1986, a total of 12 fish kills were attributed specifically to HABs with a total loss of 89 tonnes of cultured fish valued at HK$ 4.9 million[8]. A continuous red tide caused by *Gonyaulax polygramma*, lasting for three and half months from early February to May, occurred in Tolo Harbour in 1988. The collapse of the bloom in May caused the whole of the Tolo Harbour waterbody to become anoxic, resulting in a massive fish kill in the Yim Tin Tsai fish culture zone[11]. No PSP causative dinoflagellates had been identified at that time and a very low toxicity level (below 2000 mouse units per Kg tissue) was prevalent in the shellfish samples from Hong Kong waters before 1988[12].

The first recorded toxin-related HAB (caused by *Alexandrium catenella*) was in inner Junk Bay in 1989. The toxicity level of green-lipped mussel increased from 2280MU/Kg when the bloom was first detected to 13500MU/Kg within a week[13]. There is an increasing trend of paralytic shellfish poisoning (PSP) occurrences in Hong Kong. Compared to only 4 cases with 15 people severely affected in the whole of 1992, the PSP intoxication in Hong Kong rose to 32 cases involving 47 people in 1994. In a survey conducted by the Health Department of Hong Kong, 94 (14%) out of the 685 random shellfish samples collected from markets contained toxins above the safety limit of 4000 MU/Kg of tissue[14].

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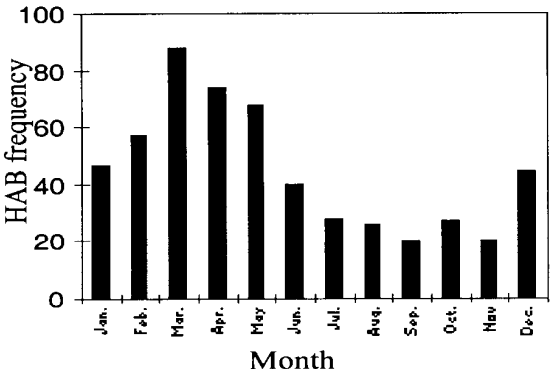

Fig. 1. Monthly HAB frequency in Hong Kong Waters (1977-1998) – data from AFCD database.

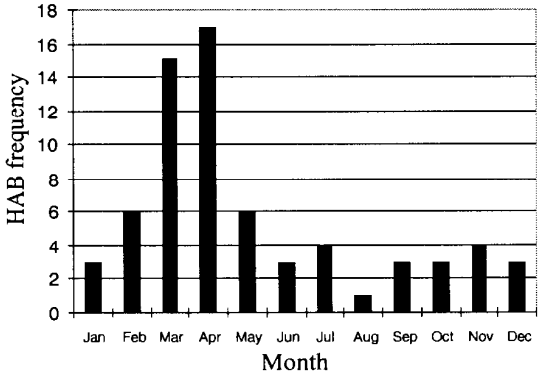

Fig.2. Monthly frequency of HABs in southern mainland China waters (1980-1998).

Over 60 algal species have been reported to be responsible for HABs in Hong Kong waters, the major species being: *Alexandrium catenella, Gonyaulax polygramma, Gymnodinium* sp., *Noctiluca scintillans, Prorocentrum micans, P. triestinum, P. dentatum, P. sigmoides,* and *Skeletonema costatum.* Only one toxin-producing species (*Alexandrium catenella*) has been well documented[3].

A red tide database from the Agriculture, Fisheries and Conservation Department (AFCD) of the Hong Kong Special Administration Region Government shows that a total of 534 identified algal blooms have been recorded from 1975 to July 1998. Of these, 377 (71%) were caused by dinoflagellates and 26 (42%) of the total 62 species responsible for the blooms were dinoflagellates. Most importantly, out of a total of 50 fish kill incidents during this time, 48 were caused by dinoflagellates, and another two were caused by combinations of diatoms and dinoflagellates. So, dinoflagellates were involved in 98% of the fish kill incidents. The mean value of monthly frequency of HAB occurrences over 23 years showed that HAB incidents increased from December on, with the highest number in March to May, and then declined from June with the lowest number in September to November (Figure 1).

Harmful Algal Blooms (HABs) in southern mainland China

With rapidly expanding mariculture and commercial development, HABs/red tides have increased in southern China waters over the last several decades. Some of these HABs/red tides have been spectacular in scale and impact. Looking only at the more recent events, a series of severe HABs/red tides occurred in 1997 and 1998. From October 1997 to February 1998, a bloom of *Phaeocystis globosa*, which lasted nearly half the year, occurred along the eastern coast of Guangdong. The area covered was over 3,000km^2, and the losses due to caged-fish mortality were estimated at over US$ 10,000,000. Another bloom of *Gymnodinium/Gyrodinium* occurred from March to April 1998 covering almost all of the waters of the Pearl River Estuary (including Hong Kong waters). Farmed-fish were devastated and losses of over US$ 20,000,000 resulted.

In a critical review of the related literature, the authors noted that 69 HABs/red tides had occurred in South China waters (exclusive of Hong Kong) since 1980, and that 20 causative species were involved. The major species were: *Noctiluca scintillans, Gymnodinium* sp., *Proboscia alata* f. *gracillima, Trichodesmium erythraeum, Ceratium furca,* and *Pseudo-nitzschia pungens.*

The monthly frequency of HAB occurrences revealed by this review (Figure 2) showed that the peak number was also in March and April.

The HAB/red tide linkage between Hong Kong and southern mainland China waters

The records of HAB/red tide incidents in both Hong Kong and southern mainland China waters show that most incidents of red tide occur from February to July each year, with the maximum in March and April. In Hong Kong waters, red tides occurring from February to July represented 84% of total incidents, and the same was true in southern mainland China waters, where the figure is 77%. Comparing the HAB/red tide incidents recorded from August to January each year, the figures were 16% and 22% respectively.

Dapeng Bay (Mirs Bay), a body of water between Guangdong and Hong Kong, is one of the places with the most frequent red tide occurrences. HABs/red tides in Dapeng Bay represented 54% of the total incidents recorded in southern mainland China waters, and 74% of the total records from Hong Kong waters in Tolo Harbour, Mirs Bay and Port Shelter.

Comparing the data of red tides occurrences from the mainland and Hong Kong side of this water body for about twenty years, *Noctiluca scintillans* was the most common red tide causative species and *Gymnodinium* species were the most harmful species in both data sets.

A special event happened in the Spring of 1998 when an historic record harmful bloom of *Gyrodinium /Gymnodinium* occurred on a large scale in both sides of Dapeng Bay almost simultaneously and resulted in large economic losses.

There are other cases indicating the close relationship between HABs/red tides occurrences in these waters. Thus, a *Gyrodinium instriatum* bloom

which occurred in Shenzhen Bay from 22 April to 2 May, 1998 was also recorded on the other side of the Bay, Deep Bay in Hong Kong, with 1-2 days difference in the time of initiation. The *Ceratium furca* bloom recorded in Port Shelter in Hong Kong in September, 1998 was also recorded on the other side of the Bay, Dapeng Bay, with several days difference in timing.

DISCUSSION

The issue remains as to whether Hong Kong red tides are linked in any way to the mainland. This is difficult to determine since it is difficult to obtain monitoring data or species identification of comparable quality to permit linkages to be established. Thus, even with the massive 1998 red tide, it is not clear if the problem originated in Guangdong and moved to Hong Kong, or vice versa. Nothing can be gained by trying to dissect the records of each past Hong Kong red tide to see whether similar events by the same species occurred first or subsequently in the mainland. The records are simply not that accurate or comprehensive, and more importantly, the exercise would ignore the fundamental fact that it is best to view the situation in the context of a single water mass that links Hong Kong to the mainland, with flow moving in both directions. Just as Hong Kong's efforts to reduce nutrient loadings in its local waters cannot ignore the contributions from the Pearl River and other mainland sources, efforts to understand and manage the red tide problem must acknowledge the possibility of blooms being carried in both directions between Hong Kong and the mainland. Monitoring and management strategies need to emphasize the need for communication and coordination with the mainland.

From a review of the available literature, a chronology of red tide occurrences in the South China Sea (excluding Hong Kong) was prepared (and is available upon request from the authors). Comparison of this chronology with the AFCD database of red tides/HABs for the period of May 1980 to May 1998 reveals that of the 67 records for red tides in southern Chinese waters, 24 events occurred in the same time slot as blooms of the same species in Hong Kong waters. Of these, 16 were *Noctiluca scintillans*, which is the most frequently recorded causative species in both data sets. Of the remainder, six were *Gymnodinium/Gyrodinium* species, and the other two were *Prorocentrum sigmoides* and *Chattonella marina*. This overlap of bloom occurrences between the two data sets underlines the need to consider HABs/red tides in Hong Kong in the context of a contiguous water mass, with flow moving in both directions between Hong Kong and the mainland. Dapeng Bay is where HABs/red tides occurred most frequently in the South China Sea records and, across from this bay are Mirs Bay, Tolo Harbour and Port Shelter, where Hong Kong's most frequent HABs/red tides have taken place.

The Hong Kong Special Administrative Region is a small place which is surrounded by south Chinese waters, and which has contiguous water with Guangdong Province. On such a small scale it is not important where the HABs/red tides originated. The fact is that HABs/red tides affected both areas by water flow in both directions between the two. It has been suggested that the major factor causing such HABs is the high level of nutrient enrichment from pollution. According to data from the Environmental Protection Agency, Guangdong, 2.9 billion tons of sewage was discharged into the South China Sea near Guangdong in 1997, and only 10% of it was treated. The result is an input of massive amounts of N and P into the seas adjacent to Hong Kong. Moreover, in aquaculture areas on the mainland, the fish are fed vastly in excess of their needs, and this leads to heavy eutrophication of the culture zones and adjacent waters. The result is over one metre of soft, organic-rich sediment in many areas. Turbulence caused by wind or other events results in N and P from the sediment becoming available as a rich nutrient supply for phytoplankton growth.

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SIMILAR BLOOMS BUT DIFFERENT RESULTS — A MITIGATION EXPERIENCE

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ABSTRACT

Harmful blooms of *Phaeocystis globosa* have occurred in Raoping, on the north-east coast of Guangdong Province, China, almost every year in recent times. Two of the biggest blooms happened in October 1997 and the summer of 1999. In the 1997 bloom, all fish and shrimp in about 4,000 culture cages died and millions of US dollars of economic losses resulted. In contrast, during the 1999 bloom, in the same area, and with the same cell density of *Phaeocystis globosa*, all fish cages were towed away from the embayments, where the bloom developed, to offshore open waters, which were not affected by the harmful bloom. Thus, only very small losses were caused by the bloom and accordingly the two blooms had markedly different results. This event provides a good demonstration that movement of cultured stock is a practical and cost effective mitigation measure for preventing the effects of harmful algal blooms and minimizing fish kills. Several lessons can be learned from this study: first, fishmen should have a good knowledge of specific harmful blooms, so that they can take action as soon as the bloom is initiated; second, fish pens should be smaller in size and easier to tow, so that they can meet the requirements for fast and cost effective action; and finally, proper and safe refuge areas should be selected.

INTRODUCTION

From October 1997 to January 1998, a large scale *Phaeocystis* bloom occurred in South Fujian and along the eastern coast of Guangdong Province, China. The seawater was discoloured dark brown, and a thick mucilaginous foam covered the water. All the fish in 3,800 culture cages were killed. The losses due to these fish kills reached US$ 700 million. This was the first record of a *Phaeocystis* bloom in China.

Phaeocystis (Haptophyta) is a cosmopolitan phytoplankton genus occurring as solitary cells or colonies. So far nine species have been identified[1]: eight colony-forming species and one solitary cell species. These nine species have been classified as two well defined species[1]: *P. pouchetii* (including presumably a junior synonym *P. globosa*), which has been well described in both the motile single cell and non-motile colony stage, and *P. scrobiculata*, described on the basis of its motile phase only[2]. Based on colony shape, arrangement of the cells in the colony, and temperature preference, *P. pouchetii* and *P. globosa* has also been considered as two different species[3]. *P. pouchetii* is a cold water species which shows preference for temperatures between 0 and 14°C, while *P. globosa* shows a preference for temperatures between 4 and 22°C. Three colony-forming species, *P. antarctica, P . pouchetii,* and *P. globosa* have been identified based on 18S small subunit ribosomal RNA sequencing[4].

The species which caused large fish kills in China was identified as *P. globosa,* s a large colony-forming species. There are two different stages in the life cycle: the colony forming stage and a single-celled motile stage. In the colony stage, hundreds of thousands of cells are embedded in a spherical mass of mucilage. The diameter of the colony can reach 3 cm, and it is visible to the naked eye. Each cell in the colony is 3-6 μm long, contains 2 parietal yellow-green chloroplasts, but is without flagella and haptonema. The cells in the motile unicellular stage contain two flagella and one short haptonema.

In July, 1999, another *Phaeocystis* bloom occurred in the same area. Fortunately fishermen had learnt from previous experience of the earlier bloom, and so there was almost no loss because the fish pens were moved away from bloom area. This resulted in different impacts from the two blooms, one involving extensive fish kills and the other no fish kills.

Study Site

The study was carried out in Zeling Bay, Raoping, Northeast of Guangdong Province, China (Figure 1).

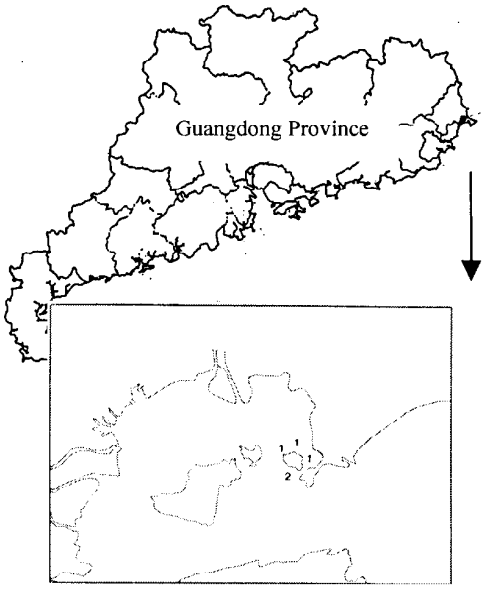

Fig. 1. Map showing the location of the blooms (Raoping), fish farms (1), and refuge area (2).

Harmful Algal Blooms 2000
Hallegraeff, G.M., Blackburn, S.I., Bolch, C.J. and Lewis, R.J. (eds)
Intergovernmental Oceanographic Commission of UNESCO 2001

RESULTS

1. 1997 bloom

This bloom was large in scale and long lasting. The bloom was initiated in October 1997, developed its maximum concentration in late November 1997, and dissipated in February 1998. The coastline of two provinces, Guangdong and Fujian, were affected. The distribution of the bloom was clearly detected by SeaWiFS[5].

The environmental conditions during occurrence of the bloom have been studied[6]. Water temperatures in November 1997, when the bloom was developing, were between 18 and 24^{0}C, which was much higher than in other years (18 to 20^{0}C). The yellow brownish gelatinous colonies of *Phaeocystis globosa* (Figure 2) aggregated in the surface seawater, and a great deal of white sticky foam with a bad smell was distributed along the coastline. The biggest colonies were 3 cm in diameter, and looked like small balloons in the water.

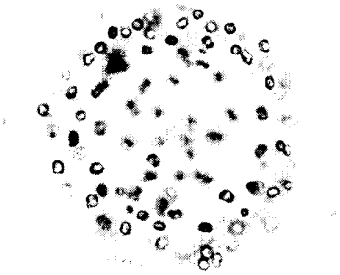

Fig. 2. Colony stage of *Phaeocystis globosa* under light microscope.

The most severely affected area was Zeling Bay, one of the biggest aquaculture areas in eastern Guangdong. Massive fish kills occurred from 20 to 27 November 1997, when the bloom developed and cells aggregated to their maximum density. The colonies were so dense that the fish cage nets were severely blocked, and almost all of the cultured fish, mainly *Cephalopholis* sp., *Pagrosomus major*, *Seriola* sp. and *Thunnos thynnus*, died.

Nothing was done about the bloom, even though there was one month from bloom initiation to fish kills. Fishmen had no idea what to do about the bloom; they did not know what had happened and what would be affected, because they had never seen such a large bloom before.

2. 1999 bloom

Another *Phaeocystis* bloom occurred in July 1999 in the same area. During this bloom, the area affected and the cell density were the same as those of 1997. However, because all fish pens were towed away from bloom affected waters, no fish kills were reported.

DISCUSSION

Phaeocystis blooms have been observed to occur more and more frequently and intensively in the North Sea over the past twenty year[7]. Studies have revealed that this increasing trend is closely related to eutrophication in the North Sea, mainly due to the increased load of nitrogen and phosphorus from the rivers Rhine and Meuse[8]. A *Phaeocystis* bloom has been reported also from Kuwait Bay, and nutrient enrichment was considered to be a key factor in the development of the bloom[9]. In Zeling Bay, enrichment of nutrients has also been noted in the mariculture area (Table 1)(Lin, per. comm.), especially phosphorus, where the mean concentration in aquacultural area waters was more than twice that in control waters. In the sediments, the mean concentration of phosphorus in the aquacultural area was almost 20 times as high as that in the control area.

Table 1. Comparison of Nutrients in Aquacuture and Control Area (_g/L)

	Aquaculture area		Control area	
	concentrations	mean	concentrations	mean
Seawater: DIN	30.1-130.62	87.86	54.32-187.74	124.6
PO_4-P	5.57-68.75	20.65	0-23.5	9.86
Sediment: DIN	1.05-1.40	1.27	-	1.16
PO_4-P	7.72-13.80	10.15	-	0.54

Phaeocystis blooms have been reported to be not directly toxic to fish[10] but the blooms are avoided by fish[11]. However, although wild fish populations can avoid *phaeocystis* blooms, caged fish do not have this ability. The large scale caged fish kills in Zeling Bay might be the biggest fish kills caused by *Phaeocystis* blooms anywhere in the world. Further study detected hemolysin from the species. The most potent component was identified as a mixture of glycolipids, with the major constituent being 1'-O-heptadecadienoyl-3'-O-(6-O-_-D-galactopyranosyl-_-D-galactopyranosyl)-glycerol[12].

After several decades of study of harmful algal blooms, more and more attention is now being paid to mitigation methods. Harmful algal blooms can kill fish, and some are shellfish and fish toxin producers. Preventing harmful blooms and minimizing their harmful effects are important objectives for HAB studies.

Several mitigation practices have been applied world-wide: Application of chemicals such as copper sulfate[13], aponin[14], and ozone etc.; spraying of clays[15]; filtration of bloom cells using siliceous earth; and

biological control such as use of zooplankton to eat the algae as food, and the use of highly specific bacteria, viruses, parasites and other algae to destroy specific algal cells.

Even though these methods have been tried in mitigating blooms, there is some difficulty in their acceptance world-wide. Chemicals are not environmentally friendly and they can cause secondary pollution to marine ecosystems. Some are very expensive and difficult to apply in open waters. Some of them are less effective. Biological control might be a good choice, but specific and effective strains (bacteria, viruses, and parasites) are very difficult to identify and isolate.

Moving mariculture pens away from waters affected by harmful blooms is a useful *in situ* mitigation method in the mariculture industry and proper application of this method can avoid fish kills. This method has been widely used by fishermen, and has been recommended in Norway[16]. Fish pen towing has been the top choice to protect fish from harmful algal blooms in Southern China waters also. It has proved easy to operate and cost effective. In the record bloom in Hong Kong in March/April 1998, almost all the fish died in culture cages in bloom affected areas. However, several fishermen towed their fish pens to safe refuge waters during the bloom, and the fish survived.

Between 1997 and 1999, two large *Phaeocystis* blooms impacted the eastern coast of Guangdong, China. Large scale fish kills and significant economic losses were recorded in the first bloom. But there were no fish kills and no significant losses in the second bloom. Why was there such a large difference?

Firstly, fishermen need to have a good knowledge of specific harmful blooms, so that they can take action as soon as a bloom is initiated, and tow their nets away before the bloom intensifies and fish kills occur. In the case of the second *Phaeocystis* bloom, the fishermen had learnt from their experience with the first bloom, and towed their fish pens to refuge waters as soon as *Phaeocystis* colonies could be seen with the naked eye.

Secondly, fish pens should be smaller in size and easier to tow, so that they meet the requirements for fast and cost effective action. In China, the commonly used fish cages are small in size (the net pen rafts are about 200 to 250 m^2), and so could be easily towed. However the cages are mostly owner-built with easily assembled materials, and thus are not strong enough for towing.

Finally, proper and safe refuge areas should be selected and the fishmen notified where they are. *Phaeocystis* blooms form a belt in coastal inshore waters where the seawater is relatively stable, and so allows the aggregation of algal cells. The strong flushing action of open waters, where cell numbers are likely to be lower is thus the best choice for refuge waters.

ACKNOWLEDGEMENT

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MITIGATION BY CYSTEINE COMPOUNDS OF RHEOTOXICITY, CYTOTOXICITY AND FISH MORTALITY CAUSED BY THE DINOFLAGELLATES, *GYMNODINIUM MIKIMOTOI* AND *G.* CF. *MAGUELONNENSE*.

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ABSTRACT

Two *Gymnodinium* species were studied for their rheotoxic and cytotoxic activity. *G. mikimotoi* was found to have marked rheotoxicity and relatively low cytotoxicity (measured as haemolytic activity), while *G.* cf. *maguelonense* showed both effects. The effects of cysteine compounds, known as mucolytic agents and as protectors against O-radical damage, were investigated as a possible mitigation tool. N-acetyl-L-cysteine and ethyl-L-cysteine ester reduced: fish mortality; rheological yield stress; haemolytic activity. Beneficial action started at 0.01 mMolar. L-cysteine is also active. The study suggests that addition of cysteine or its compounds to the water may be an economically feasible mitigation tool.

INTRODUCTION

Some phytoplankton secrete polymers that make the water more viscous [1, 2]. This effect can asphyxiate fish by reducing water flow and hence oxygen availability at their gills [3], when it is termed "rheotoxicity". Some exosecretions of harmful phytoplankton are also associated with "cytotoxicity", which can be assayed by measuring their haemolytic activity [3,4,5]. The aim of the present study has been to apply the results of previous studies on the rheology [6] and other ichthyotoxic [7,8] properties of seawater including its phytoplankton, so as to mitigate the total toxicity to fish during HABs of two ichthyotoxic *Gymnodinium* species. To assay toxicity, we used the survival time of fish. Both *Gymnodinium* species showed haemolytic activity, while the rheology of the water constituted a second indicator of its biological quality in relation to fish survival. We will show that the use of mucolytic substances, derivatives of cysteine [9, 10, 11], allowed the toxic properties to be markedly reduced in both cases.

MATERIAL AND METHODS

The culture of *Gymnodinium mikimotoi* (*G. mik*) studied (Stock GM95TIN) had been isolated at Tinduff (Rade de Brest, France) in 1995, while that of *G.* cf *maguelonense* (*G. mag*), isolated by E. Erard-Le Denn, originated from Tunisian waters, north of Sfax in the Gulf of Gabès in 1994, after an intensive fish kill. Both cultures were cultivated at 18°C, and illuminated 12h/12h at 60µmole quanta.$m^{-2}.s^{-1}$ at IFREMER, Brest. Enriched f/2 medium was used, made using ocean water of salinity 36 ppt. All cultures were investigated at the end of their exponential phase. Cells were enumerated by optical microscopy.

Seabass, *Dicentrarchus labrax*, of about 80 g, reared under controlled conditions on untreated feed by the Department of Living Resources, IFREMER, were used for investigation both of survival and of flow through the gills.

Fish survival tests were carried out on batches of three fishes in 3 litres of algal culture or filtered seawater under a battery of fluorescent lamps. Investigations were furthermore done with and without aeration, so that information about the effect of the oxidative environment on fish survival might help elucidate any role of free oxygen species associated with algal toxicity. Survival time was taken as that until the fish turned ventral side up for the first time. Survival time in seawater without aeration was considered to be limited by available oxygen content.

Haemolytic activity in cultures was determined using the method of Arzul *et al.* [12], slightly modified to obtain a more sensitive repsonse. This test reveals the presence of substances able to lyse cell membranes. We used horse red blood cells (RBCs) from *Pasteur-Sanofi*, Paris, or human RBCs when horse RBCs were unavailable.

Some *G. mik* culture was subject to sonification using a Deltasonic® apparatus for 8 minutes at 42 kHz (Table 2b).

Rheological measurements of flow through fish gills under differing hydrostatic pressure differences were made following Jenkinson & Arzul [3]. The same gill-flow apparatus was used. We estimated the yield stress y of the culture or seawater, by solving for y in the equation

$$P_t = (P_0 - y)e^{-(k/\eta)t} + y$$

where P_0 and P_t are the hydrostatic pressure at times 0 and t respectively. η and k (both assumed to remain constant during an individual flow trial) are the dynamic viscosity (in the limit of high shear rate) and k is a flow coefficient for the geometry of the gill passages.

The L-cysteine (C), ethyl L-cysteine ester (EC) and N-acetyl-L-cysteine (AC) were provided by Sigma and used as dilutions of stock solutions in seawater.

RESULTS

Effects of aeration, AC and EC on fish survival in Gymnodinium *culture*

We made a first batch of experiments to investigate the effects of aeration and EC. In this series of experiments, the *G. mik* was of concentration 21 million cells L^{-1}, and that of *G. mag* was 6.4 million. Both were in stationary phase. Table 1 shows the results.

In reference trials using filtered seawater instead of culture, and no aeration, the fish survived 90 or 95 minutes, with the appearance of asphyxia, the opercula being open and rigid. It is considered that death was induced by oxygen depletion in the 3-L volume of water. In 21 million .L^{-1} of *G. mik*, aeration (with or without EC) eliminated observed mortality, while EC without aeration also prolonged survival.

In contrast, *G. mag* was the more toxic. 3.0 million L^{-1} (Table 1*a*) and 6.4 million L^{-1} gave only ~25 minutes' survival (Table 1*b*). Importantly, the respective modulation by aeration and EC on the effects of *G.* cf *mag* were practically reversed with respect to those of *G. mik*: aeration increased survival only moderately, while EC prolonged survival markedly (with or without aeration).

At 0.1, 1 and 5 mM EC, fish survived in *G. mik* culture, (*G mag* was not tested at this concentration) 132, 88 and only 7 minutes respectively (Table 1b), suggesting toxicity by the compound itself.

Effects of AC and EC on Gymnodinium *survival*

In preliminary trials, using 18 million L^{-1} G. *mikimotoi*, the fishes showed high agitation, perhaps caused by irritating material produced by the algae.

In this series of experiments, no aeration was employed, but the flasks were brightly illuminated by fluorescent lamps. The *G. mik* was of concentration 23 million cells L^{1}, and that of *G. mag* was 3.0 million. Cells were counted 1 hour after addition of compound. AC was tested up to 0.1 mM, and had little effect on cell numbers of either *Gymnodinium* species. EC was tested up to 5 mM, and between 0 .01 and 0.1 mM, no cells *G. mag* were apparent, but those of G. *mik* fell less markedly: by 30% at 0.01 and 0.1 mM, by 48% at 1 mM and by 57% at 5mM. However, it was noticed that in 1 mM EC, numerous cells of both species rounded up and became immobile.

Haemolytic activity of the Gymnodinium *spp., and its reduction by cysteine compounds*

The haemolytic activity of cultures after addition of three cysteine compounds was tested. *G. mik* at 23 million cells L^{-1} in stationary phase, and *G.* cf *mag* at 10 million cells L^{-1} in exponential phase were used. Horse red blood cells (RBCs) were employed. *G. mik* showed no detectable haemolytic activity. By contrast, *G.* cf *mag* was markedly haemolytic.

Figs 1A and 1B show the haemolytic activities measured in cultures of both *Gymnodinium* species in the presence of cysteine compounds. C at 0.1 and 0.5 mM reduced haemolytic activity, as did EC at levels from 0.1 to 0.5 mM. Haemolytic activity was completely suppressed by 10 and 20 mM EC, as well as by AC at 0.1 and 0.5 mM. At the highest concentrations tested C and AC themselves became haemolytic. *G. mik* culture, although only weakly haemolytic, showed the same trends. At the lowest concentrations tested: 0.01 mM, EC and AC were only weakly haemolytic (data not shown).

TABLE 1. Fish survival in Gymnodinium *spp. culture.*
a) Batch 1 of experiments : effect of EC and AC

Material	Survival (min)	*n*	SD	*P*
Seawater	90	3	10	
G. mik 23 million/l	53	3	12	Cont.
G. mik + 0.1 mM EC.	132	3	43	0.04
G. mik + 1 mM EC.	88	3	11	0.01
G. mik + 5 mM EC	7	3	0.5	10^4
G. mik + 0.01 mM AC	81	2	4	N.S.
G. mik + 0.1 mM AC	129	3	33	0.02
G. mag 3.0 million /l	24	3	4	Cont
G. mag + 0.01 mM EC	55	3	2	0.0004
G. mag + 0.1 mM EC	88	3	19	0.005
G. mag + 0.01 mM AC	26	3	3	N.S.
G. mag + 0.1 mM AC	58	2	10	0.01

b) Batch 2 of experiments : Effect of EC and aeration

Material	Survival (min)	n	SD	*P*
Seawater	95	3	68	
G. mik 21 million/L	34	3	0.6	Cont.
G. mik + bubbling	No mortality (n=3)			
G. mik + 0.7 mM EC	55	3	6	0.004
G. mik + 0.7 mM EC + bubbling	No mortality (n=3)			
G. mag 6.4 million/L	<25	3		Cont.
G. mag + bubbling	<25	3		
G. mag + 0.7 mM EC	No mortality (n=3)			
G. mag (Treatment 1)	105	2	46	

Treatment 1: Fishes in previous class then transferred to bubbling without EC ; Cont.: control.

Effect of cysteine compounds on flow of Gymnodinium *culture through fish gills*

For *G. mik*, the yield stress, *y*, (corrected for filtered seawater zero) varied from 3.3 to 3.8 mm water for 21 and 23 million cells L^{-1} respectively (Table 2a,b). Previous values for the same algal stock [3] were similar for slightly denser culture. For *G. mag*, corresponding values were comparable, 0.7 and 2.2 mm water for 10 and 21 million cells L^{-1}, respectively.

Addition of 0.01 mM AC reduced y by 80% in *G. mik* and 66% in *G. mag* culture, while 0.01 mM EC reduced y 84% also in *G. mag* culture. Bubbling reduced *y* in *G. mik* culture 65%.

Table 3 summarises the action thresholds of the cysteine compounds tested.

Price of cysteine

For L-cysteine anhydrous and L-cysteine monohydrate, we were quoted lowest prices of 8.50 to 13 US dollars per kg, usually with a minimum order of 1 or 1.5 tonnes.

TABLE 2a. Yield stress of culture (y). Batch 1

Material	*y* (mm water)	n	SD	*P*
G. mik 23 million L^{-1}	+ 3.80	1		N.S.
G. mik + 0.01 mM AC.	+ 0.76	3	0.008	0.016
G. mik + 0.1 mM AC.	+ 0.69	3	0.097	N.S.
G. mag 10 million L^{-1}	+ 2.20	3	0.045	0.001
G. mag + 0.01 mM AC	+ 0.75	3	0.016	0.05
G. mag + 0.01 mM EC	+ 0.36	3	0.07	N.S.

TABLE 2b. Yield stress of culture (y). Batch 2

Material	*y* (mm water)	n	SD	*P*
Seawater	0.22	6	0.31	
G. mik 21 million L^{-1}	3.50	2	0.50	$<10^{-4}$
G. mik + 0.07 mM EC.	0.05	4	0.57	N.S.
G. mik after bubbling	1.15	2	0.05	0.01
G. mik bubbl + sonific'n.	0.25	2	0.25	N.S.
G. mag 6.4 million L^{-1}	0.93	3	0.31	0.02

P: *Probability that value differs from seawater; y is expressed as difference from seawater in* a, *but in absolute values in* b.

SCIENTIFIC DISCUSSION

Comparable concentrations of *G. mik* gave rheological yield stress similar to that of *G. mag* (Table 3), but showed markedly less haemolysis-associated cytotoxicity. However, *G. mik* may attain higher bloom concentrations and therefore be of comparable danger to fish and shellfish.

At the lowest concentration tested, 0.01 mM, both AC and EC reduced firstly fish mortality *in vitro*, secondly *y*, and thirdly (as did cysteine) haemolytic activity. Even lower concentrations should be tested in future.

That aeration reduced both *y* and fish mortality in *G. mik* culture (Tables 1b, 2b) may reflect firstly mechanical rupture of polymers and increased O_2 levels reducing the effect of poor gas exchange at the gills. In contrast, bubbling did not reduce fish mortality in *G mag* culture. The beneficial affects on O_2 availability to the fish and polymer rupture may have been offset by a greater tendency to free-radical-mediated cytotoxicity associated with haemolytic activity [13]. So aeration as a mitigation strategy may be inappropriate in haemolytic blooms with free O-radical activity.

AC is prescribed for humans to dissolve mucus at a dose of 0.6 g/day for persons >7 years old [14]. Assuming a retention time of 1 day and a soft tissue weight of 30 kg, this gives a tissue concentration of 3mM. Thus, use of 3mM or less to aquaculture water is likely to pose little danger to fish consumers. Also severe harm to the environment seems unlikely.

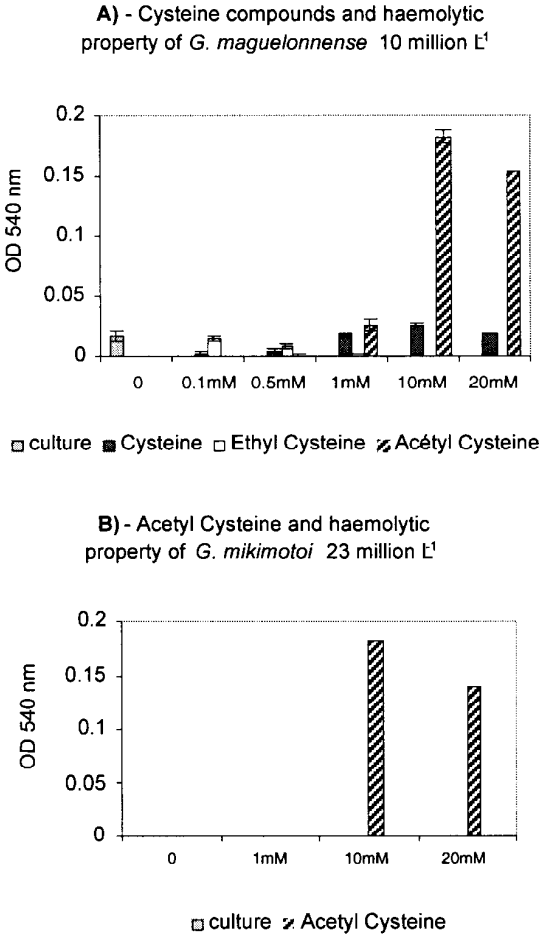

Fig. 1. Reduction in haemolytic activity (measured as optical density at 540 nm) of Gymnodinium mag *(A) and* Gymnodinium mik *(B) with different cysteine compounds.*

These results show that both *Gymnodinium* species were rheotoxic and cytotoxic. Furthermore, all the cysteine compounds tested, not only reduced fish mortality, but also reduced both haemolytic activity (related to cytotoxicity) and rheological thickening. These cysteine compounds were found to protect fish against lethal factors secreted by both algal species, probably mainly by acting both physically and chemically on these secretions.

The live and dead fish used in this study always had very clean gills before contact with algal cultures. Thus our rheological results reflect dissolution by cysteine compounds only of algal polymers and not of fish mucus. When irritated by harmful algae, mucus secreted by fish has frequently been suggested as causing asphyxiation [14]. As cysteine compounds lyse mammalian and algal mucus, they may furthermore dissolve fish mucus, adding to their mitigation power. So cysteine compounds should also be tested for dissolution of fish mucus. As when added to feed [11], cysteine compounds added to the water might then protect fish also from the effects of

TABLE 3. *Threshold concentration (mM) at which cysteine compounds start to eliminate the effects due to both Gymnodinium species*

Compound	Haemolysis		Gill flow		Fish survival		Action on *Gymnodinium*	
	G. miki	*G. mag*	*G. miki*	*G. mag*	*G. miki*	*G. mag*	*G.miki*	*G.mag*
AC	(1)	≤0.1	≤0.01	≤0.01	≤0.01 to 0.1 (2)	0.01 to 0.1	≤0.01	>0.1
EC	(1)	0.1 à 0.5	n.t.	≤0.01	≤0.1	≤0.01	0.1<1<5 (2)	0.01 à 0.1
C	n.t.	≤0.1	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.

Notes: (1) - Culture not haemolytic ; (2) - effect gradual ; n.t. - not tested.
AC: L-acetyl cysteine ; EC: Ethyl cysteine ester ; C: Cysteine

excess gill mucus, secreted in respons to diatom-spine irritation [11].

ECONOMIC CONCLUSIONS

Estimated cost to treat 1 m^3 water

Since the mitigation effects of AC and EC against *Gymnodinium* spp. rheotoxins and cytotoxins start at 0.01 to 0.1 mM, that is ~1.55 to ~15.5 g/m^3. If cysteine produces the same effects for the same concentration, the price will be 1.4 to 14 US cents/m^3 of water.

Estimated cost to treat 1 tonne fish

Salmonids are frequently stocked at densities ~10 kg/m^3. The cost of treatment would then be 1.40 to 14 USD/tonne of fish. The possible error is large, so pilot trials are needed, as well as investigation of cysteine residence time in the environment.

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AN INTERNATIONAL AND AUSTRALIAN AGENDA FOR MINIMISING THE SPREAD OF HARMFUL ALGAL BLOOMS VIA SHIPS BALLAST WATER

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ABSTRACT

One of the principal ways in which the microscopic organisms that make up algal blooms are spread is via ships ballast water. Ships must take on ballast water, usually when not carrying cargo or carrying only a small amount of cargo, to enable them to operate safely and efficiently. When taking on cargo, usually in a port far removed from where they took on the ballast water in their tanks, they discharge part or all of this water. This ballast water frequently contains a variety of marine species and organisms which often survive the original uptake, voyage and discharge and may, if conditions are suitable, then establish in the new environment. This link between ships ballast water and translocation of marine species is well documented [1].

The means by which the consequences of spread of marine species by ships ballast water can be minimised has been the subject of much international, and Australian, scientific and management effort over many years. Real progress has been made in recent years and this, along with the future agenda, are discussed.

INTRODUCTION

Algal blooms are one of the more prominent and major concerns arising from the thousands of marine pests and diseases that are translocated throughout the world daily by ships. Indeed, because of the way modern ships ballast (take on ballast) and deballast (discharge ballast water), the construction of modern ballast tanks, the increasing speed of ships and therefore shorter time intervals between ports, and the general increase in world shipping movements, the extent of such translocations, and their consequences are increasing considerably.

However research has demonstrated that there is no quick fix to the problem. Of course, shipping remains fundamentally important to global commodity trade. In fact it carries over 80% of international trade [2]; in the case of Australia it is some 97% [3]. Accordingly, the challenge is to find a means of rendering the ballast water harmless through treatment and/or management processes that are safe, practical, cost effective and environmentally acceptable.

Ships must take on ballast water to keep them stable and enable them to operate efficiently during voyages when they have little or no cargo on board.

- each year an estimated 10 billion tonnes of ballast water is carried around the globe by international vessels [4].
- it is estimated that on average, more than 3,000 species of plants and animals are being transported daily throughout the world in ships ballast tanks [5].
- in Australia's case over 11,000 vessels visit our shores each year from over 600 overseas locations arriving at 65 ports around the country. These vessels alone discharge over 150 million tonnes of ballast water; some vessels discharging over 100,000 tonnes each voyage. On top of this we have coastal voyages where there is a further 35 million tonnes of ballast water, and associated hitchhikers, translocated between Australian ports [6].

CONSEQUENCES OF INTRODUCED MARINE PESTS

Now whilst not all introduced organisms have the potential to cause harm to the marine environment, or to human health, many countries have already suffered severe impacts from those that do cause harm; for example:

- the comb jellyfish (*Mnemiopsis leidyi*) from the Atlantic which eliminates plankton and destroys fisheries; as has occurred in the Black and Azor Seas causing the near total collapse of the sprat and anchovy fisheries;
- the zebra mussel *(Dreissena polymorpha)* which has cost billions of dollars for pollution control and cleaning of fouled underwater structures and water intakes in the Great Lakes area of North America;
- the Chinese mitten crab *(Eriocheir sinensis)* which has reached plague proportions in some waterways in Europe;
- and there are many more including toxic dinoflagellates, such as *Gymnodiun catenatum*, which result in serious algal blooms in various parts of the world especially in SE Asia and pose a potentially serious threat to seafood production and public health.

Australia has not been immune from the problem and in some locations is suffering severely as a consequence. We now have well established populations of several serious and harmful introduced marine invaders including the Northern Pacific Seastar *(Asterias amurensis);* Japanese kelp *(Undaria pinnatifida)*; Mediterranean fan worm *(Sabella spallanzanii)*; toxic dinoflagellates, including *Gymnodinium catenatum, Alexandrium catenella* and *Alexandrium minutum,* and several others.

Indeed a recently released study [7] of Port Phillip Bay in the State of Victoria revealed that up to 178 exotic marine species have been introduced into that Bay and that on average 2-3 new exotic species have been establishing there each year. Several of the introduced species are classified by Australia as target 'pest' species [8], having known or potential economic and/or ecological impacts in Australia or overseas.

Of particular concern are toxic dinoflagellates, such as *Gymnodinium catenatum, Alexandrium catenella and Alexandrium minutum* which affect shellfish aquaculture farms by contaminating stocks with toxins thus resulting in economic loss to commercial operators and potentially human health concerns, unless properly managed. Periodic closure of commercial shellfish farms have

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occurred particularly in the Derwent and Huon estuaries in Tasmania.

The principal underlying threat from introduced marine pests that makes them quite different from almost all terrestrial invaders, is that once a species has become established it is virtually impossible to eliminate it and there is almost always lasting adverse consequences. The International Maritime Organisation has stated that the consequences of the translocation of invasive marine species by shipping is worse than that of oil, sewerage and garbage pollution by shipping [9]. Unlike these pollutants which can normally be cleaned up, invasive marine species once established are virtually impossible to safely and cost efficiently eradicate.

MINIMISING THE RISKS - AN AUSTRALIAN PERSPECTIVE OF AN INTERNATIONAL PROBLEM

Now after many years of concerted effort, we know that there are ways of minimising the risks of ballast water translocating unwanted marine pests, including dinoflagellates, which can subsequently establish populations in new territories with sometimes drastic consequences.

Australia has been heavily committed over the last decade, both domestically and internationally, in developing management measures in cooperation with stakeholders to achieve this, whilst at the same time investigating longer-term solutions - the so-called 'silver bullets'.

These management measures can be taken at one or several points in the 'continuum of ballast water management' (this refers to the three principal steps in the ballast water cycle – uptake, transportation, discharge).

The actions that may be able to be taken at each of the three steps include:

(1) At uptake of ballast water - not taking on ballast when there is an algal bloom; when there is a known outbreak or colonisation of a harmful unwanted marine pest; not taking up sediment from the bottom of the port.

(2) During the voyage – exchanging ballast in mid ocean (there are acceptable means of doing this for most vessels); by treating the ballast water (technologies such as heat treatment and use of UV are beginning to emerge).

(3) At discharge of ballast water – by not discharging in enclosed or shallow waters or near seafood production facilities.

Whilst none of these actions is in itself a total solution, action taken at one or more of these three points in the 'continuum of ballast water management' can greatly reduce the risk.

Additionally a range of other treatments – such as micro filtration; UV treatment; hydro cyclonic separation; and many more – are under active development throughout the world at this time, with some showing considerable potential.

Australia has been one of the leading nations both individually and as a member of the International Maritime Organisation (the IMO) for the last 10 years in addressing this matter. While generally good progress has been made internationally (and this is where it must occur, with shipping being international in nature) we are having a lot of difficulty getting over the next major hurdle – that is, the IMO putting into place a legally binding international arrangement for ballast water management. However, progress towards this objective is continuing and we should expect to see it in the next two to three years.

KEY INTERNATIONAL AND AUSTRALIAN DEVELOPMENTS

Let's now look at the key initiatives taken internationally and by Australia over the last 10 years.

International Action

As mentioned earlier, because this is an international issue it requires international action; this has been occurring principally through IMO and its Marine Environment Protection Committee (MEPC). The IMO, a United Nations organisation, is charged with the responsibility for international shipping and its affect on the marine environment.

Whilst IMO first recognised the problem in 1973 little tangible change occurred until the 1990's, when in 1991 the MEPC adopted its "Guidelines for Preventing the Introduction of Unwanted Aquatic Organisms and Pathogens from Ships Ballast Water and Sediment Discharge"[11] 'the Guidelines'. Incidentally, these guidelines were based on Australia's guidelines introduced in 1990. Since then a range of other important initiatives has been taken.

1973	IMO first recognises the so-called 'ballast water problem' during the International Conference on Marine Pollution [10].
1989	Issue first placed on agenda of MEPC.
1991	MEPC completes and adopts the Guidelines [11].
1993	IMO adopts the 1991 MEPC Guidelines [12].
1993	IMO/MEPC establish a specific working group to consider the ballast water issue.
1997	Revised/enhanced Guidelines completed and adopted by IMO [13].
1999	MEPC sets target of 2000/2001 for introduction of mandatory international requirements.
2000	MEPC continues to consider the issue and draft possible legally binding arrangements.

Australian Action

1989	Australia starts to experience the first affects of the problem, addresses it as a quarantine (introduced marine species) issue and refers it to MEPC.
1990	Australia introduces world's first national ballast water management guidelines [14] for all international shipping visiting its ports.
1990	Australia commences a sampling and testing program of international vessels visiting its ports.
1994	Lead Australian agency for this matter, the Australian Quarantine and Inspection Service

(AQIS) convenes a national Ballast Water Symposium to bring together all stakeholders to address the need for an integrated approach.

1996 Australian Ballast Water Management Strategy released by the Federal Government.

1996 Australian Ballast Water Management Advisory Council established.

1996 New R&D program established by AQIS.

1996 Coastal Ballast Water Working Group established, to examine the role of coastal shipping in translocations from port to port within Australia.

1998 Australian government, through its Oceans Policy Statement [5], made a series of announcements concerning their concern at the increasing introduction of invasive marine species and organisms in ships ballast water.

1998 Australian shipping industry begins paying a levy to support ballast water R&D.

1999 AQIS releases "Ballast Water Requirements Australia", a maritime awareness package [16] advising international shipping of Australia's requirements.

1999 Australian government announced mandatory ballast water arrangements for international shipping would be introduced by mid 2001.

1999 Australia, introduces mandatory reporting by all vessels visiting Australian ports regarding handling of their ballast water. AQIS also introduces associated compliance monitoring requirements.

2000 Australia, designing and trailing a ballast water Decision Support System (DSS) to support its intention to introduce a full mandatory regime by mid-2001.

A few explanations are required of some of the foregoing initiatives. Firstly, it is to be noted that early Australian efforts largely concentrated on international shipping ballast water and with a specific focus on toxic dinoflagellates. This was based on the belief that ballast water was the principal vector and toxic dinoflagellates the major concern, in light of their potential effect on human health. However, in more recent years it has become obvious that translocation of marine species between Australian ports, still principally by ballast water, is an equally serious issue and hence greater efforts are now being focussed in this direction. The target list of species of major concern to Australia (and which underpin Australia's Ballast Water Management Strategy)[17] has also been extended to include other vectors – principally hull fouling.

In the early 1990s AQIS began a substantial sampling and testing program of international shipping visiting Australia. This involved taking ballast water samples from targeted international vessels and testing them for a variety of species, but principally toxic dinoflagellates. This enabled us to build up somewhat of a profile of risk based on ports, voyages, ships etc and hence reach some prima facie conclusions concerning the risk, both direct and comparative, to Australia of individual ships on each voyage. This sampling and testing program was an important element of the R&D program as well as providing base data to assist putting in place appropriate management measures to reduce the risk.

The Australian Ballast Water Management Guidelines introduced by Australia in 1990/91 was, at that time, the first fully national approach by any country in the world.

These Guidelines have subsequently been improved, some aspects of them – the requirements dealing with ships reporting of their management of ballast water – have now become compulsory and from 1 March 2000 Australia introduced a verification method – known as the 'Newcastle' verification method to ensure the accuracy of vessel's claims concerning their ballast water management.

While these developments were taking place, Australia was developing a national strategy (first released in 1996) which is an essential blueprint for concerted national action; and establishing an expertise and key stakeholder based Council, the Australian Ballast Water Management Advisory Council (ABWMAC), to advise AQIS and the Government. It is to be noted that this is a cooperative partnership approach between Government and key industry stakeholders. This cooperative partnership is, we believe, an essential element of any truly effective approach to this issue.

We have also maintained a comprehensive R&D program covering improved ballast water management practices; studying many aspects of invasive marine species such as their relative threat to Australia and their survivability in ballast water; and searching for improved longer term solutions.

We established a methodology and commenced a program of port surveys in Australia, in conjunction with the CSIRO's Centre for Research on Introduced Marine Pests (CRIMP) – to determine just what species were present in our major ports.

A specific point to be noted is that since Australia began working on this issue we have adopted a targeted, risk based approach. We have, for example, therefore focussed on a Target List of Marine Species [8] of major concern to us. This doesn't imply that these are the only species, but rather it was, and is, our assessment that, firstly, they pose the greatest economic and/or ecological threat and secondly, that each one – and toxic dinoflagellates were our first target – represents a category or group of potential marine invaders. Hence, successful control or management measures for a target species or organism are likely to be effective for the group it represents.

By the mid 1990s we were convinced however, that domestic or coastal translocation of introduced marine pests, was, for Australia, an issue as serious as, or close to, that posed by international movement. We therefore looked to address this and in 1996 devised and trailed voluntary Australian Coastal Ballast Water Management Guidelines [18].

In the belief that we need to approach this matter from the perspective of having a single national management arrangement (rather than separate national and state arrangements) we have recently developed an Action Plan [19] for such an arrangement. Additionally,

following a potentially serious outbreak of the Black Striped Mussel (*Mytilopsis sallei*) in Darwin in early 1999, the Australian and New Zealand Environment and Conservation Council (ANZECC) and the Ministerial Council on Forestry, Fisheries and Aquaculture (MCFFA) established the National Task Force on the Prevention of Marine Pest Incursions. Its report [20] which is still the subject of Commonwealth and State government consideration endorses the approach taken by Australia and AQIS to date but makes many very useful recommendations that will, if agreed, not only enhance current arrangements but result in an integrated single national management regime for all aspects of invasive marine species management in Australia.

We are well advanced in the design and construction of a Decision Support System that will enable us to more effectively assess the potential risk of every vessel voyage to Australia (and eventually between Australian ports), and from this determine the required management action. This also will be a world's first in this area and it is expected to be in trial by early 2001. It will be accompanied by a comprehensive compliance monitoring program for shipping. This will ensure, through a series of enquiries, observations and where necessary testing, that action required of ships in handling their ballast water, have been taken.

While the Australian requirements for ships to manage their ballast water in accordance with the assessed risk are important, further advances will only be made if research both of a scientific nature and improved management arrangements continues. Accordingly Australia has had a very active Ballast Water Research and Development Program over the past 7 years. This has made significant contributions to:

- gaining a better understanding of the characteristics of ballast water as a vector for the introduction of harmful marine pests and diseases; including key issues such as species survivability and pest similarities;
- studying individual species of specific concern to Australia such as the Northern Pacific Seastar (*Asterias amurensis*); Japanese kelp *(Undaria pinnatifida);* cholera *(Vibrio cholerae);* toxic dinoflagellates and several others;
- developing scientifically based risk assessment decision support systems to support decision-making pertaining to vessels;
- preparing and disseminating a Maritime Awareness Package for the shipping industry;
- assessing potential verification approaches (to ensure action required by ships is taken by them); and
- new treatment and verification methodologies and the study of other vectors – such as hull fouling.

This comprehensive strategy led to the position where the Australian Government announced in September 1999 that it would apply mandatory ballast water management arrangements for international shipping visiting Australian ports from mid-2001. These arrangements will involve the relevant Australian agency, AQIS, carrying out a risk assessment of each vessel on every voyage to Australia, and based on the assessed risk, those vessels deemed to present a "high risk" will be required to undertake an accepted ballast water management arrangement. This will usually involve exchange of ballast water by one of several internationally accepted means, or treatment of the ballast, non-discharge of ballast, or discharge in a 'suitable area' before being permitted entry into a port. The proposed arrangements will be consistent with the approach being taken by IMO.

CURRENT AUSTRALIAN POSITION

So where are we at? In summary, Australia now has :

- the world's most comprehensive operational ballast water management arrangements for both international and coastal vessels;
- the Australian government has announced that a fully mandatory regime for international shipping will come into place from mid-2001;
- the Federal and State Governments are presently considering a significant proposal for an integrated national system of management covering all vessels (international and Australian coastal) and all vectors;
- we have a comprehensive, largely industry funded, R&D program;
- we have an established methodology for, and are carrying out, port surveys to determine what species presently reside in our national waterways;
- we are well advanced in developing a world's first risk assessment based decision support system (the so-called DSS), to enable us to operate a scientifically and risk based management system for all vessels;
- we are establishing standards for assessment of potential verification and treatment methodologies;
- we have a compliance monitoring regime in place;
- we have a national strategy to guide Australia's approaches to ballast water management, encompassing policy, operational and research perspectives;
- and, Australia continues to lead the push in the IMO for urgent finalisation and adoption of a legally binding international management regime for ballast water.

And lastly, but importantly, Australia's Oceans Policy [15] has restated the Government's commitment to the pursuit of ballast water management measures and integrated national regime for international and coastal shipping in Australia's waters.

CONCLUDING REMARKS

Introduced marine species, including those that may result in algal blooms, is an issue of major environmental, economic and social concern. It is an issue that particularly affects Australia with our relatively pristine marine environmental status and, on the one hand, our use of our waterways for recreation and seafood production, and on the other hand, our dependence on shipping, which is the greatest single means of translocating harmful, invasive marine species between the world's ecosystems.

There has been considerable action taken by Australia to reduce the threat and firm plans are in place

both by the IMO and Australia to take further action to stem the tide of invasive marine species.

There is an international and Australian agenda but it is a long and complex agenda and will not resolve the problem overnight.

But to conclude, a note of caution. Actions both in Australia, by other individual countries and by the peak international shipping industry organisation, the IMO, must be tempered by the more broadly-based needs of the international community, and must seek to reach an appropriate balance between environmental protection, economic well-being, practicality, cost effectiveness and safety.

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BALLAST WATER EXCHANGE: TESTING THE DILUTION METHOD (PETROBRAS, BRAZIL)

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ABSTRACT

The ballast water exchange method devised by PETROBRAS (Brazilian State Oil Company), the dilution method, is based on ballasting through the top of the tank while unloading by gravity through the bottom. This method is safe, even at high seas, for the ship's structure and crew members. In June 1998, a full-scale experiment was performed to assess the efficiency of this method on a segregated ballast tank of the oil carrier *M/V Lavras* (2,286m^3). A simulation model of its theoretical performance established sampling points in the tank, representing areas of different exchange rates. Sampling was done with a pneumatic pump (10L/min, 20mm-diameter hoses) which was efficient for phytoplankton (concentrated in a 20μm mesh), but zooplankton sampling required tows (200μm mesh) through a manhole. Sediment from the empty tank was sampled before and after the experiment. The amount of the original water that remained after exchanging 3 tank volumes (21 hours) depended on the parameter analyzed: chlorophyll *a* (14%), methylene blue (10%), density of phytoplankton cells (4%); only oceanic zooplankton groups were found, with dominance of oceanic copepods; and microalgae cysts/resting spores were close to non detectable in the water column. Sediment was not quantified, but visual observation after deballast showed that the thick layers previously present were partly washed out. Cysts/resting spores that remained in the tank (1-2 x 10^5.L^{-1}) indicate that sediment in ballast tanks represents a problem for further investigation.

INTRODUCTION

Domestic and international shipping is the major cause of the introduction of exotic species in aquatic environments, because vessels provide habitats for organisms that live in their ballast water, in sediments in the ballast tanks, and as hull fouling [1]. Potentially harmful algae, especially those that survive the voyage as resting cysts, have been introduced as exotic species [2].

The International Maritime Organization (IMO) has developed voluntary guidelines as a first step to address control and management of ships' ballast water. A working group of the Maritime Environmental Protection Committee (MEPC) is working on regulations for acceptance and implementation by all IMO member nations. These guidelines seek to establish management and treatment options that are efficient, safe for the ship and the environment, and cost effective.

A review of management strategies [3] indicated mid-ocean ballast exchange on route as one of the most promising options at present. Ballast exchange while in oceanic water, with increased salinity and oligotrophy, decreases the likelihood of introducing exotic species viable in coastal waters. There exist two methods tested to date: (1) the complete deballast-reballast procedure, which is not always possible, especially at high seas; and (2) the continuous flow-through exchange method that overfills the tank and floods the deck, which can pose problems to routine deck operations unless extra pipework is installed to overcome the problem.

The dilution method, devised by PETROBRAS (Brazilian State Oil Company), is based on loading ballast through the top of the tank while unloading by gravity through the bottom, at the same flow rate. A full-scale experiment was performed on the oil carrier *M/V Lavras* (PETROBRAS) to assess the efficiency of this method. The results presented here were submitted to the 42nd MEPC session and are now part of the "tool box" of ballast water management options under consideration by the Ballast Water Working Group.

MATERIALS AND METHODS

The experiment took place from 26 to 28 June 1998. The ballast water was taken while the ship was anchored close to the mouth of the Amazon River (lat. 00° 28.7 S; long. 047° 25.8 W; local depth =15m). It took 7 hours to ballast the tank. The ballast exchange was carried out en route, after the ship reached 200 n m offshore (depth > 2000m). It took 21 hours to exchange 3 tank volumes (one tank = 2,286m^3).

Characterization of coastal and oceanic waters used as controls was done by casting sampling devices from mid ship during ballasting (coastal) and between the second and the third ballast exchange (oceanic). Parameters used to indicate the efficiency of the method were: methylene blue, salinity, chlorophyll *a*, phytoplankton and zooplankton populations, and microalgae cysts/resting spores (water and sediment).

The *M/V Lavras* is a double hull vessel of 66,500t dw. The experiment was done in one of its 7 segregated ballast tanks (starboard, number 4). The tank cleaning system of this ship has an independent pump, online with the sea chest that connects to one deck line with valves

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that can feed water to the ballast tank through 3 manholes that are 65cm in diameter. Three steel pipes (2m long, 15cm in diameter) were designed and manufactured for water injection. An orifice plate was manufactured and fitted online with the tank cleaning system so as to add a known concentration of methylene blue (dye used as tracer) to the water as it was being pumped to ballast the tank. Methylene blue was measured with a HACH's spectrophotometer (DR 2010).

A computer-simulated model of the performance of the method guided the placement of sampling points in the tank (Fig.1): 1, 2, 3, represented areas of higher exchange, while points A, B, C were "shadow" areas.

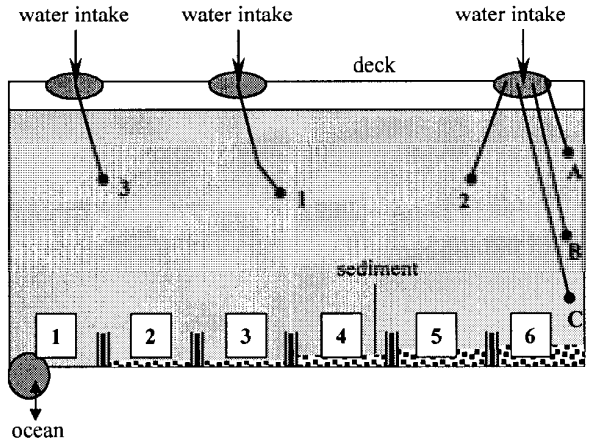

Fig. 1. Experimental tank (length=22m; hight=15m; width=6m): water column sampling points (1, 2, 3, A, B, C), sediment sampling points (boxes 1-6), water intake through manholes, and tank-ocean connection are shown.

Water from the tank was sampled by a pneumatic pump that delivered water through a 20mm-diameter hose (10L/min). The hoses were placed in the empty tank, before ballasting. Sampling was done before the beginning of the exchange (T_0), and at 3 other sampling times (T_1, T_2, T_3), that is, after each exchange of one complete tank volume. Each sampling lasted ca. 2 hours. This flow rate and diameter of the hose were efficient for phytoplankton, but not for zooplankton (see below).

Salinity was determined by Mohr-chloride titration and chlorophyll *a* was determined by a Turner® TD-700 fluorometer, after filtration through cellulose membrane (0,45µm) and extraction with 90% acetone [4].

Phytoplankton samples from the tank were collected by the pump (at least 100L) and concentrated by a 20µm-mesh net, while 1-L water samples were collected from the environment. Organisms larger than 20µm, with or without chloroplasts, were counted and identified by the settling technique. Average values of counts done in triplicate are reported (coefficient of variation <18%).

Zooplankton samples were taken from the tank by the pump and concentrated by a 200µm-mesh net. On-board observations showed that this sampling was not effective (discussed below). Samples were then collected directly in the tank with a 200µm-mesh net from 12m to the surface, while tows from the environment were taken from ca. 10m to the surface, all in triplicate. The organisms were sorted, identified and counted using a stereomicroscope through standard procedure [5].

Splits from the pump+net samples collected in the tank for phytoplankton were used for cysts/resting spores. Samples from the coastal environment were concentrated by a 20µm net from a known volume. Sediment samples in the empty tank were taken before and after the experiment by scraping only the surface layer to avoid material from the anoxic layer below (one sample for each of the 6 sections) (Fig.1). Mud deposition was limited to the sides of the bottom of the tank, where samples were collected, and on the longitudinals of the forward section. All sediment samples were processed for quantitative analysis [6] and counts were done by the settling technique.

RESULTS

The water used to ballast the tank (coastal) was considered markedly different from the water used for the exchange (oceanic) (Fig.2). The influence of the nutrient-rich discharge from the Amazon River was detected closer to shore through lower salinity, higher chlorophyll *a* and greater phytoplankton densities (with some freshwater species). The coastal phytoplankton revealed the dominance of chain-forming diatoms, while the oceanic was composed of naked and large-size armored dinoflagellates, coccolithophores and small pennate diatoms. Zooplankton groups present only on the coast were Gastropoda Larvae, Bivalve Larvae, Mysidacea, Hydromedusae, Echinodermatha Larvae, and Stomatopoda Larvae, while Foraminifera, Polychaeta, Fish Eggs, Fish Larvae, Salpidae, Amphipoda, Cladocera, and Pteropoda were restricted to oceanic waters. Cysts/resting spores in the water of the coastal site increased from surface ($34.L^{-1}$) to a depth of 7m ($108.L^{-1}$). Diatom resting spores comprised the bulk of the cells found.

In the tank, the average salinity increased from 31.61 (T_0) to 35.95 (T_3) (Fig.2). A trend of lower salinity at sites A, B and C suggest the presence of the "shadow" area indicated by the simulation model.

The amount of the original water that remained after exchanging 3 tank volumes varied according to the parameter analyzed (Fig.2): chlorophyll (14%), methylene blue (10%) or phytoplankton > 20µm (4%). These differences are expected and can be attributed to distinct behavior of each parameter in the tank and their methods of analysis. In the best case scenario (4%), only cells larger than 20µm were considered. In the worst case scenario (14%), all photosynthesizing organisms larger than 0.45µm were included, but most of the chlorophyll *a* (in average 80%) was present as phaeophytin throughout the experiment, indicating that organisms were photosynthetically inactive.

Ninety-one phytoplankton species were identified, but only 6 were common to both coastal and oceanic environments (their occurrences were not considered in the analysis). Two freshwater diatom species that were not in the coastal sample (*Aulacoseira granulata* and *Polymixus coronalis*) were detected in the tank water from T_0 to T_3. These species, commonly associated to sediments, were probably revolved from the bottom of the tank during ballasting. This ballast tank had not been cleaned for ca. 5 years, so that its sediment and associated biota represented a composite from different

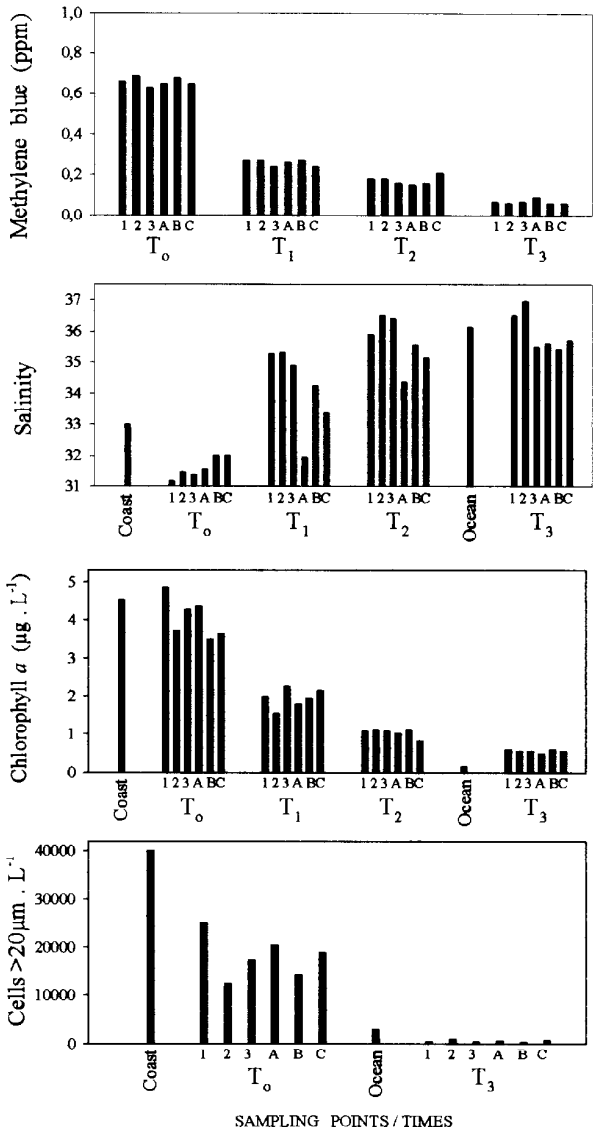

Fig. 2. Parameters analyzed during the ballast exchange: (a) methylene blue, (b) salinity, (c) chlorophyll *a*, (d) phytoplankton. For X-axes: coast and ocean are the controls from the environment; sampling in the tank was done before the exchange started (To), and after each exchange of one tank volume (T1-T3).

ports of the eastern coast of South America, from Brazil to Argentina, some of them located in rivers.

The pump greatly underestimated the concentration and composition of the zooplankton so that direct net hauls in the tank were also used (Tab.1). A stronger hose with greater diameter and higher flow rate could have counteracted the capability of larger and stronger swimmer species to escape from the pump sampling. Because of these methodological difficulties, only qualitative data is presented for the zooplankton.

Table 1. Zooplankton: comparison of sampling methods.

DATA PER SAMPLING TYPE	PUMP	NET TOWS
Time required	≅ 2 hours	≅ 10 min
Volume filtered	3.6 m^3	9.9 m^3
Mean density	33.8 $org.m^{-3}$	53.4 $org.m^{-3}$
Mean n° of groups	6	12
Size of organisms	200 - 2400 µm	200 - 7000 µm

Twenty-three zooplankton groups were found. Twelve groups were exclusively in the coastal or in the oceanic environment. Eleven groups were found in the tank: Copepoda, Decapoda, Cirripedia, Chaetognatha, Isopoda, Polychaeta, Siphonophorae, Foraminifera, Gastropoda, Echinodermata and Engraulidae. Of the groups found in the tank, three are considered predominantly coastal (Cirripedia Nauplius, Gastropoda Larvae and Echinodermatha Larvae) and two are preferentially oceanic (Foraminifera and Siphonophorae) [7]. All coastal groups were found at T_0, coastal and oceanic ones were present at T_2, but only Foraminifera and Siphonophorae were found at T_3. Copepods, found in all samples, confirmed this trend: the coastal species *Acartia lilljeborg* and *Pseudodiaptomus acutus* dominated at T_0, while the oceanic species *Farranula gracilis* and *Clausocalanus furcatus* dominated at T_3.

The microalgae cysts/resting spores found in the sediment and in the water column were mostly composed of diatom resting spores, with a minor contribution of dinoflagellate cysts (Fig.3). The concentrations in the water were three orders of magnitude lower than in the mud (10^2 and 10^5 $cells.L^{-1}$, respectively). Concentrations in the water decreased during the experiment. Sampling sites A, B and C, located in the section which originally had more accumulated sediment, showed the highest cyst/resting spore concentrations found in the water, probably due to ressuspension. The sediment was not quantified, but visual observation after deballast showed that the thick layers previously present, especially in section 6, had been partly washed out. The concentration of cysts/resting spores in the mud, however, increased in all sections investigated. Since the oceanic water can be considered a diluting agent rather than a source of cysts/resting spores, we speculate that their increase in the sediment may be attributed to their mobilization and redistribution from the sediment of the tank itself, due to turbulence caused during water exchange.

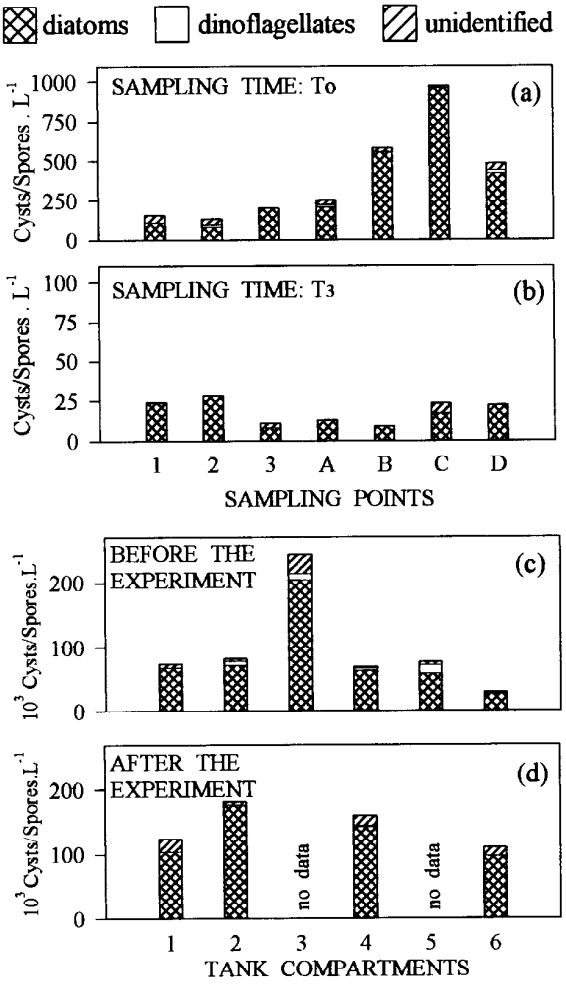

Figure 3. Cyst/resting spore in the tank: (a-b) in the water column and (c-d) in the sediment.

DISCUSSION

The dilution method is safe, even at high seas, for the ship's structure and crew members. The worst case scenario when assessing the effectiveness of the water exchange (14% of the chlorophyll *a*) showed that phytoplankton was photosynthetically inactive (present as phaeophytin). This was probably due to mechanical stress and darkness in the tank, as has been shown in studies of survival of phytoplankton in ballast tanks [8,9].

Species composition in the tank can be important for the degree of efficiency of the mid-ocean exchange, since diatoms are expected to sink while flagellates can remain in the water column [10]. In this trial of the dilution method, there was no stratification of the diatom-dominated community in the tank (no difference between sites A,B,C), probably due to the turbulence caused by the injection of the water through the manhole.

The turbulence in the tank was also important for the re-suspension of sediments (and its associated biota) that could, therefore, be discharged offshore. Nevertheless, high numbers of cyst/resting spores in the remaining sediment may still be delivered to the environment during further deballasting procedures, acting as a inoculum for species proliferation. Even complete deballast/reballast does not eliminate, for any type of vessel, all water and sediment from the tank. This residual water often contains more planktonic organisms which can be concentrated through sinking during the voyage, before the deballast/reballast procedure [11]. The amount of water exchange varies with tank design and older vessels do not seem to be as efficient as newer ones [12,13]. Once again, the turbulence induced by the dilution method could counteract, in part, this problem.

Salinity, the parameter representing the dissolved fraction in the tank, confirmed the simulation model (that is, section 6 of the tank represented a "shadow" area where dilution was more deficient). Future modeling can take into account the behavior of particles in the tank and further improve the degree of efficiency of the dilution method. Both the flow rate and the water intake system can be adjusted to adapt the method to other tank designs. The dilution method can be used in conjunction with other ballast management options (e.g., filtration, heat).

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THE QUANTITIES OF *HETEROCAPSA CIRCULARISQUAMA* CELLS TRANSFERRED WITH SHELLFISH CONSIGNMENTS AND THE POSSIBILITY OF ITS ESTABLISHMENT IN NEW AREAS

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ABSTRACT

Populations of the harmful dinoflagellate, *Heterocapsa circularisquama*, have been expanding rapidly in coastal areas of western Japan since 1988, causing mass mortality of shellfish. Since large quantities of pearl oysters and oysters are frequently transported to the many culture grounds within Japan, we examined the quantities of cells transferred together with the shellfish consignments and the possibility that the dinoflagellate could become established in new areas. The results demonstrated that 1) the number of *H. circularisquama* cells within the shellfish increased in logarithmic proportion to the concentration of cells to which the shellfish were exposed, 2) immotile cells of *H. circularisquama* were found in shellfish collected from areas where motile cells had been observed, and 3) immotile cells reverted to motile cells when there were few diatoms in the unfiltered seawater medium, although they remained immotile when diatoms proliferated in the medium. The possibility that the dinoflagellate could become established in new areas of western Japan is discussed.

INTRODUCTION

Populations of the harmful dinoflagellate, *Heterocapsa circularisquama* Horiguchi [1], have been expanding rapidly in coastal areas of western Japan since 1988, causing mass mortality of shellfish such as pearl oysters (*Pinctada fucata*) and Pacific oysters (*Crassostrea gigas*) [2, 3, 4]. These shellfish are frequently transferred from seedling production grounds to rearing grounds and from red tide areas to other areas by shipping or trucking. In a previous paper we suggested, based on cultured strain, the potential for the accidental transfer of *H. circularisquama* to new areas [5]. In the present paper we examine the relationship between the concentration of cultured *H. circularisquama* to which shellfish were exposed and the number of immotile cells released by the shellfish, the number of immotile cells transferred together with shellfish from several areas, and the possibility that the dinoflagellate can become established in new areas.

MATERIALS AND METHODS

A strain of *H. circularisquama*, isolated from Kusuura Bay, Kumamoto Prefecture in September 1994, was cultured in modified SWM-3 medium under 25±°C, 65μE $m^{-2}s^{-1}$ in a 12 h light : 12 h dark cycle. Pearl oysters (one- and two-year-old) and Pacific oysters (spat and two-year-old) were exposed to suspensions of the dinoflagellate adjusted to 16 to 10,000 cells ml^{-1}. After 10 minutes the shellfish were removed from the suspension and permitted to stand in empty beakers for 4 h. Each pearl oyster and oyster was then immersed individually in fresh culture medium and the number of immotile *H. circularisquama* cells released by the shellfish were counted.

In 1997, samples of shellfish from several areas (Ago Bay and Gokasho Bay, Mie Prefecture; Hiroshima Bay, Hiroshima Prefecture; Hinase coastal area, Okayama Prefecture; and the western part of Suo-nada, Fukuoka Prefecture) were collected and sent to Kyushu University within 24 h by trucking, where they were immersed in fresh medium. The numbers of released immotile cells were counted.

Samples of pearl oysters that were suspended for 4 hours in the layer in which the dinoflagellate was blooming in Gokasho Bay were sent to the University and were immersed in fresh medium. The number of released immotile cells was counted and was inoculated individually into small chambers containing 200μl of unfiltered seawater including natural phytoplankters or filtered seawater with or without addition of nitrate and phosphate. Morphological changes from immotile to motile cells under the experimental conditions were observed to confirm the possibility that the ingested dinoflagellates were capable of colonizing new areas.

RESULTS AND DISCUSSION

The number of *H. circularisquama* cells released by the shellfish exposed to suspension of cultured cells increased in logarithmic proportion to the concentration of cells to which the shellfish were exposed (Fig. 1 (A) and (B)). The average number of cells released by one- and two-year-old pearl oysters and two-year-old Pacific oysters was about 10 times the concentration of cells to which they were exposed (Fig. 1).

Table 1 shows the number of immotile cells released by shellfish from each area. Immotile cells were observed from shellfish in all areas except Hinase coastal area and the western part of Suo-nada. Particularly, pearl oysters suspended in a dinoflagellate bloom in Gokasho Bay released an average of 80,000 cells per individual. We found 100 immotile

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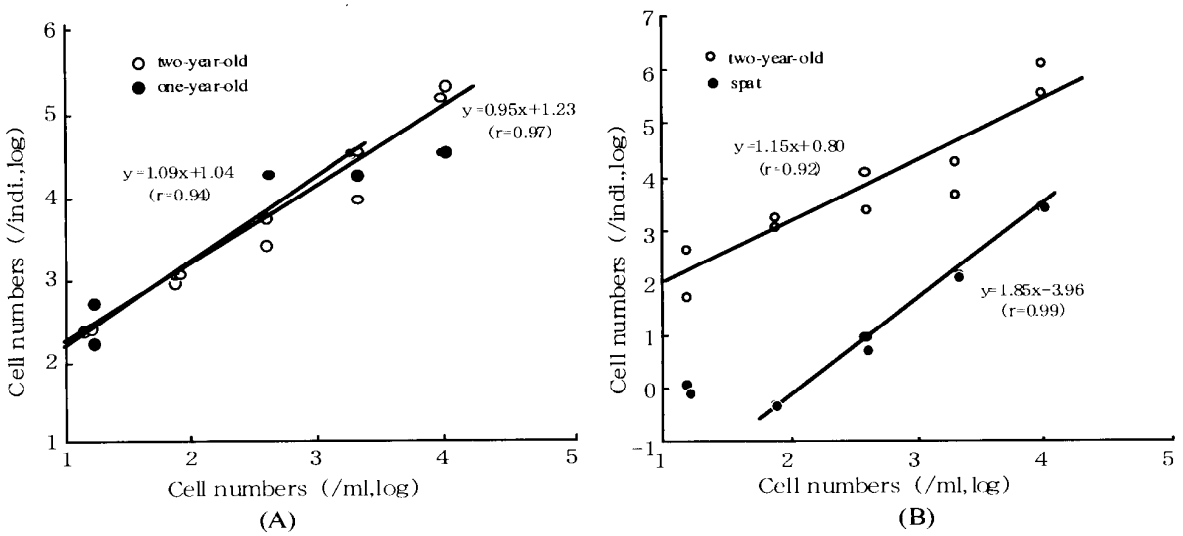

Figure 1. (A) Relationship between concentration of *Heterocapsa circularisquama* motile cells exposed and number of immotile cells released by pearl oysters, (B) same as (A), but by Pacific oysters.

cells in Japanese oysters (*Crassostrea nippona*) collected from Ago Bay, although these were permitted to stand in the laboratory for 4 days until the observation. Thus, immotile cells were found in all the shellfish we collected from areas where motile cells were observed. *H. circularisquama* cells were not detected in 1-ml samples of seawater in Ago Bay on 31 October and 4 November 1997. However, the pearl oysters and Japanese oysters released small numbers of immotile cells. These results suggest that we can obtain useful information on the occurrence of *H. circularisquama* at low cell densities in seawater by looking for immotile cells released by shellfish.

Table 2 shows the results of individually inoculating *H. circularisquama* immotile cells from Gokasho Bay into small chambers containing unfiltered seawater or filtered seawater with or without the addition of nitrate and phosphate. Regardless of nutrient enrichment, the immotile cells reverted to the motile form when diatoms did not grow in the unfiltered seawater medium, but remained immotile when diatoms proliferated in the medium. Uchida et al. [6] reported that the growth of diatoms is not affected by association with *H. circularisquama* but the growth of *H. circularisquama* is suppressed in bialgal cultures with diatoms.

The shellfish are frequently transferred to many culture grounds within Japan by shipping or trucking. Unfortunately, the dinoflagellate had high resistance to rapid changes in water temperature and salinity by

Table 1. Number of motile cells in seawater from which shellfish were collected in 1997, and the number of immotile cells released by the shellfish from each area.

Collection date	Water area	Number of motile cells in seawater (/ ml)	Number of immotile cells released (/ individual)*
Pearl oyster (two-year-old)			
21. Oct.	Gokasho Bay (CPO)	—	80,000
4. Nov.	Ago Bay (CPO)	ND	80
25. Nov.	Ago Bay (CPO)	930	500 - 2,500
Oysters			
20. Oct.	Hiroshima Bay (CPO)	10	200 - 500
31. Oct.	Ago Bay (SJO)	ND	100**
4. Nov.	Ago Bay (SJO)	ND	30
4. Nov.	Hinase coastal (CPO)	ND	0
5. Nov.	Suo-nada (CPO)	—	0
25. Nov.	Ago Bay (SPO)	930	100 - 2,000

CPO: cultured Pacific oyster, SJO: spontaneous Japanese oyster, SPO: spontaneous Pacific oyster.
* Five individuals in each examination were used.
** The sample was left in the laboratory for 4 days.

Table 2. Time (hours) required for inoculated immotile cells to regain motility in unfiltered and filtered seawater with or without the addition of nitrate and phosphate. Immotile cells used in this experiment were obtained from pearl oysters that were suspended in the layer in which the dinoflagellate was blooming in Gokasho Bay.

Culture medium	Time (h) after inoculation			
	1	17	20	41
Unfiltered seawater	I I	I I	M M	M M
Unfiltered seawater + N, P	I I	I I → Proliferation of diatoms	I I	I I
Filtrate	I I	M M	M M	M M
Filtrate + N, P	I I	M M	M M	M M
SWM-3 culture medium	I I	M M	M M	M M

M: reverted to the motile form, I: remained immotile form.

reverting to immotile cells, which are temporary cyst [5]. Also, *H. circularisquama* did not die in seawater in which diatoms and *Gymnodinium mikimotoi* were growing at high cell densities [6, 7] or within a Japanese oyster that was permitted to stand in the laboratory for 4 days. Thus, our results strongly support the view that *H. circularisquama* can become established in new areas by transfer in shellfish consignments and can expand rapidly in the coastal areas of western Japan. *H. circularisquama* produced maximum growth at 30°C and did not grow at or below a temperature of 10°C [8]. Hence, this species is unlikely to reside in the coastal areas of northern Japan.

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RECENT INTRODUCTION OF *GYMNODINIUM CATENATUM* TO PORT LINCOLN, SOUTH AUSTRALIA

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ABSTRACT

In association with a major tuna kill in Boston Bay, Port Lincoln, SA, in 1996, an intensive phytoplankton survey identified the presence of the toxic dinoflagellate *Gymnodinium catenatum* for the first time. This alga had not previously been recognised in SA and has only been identified from Tasmania and a few sites on the mainland of Australia, notably Victorian coastal waters. Genetically and toxicologically the Port Lincoln and Tasmanian dinoflagellate populations are indistinguishable and therefore, this project seeks to determine whether the Port Lincoln site is the origin of the recently introduced Tasmanian population (since 1971), for instance by a known domestic shipping link, or whether the population at Port Lincoln has been secondarily introduced from Tasmania. Three sediment cores were taken from Boston Bay. Each contained abundant *Gymnodinium catenatum* cysts in the top 6 cm but these disappeared rapidly with depth. ^{210}Pb profiles indicate that bioturbation extends down to no more than 5 cm and that the sedimentation rate is approximately 0.2 cm/yr. Modelling of the cyst and ^{210}Pb profiles suggests that the observed profiles are consistent with an introduction event within the last 40 years. This approach confirms that *G. catenatum* was introduced to Port Lincoln but does not provide the resolution to determine whether or not this was the source of the Tasmanian populations.

INTRODUCTION

Following a major fish kill in the Port Lincoln area in 1996 in which an estimated 1700 t of caged tuna died, an investigation of the sediments revealed the presence of cysts of the dinoflagellate *Gymnodinium catenatum*. Although this organism was in no way implicated in the fish kill, it was the first time it had been reported from this area and one of the few reports from mainland Australia. *Gymnodinium catenatum* first appeared in Australia in Tasmania in the late 1970's and it the most likely vector of introduction. An analysis of the cyst distribution in sediment cores from this location confirmed that *G. catenatum* was not present in Tasmania prior to this time and that its presence was most likely to have resulted from a recent introduction [1]. has been suggested that ballast water import was Genetically and toxicologically the Port Lincoln and Tasmanian dinoflagellate populations are indistinguishable [2] and therefore, this project seeks to determine whether the Port Lincoln site is the origin of the recently introduced Tasmanian population (since 1971), for instance by a local known domestic shipping link, or whether the population at Port Lincoln has been secondarily introduced from Tasmania.

Gymnodinium catenatum produces a distinctive microreticulate cyst that is quite resistant to chemical weathering and so preserves well in sediments. The oldest *G. catenatum*-like cysts found so far are from approximately 2,000 years ago in the Skagerrak and Kattegat region of northern Europe [3], although these are now considered to be *G. nolleri*. In Australia they have been reported principally from around Tasmania but also from Port Phillip Bay [4], Broken Bay and Port Lincoln. The oldest reliably dated Australian cysts are those from Deep Bay, Tasmania, which appeared in the early 1970s [1].

Interpreting sediment sequences in which there has been minimal surface mixing is relatively straightforward. Once the sedimentation rate in the core has been determined, dating of events can be achieved simply by applying the sedimentation rate to the depth of the event. However, in most marine sequences bioturbation and physical mixing of the upper 5-30 cm is normal. This mixing severely limits the resolution of events and makes recognition of dates with an error of less than at least a decade rare. Here we apply a simple mixing model to constrain the maximum age of introduction.

METHODS

Triplicate sediment cores (15-35 cm long, 4.5 cm diameter) were collected in February 1996 from Boston Bay at Port Lincoln, SA, Australia, using divers. The cores were collected from depths between 16 and 18 m and were frozen on retrieval for storage and transport. Alternate 1 cm thick sections were sent to either ANSTO for 210Pb analysis or Laola Pty Ltd, Perth, for cyst extraction. This involved disaggregation in HF, density separation in a ZnBr2 solution (specific gravity 2.1),

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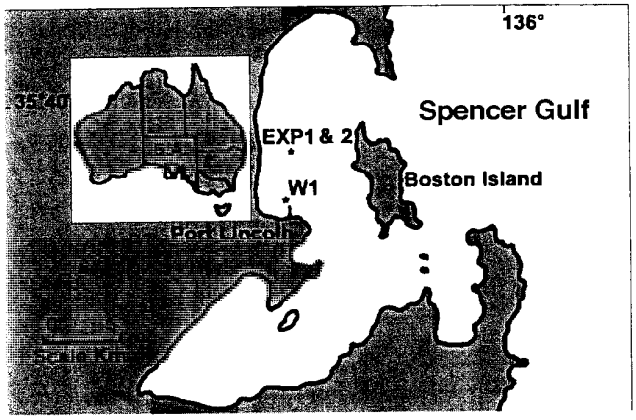

Fig. 1 Location of coring sites at Port Lincoln

sieving on an 8 μm filter, and mounting in a permanent mounting medium (Eukitt). A *Lycopodium* tracer was added to enable the quantification of cyst numbers. All cyst abundances are based on cysts per g dry weight.

Slides prepared for dinoflagellate cyst analysis were examined with a Zeiss Axioskop microscope and estimates of dinoflagellate cyst abundance were made from 3 replicate counts of 400 specimens.

Procedures for ^{210}Pb analysis follow McMinn et al. [1], using the Constant Rate of Supply (CRS) model to determine the age profiles. Numerical abundances (cysts/g) were converted to cyst flux rates (cysts $m^{-2}yr^{-1}$) for the purpose of modeling.

Surface mixing, due to a combination of physical and biological processes, was assumed to be instantaneous. When a layer of cysts is delivered to the sediment surface it is evenly mixed to the depth of the mixed zone. After an increment of time (i.e. 1 year) an addition layer of sediment has been added to the sediment surface. This removes an equal thickness of sediment from the bottom of the mixing layer. The lower interval of sediment has a cyst density equivalent to that before it was mixed with the additional sediment at the surface. The cyst density of the mixed zone is reduced as it has now been mixed with the additional sediment at the surface. Hence a pulse of cysts at the surface will leave a cyst density maxima at a distance equivalent to the mixed zone beneath the surface.

RESULTS

Each sediment core contained an unlaminated sequence of muddy sands. Abundant, diverse and well-preserved dinoflagellate cyst assemblages were recovered from all samples. Species present included *Gymnodinium catenatum, Gymnodinium microreticulatum, Lingulodinium machaerophorum, Protoperidinium conicum, Protoperidinium pentagonum, Protoperidinium oblongum, Protoceratium reticulatum, Spiniferites bulloideus, Spiniferites membranaceous, Spiniferites mirabilis, Spiniferites ramosus,* and *Tuberculodinium vancampoae*. Numerical abundance (cysts/g) of *G. catenatum* is shown in Figure 2.

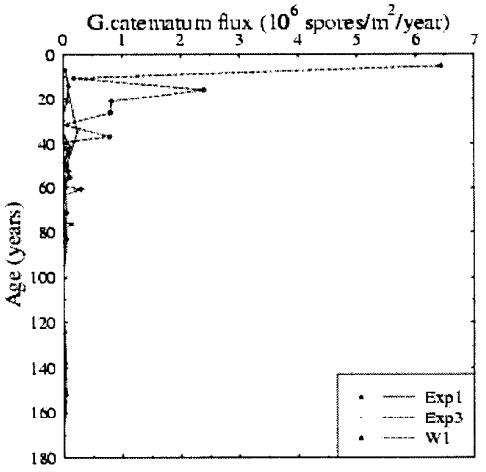

Figure 2. Distribution of G. *catenatum* cysts with depth in all three Port Lincoln cores

In each of the three cores *Gymnodinium catenatum* is considerably more abundant at the top of the core than the bottom; in two of the cores it is completely absent from the bottom. Numerical abundances of the most abundant cyst types (i.e. *P. reticulatum, S. mirabilis, S. bulloideus*) do not show any systematic variation with depth.

^{210}Pb Dating: Two of the three cores yielded good ^{210}Pb profiles. These both indicated a surface mixing depth of approximately 6 cm and sedimentation rates of 0.2 cm yr^{-1}. Results from the third core were poor and so the mixing depth and sediment rate determined from the other two cores were applied.

DISCUSSION

We have applied a simple sediment model to assist in the interpretation of the down core distribution of the dinoflagellate cysts in the three marine cores from Port Lincoln. This is illustrated in Figure 3. If a pulse of a tracer, such as dinoflagellate cysts, is added to the surface of the sediment (Fig 3A) it is rapidly mixed to give an even distribution within the mixed zone (Fig. 3B). When further sediment is added (Fig. 3C) it will be mixed with underlying, cyst-rich sediment. A layer of sediment at the bottom of the mixed zone will fall below the depth of mixing and the cyst concentration in the mixed zone will be reduced (Fig. 3D) as a result of dilution from the addition of the fresh sediment. The sediment that now moves below the mixed zone will have the highest concentration of cysts as it was not further diluted with fresh sediment.

Figure 3. Effect of a mixed zone on the depth of maximum cyst abundance. A, addition of cysts; B, complete mixing in mixed zone; C, addition of more sediment – lower sediments pushed below mixed zone; D, complete mixing in mixed zone; E addition of more sediment - lower sediments pushed below mixed zone; F, complete mixing in mixed zone.

Addition of more sediment will lead to an increasingly diluted mixed zone and a declining cyst abundance in the sediment beneath the mixed zone (Fig. 3E,F).
Results of a simulated single input of cysts are shown in Fig. 4. It can be clearly seen that the maximum cyst abundance is at a depth equivalent to the thickness of the mixed zone below the point of introduction. Thus, in determining the timing of cyst introduction, the thickness of the mixed layer needs to be taken into account. Sediments studies of dinoflagellate cysts from marine or estuarine environments need to apply an equivalent sediment model if reliable age reconstructions are to be obtained.

In each of the three sediment cores from Port Lincoln *G. catenatum* cysts were abundant at the top and decreased, approximately exponentially, towards the bottom. When the sediment rate alone was applied to determine the time of appearance of the cysts, a date of approximately 1930 was determined.
However, application of the sediment model revealed that the depth of the mixed layer was critical to the determination of age. In particular, the estimated age should be equal to the age estimated from the application of the sedimentation rate alone minus the length of time taken for an input pulse to cross the mixed layer, i.e. mixed zone.

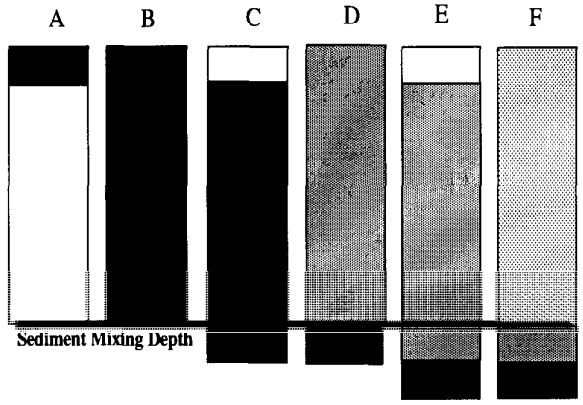

Figure 3. Effect of a mixed zone on the depth of maximum cyst abundance. A, addition of cysts; B, complete mixing in mixed zone; C, addition of more sediment – lower sediments pushed below mixed zone; D, complete mixing in mixed zone; E addition of more sediment - lower sediments pushed below mixed zone; F, complete mixing in mixed zone

Age = sedimentation rate determined age - (mixing depth thickness /sedimentation rate)

At Port Lincoln

Core Exp1: Age = 80 - (6/0.15)= 40 years ago (or 1960)

Core Exp3: Age = 67 – (5/0.12)= 42 years ago (or 1958)

Core W1: Age = 55 – (10/0.38)= 29 years ago (or 1971)

Thus the best estimate for the time of introduction of *G. catenatum* at Port Lincoln is approximately 1960 with an error of ± 20 years. This date of appearance is intriguingly similar to the time of its appearance in Tasmania. Unfortunately, however, the dating error that has resulted from using a mixed sediment column has reduced the precision of the dating and made it impossible to determine which introduction was first, Tasmania or Port Lincoln. That they are both so similar in age, does, however, suggest a similar mode of introduction.

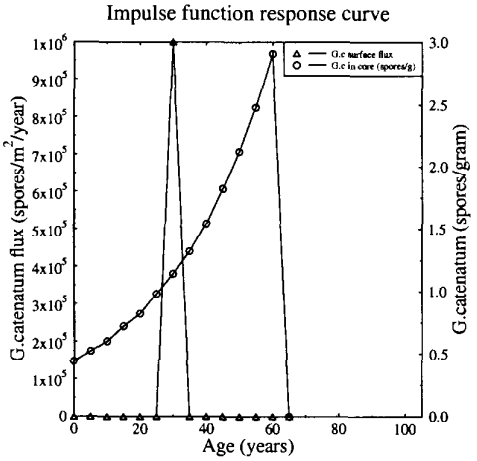

Figure 4. Model output of the distribution of cysts after a pulse input

ACKNOWLEDGEMENTS

We wish to thank AINSE for a grant to cover the cost of the ^{210}Pb analyses.

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WORLDWIDE OCCURRENCE AND HOST SPECIFICITY OF *PARVILUCIFERA INFECTANS*: A PARASITIC FLAGELLATE CAPABLE OF KILLING TOXIC DINOFLAGELLATES

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ABSTRACT

Parvilucifera infectans Norén et Moestrup, 1999 has been shown to kill several species of toxic dino-flagellates. Based on our present knowledge we know 26 microalgal species which are susceptible to infection. A report is provided on the known geographical distribution of *Parvilucifera* which seems to be worldwide from Australia in south to Norway in north.

INTRODUCTION

Together with an increased scientific interest in harmful algae, such as toxic dinoflagellates, the importance of potential predators has also been intensified. Intracellular predators such as viruses and bacteria have been reported [1] together with eukaryotic forms such as the dinoflagellate *Amoebophrya* infecting other dinoflagellates [2]. Recently a new organism was found that infected and killed several thecate dinoflagellates in field samples and *Alexandrium* in culture. It was studied and described using morphological and molecular methods and named *Parvilucifera infectans* [3] (Fig. 1). Based on the results it was transfered to a new taxon, Perkinsozoa, at the level of phylum. It is related to the dinoflagellate, apicomplexan and ciliate phyla within the alveolate superphylum. During the last three years it has been found independently in many parts of the world by different groups of researchers. This could be a result of the awakened scientific interest in the host species with more researchers and more directed efforts to reveal the ecology of the organisms. There is also a possibility that a possible increase in dinoflagellate populations, noted as more blooms, could cause an increase in parasite/predator populations accompanying them. A third possibility is that *Parvilucifera* is an introduced species from an area lacking intensive protistological research and been previously overlooked. Early stages of *Parvilucifera* is easily misinterpreted as food vacuoles and late stages, i.e. the sporangium stage, sinks to the bottom and is not noticed in plankton-net hauls. The purpose of this paper is to summarize the geographical distribution of *Parvilucifera* and list the host species that can be infected.

MATERIAL AND METHODS

Geographical distribution

Information on occurrence are taken from other researchers that have found *Parvilucifera* sp. and positively recognized them from pictures and descriptions (for occurrence in India, Norway and eastern North America). In three published papers we conclude they have found *Parvilucifera* sp, based on pictures shown and descriptions of the life cycle. One of these is an erroneous report of *Parvilucifera* as a sexual phase of *Dinophysis* [4], another

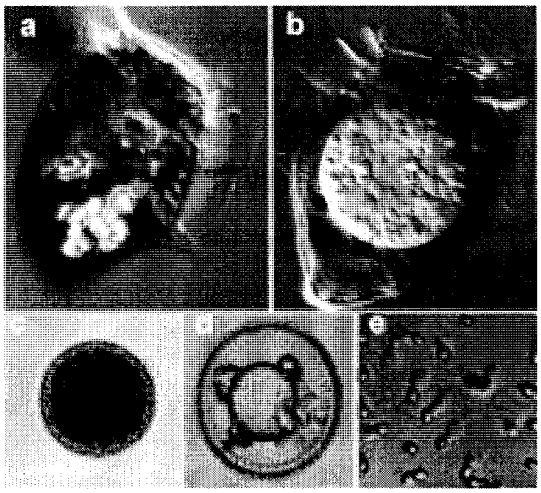

Fig. 1a-e. *Parvilucifera infectans* infection in *Dinophysis norvegica*. **a**. Early infection ~1 day. **b.** late infection ~2 days. **c**. mature sporangium after death of *Dinophysis*. **d**. Empty sporangium after release of swarmers. **e**. Swarmers, length 5 _m. In Fig. 1b-d the diameter of the sporangium is 28 _m.

paper is describing the occurrence of a new organism that infects *Alexandrium minutum* [5] and the last is from a news-letter reporting a parasite in *Alexandrium catenella* [6]. For detection of *Parvilucifera* from Tasmania, Australia, natural samples were taken from surface water in the port of Hobart (18°C, approx. 30 psu, 19 Feb 2000) and transported in a water bottle to a laboratory in Sweden. On arrival the sample was kept in 18°C and 14:10 L:D regime. The sample was analysed with a Zeiss Axiovert microscope and sporangia similar to *Parvilucifera* were compared with type material. An infection test was made on a culture of *Alexandrium ostenfeldii* (K287, see below for source) to compare with type material.

Species infected

For infection NUNC multiwell dishes (2 ml wells) were filled with 1.5 ml host algae culture. Algal cultures originate from the Scandinavian Culture Collection of Algae and Protozoa (denoted with a K-number) or from Jacob Larsen's cultures (denoted with a J-number). Micropipetted *Parvilucifera* sporangia were added in five replicate wells and compared with five control wells without added sporangia. The sporangia originates from cultures grown in *Alexandrium ostenfeldii* (K287) or *A. fundyense* (K271). All the cultures were grown under non-axenic conditions. They were kept at 18°C (except for the

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warm water species *Gymnodinium breve, G. sanguineum, Prorocentrum mexicanum* and *P. lima* which were kept at 25°C) and under 14:10 L:D cycle. The infection experiment was conducted for five days. Species infected reported by Erard-Le Denn et al. [5] and Norén et al. [3] are also included for completeness.

RESULTS AND DISCUSSION

Geographical distribution

The geographical distribution is presented in Fig. 2. For North Sea waters *Parvilucifera* sp. is reported from the Swedish west coast [3] and from Trondheimsfiord in mid west Norway (Catherine LeGrand, Kalmar University, pers. com.). From European waters it is reported from Atlantic coast [4, 5] and from the Mediterranean [6]. From Indian waters, Goa region, *Parvilucifera* sp. is reported by Anna Godhe, Göteborg University (pers. com.) and from eastern North America it is reported from Narragansett Bay by Lucie Maranda (University of Rhode Island, USA, pers. com.). *Parvilucifera* was found in Tasmania and studied in laboratory. Compared with type material it has the same morphology in the LM, but this strain will also be gene sequenced in the near future to study possible dissimilarities between geographical different strains. Are they the same species or are they different species? The Tasmanian strain was positively identified by making a successful infection in *A. ostenfeldii*. It seems that *Parvilucifera* has a wide distribution and is not a regional phenomenon and increased awareness may stimulate further findings around the world.

Species infected

The species susceptible to infection are presented in Table 1 which is a summary of this work and published findings [3, 5, 6]. There is agreement between the studies for four species and one noncorrespondence for *Gymnodinium mikimotoi*. It is notable that some species, *Heterocapsa triquetra* and *Prorocentrum micans* is found to be infected in nature but not in laboratory cultures. This has to be further elucidated but one explanation could be that there are different strains with different host preferences. The notion that only dinoflagellates are infected has only been tested by Erard-Le Denn [5] but our results support this. When studying natural samples, only dinoflagellates are infected and sporangium development of *Parvilucifera* in diatoms or other plankton groups has never been seen. The grouping of the infected species *Gymnodinium chlorophorum* and *Lepidodinium viride* is because lack of TEM studies which is needed for proper discrimination between these species.

As with the geographical distribution, it seems that the future will reveal several more species that could be infected by *Parvilucifera*, and so enhance our knowledge of this most interesting organism and its ecology.

Table 1. Species infected. References; 1. Norén *et al.* (1999), 2. Erard-Le Denn (2000), 3. Delgado (1999), 4*.

Species infected in laboratory	Reference
Alexandrium affine	3
Alexandrium andersonii (JL24)	4*
Alexandrium catenella (JL 47)	3+4*
Alexandrium fundyense (K271)	1+2
Alexandrium minutum	2+3+4*
Alexandrium ostenfeldii (K287)	1+4*
Alexandrium tamarense (K56)	1+2
Dinophysis acuta, D. acuminata, D. norvegica,	1
Dinophysis dens	1
Gymnodinium catenatum (JL43)	4*
Gymnodinium mikimotoi	2
Gymnodinium sanguineum (JL 36)	4*
Scripsiella trochoidea	2
Species infected in nature	
Alexandrium catenella, A. minutum	3
Ceratium furca, C. tripos	4*
Diplopsalis sp.	1
Gymnodinium chlorophorum / Lepidodinium viride	4*
Heterocapsa triquetra	1
Prorocentrum micans	1
Protoperidinium cf *brochii, P* cf *divergens*	1
Protoperidinium cf pellucidum	1
Protoperidinium bipes	3
Scripsiella trochoidea	3
Species not infected in laboratory	
Alexandrium taylorii	3
Amphidinium carterae (JL28)	1+4*
Chaetoceros gracile	2
Coscinodiscus cf *radiatus*	3
Gymnodinium mikimotoi (JL21), *G. breve* (JL32)	4*
Gyrodinium aureolum (K260), *G. impudicans* (JL41)	4*
Gyrodinium corsicum	3
Heterocapsa triquetra	1
Heterosigma akashiwo	2
Prorocentrum micans	1
Prorocentrum lima, P. mexicanum	4*
Prorocentrum minimum (K295)	4*
Skeletonema costatum	2
Thalassiosira weissflogii	3

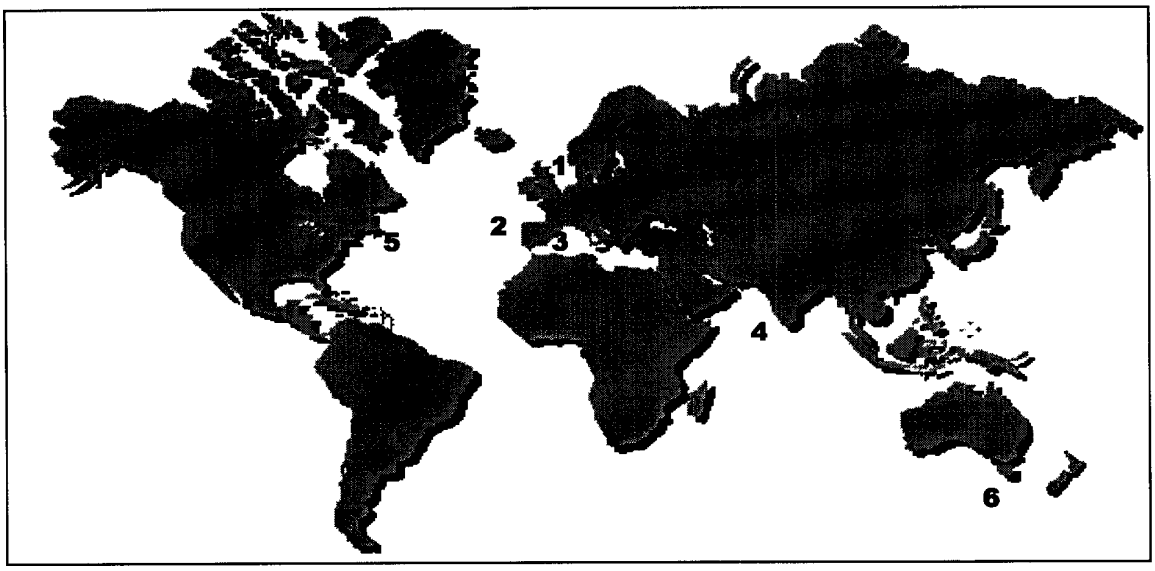

Fig. 2. Geographical distribution of *Parvilucifera infectans*. **1**. North Sea waters; Swedish west coast and Norwegian west coast. **2**. European Atlantic coast; Northern Bretagne and Spain. **3**. Mediterranean: French south coast. **4**. India: Western part. **5**. North America: Narragansett Bay. **6**. Australia: Tasmania.

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PROGRESS OF HARMFUL ALGAL BLOOM (HAB) MITIGATION WITH CLAYS IN CHINA

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ABSTRACT

Removing harmful algal bloom (HAB) organisms with clays is a promising method used to control HABs in the world. This paper reviews our studies on HAB prevention with clays in the last few years, which mainly included mechanism of bloom coagulation with clays, clay modification methods for higher removal efficiency, coagulation kinetics and environment impact. Finally, some suggestions were proposed for the further study in this area.

Coagulating HAB organisms with clays has the advantages of sufficient source, lower cost and non-pollution. Hence, it is considered a promising and attractive direct-control option to eradicate HABs. This control method has been used in some HABs in maricultural and coastal areas [1]. But there were not enough studies on the control mechanism, optimal loading, environment impact etc., which seriously limited control efficiency and method application. Using some dominant HAB species in Chinese coastal waters as models, we systematically studied the interaction mechanisms between HAB organisms and clay particles and found some key factors that limited removal efficiency. Furthermore, we proposed the theory and method of clay surface modification, which dramatically improved the coagulation capability of clays, to further develop this HAB control method, and to lay a foundation for further application.

COAGULATION OF HAB ORGANISMS WITH CLAYS

The principle of clay controlling HAB is actually clay particles coagulating HAB cells and settling them down. So the capability of clay particles to coagulate cells is very important for removal efficiency, which varies with various kinds of clays and organism.

Various HAB species

Four dominant species of HAB organisms in Chinese coastal waters were studied based on coagulation with montmorillonite [2]. Because of differences in cell structure, shape, size, habit, movement etc., their capability for coagulation with clays was also different. Fig. 1 showed several representative removal curves describing the coagulation of the above four species with various concentrations of montmorillonite. The capability of montmorillonite to coagulate the four species was *N. pungens* > *S. costatum* > *P. minimun* > *N. scintillans*. The removal efficiency of *N. pungens* and *S. costatum* (belonging to Bacillariophyta) reached more than 80% when the concentration of clay was 270 and 500 $mg \cdot l^{-1}$ respectively, but was much higher than that in

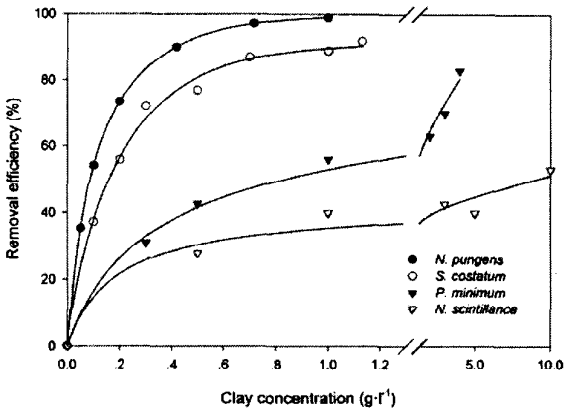

Fig.1. Coagulation of montmorillonite with different HAB organisms at pH=8.5 and 18°C.

the case of *P. minumum* and *N. scintillans* (belonging to Pyrrophyta). The higher removal efficiencies of the two diatom species were due to strongly adhesive characteristics of the cell surface, such as the more specific surface area, a slime sheath by excreted gelatinous materials [3]. In addition, the difference in the degree of surface ionization, resulting from surface functional groups of the species, was another cause. In general, the functional groups on the surface of diatom cells have a lower pK_a, which means that the electrostatic interaction is stronger between the cells and clay particles [4].

Clay type

Some studies have shown that montmorillonite is the most effective clay, while the wide-spread and plentiful kaolin had very little removal capability [5]. Yu et al. [6] found that the kaolin used in their experiments had greater ability to remove HAB organisms, which was completely contrary to the above viewpoint. The ability of kaolin and montmorillonite to remove *S. costatum, P. minimum* and *N. scintillans* is illustrated in Fig.2. The ability of kaolin to remove all the above HAB organisms was greater than that of montmorillonite and acid-treated montmorillonite. The removal efficiency of *N. scintillans* by kaolin, for example, was three times as much as that by montmorillonite and 20% more than that by acid-treated montmorillonite. Such an experimental phenomena had not yet been reported in previous literature. According to analysis of structure, chemical composition, pH experiments, as well as simulation on the coagulation model of clay particles and HAB cells [4], it was concluded that the higher removal efficiency kaolin was due to the lower negatively charged surface than montmorillonite. Based on the study, we proposed clay surface modification theory and method to improve

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its coagulation capability with HAB cells.

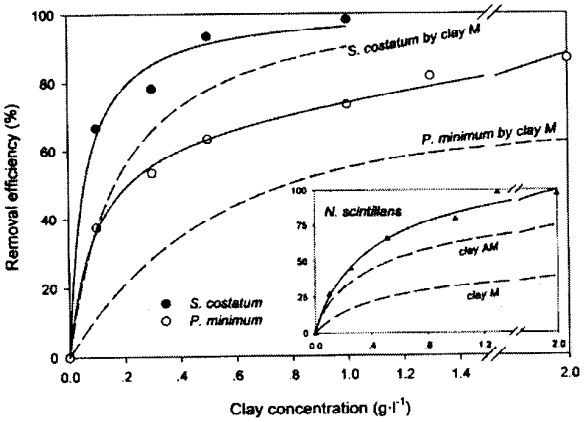

Fig.2. The efficiency of kaolin removing *S. costatum, P. minimum* and *N. scintillans*, compared with montmorillonite (M) and acid-treated montmorillonite (AM).

CLAY SURFACE MODIFICATION

Clays are negatively charged in seawater because of isomorphic substitution, lattice imperfections and exposed structural hydroxyls. HAB organisms have negative charges on their cells as well. The repulsive potential between the like charges of the clay particles and algal cells resulted in the lower efficiency, and accordingly, higher clay loading to remove HAB organisms. To enhance the capability of clay particles coagulating HAB cells, the key step was the conversion of clay particle surface charge from negative into positive, which reduced the repulsive potential between clay particles and algal cells. Based on the principles of colloid chemistry, and our findings, we proposed the following surface modification methods, by which we made positively charged clays that have much higher coagulation capability with HAB cells [7].

Surface adsorption method

This method was based on the adsorption of inorganic polymers with highly positive charges and long molecular chains onto clay particles to convert the original surface charge and to increase contact radius. Our study showed that PACS (polyhydroxy aluminum chloride) and MMH (mixed metal layered hydroxide) were this kind of inorganic polymers. Under a certain preparation condition, we synthesized both compounds with highly positive charges and high molecule weight in laboratory. By means of colloid chemistry methods, we first obtained positively charged clays respectively with PACS and MMH [7], [8]. The surface potentials of clay particles were converted from original −20 mV respectively into +19 mV by PACS modification and +25 mV by MMH. The removal efficiencies of modified clays respectively increased almost 20 times by PACS (Fig.3) and 3 times by MMH [8]. The results further

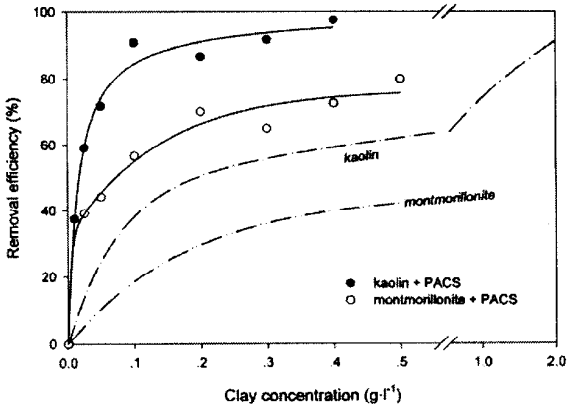

Fig.3. The enhancement of the coagulation of clays with *P. minimum* by PACS modification (PACS: 6 mg·l^{-1}).

proved the surface modification theory that surface characteristics of clay particles determined their coagulation capability with HAB organisms.

Insertion reaction method

This method was based on the principle that Mg^{2+} was inserted into both of the clay lattice and $Al(OH)_3$ layer precipitation on the clay under proper conditions, resulting in the conversion of the charge on the clay to a positive charge. Insertion of Mg^{2+} was a key step in the preparation, while the oxidization extent of the charge on the clay was related to Mg^{2+} concentration and temperature. As shown in Fig.4, when Mg^{2+} concentration was 2 mol·l^{-1} and temperature was 70°C,

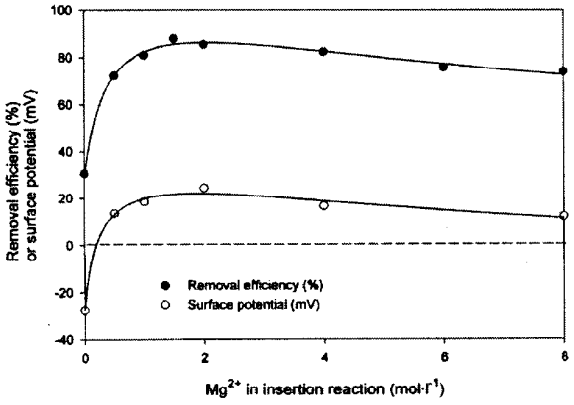

Fig.4. The effects of surface modification by insertion reaction on surface potential of montmorillonite and on removal efficiency of *Heterosigma akashiwo* (clay: 1 g·l^{-1}).

the zeta potential reached the maximum of 24.5mV, which was thought to be the optimum preparation condition. The removal efficiency of *Heterosigma akashiwo* by the montmorillonite modified under the optimum condition reached a maximum of 88.7%, an increase of nearly threefold of the original clay. Correlation analysis showed there was a positive

correlation between removal rates and surface zeta potential, which indicated that the positive charge property of the clay surface was the major reason for the higher removal efficiency of the algal cells [9].

COAGULATION KINETICS STUDY

In order to profoundly study coagulation mechanism between clay particles and HAB organisms, we developed a kinetic approach to study their coagulation rates. A model of the kinetics of organism coagulation with clays was established [10], which theoretically addressed the factors affecting coagulation rates and suggested ways to increase coagulation rates. The experimental results showed that the coagulation rates were more rapid in the system of kaolin than in that of montmorillonite. The kinetics in both systems could be described as bimolecular reaction, in which the rate constant was varied with particle sizes, interaction energy as well as species and clay type in the process of coagulation. The potential energy and radius of interaction between clay particles and organisms were the major factors controlling the coagulation rate. It was found that the increase of clay loading could accelerate coagulation, however, it was not recommended because it would cause more self-coagulation of clay particles resulting in a lower efficiency in use. It was found that modifying clays by PACS was a very effective way of increasing the coagulation rate because of the increase both in contact radius and interaction energy between clay particles and HAB organisms (Fig.5).

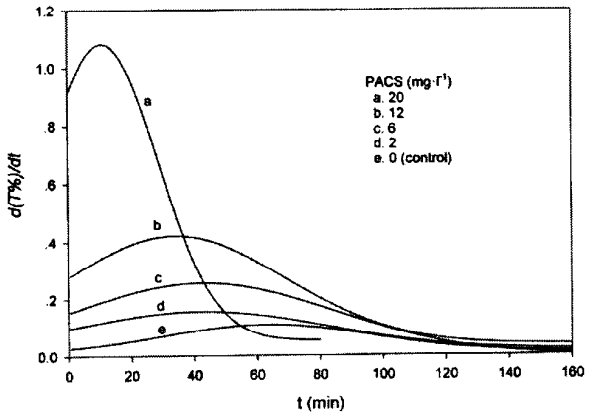

Fig.5. The effect of PACS concentrations on coagulation rate of kaolin (0.1 $g \cdot l^{-1}$) with *P. minimum* at 20°C.

IMPACTS ON EUTROPHICATION, HAB TOXIN AND MARICULTURED SPECIES

Some clays could not only remove HAB organisms, but also could adsorb nutrients in seawater decreasing eutrophication in waters. Studies [11] showed that phosphate adsorption capability of kaolin was greater than that of montmorillonite (Fig.6). It was explained

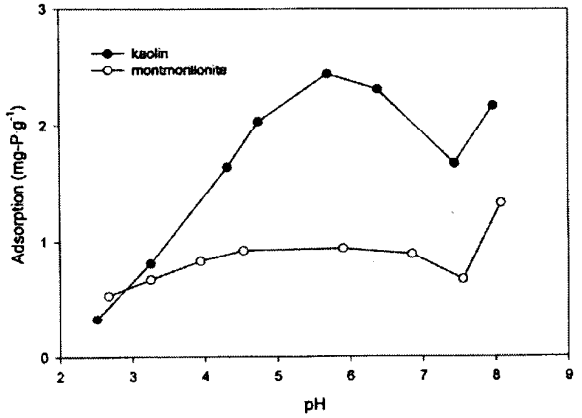

Fig.6. The adsorption of phosphate on kaolin and montmorillonite (2g-clay·l^{-1}, 25°C).

that the alumina-silica ratio of clays was the dominant factor for phosphate adsorption (i.e. the more the alumina content in clay surface layer, the stronger the phosphate adsorption). The study of nitrate adsorption on clays showed that the adsorption per centage was generally lower than 6%, and so its adsorption on clays could be negligible. We also studied the impacts of clay (halloysite) on growth of the HAB organisms, *Pseudonitzschia pungens f. multiseries*, and on the production of domoic acid [12]. The clay particles could cause a "mutual shading" effect on the growth of algal cells, resulting in the decrease of the maximum growth rates. Our experimental results also showed that the clay could inhibit domoic acid production and the maximum inhibition efficiency was more than 30% (Fig.7). We

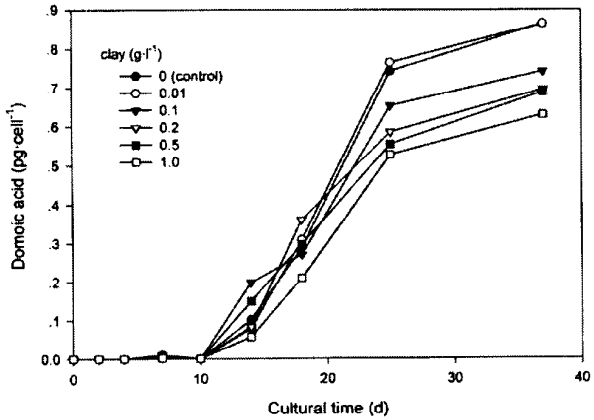

Fig.7. The effect of halloysite clay on domic acid production from *Pseudonitzschia pungens f. Multiseries*.

only examined the impact of clays on one species of maricultured shrimp. Mortality was not found during a 30-day experimental period. So, all above results indicated that the clay could not only control HAB, but could also prevent it from rupturing and decrease the harm from HAB toxins. Hence, the use of the clay is the first option for HAB mitigation.

THE ISSUES FOR FURTHER STUDY

Further research in laboratory on coagulation of clays with HAB organisms

Clay is a kind of mineral with a lot of varieties. HAB organisms also have many species. Because removal efficiency varied with the difference in clay variety and HAB species, we need more coagulation study on clay variety and HAB species in order to effectively control various HABs in the world. Also pretreatment method of original clays could result in very big difference in their coagulation capability with HAB organisms (e.g. up to 10-100 times). So clay modification will be another important study in laboratory in the future.

Environmental impact

An environmental assessment is a precondition for clay to be used in field. Environmental impact of clay loading can result from two parts. One is the impact of clay loading on biologic community (especially on benthic organisms and environment). Another is the impact of components released from clays on environment. Although clays are generally considered as a kind of non-polluting and safe material widely, being used in food and medical industries, there is still a lack of data and evidence to confirm that there is on negative impact on marine ecology and environment, especially under a large amount of clay loading. So, the environmental impact should be done in further studies.

Loading technology

Our studies showed that clay loading method and technology affected removal efficiency. Some experimental results in the laboratory could not be compared with that in field work because of different loading methods. Some special loading equipment used in field should be studied and designed. Site selection, clay storage and spraying method should be considered thoroughly prior to its application.

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COSTS ASSOCIATED WITH ALGAL TOXINS IN SEAFOOD IN CANADA

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ABSTRACT

In Canada, the medical and lost productivity costs for the estimated 150 paralytic shellfish poisoning (PSP) cases is probably over $525,000 each year, with the cost per case of about $3,500. In 1988, the PSP monitoring program cost $3,000,000. There are an estimated 57 cases of diarrhetic shellfish poisoning (DSP) or DSP-like illnesses costing about $108,000 each year or about $1,900 per case. Monitoring of DSP is limited to areas where the risk of DSP is greatest. On the assumption there may be as many as 133 ciguatera poisoning cases, the cost is over $684,000 each year with about $5,145 per case. Therefore, the total annual costs associated with the estimated 340 illnesses caused by seafood toxins of algal origin is about $1,318,000, and the product monitoring and analytical costs are about $2.7 million. Only one episode of amnesic shellfish poisoning (ASP) caused by ingestion of domoic acid has been documented in Canada (145 cases and 3 deaths) with illness-related costs and lost business of $8.4 million. In 1988, the cost for implementing a domoic acid monitoring program was $1,390,000. Because of the effectiveness of the monitoring program no further illnesses or public concern for ASP has occurred. These types of costs may be used to evaluate the economic impact of seafood-associated diseases in order to consider control measures.

INTRODUCTION

Case numbers and costs associated with some seafood toxins were estimated for Canada 5 years ago [1], and they need updating. Although the burden of illnesses associated with these toxins has had limited attention, other studies may give an indication of the extent of their impact. For instance, even in industrialized countries cases of infectious intestinal disease are not reported frequently. In a recent study in England, one case in 6 visits a physician and only one case in 136 cases reaches the national surveillance system [2]. Also, Mead et al. [3] estimated that 76 million cases of foodborne disease occur each year in the United States, an increase over the 6 - 33 million previously estimated [4]. For each foodborne disease, they gave a multiplier for reported cases ranging from 2 to 45 times [3]. Seafood toxins were not specifically mentioned by them but since well-known toxin-producing *Staphylococcus aureus* and less well-known marine *Vibrio* species were underreported by an estimated 20-38 times, based on epidemiological and laboratory studies [3], a similar range could be considered for seafood toxin illnesses (assumption:20 x for the better-known PSP and 38 x for the lesser-known DSP and ciguatera poisonings) but an extreme position could be 136 x the number of reported cases from Wheeler *et al.* data [2].

Illness costs include those affecting the infrastructure (societal), individuals (private) and industry. In Canada, inflation has been generally low over the past decade with most incomes not increasing, but medical costs have gone up. The following are approximate costs for today, but vary from location to location. Societal costs include those for medical care (estimate of $250 for physician visits and treatment), hospitalization (bed care, $800/day or $600/overnight, and intensive care, $3,000/day), emergency transportation (ambulance and special air flights, $1,500), laboratory testing (chemical or bioassays, $600), and illness investigation (patient interviews, epidemiological analysis of data, communication with other health and fisheries authorities and report writing, $1,500). Private costs to patients, relatives and friends include productivity losses through unearned wages, lost vacation or leisure time, and transportation to and from hospital. In some situations productivity losses are borne by employers or insurance companies, but they are treated as private losses here. The value of lives lost is estimated at $2,000,000; deaths are rare but may occur very occasionally from PSP or ciguatera poisoning. A figure of $160 per day has been used (about $42,000/year) for earnings of a typical person eating seafood, but there is a likely range from recreational harvesters on welfare to rich individuals who eat shellfish in exclusive restaurants. Estimates for transporting patients are from $200 to $1,500 depending on the severity of the illness and the distance to travel. No value has been attributed to indirect costs such as pain, grief or suffering. Costs to companies include lost revenue through bans on harvesting, embargo, recall, destruction of product, lost business, and legal action. Information on these are generally lacking for seafood toxin-associated illnesses and overall rough costs are given. Rounding up or down has been done with some costs to avoid giving the impression that these figures are accurate.

PARALYTIC SHELLFISH POISONING

PSP on both the east and west coasts of Canada have been reported almost every year since the late 19th century, but the number of illnesses has diminished recently. A typical case number of about 7.5/year for

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today is more appropriate based on later reports (114 from 1980 to 1995;[5]). An estimate of the annual incidence of PSP in Canada, therefore, is 7.5 x 20 = 150 cases. There were three fatalities during the 1980-89 decade [5], and none since, but the potential remains for fatalities [6]. Since most of the unreported cases would be mild, it is assumed that there would be no deaths associated with these cases. Therefore, each year there are probably 150 cases (7.5 reported and 142.5 unreported), most with mild symptoms, and less than one death (2 in 10 years). For each case the length of illness averaged 4.5 days and 36 % were hospitalized for one day or longer [7]. Societal and private costs are estimated at $79,990 (14.8%) and $429,200 (79.6%), respectively (Table 1). Deaths, even although rare (potentially 2 in every 10 years) account for most of the private costs. Industry losses are expected to be small, since no recent PSP episode involved commercial product. However, occasionally some locally harvested toxic shellfish may be served in restaurants and there may be some loss due to recalls and local adverse publicity; a nominal figure of $30,000 (5.6%) is given. The total estimated costs for PSP is, therefore, $539,190, with an average cost per case of $3,600. The cost for the control program to the Canadian Food Inspection Agency on both east and west coasts was $3,000,000 in 1988 including start-up costs for new mouse bioassay units. Slightly less money was spent in subsequent years, about $2.5 million for both PSP and ASP. Without this control program, illnesses and their associated costs would be much higher. No costs have yet been determined for potential illnesses arising from consumption of lobster hepatopancreases containing PSP toxins and monitoring of lobsters for toxicity [8]

Table 1. Estimated annual numbers of PSP cases and costs in Canada.

Cases = 7.5 reported; estimated annual number = 150
Deaths = 0.2 reported (2 per decade)

Medical costs: 25% of those ill would seek medical help = 37.5 x $250	$9 375	
Hospitalization: 36% of those seeking medical help = 36% x 37.5 =13.5 x $800/day x 1 day	$10 800	
10% of those hospitalized in are intensive care = 10% x 13.5 =1.35 x $3,000/day x 4 days	$16 200	
Emergency transportation for severely ill (some ending in intensive care) patients = 5 x $1,500	$7 500	
Laboratory testing: 7.5 reported x $600	$4 500	
Investigation: 25% of suspected and confirmed cases seeking help = 25% x 37.5 x $1,500	$14 063	
Total societal costs	$62 438	11.9%
Lost productivity: 12.15 hospitalized (not in intensive care) x $160/day x 4.5 days	$8 748	
Lost productivity: 1.35 hospitalized (in intensive care) x $160/day x 6 days	$1 296	
Lost productivity: 136.5 unreported x $160/day x 1 day	$21 840	
Personal transportation costs for those ill	$1 000	
Value of lives lost = 0.2 x $2,000,000 each	$400 000	
Total private losses	$432 884	82.4%
Losses to harvesters/restaurants, etc.	$30 000	5.7%
Total costs	$525 322	100.0%
Costs per case	$3 500	
Control program costs (monitoring and staff costs)	$3 000 000	

DIARRHETIC SHELLISH POISONING

Two DSP outbreaks have been documented in Canada, both from Nova Scotia [9,10]. DSP-like illnesses are reported from the Maritime Provinces practically every year but they are not necessarily diagnosed, since DSP resembles many other types of gastrointestinal illnesses, and could easily go unrecognized. These illnesses may be caused by DSP toxins other than okadaic acid or dinophysistoxins. If we consider there are at least 1 to 2 cases of DSP or DSP-like illnesses every year, a multiplier of 38 would estimate the number of affected persons to be 57. No serious conditions or deaths, however, are expected. Costs to society ($38,290, 34.2%) and individuals ($18,740, 16.7%) are less than for PSP. However, costs to companies are expected to be higher ($55,000, 49.1%), based on the harvesting bans and illnesses in restaurants that have already occurred. The total annual costs are $112,030 with $1,965 per case. There is no established control program, although limited monitoring of shellfish in areas known to have *Dinophysis* is carried out in the summer months. Rapid follow-up on consumer complaints is one mechanism to identify problems. About 500 samples are collected each year at a cost of $300 per sample including collection and shipping for a total of $150,000.

CIGUATERA POISONING

From 1983 to 1997 there were 53 cases in 15 episodes documented in Canada [5]. This is the equivalent of 3.5 illnesses each year. Because ciguatera is not easy to diagnose, we can expect at least 38 times the number of recorded illnesses, i.e., 133 each year. Societal costs are high because of degree of treatment required ($88,915, 13.0%), and even more so for private costs ($295,400, 43.2%) because of the long duration of the illness. The consequences of a ciguatera incident associated with a commercial establishment mean possible recalls, loss of business, or legal suits and $300,000 (43.8%) has been assigned to cover these costs. Legal action has been taken in some ciguatera incidents [11,12] with one settlement being for $900,000 cash plus $5,000 a month for the rest of the plaintiff's life assumed to be 17 years for a total of $2 million [13]. The total annual costs are estimated at $684,515, with $5,415 per case. There is no specific control program for ciguatera poisoning, but the Canadian Food Inspection Agency responds to consumer complaints and implicated fish will be removed from the market. About 10-30 samples of suspected fish are analyzed each year for a total of $3,000 - 9,000.

AMNESIC SHELLFISH POISONING

One episode of ASP occurred in Canada after cultured mussels from Prince Edward Island containing domoic acid originating from *Pseudonitzschia multiseries* were consumed in 1987 [14,15]. At least 145 persons were affected (although only 107 met the strict case definition) and there were three deaths. Costs totalled $8,363,000, with $2,012,500 for medical care and hospitalization (24.1%), $300,000 for the value of deaths (3.4%), $87,000 for lost productivity and leisure time (1.0%), $1,600,000 for research, recall and investigation costs (19.1%), and $4 ,363,500 for losses to the seafood industry (52.2%)[16]. Because of the control program to test shellfish for domoic acid that was initiated at that time, no further illnesses have occurred or are expected to occur from consumption of any commercial product. It is always possible, however, for recreational harvester to ingest seafood containing domoic acid, as occurred in Washington State [15], or the identification of a new source of domoic acid associated with seafood, both of which may result in affected persons, but no costs attributed to possible illnesses have been made. The annual monitoring program costs were about $1,390,000 for a few years after the episode [16] but are somewhat reduced today ($2.5 million for both PSP toxins and domoic acid monitoring).

CONCLUSION

It is important to try and estimate illnesses from seafood toxins in order to determine cost-benefit studies for mitigation strategies. In the current estimates on an annual basis, there are 150 PSP illnesses costing about $525,000, 57 DSP or DSP-like illnesses costing about $108,000, and 133 ciguatera illnesses costing about $684,000. No illness-related costs have been assigned to ASP. The total cost, therefore, for these estimated 340 intoxications is about $1,318,000 with an average cost per case of $3,880. The average annual cost per case for the specific diseases are ciguatera, $5,145; PSP, $3,500; and DSP $1,900. On an overall basis the control programs by the Canadian Food Inspection Agency for all these toxins total at least $2.7 million each year. (Other illnesses associated with non-algal toxins (histamine and tetramine cost about $310,000 annually but are not discussed further here).

Therefore, the total impact of illness-related costs associated with seafood toxins is $1.3 million together with the monitoring and analytical costs of $2.7 million for a total burden of $4.0 million. If a case multiplier of 136, based on the study of Wheeler *et al.* [2], is used instead of the 20 (PSP) or 38 (DSP, ciguatera) chosen, the number of cases and costs would increase substantially (about 6,400 cases at a total cost of at least $9.7 million). However, these numbers seem to be unrealistically high. The monitoring for algal toxins is effective in preventing contaminated product reaching the commercial trade, but cannot stop recreational harvesters from fishing in closed areas. Domestic cases of ciguatera poisoning, the most economically significant of the seafood toxin diseases, are more difficult to predict and there is no screening program in place to test tropical fish. Better consumer awareness for the different seafood toxins is another area where programs could be developed.

Can these Canadian cost data be used in other countries to estimate their losses and program costs? Each situation usually has specific costs but generalizations can be made. These include medical costs, the typical wage or range of wages of those affected, and the cost of a death. Losses to industries and local economies, however, are better measured individually. One attempt to use existing data for estimating costs of foodborne disease was done in Croatia based on US, Canadian, UK and Swedish data [17]. They multiplied the costs of different foodborne diseases by the Gross National Product per capita in Croatia divided by those in these other countries that were relatively similar, e.g., a salmonellosis case costing $1,350 in Canada or the U. S. would only cost $280 in Croatia. If their assumption is correct, it would be possible to take the Canadian data and estimate the costs in countries where costing might be difficult. However, it would be wise to do at least some economic analysis locally in order to verify this approach. As risk management becomes a tool for improving the safety and quality of food, increasing weight will be put on cost-benefit analysis. Costing of diseases and their control measures allow comparisons to be made to support specific intervention strategies. These estimates have been based on point estimates of

cases and costs. Monte Carlo analysis may make it possible in the future to consider ranges of data and case and cost probabilities with median and 5th and 95th percentile values.

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A BACTERIAL RISK ASSESSMENT AS A POSSIBLE MODEL FOR ASSESSING RISKS FROM ALGAL BLOOMS

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ABSTRACT

A risk assessment is a process of determining the probability of occurrence of adverse health effects resulting from exposure to a hazard. There are four steps: 1) hazard identification; 2) exposure assessment; 3) hazard characterization, including a dose-response assessment; and 4) risk characterization. Ideally, for harmful algal blooms, exposure assessments should begin with toxigenic plankton in the sea and end with a probability of illness after ingestion of a seafood containing toxin. Microbiological risk assessments have been developed with models from an animal host through to the consumer, where there is limited information to connect the reservoir with the hazard in the food. One of these is suggested as a model to build upon for algae. The hazard is *Vibrio vulnificus*, a bacterium that grows well in warm seawater. Oysters in the Gulf of Mexico that concentrate this organism through filter feeding have been implicated in illnesses. The assessment considers information from the *Vibrio* in the sea to the ingestion of an oyster meal. A model was developed to consider the prevalence, numbers and seasonality of *V. vulnificus* in oysters, and the influence of meal sizes. From these data, a simulation computed the probability of illness in a healthy individual living in Canada. The average probability of illness from eating a raw oyster meal ranged, month-to-month, from 2.2×10^{-6} to 5.5×10^{-3}. Median values ranged from 2.8×10^{-9} to 5.8×10^{-5}. Assumptions have been made based on existing knowledge and an allowance has been made for uncertainty. The bacterial model would have to be adapted for specific algal situations.

INTRODUCTION

Risk assessments are used to determine the probability of occurrence of adverse health effects resulting from exposure to a hazard, and if it is found to be significant, to help prioritize options for reducing the impact of the hazard. The reason for doing these assessments is to prioritize management programs to limit exposure of the population to pathogen or toxin levels that might cause illness. There are four steps in the process: 1) hazard identification; 2) exposure assessment; 3) hazard characterization, including a dose-response assessment; and 4) risk characterization. Ideally, for harmful algal blooms the assessment should begin with organisms in the sea and end with a probability of illness after ingestion of the seafood. However, this is difficult to do since the relationship between the number of algae and levels of toxin in seafood has not been clearly demonstrated. Microbiological risk assessments have been developed with models from an animal host through to the consumer where there is limited information to connect the reservoir with the hazard in the food. One of these involving a human pathogen, *Vibrio vulnificus*, is suggested as a model, after modification, for toxic algal blooms.

The hazard identification step

Gulf of Mexico Oysters containing *V. vulnificus* and eaten uncooked have been implicated in gastroenteritis and for some individuals septicemia and death. Most illnesses occur in the summer and fall months, when the organism reaches its highest concentration in warm Gulf waters. As the pathogen numbers decrease during the cooler winter and spring, the number of illnesses declines. This is in some way similar to dinoflagellates *Alexandrium* spp. or *Dinophysis* spp. in shellfish in other parts of the world. A model was developed to consider the prevalence, numbers, seasonality and virulence of *V. vulnificus* in oysters. Assumptions were made based on existing data with uncertainties being accounted for by using a Monte Carlo simulation [1], here, using Analytica™ (Lumina Corporation).

The exposure assessment step

In the *V. vulnificus* model, the level of exposure is the number of organisms consumed in an individual meal, represented by

Individual exposure = (organisms/g) x (oyster amount in g) x (oyster/serving).

The average weight for Gulf oysters is assumed to be Uniform(22, 26) g. Meal sizes are assumed to be Uniform(1, 24) oysters. An important step in quantifying exposure levels is to characterize the seasonal fluctuation of the concentration of organisms in harvested oysters. Temperature is an important controlling factor in the generation of *V. vulnificus*, with the best growth conditions occurring above 20E C. Concentrations of *V. vulnificus* in oysters range from as little as <1 MPN/g (Most Probable Number per gram) from December to April to highs of 10^5 MPN/g in the summer months [2, 3, 4]. Here, a simple step function is used to characterize changes in mean concentrations ($\log_{10}$ MPN/g) from winter

(Normal(1.7, .05)) to summer levels (Normal(5.0, .05)), with transition steps up in April and down in November (Normal(4.0, .05)). As well, concentrations are assumed to vary from oyster to oyster in a Poisson manner. For harmful algal blooms, different population dynamics should be substituted to include the effects of nutrient, light, turbulence, season, temperature and random climatic events [5, 6], and could also make use of chaotic dynamic models [7] or a model based on Brylinsky, 1997 [8].

Growth of *V. vulnificus* in harvested oysters can be easily controlled by storage temperature. In addition, concentrations in oysters can be reduced by cooking. This is in contrast to biotoxins which are resistant to heat. Since there are few barriers between seafood contaminated with biotoxins and the consumer, we use the consumption of raw oysters as equivalent for the purposes of exposure characterization. With this model, there is no increase or decrease in the level of contamination in a single oyster from harvest to consumption.

The model does not account for variability in the frequency of consumption of Gulf oysters in Canada over the year among different groups in the population. During summer months, shellfish from east and west coasts of Canada are commonly available. Imports of Gulf oysters tend to be more prevalent in October to December than other times of the year. Also, shellfish consumption is more popular in some Canadian provinces than in others. Some persons might choose to avoid eating raw oysters at particular times of the year.

Dose response modelling

The dose response model computes the probability of illness for an individual based on the determined level of exposure. Because no threshold can be clearly identified, the model provides a smooth transition from zero probability of illness at zero exposure, to very small (but positive) probabilities of illness at low exposure levels of the pathogen.

The model attempts to incorporate the identified sources of uncertainty. Although the most common source of exposure to *V. vulnificus* is through the consumption of contaminated food or water, wound infections are also possible. In either case, the infectious dose is not known, and reported exposures vary considerably. This lack of knowledge is an important source of uncertainty, which must be incorporated into any model that predicts the probability of illness given a particular level of exposure. Although this condition does not affect models for biotoxins, it illustrates some of the difficulties in establishing thresholds of human toxicity and how uncertainties must be considered

Which strains of *V. vulnificus* are virulent, how prevalent they are and what degree of virulence they possess is largely unknown. Given that there are large numbers of *V. vulnificus* present in oysters in the summer months and relatively few illnesses reported, the proportion of highly virulent organisms is likely to be small. Using the prevalence of *cth* and *viuB* genes in *V. vulnificus* from Gulf Coast oysters [9], we assume that the virulence fraction is slightly higher than 1/2 during warm months (ß(33, 33) + ß(5, 61)) and slightly lower than 1/2 for cool months (ß(33, 33)). This uncertainty parallels the uncertainty in predicting the amount of toxin per algal cell because of the variation in toxin production by algal strain and by season. The inclusion of virulence factors in bacterial risk assessment can be replaced with models for toxin accumulation [10].

For bacterial risk assessments, infection is usually assumed to be a precursor to illness. Because of the lack of information on the transition from infection to illness, we assumed that all of those infected became at least mildly ill, and used an infectivity model for the dose response calculations. The form of the infection dose response model is the ß-Poisson [11], which is often used in microbiological risk assessments [12, 13, 14]. The ß-Poisson model recognises variability in the response among doses and among hosts at the same dose [15]. Under that model, given the number of organisms ingested (d), the probability (P(d)) that an individual becomes infected or mildly ill is $P(d) = 1 - \left[1 + d/\beta\right]^{\alpha}$. In absence of other information, the model sets the α parameter to 0.2, which effects a rather flat transition from near 0 to near 1 probability of infection over a wide range of doses d. Using a stochastic ß parameter value, ln ß~Normal(24.5, 3.5), gives a range of values for ß that describe variability and uncertainty in susceptibility in the population, and a dose response function that reflects available epidemiological data [16].

Though subject to considerable uncertainty, attack rates based on epidemiological evidence can be used to calculate proportions of individuals with increasingly severe outcomes, such as hospitalization or death. *V. vulnificus* attack rates differ significantly among individuals with different risk factors such as chronic liver disease, prior gastric surgery, and diabetes mellitus [16].

Risk characterization step

The final step in any quantitative risk assessment is the assessment of the model and the computation of risk, the numbers of illnesses resulting from exposure to virulent *V. vulnificus* through eating raw oysters, either for specific areas of interest or over all scenarios considered in the simulation model. Various factors can affect the outcome of the model, as shown in the influence diagram (Fig. 1).

In the following scenario, the exposed population is assumed to consist of healthy individuals. Probabilities are simulated for each month of the year, with results

based on 10,000 iterations of a simulation model. The simulated values of the probability of illness from a single meal for the month of July averaged $5.5x10^{-3}$, with 5th, 50th and 95th percentiles $2.5x10^{-7}$, $5.8x10^{-5}$ and $1.2x10^{-2}$, respectively (Fig. 2).

The average probability of illness from eating a raw oyster meal ranged, month-to-month, from 2.2x10-6 to 5.5x10-3. Median values ranged from 2.8x10-9 to 5.8x10-5 (Fig. 3).

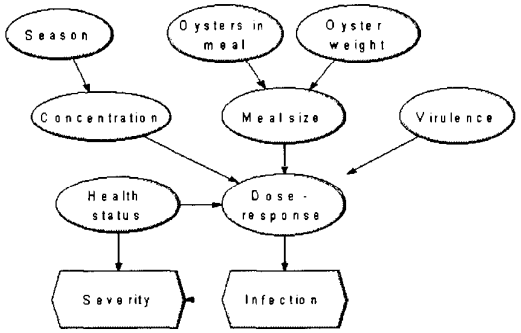

Figure 1. Factors that influence the outcome of the model in determining the probability of illness.

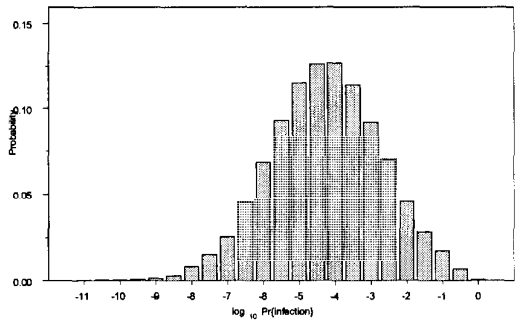

Figure 2. Simulated distribution of probability of infection arising from consumption of one meal containing virulent *V. vulnificus* in oysters harvested during July from the Gulf of Mexico and consumed in Canada.

This range shows the marked influence of seasonality on the probability of illness. The 5th and 95th percentile values reflect the degree of uncertainty in the model. The probability of illness from consuming a single raw oyster is approximately 10^{-2} times the probability of illness from a single meal; the average probability of illness ranged from $1.5x10^{-8}$ during the period December to March, to $6.6x10^{-5}$ in May to October.

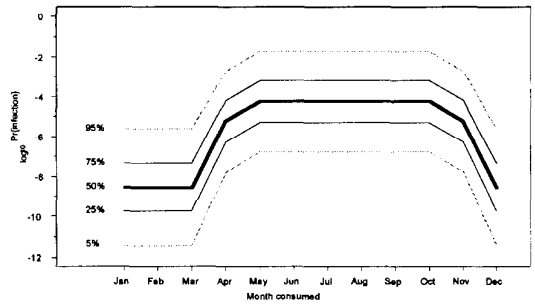

Figure 3. Range of simulated values of the probability of infection arising from consumption of one meal containing virulent *V. vulnificus* harvested from the Gulf of Mexico during the 12 months from January to December and consumed in Canada.

CONCLUSION

A model was designed to predict the probability of illness from eating Gulf oysters contaminated with *V. vulnificus*, even with limited data. The uncertainty elements are large and can be identified as areas needing additional research to improve the data going into future modifications. This model could be changed for toxins in shellfish arising from harmful algal blooms, in which the algal cell concentration could be used to estimate toxin levels in meals, and a different dose response model used to determine probability of illness.

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MONITORING A TOXIC CYANOBACTERIA BLOOM IN LAKE BOURGET (FRANCE) AND ITS CONSEQUENCES FOR WATER QUALITY

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ABSTRACT

This work describes the appearance of a toxic cyanobacterial bloom in Lake Bourget (France) in the winter 98/99 and its effect on the quality of the water that supplies two population centers. The horizontal distributions of the *Planktothrix rubescens* filaments were very similar in the three sampling stations and the vertical distributions were homogeneous from 0 to 140 m depth after complete mixing of the lake (January 1999). Before that, *P. rubescens* filaments were mainly located in the epilimnion and metalimnion. A microcystin-RR was identified by HPLC analysis. The RR-microcystin concentration in the lake was higher than 4 µg l^{-1} at the maximum of the bloom, while the concentrations in drinking water were always below 1 µg l^{-1}. This event shows that a large decrease in phosphorus concentration in the lake over the past 10 years, and pumping deep water (30 m) for water supply are not sufficient to avoid problems with cyanobacteria.

INTRODUCTION

Planktothrix (Oscillatoria) rubescens is very common in sub-alpine and other lakes in central and northern Europe [1]. This species can form blooms and produce toxins such as microcystins and anatoxin-a [2]. Thus, a *P. rubescens* bloom in a lake whose water is used for human consumption or for recreation must be monitored.

There have been problems with this organism in Lake Bourget over the past two years. A *P. rubescens* bloom in November 1998 sharply increased the turbidity of the water in treatment units providing drinking water. These central supplies obtain their lake water from a depth of 30 m. We have surveyed this new phenomenon by studying the spatial and temporal distributions of *P. rubescens* in the lake and estimated the microcystin concentrations before and after water treatment.

MATERIAL AND METHODS

Lake Bourget is a large (45 km_), deep (140 m at the point B) lake in the French Alps (Fig. 1). Water samples were taken at 3 points (B : northern lake, M : middle of the lake and T : southern lake) twice a month, to evaluate the horizontal distribution of *P. rubescens*. Samples (2 liters) were taken at depths of 3, 10, 15, 30, 45 and 65 m. 300 ml of each of them were preserved in Lugol's iodine solution for microscopic enumeration. 200 µm units of *P. rubescens* filaments were counted using the Utermöhl inverted microscope technique after sedimentation of 25 or 50 ml water. Estimation of the number of cells was obtained knowing that the mean length of a *P. rubescens* cell (estimated on 100 measurements) was 5 µm.

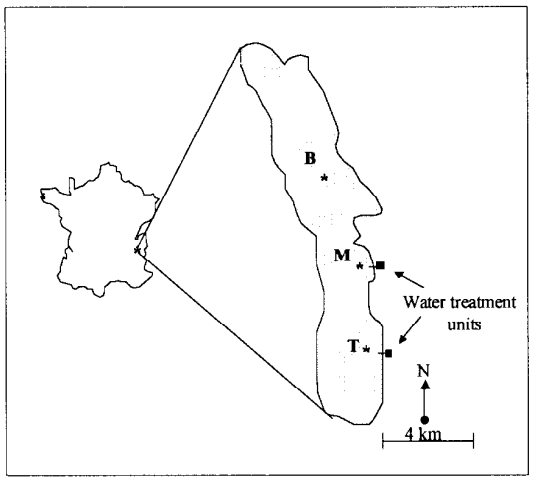

Fig. 1. Location of Lake Bourget in France. B, M (Mémard) and T (Tresserve) are the three sampling stations for this study

Microcystins were extracted from 1 liter of water by the protocol of Feuillade *et al.* [3]. The samples were taken in the water treatment units, before (water intake in treatment unit) and at the end of the treatment steps (in water reaching the consumers). Intracellular microcystins were identified and quantified by high-performance liquid chromatography (HPLC). The eluting compounds were detected by photodiode-array UV spectroscopy (Waters). Identification of toxins was realized on their retention time and on their UV spectra (absorbance maximum at 238 nm). The amount of microcystin was estimated by measuring the area of the UV absorption peaks at 238 nm and quantitation (Millennium Software from Waters) was made using a mcyst-RR and a mcyst-LR calibration curve. These two microcystins were purchased from SIGMA.

RESULTS AND DISCUSSION

The vertical distributions of *P. rubescens* in the three sample areas (M, T & B) were very similar (Fig. 2). In December 98, the filaments were located above the thermocline with a maximum density (> 20000 cells ml^{-1}) at 30 m depth. There was a small difference in the depth of the thermocline at the

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beginning of the month, deeper at points M and T (50 m) than at point B (35 m). This was due to the strong dominant north wind at this time of year (data not shown), which deepens the thermocline in the southern lake.

From January to the end of the bloom in March 99, the cell concentration, similar throughout the water column, slowly decreased. At point B, the density was the same from top to bottom (140 m) from January to March as a result of complete mixing of the lake.

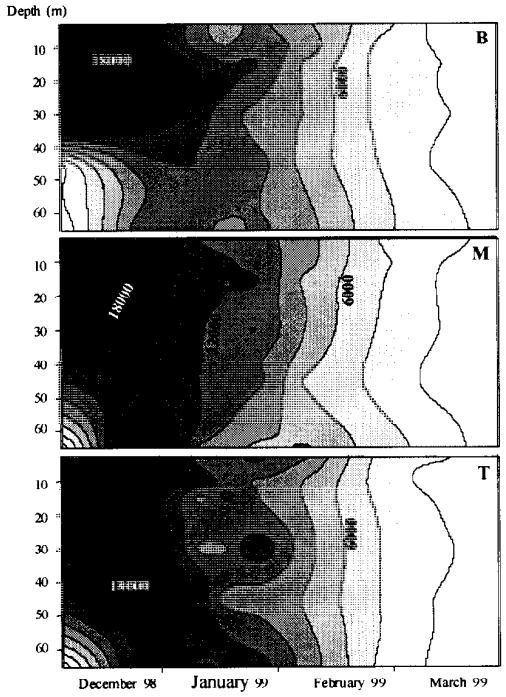

Fig. 2. Spatial distributions of *Planktothrix rubescens* at the three Lake Bourget stations during the bloom (98-99). The cyanobacterial concentration is expressed in number of cells per milliliter of water.

The spatial distribution of *P. rubescens* in the water column is very interesting as there are no published reports of a homogeneous distribution of this cyanobacterium from the surface to the bottom in a deep lake. Micheletti *et al.* [4] showed that *P. rubescens* occupies the metalimnion (10-15 m) in lake Zürich in summer and gradually spreads to the epilimnion in autumn. Filaments were found in the epilimnion throughout the year in lake Nantua [5].

The cell concentration of *P. rubescens* in the untreated water of the two central water supplies (Fig. 3) was significantly ($p < 0.05$) correlated with the one estimated in the lake at 30 m (Pearson correlation coefficient $r = 0.94$ for Mémard and $r = 0.98$ for Tresserve supplies respectively).

There was also a significant correlation ($p < 0.05$) between the numbers of cells before and after water treatment in the Mémard central supply ($r = 0.9$), but not in Tresserve ($r = 0.6$) (Fig. 3). This phenomenon was probably due to differences in the performances of water treatment protocols used in the two treatment units. Differences in the cell concentrations were especially significant from January to March, after the treatment protocol in the Tresserve unit was changed (Fig. 3). These changes concerned the washing of the sand filters (every day instead three times a week) and the quantity of ozone used (4 g/m^3 O_3 instead of 3 g/m^3). This change was followed by a strong decrease of the cell concentration in the water at the 01/13/1999.

Most of the microcystin (> 90 %) found in the cellular extracts was mcyst-RR. There was a good linear correlation (Mémard, $r = 0.8$; Tresserve, $r = 0.9$; $p < 0.05$) between the number of *P. rubescens* cells and the microcystin-RR concentration in water before treatment (Fig. 3). There were several intracellular microcystin-RR concentrations above 0.5 µg l^{-1} in treated water during three weeks in December 1998 and January 1999 but no extracellular toxin was detected by HPLC, in treated water throughout the study period (data not shown).

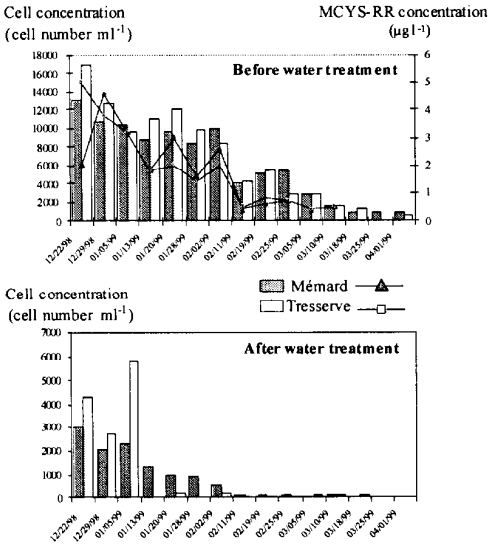

Fig. 3. *Planktothrix rubescens* cell concentrations (bars) and microcystin-RR concentrations (lines) in water before treatment, and cell concentrations after water treatment in the two water supplies (Mémard and Tresserve).

A drop in the level of the ground water has resulted in surface water being increasingly used as a source of drinking water. Falconer et al. [6] have recommended that the water body used for domestic consumption should be selected on the basis of its history (cyanobacterial blooms), nutrient concentration (total phosphorus < 10 µg P l^{-1}) and hydrodynamic regime. In Lake Bourget, the total phosphorus is over 10 µg P l^{-1} after complete mixing of the water in winter. But phosphorus concentration was six times higher in the Eighties and there were never any cyanobacterial blooms. So, the reduction

of phosphorus concentration is not always sufficient to prevent cyanobacterial bloom formation. In Lake Nantua, the decline in phosphorus inputs has resulted in *P. rubescens* population sinking to the metalimnion rather than causing a major reduction in the cyanobacteria biomass [5. In the same order, Mez *et al.* [7] have observed cyanobacterial blooms in oligotrophic lakes from Switzerland.

Hrudey *et al.* [8] pointed out that the vertical movement of cyanobacterial populations must be known in order to select the best depth for withdrawing drinking water to avoid contamination. Taking water from depth generally avoids problems of cyanobacteria. The bloom in Lake Bourget shows that this generality does not always overcome problems with cyanobacteria in deep lakes because complete mixing of the water in winter distributes the filaments throughout the water column.

The concentrations of intracellular microcystin-RR in drinking water were always below the maximum dose (1 $\mu g\ l^{-1}$) of microcystin-LR recommended by the WHO [9], which is considered to be more toxic than microcystin-RR [10]. However, the human health risk resulting from chronic exposure to this toxin is very difficult to estimate. We are therefore preparing an Alert Levels Framework (monitoring and management action sequence) for Lake Bourget and the pumping of ground water has been re-instituted for use during the blooms.

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THE EFFECT OF THE DAWESVILLE CHANNEL ON CYANOBACTERIAL BLOOMS AND ASSOCIATED MICROALGAE IN THE PEEL-HARVEY ESTUARINE SYSTEM, WESTERN AUSTRALIA

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ABSTRACT

The Peel-Harvey, a shallow micro-tidal estuary 70 km south of Perth, has been subjected to inputs of excessive agricultural nutrients over several decades and the repeated occurrence of cyanobacterial blooms. The Dawesville Channel constructed in 1994 to improve flushing appears to have led to a reduction in microalgal densities, altered community composition and succession in the estuary.

All spring blooms (composite max. ca. $5x10^5$ cells per ml) of the hepatotoxic cyanobacterium *Nodularia spumigena* (Mertens) have occurred almost annually since 1978 but now have ceased in the estuary, probably because of the maintenance of higher minimum salinities and reduced nutrient availability. Since the opening of the Dawesville Channel, annual rainfall and runoff to the estuary have been low and thus an assessment of the effectiveness of the management strategy cannot be completed until tested against a series of wetter years.

In the absence of *Nodularia* blooms, the estuary continues to experience occasional diatom blooms and low densities of *Nodularia* are episodically translocated from riverine reaches where severe annual blooms continue.

The absence of recent *Nodularia* blooms in the estuary is cause for optimism that the Dawesville Channel has achieved its aims. In the Serpentine River estuary annual cyanobacterial blooms as cause for continued concern and the first recorded bloom of *Prymnesium parvum* (N Carter) was recently observed.

INTRODUCTION

Prior to 1994, the 136 square kilometre shallow Peel-Harvey estuarine system (Fig.1) at Mandurah, 75 kilometres south of Perth in Western Australia, had a single restricted connection to the ocean and the estuary experiences only 15% of external oceanic tidal fluctuation. The region has a Mediterranean climate with hot dry summers and mild wet winters. Ninety five percent of the annual runoff occurs between May and October [1].

The estuarine system experienced severe *Cladophora* blooms from the 1950's through to the 1970's, which emitted hydrogen sulphide as they decomposed and left the beaches with a foul black sludge [2]. This was later followed by toxic cyanobacterial *Nodularia* blooms in the estuary that became more frequent from the 1970's to early 1990's. Blooms of *Nodularia* and other blue green algae also occurred in the Serpentine River estuary that flows into Peel Inlet. Blooms of *Nodularia* in the estuary were associated with fish and crab deaths where *Nodularia* scum accumulated and rotted [2]. These blooms were the visible symptoms of nutrient enrichment, caused by the oversupply of phosphatic fertilisers and wastes from the surrounding 11,434 square kilometre catchment.

During winter, before the construction of the Dawesville Channel, blooms of freshwater diatoms *Cyclotella atomus* (Hust) and *Skeletonema* spp. occurred in the Peel-Harvey estuary. These diatom blooms utilised much of the phosphorus entering the Peel-Harvey during winter-spring when the salinity was low. The freshwater diatom bloom then collapsed and became part of the sediment bank [3]. When the estuarine salinity increased in spring, the sedimented nutrients were released back into the overlying water column as bottom oxygen levels decreased. The released nutrients were then available for uptake by both macroalgae and estuarine microalgae during the spring-summer.

In the early and late 1970's, and almost annually in the 1980's and early 1990's [4], toxic blooms of *Nodularia* dominated both the Peel Inlet and Harvey Estuary. The blooms frequently exceeded a 3-site average peak integrated density of 500,000 cells ml^{-1} in both the 20 km long Harvey Estuary and the 10 km wide Peel Inlet. Tests on edible mussels *Mytilus edulis* growing in the Mandurah channel confirmed the toxicity of the bloom [5]. Toxicity was also confirmed by the Western Australian Department of Agriculture using mouse bioassay [6].

In 1988 a formal environmental impact assessment processes reviewed a number options to resolve the problem [4].

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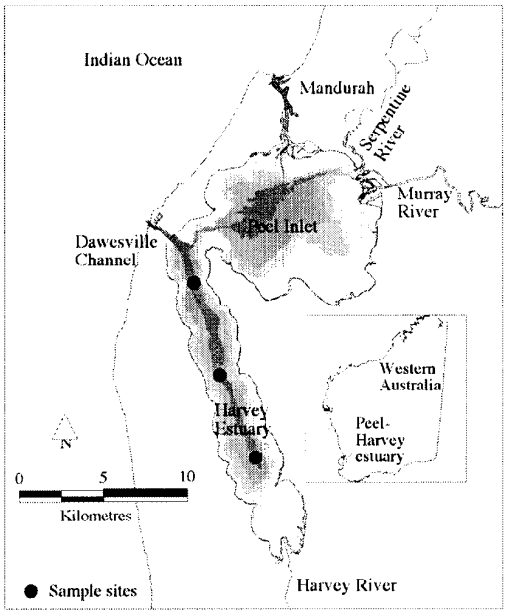

Figure 1 Map showing the location of the Peel-Harvey estuary in Mandurah Western Australia (inset) and the location of the Dawesville Channel.

The preferred management strategy included improved flushing of the estuary and reduction of nutrient exports from the surrounding catchment. Flushing was to be increased through dredging of the existing Mandurah Channel and construction of a new channel at the junction of the Peel and Harvey estuaries [2].

In April 1994 a 2.5km long and 200m wide channel that connected the Harvey Estuary to the ocean was commissioned (Fig.1). The aim of the Dawesville Channel was to reduce *Nodularia* blooms to once in every 10 years [7], by increased export of soluble and particulate phosphorus contained in winter runoff directly to the ocean and through reduced nutrient regeneration because of increased dissolved oxygen concentrations in bottom waters [4].

The improvement in marine flushing of the estuary maintained higher winter salinities thus reducing the likelihood of *Nodularia* akinetes germinating in the sediments. Salinities above 20‰ have been found to greatly decrease akinete germination [8]. Viable *Nodularia* akinetes were found to be important in the rapid onset of blooms and these were found to be present to a sediment depth of 35cm [9].

In addition to environmental benefits expected to accrue from the Dawesville Channel, there were also some predicted adverse impacts. These included: the potential for increased mosquito and Ross River virus infection associated with the grater tidal inundation of fringing marshlands; impacts on fringing vegetation; reductions in commercial and recreational fish species such as cobbler and king prawns; increased current speeds, scouring and siltation of navigable channels [1,4, 7].

This paper reviews the changes in microalgal species composition and densities in the Peel-Harvey estuary for the period 1983 to 1999 and associated waterways assessed by microalgal monitoring by the Water and Rivers Commission.

MATERIALS AND METHODS

Integrated water samples were collected from three sites each in the Peel Inlet and the Harvey estuary (Fig.1) at intervals of between 7 to 28 days from June 1983 to the end of December 1999. The depth of all sites was less than 2.5metres.

Integrated water samples were collected between 0800 Hrs and 1300 Hrs on all occasions and immediately preserved in Lugol's solution. To observe delicate species that may have been damaged by the fixative and to help identify rarer species, estuary water was passed through a 5 micron net (Swiss Screens) to concentrate live microalgal cells. These fresh samples were kept cool in an esky and examined within 24 hrs of collection.

Between 1983 and 1996 microalgal cell counting of the preserved cells was undertaken at X125 magnification using a 1 ml volume plastic Sedgewick Rafter counting chamber. From 1996 a glass Sedgewick Rafter counting chamber was used with a long working distance objective lens (X400). In 1990 a significant bloom of picoplankton (cyanobacterium) *Synechococcus* spp. was enumerated using a glass Neubauer cell.

RESULTS AND DISCUSSION

There have been no observed reductions in total phosphorus concentrations [10] or loads to the estuary prior to or after the Dawesville Channel was completed (Fig.2). The success of the Dawesville Channel in reducing blooms of *Nodularia* can be seen for the years 1996 and 1998 (Fig.2). No blooms occurred in these two wetter years even though these TP loads probably would have triggered a bloom prior to the Dawesville Channel.

Improved flushing resulting from the Dawesville Channel has altered the seasonal patterns of salinity in the Harvey estuary (Fig.3). Average winter minimum salinities and summer maxima have been attenuated. Fresh water inputs from catchment runoff now result in a short-term reduction in estuarine salinities followed by a rapid return to higher salinities. Before the Dawesville Channel, winter runoff inputs caused a longer term reduction in estuarine salinities.

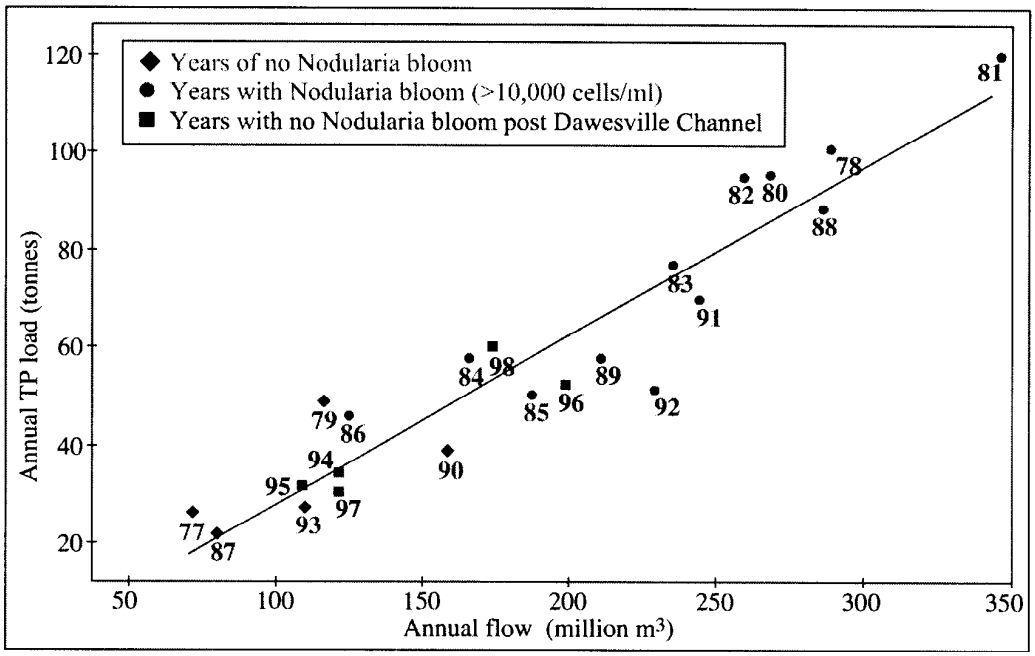

Figure 2 Correlation between flow and total phosphorus (TP) loads for the Harvey River and the relationship to blooms of *Nodularia* in the estuary.

Since the opening of the Dawesville Channel in April 1994, there has been a notable absence of cyanobacterial blooms in the Peel-Harvey estuary (Fig.4). The density of total microalgae has also been much lower.

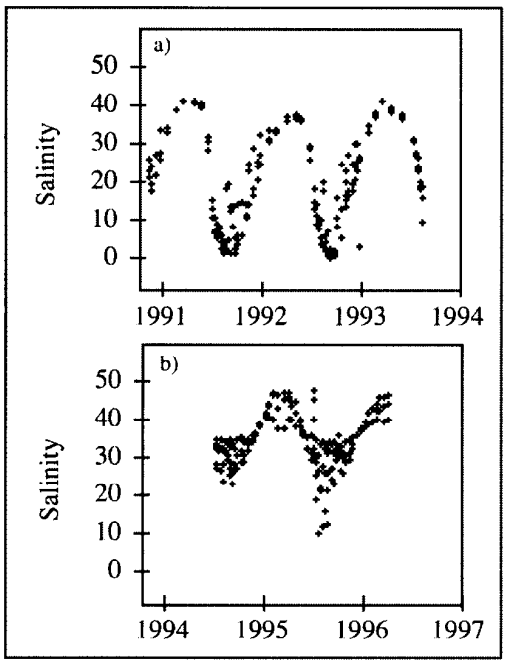

Figure 3 Salinity of Harvey estuary. a) Prior to Dawesville Channel (1991 -1994). b) After Dawesville Channel (1994 - 1996).

Peak mean *Nodularia* densities were originally over $6x10^5$ cells per ml. In years when *Nodularia* blooms occurred, the blooms were preceded by high levels the diatoms *Cyclotella atomus* and *Skeletonema potamos* that were favoured by low winter salinities.

Nodularia blooms occurred from October to January while salinities were between 10‰ to 30‰. When estuarine salinities exceeded 30‰ in summer, *Nodularia* blooms declined and a marine diatom bloom followed (*Chaetoceros spp.*, *Cerataulina pelagica*, *Asterionellopsis glacialis* and *Rhizosolenia setigera*). Total microalgal densities declined to their lowest levels in autumn when estuarine and marine dinoflagellates and other species became dominant. In 1987 and 1993 when *Nodularia* failed to become established (Fig.4) probably due to a combination of low rainfall and low total phosphorus loads, the level of phytoplankton activity remained low and estuarine species remained dominant in the winter.

In 1990 a bloom of the cyanobacterium *Synechococcus* occurred reaching a maximum density of $37x10^6$ cells per ml (Fig.4). The extremely small size of these species cells (ca. 1micron) meant that even though the numeric cell density was extremely high the biomass was significantly less than of regular microalgae. In the late summer/autumn period when the biomass of microalgae was at its lowest and the salinity high, the waterway was usually dominated by dinoflagellates and other minor species (Fig.4).

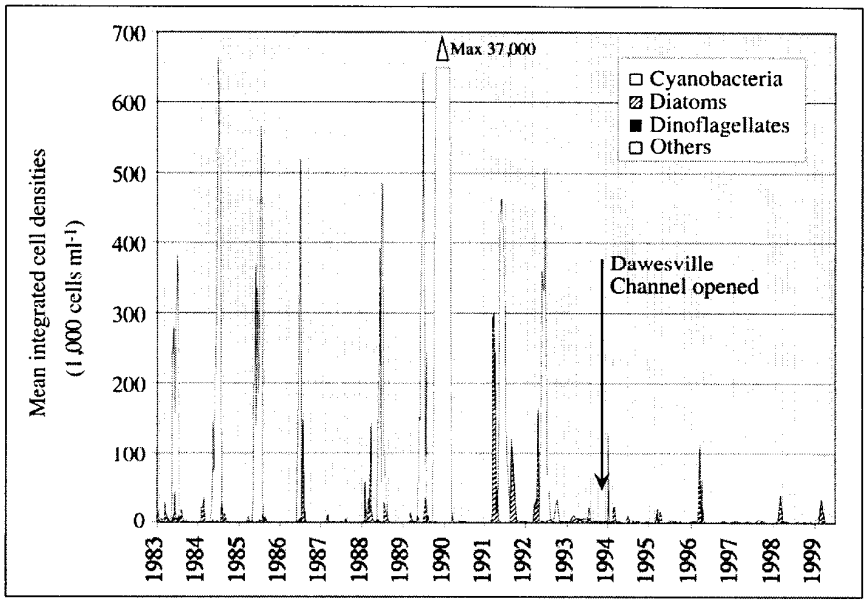

Figure 4 Mean integrated microalgal composition and densities in Harvey Estuary from 1983 to 1999.

CONCLUSIONS

It is concluded that the construction of the Dawesville Channel has stopped *Nodularia* blooms in the Peel Harvey estuary. The post-Dawesville Channel results have also shown a reduction of total integrated microalgal biomass. The years 1996 and 1998 had annual rainfall totals and phosphorus loads that exceeded the levels that would normally have resulted in a *Nodularia* bloom prior to the Channel opening.

This is consistent with predictions that widespread *Nodularia* blooms would only occur in the estuary in the very wettest years and that in normal years, the growing season would be markedly restricted [4].

In addition to the Dawesville Channel, the preferred management strategy also required significant reductions in nutrient export from the catchment. Nutrient exports from the catchment have not been recorded [10], and even though *Nodularia* blooms have not been observed in the Peel-Harvey estuary, the Serpentine River has undergone regular recreational and contact closures because of an annual blooms of cyanobacteria and other potentially harmful species (*Prymnesium parvum).*

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DEVELOPMENT OF POTENTIALLY TOXIC CYANOBACTERIA AND BACTERIA DURING ARTIFICIAL RECHARGE OF GROUNDWATER

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ABSTRACT

The occurrence of cyanobacterial blooms is typical for the Lake Mazais Baltezers, the water source for the artificial groundwater recharge plant supplying up to 25% of drinking water for Riga City, Latvia. In 1997-98, investigations of algae and bacteria were carried out in the lake water as well as in water and sediments from one infiltration basin and in sand from bore-holes between infiltration basin and a siphon-pipe, i.e., in the course of artificial recharge of groundwater.

In 1997, cyanobacteria appeared in water of the lake and the infiltration basin in July (0.381 and 0.847 mg L^{-1}, correspondingly) with *Microcystis* spp. dominating, and in August (0.847 and 1.925 mg L^{-1}, correspondingly) with *Aphanizomenon flos-aquae* (L.) Ralfs. The development of algae including cyanobacteria as well as bacteria (measured as aerobic heterotrophic oligocarbophillous bacteria and total bacterial number) in water of the infiltration basin was substantially higher than in the lake indicating favourable conditions for development of biota. Cyanobacteria were observed also in sediments to a depth of 3 cm below the bottom's surface of the infiltration basin. In contrast to water, cyanobacteria in the sediments were detected not only in late summer, but also during other seasons. The investigations of sand taken from 10 bore-holes between infiltration basin and siphon-pipe to a depth of 900 cm showed that cyanobacteria developed to a depth of 200 cm in the bore-hole under the infiltration pond while bacteria were found a depth of up to 900 cm in all bore-holes. A positive correlation was observed between cyanobacteria and total bacterial number in sediments from the bottom's surface of the infiltration basin ($r=0.82$; $n=4$) and in sand from the bore-hole under the infiltration basin ($r=0.87$; $n=8$, $\alpha=0.01$), and further investigations are needed to find the explanation of this phenomenon. A direct link between development of cyanobacteria and heterotrophic bacteria was neither established in water nor in the sediments or sand of the infiltration system.

Consequently, this study revealed that at low quantities of cyanobacteria interaction between them and heterotrophic bacteria was not found in the course of artificial recharge of groundwater and thus the influence of cyanobacteria on the biodegradation of organic matter was not established.

INTRODUCTION

Artificial recharge of groundwater is being used for drinking water production (about 25% of total) in Riga, Latvia. In the process of infiltration, surface water from Lake Mazais Baltezers (M. Baltezers) is pumped to the infiltration basins from where it percolates down to the groundwater. After underground transport (retention time 30-180 days) the recharged groundwater is abstracted by a siphon system (production wells are connected to vacuumed siphon-pipes), disinfected and pumped to the city water distribution.

Lake M. Baltezers is located in the vicinity of Riga City. Its trophic state is eutrophic. In comparison with the lake in the beginning of the century concentrations of organic matter and biogenic substances in water as well as in sediments has increased [1]. In 1996, mineral nitrogen and phosphorus concentrations in lake water varied from 1.0 to 1.83 mg L^{-1}, and from 0.015 to 0.055 mg L^{-1}, respectively. The major part of phosphorus (64-97 % of total phosphorus) in the lake sediments was found bound to organic substances [2]. Nitrogen is more abundant than phosphorus in the lake, and the N:P ratio exceed 12 [3], suggesting that the algal production was limited by phosphorus. While the development of *Aphanizomenon flos-aquae* (L.) Ralfs. and *A. scheremetievii* Elenkin was scanty in 1951, potentially toxic blue-greens *Microcystis aeruginosa* Kütz., *Anabaena flos-aquae* (Lyngb.) Breb. and *A. scheremetievii* Elenkin were found in very high amounts in the 1990's [4, 5]. *Microcystis aeruginosa* Kütz. has previously been found to be dominant in phytoplankton from eutrophic and hypereutrophic lakes in Latvia with minor amounts of *Anabaena* spp., diatoms and green algae [6].

With increasing eutrophication toxic cyanobacterial blooms can be expected to cause a risk for drinking water [7]. In 1995, toxic cyanobacterial blooms occurred in the lake M. Baltezers at the end of the summer and microcystin-LR was found in the algal biomass [5].

Further filtration through sediments and sand results in a rather efficient removal of cyanobacterial toxins and cells except when abundant cyanobacterial blooms occurred [8]. Toxins from water are removed mainly as a result of microbial degradation or photolysis in the presence of humus [9,10]. Besides the fact that toxins excreted by cyanobacteria in the basin directly threaten the health of drinking water consumers, they may influent metabolism of biota in the water body [11], and

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subsequently the degradation processes of organic matter in the recharged groundwater.

Organic substances in drinking water may form potentially carcinogenic compounds upon chlorination [12], and serve as a substrate for bacterial re-growth in the water distribution system [13]. In the process of artificial groundwater recharge, organic substances are mostly removed during water passage through the subsurface from the infiltration basins to the production wells. Adsorption on sand and biodegradation by heterotrophic bacteria living in the subsurface are the two major processes responsible for this reduction [14].

The groundwater quality largely depends upon the input from Lake M. Baltezers and sediment biology. Potentially toxic cyanobacteria and bacteria living in the infiltration ponds and in the sand between the infiltration ponds and drinking water production wells influence self-purification process of the recharged groundwater. It is therefore, the knowledge about development of cyanobacteria and bacteria, and interactions between them are important. This study attempted to find whether the cyanobacteria significantly influenced the development of bacteria in the course of artificial recharge of groundwater.

MATERIALS AND METHODS

The samples for bacteriological and algological analyses were collected from water with a Ruttner water sampler and from sediments with microbenthometer from Lake M. Baltezers (M. Baltezers) and from one infiltration basin (May, July, September 1997). Sand cores were taken with a special vibrator-drill sampler from 10 sampling bore-holes between the infiltration basin and the siphon-pipe (October 1998) (Figure. 1).

In samples from water, sediment and sand the total bacterial number (TBN) was determined by direct count method. The number of aerobic oligocarbophillous heterotrophic bacteria was determined by the pour-plate method with specific nutrient medium [15, 16]. Algal analyses were conducted according to Standard Methods [16].

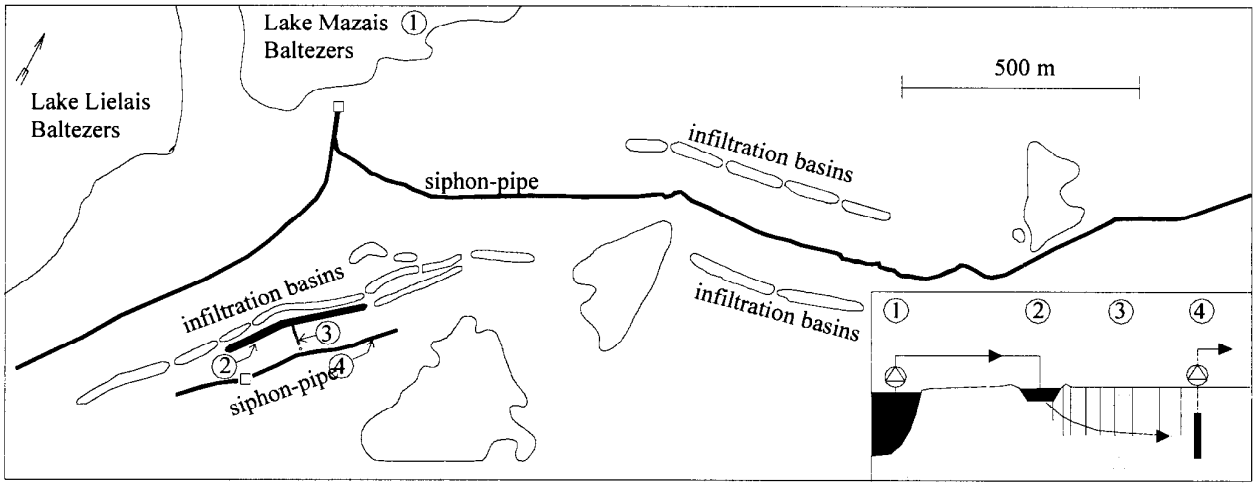

Figure 1 Map of the study site and a principal scheme of water passage during artificial recharge of groundwater - Lake M. Baltezers (1), infiltration basin (2), sand sampling bore-holes (3), and production wells connected to a siphon pipe (4).

RESULTS AND DISCUSSION

During the period of investigation (May-September 1997) cyanobacteria occured in the lake at the input sites for artificial groundwater in July (0.381 mg L^{-1}) when *Microcystis* spp. was dominant, and in early September (0.847 mg L^{-1}) when *Aphanizomenon flos-aquae* (L.) Ralfs. was dominant. In 1997, contrary to previous years [4, 5] a development of cyanobacteria was less pronounced. In 1997, TBN in lake water was high, although low mesophillous saprophytes and the coli index characterized the water as clean [17].

In the water of the infiltration basin, the development of cyanobacteria and bacteria was substantially higher than in the lake (Table 1).

The overall composition of algae in the infiltration basin was similar to that of the lake, but the phytobenthic species *Oscillatoria tenuis* (AG.) Kütz. apparently washed in from the lake was overgrown the bottom of infiltration basin. Bacillariophyceans dominated the upper sediment layer (to 10 cm) known to reduce the infiltration rate of water by clogging pores between sediment particles [18]. At the end of the vegetation period (September), diatoms together with decayingcyanobacteria (*Microcystis* spp., *Aphanizomenon flos-aquae* (L.) Ralfs.) formed a crust on the surface of the sediment, further reducing the infiltration process.

Table 1. Abundance of heterotrophic oligocarbophilous bacteria (HB), total bacterial number (TBN) and cyanobacteria in the water of Lake M. Baltezers and one infiltration basin, May-September 1997

Date	HB, cells mL^{-1}		TBN, cells 10^6 mL^{-1}		Cyanobacteria, cells L^{-1}	
	lake	inf. bas.	lake	Inf. Bas.	lake	inf. bas.
7.05.	2 100	2 930	5.6	8.4	0	0
26.05.	150	790	3.5	9.3	0	7740
15.07.	120	1 500	6.1	9.2	304000	2348800
17.09.	110	490	8.0	8.0	888200	16836850

Cyanobacteria were observed to a depth of 3 cm and in contrast to the overlying water they were detected not only in the late summer but also during other seasons. A similar trend of development of cyanobacteria and bacteria on the surface of sediments, and a correlation was found between numbers of cyanobacteria and TBN ($r=0.819$; $n=4$). Correlations between numbers of cyanobacteria and oligocarbophillous heterotrophic bacteria and TBN were neither established in the water nor in deeper layers of sediment.

In the sand taken from 10 bore-holes between the infiltration basin and the siphon-pipe (depth down to 900 cm), the TBN was related to the concentration of NO_3^- ($r=0.862$; $n=7$, $\alpha=0.05$) and to organic matter ($r=0.987$; $n=7$, $\alpha=0.01$). In October 1998, algal cells were found to 700 cm only in the bore-hole below the infiltration basin, dominated by Bacillariophyceae.

The Cyanobacteria *Oscillatoria tenuis* (Ag.) Kütz were found to a depth of 200 cm, and also *O. princeps* Vauch., typical for autumn, as well as *Lyngbia* sp. occurred (Table 2).

Table 2 Distribution of cyanobacteria in sand from bore-hole below an infiltration basin to a depth of 900 cm

Depth, cm	*Cyanophyta*		
	Oscillatoria princeps	*Oscillatoria tenuis*	*Lyngbia sp.*
0	+	+	+
10	+	+	+
20	+	+	+
30	+	+	0
40	+	+	0
50	+	0	0
100	+	0	0
200	+	0	0
300	0	0	0
400	0	0	0
500	0	0	0
600	0	0	0
700	0	0	0
900	0	0	0

In the sand from bore-holes a positive correlation between the concentration of cyanobacteria and TBN was found: $r=0.87$ ($n=8$, $\alpha=0.01$) (Figure.2) but correlations between cyanobacteriacea and heterotrophic bacteria were not observed.

In general, the presence of cyanobacteria in the course of artificial recharge of groundwater appears to be determined by their development in Lake Mazais Baltezers. The numbers and the biomass of cyanobacteria as well as the numbers of total bacteria increased substantially in the infiltration basin indicating favourable conditions for development of both algae and bacteria. A correlation was found between the concentration of cyanobacteria and the total bacterial number in the sediments from the infiltration basin and in the sand from bore-hole below this basin.

At the same time significant correlations between cyanobacteria that occurred in 1997 at low quantities and heterotrophic bacteria in the infiltration basin and in the sand from bore-holes were not found, indicating that there was no significant interaction between algae and heterotrophic bacteria. Thus the biodegradation of organic matter in artificially recharged groundwater was not considerably influenced by the cyanobacteria.

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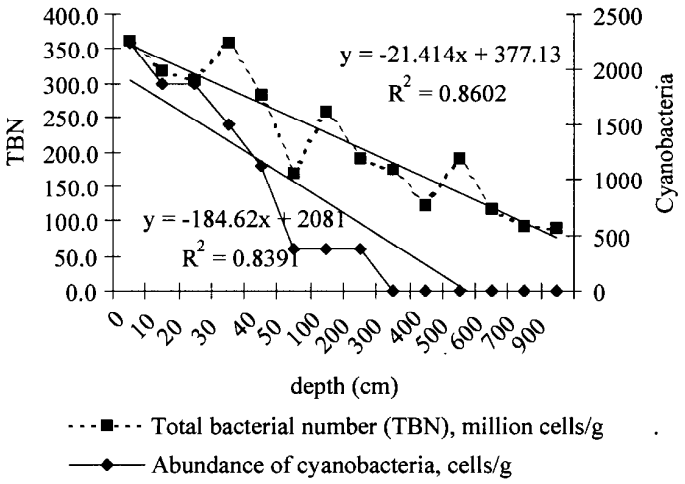

Figure. 2 Changes in total bacterial number (TBN) and abundance of cyanobacteria with the depth in sand of the bore-hole below the infiltration basin

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INVESTIGATION INTO THE FORMATION OF TRIHALOMETHANES, CHLOROPHENOLS AND DIOXINS AFTER CHLORINATING WATER CONTAINING THE CYANOBACTERIAL TOXIN CYLINDROSPERMOPSIN

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ABSTRACT

Cyanobacterial toxins such as cylindrospermopsin and microcystins are potent toxins, but recent studies have demonstrated that these compounds can be effectively degraded using chlorine treatment, providing that the residual chlorine levels are sufficiently high. However, chlorine treatment is associated with formation of a wide variety of disinfection by-products that are associated with a series of health effects including carcinogenesis. The aim of this study was to assess formation of disinfection by-products during chlorine treatment of water, which was spiked with cell free extract (CFE) material of toxic cyanobacteria. In this study the formation of trichloromethane and chlorophenols was associated with chlorination of CFE of *C. raciborskii*, while the formation of other trihalomethanes, chlorinated benzenes or polychlorinated dibenzodioxins and dibenzofurans was not detectable. With respect to THM formation, data from this study indicate that the increase in trichloromethane concentration was primarily the result of chlorination of organic matter other than cyanotoxins. Furthermore the levels of trichloromethane formation associated with degradation of high levels of cyanobacterial material is relatively low when compared to the levels which are associated with current drinking water in Brisbane and with the Australian drinking water guidelines. In summary the results from this study did not identify any significant risk associated with the chlorine treatment of water containing the cyanobacterial material containing the cyanotoxin, cylindrospermopsin.

INTRODUCTION

Toxic cyanobacteria such as *Cylindrospermopsis raciborskii* (Woloszynska) Seenayya et S. Raju, and *Microcystis aeruginosa* (Kutzing) are common and have been associated with human health problems [1,2]. Dissolved cyanotoxins are not readily removable from water using general drinking water treatment methods (flocculation, sedimentation and filtration) [3]. Cylindrospermopsin is more often in the dissolved fraction than microcystins, hence subsequent treatment steps such as use of activated carbon may be needed to reduce dissolved cyanotoxin concentration [4,5]. Chemical oxidants such as chlorine have also been shown to be effective for degradation of microcystin-LR [6] and cylindrospermopsin [7]. Chlorination can cause the formation of potentially toxic disinfection by-products (DBBs) such as the volatile trihalomethanes (THMs). DBBs are formed from precursors such as humic substances and phytoplankton metabolites [8]. Epidemiological studies have found evidence of a causal link between consumption of chlorinated drinking water and in particular THM levels, and elevated cancer occurrence (i.e. bladder and colorectal) [9]. Other potentially harmful DBBs include chlorinated phenols which have been associated with embryotoxicity and tumor promotion [10,11] and an elevated risks of non-Hodgkin's syndrome and soft tissue sarcoma [12]. In addition to traditional DBBs, formation of polychlorinated dibenzodioxins and dibenzofurans (PCDD/Fs, commonly referred to as dioxins), which include some of the most toxic compounds known, has been associated with chlorine oxidation for example in paper bleaching.

The aim of this study was to assess formation of THM's, chlorophenols and dioxins as a result of chlorination of toxin containing cyanobacterial extracts. This is essential to assess risk management steps related to the degradation of cyanotoxins. At the time of experimentation, there was very little cyanobacterial activity in reservoirs used in this study.

MATERIALS AND METHODS

Cyanobacterial material. Cultures of *C. raciborskii* were harvested and prepared for experimental work as set out in Senogles *et al.*, [7]. Microcystin was obtained from a natural occurring *M. aeruginosa* bloom in Brisbane. Microcystin-LR was the dominant toxin present, with few other microcystins present at relatively low levels.

Chlorination. Sodium hypochlorite was used as the chlorine source for this work. Chlorine residuals were determined by DPD calorimetric analysis [13], where a 25 mL sample was collected and analysed on a HACH DR-2000 spectrophotometer.

Organic carbon quantification. A standard Dissolved Organic Carbon (DOC) working solution of 50 mg/L potassium hydrogen phthalate in water was used as the source of OC in experimental samples. Total organic carbon was determined by acidifying samples to a pH $\leq$ 2 upon collection. A Shimadzu TOC-5000 organic carbon analyser was used for DOC quantification.

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Solution pH. The effects of solution pH were not measured in this study. The pH was unbuffered and varied with the addition of chlorine, pH 6.7±0.5.

Protocol

Experiments 1-2: Trihalomethane formation in low volume water samples. In Experiment 1, 250 mL of reverse osmosis (RO) water was spiked with *C. raciborskii* CFE material, and then chlorinated with a chlorine dose of 40 mg/L which is more than 10 times in excess of the dose required to degrade cylindrospermopsin (CYN) [7]. Blanks consisting of RO water, RO water containing no CFE, but which was chlorinated with 40 mg/L, RO water containing CFE but no chlorine and water from the tap were included.

In Experiment 2 the study was altered to include dissolved organic carbon (4 mg/L which is similar to the DOC in the cyanobacterial samples). Furthermore the chlorine dose was reduced to relevant doses of 4 and 8 mg/L of chlorine. Controls included water from the tap and water containing the CFE or OC, but which were not chlorinated. Expt's 1 and 2 were carried out in triplicates and subsamples were analysed for THMs.

For analysis of THMs in the samples, subsamples (10 mL) were transferred into calibrated centrifuge tubes and after the addition of 2 mL of pentane shaken for 3 min in an automated shaker. The supernatant phase was then carefully transferred into vials and analysed using GC-ECD. Quantification was performed using external calibration.

Experiment 3 Dioxins and chlorophenols. Experiment 3 focused on the more potent toxicants such as PCDD/Fs and also potentially unknown DBBs specifically related to cyanotoxin degradation, 40 - 80 L of water was spiked with CFE of *C. raciborskii* or *M. aeruginosa.* After chlorination (30 min contact time) compounds of interest were collected using a filter/sorbent system which was designed to enrich lipophilic organic chemicals from water [14], where compounds associated with suspended particles are collected on glass fibre filters and dissolved phase compounds are enriched on XAD-2 resin. For chlorophenol sampling the water was acidified to a pH < 2 prior to sampling.

For chlorophenol analysis (Expt. 3) filters and resin were spiked with a cocktail of deuterated 3-5 ring PAHs. The filters were extracted in an ultrasonic bath using first acetone (50 mL) and dichloromethane (2X). XAD-2 cartridges were rinsed on the outside with acetone and then transferred into soxhlet. Then about 200 mL of acetone and 200 mL of dichloromethane were carefully added onto the cartridge, and the samples were soxhlet extracted for at least 10 hours. Both, the extracts from the filters and the extracts from the XAD-2 were then concentrated to about 50 mL and subject to liquid/liquid partitioning adding n-hexane. The water phase was washed 2 more times with n-hexane and the combined solvent phase was filtered through anhydrous sodium sulfate and then concentrated to about 1 mL. The samples were analysed on a GC-MS operated in full ion scan. Chromatograms were qualitatively examined with respect to the occurrence of specific known compounds such as chlorobenzenes and chlorophenols as well as unspecified compounds with a mass-fragmentation typical for chlorine substitution.

For analysis of PCDD/Fs filter and resin extracts were combined from the individual treatments and the samples were sent to ERGO-Forschungslabor in Germany. At ERGO the samples were transferred into n-hexane spiked with a known quantity of 12 carbon labeled tetra- to octachlorinated PCDD/Fs. Samples were purified on an acid/base activated silica column followed by a fractionation on a column filled with basic aluminium oxide. Samples were then concentrated almost to dryness, transferred into microvials and taken up into 20 µL of toluene containing known amounts of 1,2,3,4-TCDD as a recovery standard. Samples were analysed on a GC coupled to VG-Autospec at a resolution of approximately 10,000.

RESULTS AND DISCUSSION

Reproducibility

Reproducibility of the experimental set-up was tested for experiments 1 and 2 where samples were analysed in triplicates. Trichloromethane was the key analyte which was detectable in all samples, and which varied significantly between different treatments. The mean coefficient of variation (CV) of trichloromethane was 11 %. In 85 % of the treatments the CV was smaller than 10 %. This demonstrates that the methods used were appropriate considering that the reproducibility includes variations in sample set-up, extraction and the analytical method. Experiment 3 which required relatively large quantities of the cyanobacterial material as well as other resources was not replicated.

Experiment 1 and 2

The formation of trihalomethanes at chlorination levels of 40 mg/L which is at least 10 times greater than that required to remove CYN [7] was analysed. Trichloromethane was elevated with 140 (± 4.0) µg/L in samples with chlorinated CFE compared to the concentrations in the non-chlorinated treatments which were 11 and 9.6 (± 0.3) µg/L for the controls (i.e. RO water only and RO water with cylindrospermopsin, respectively) (Fig. 1).

A minor increase in dichlorobromomethane from about 6.2 (±0.16) µg/L (RO water) and 5.7 (±0.11) µg/L (RO water with only CFE) to 7.5 (±0.17) µg/L in the chlorine treated solution which contained *C. raciborskii* material was observed. Chlorodibromomethane and tribromomethane were below the detection limit in all

treatments, however, chlorodibromomethane concentrations in tap water was 12.2 (±0.32) µg/L, at least a factor of 6 greater than those in the chlorine treated *C. raciborskii* sample or any of the control samples (detection limit 2 µg/L).

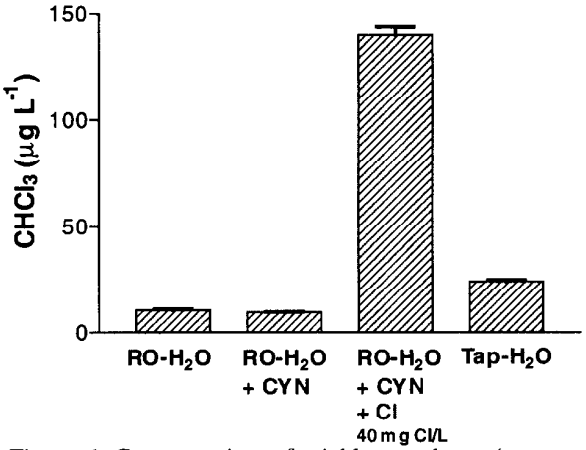

Figure 1 Concentration of trichloromethane (mean and standard deviation) in control samples (RO water and RO water with CYN as CFE) compared to water with CYN as CFE and chlorine treatment and tap water.

To test whether significant formation of THMs is observable in water containing CFE material from toxic *C. raciborskii*, more realistic doses of chlorine (4 or 8 mg/L) were examined. In addition comparisons of THM formation between the chlorination of CYN and a standard OC solution were examined. In Expt. 2, trichloromethane was the only trihalomethane which was detectable in all treatments while dichlorobromomethane, chlorodibromomethane and tribromomethane were only detectable in the tap water. Trichloromethane concentrations in Expt. 2 were lower than in Expt. 1. Mean concentration of trichloromethane in all non-chlorinated treatments (i.e. RO water only, RO plus CFE of *C. raciborskii* and RO plus OC) were 1.3 to 1.7 µg/L. Addition of 4 and 8 mg/L of chlorine resulted in a successive increase in the trichloromethane concentration for all treatments as may be expected (Fig. 2). However THM concentrations in the chlorinated controls (only RO water, open bars) were similar or even higher compared to the chlorinated *C. raciborskii* samples (grey bars). This indicates that increased THM formation (as opposed to non-chlorinated samples) is not a result of the chlorination of cyanobacterial material specifically. The OC present will lead to THM formation as seen with samples containing a standard source of OC. Hence THM formation is a result of chlorinating OC not the transformation of cyanotoxins. It is further noteworthy that the trichloromethane concentration in tap water was between 25 and 35 µg/L and thus significantly higher than those observed in all treatments from Expt. 2. This suggests that the THM related risk associated with degradation of toxic CFE material was low compared to the existing THM risk.

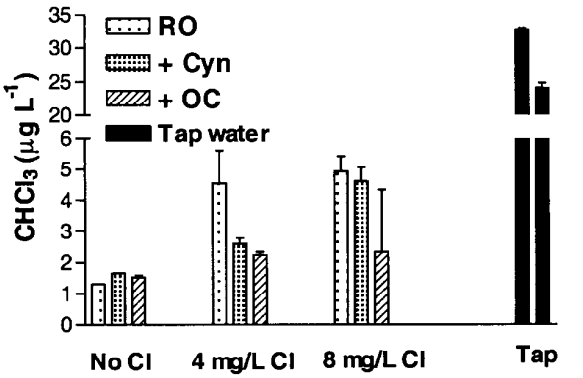

Figure 2 Concentration (mean and standard deviation of n=3) of trichloromethane in various treatments (i.e. with CYN as CFE or a commercial organic carbon source (OC) and at various chlorination levels) and water at the tap in Brisbane.

Experiment 3

In experiment 3 formation of more potent toxicants which require more sensitive analytical methods were examined. The initial focus was to analyses the samples using GC-MS (full ion scan) to identify compounds with mass-fragmentations typical of chlorine substituted compounds. No lipophilic chlorinated compounds such as chlorinated benzenes could be detected. Chlorophenols, and in particular dichloro- and trichlorophenols, were the only compounds of interest which could be identified, and which were elevated in the chlorinated *C. raciborskii* CFE samples compared to the non-chlorinated control samples. An increase in chlorophenols was also observed in chlorinated RO water compared to the control samples. The chlorophenol data from Expt. 3 should be seen mainly as qualitative data since to date these experiment have not been replicated and the extraction efficiency could not be quantified with certainty. It is thus not clear, whether the observed difference in, for example trichlorophenol concentration is significant in the treatments which received extra chlorine (i.e. with *C. raciborskii* or just RO water) (Fig. 3).

The water extracts from Expt. 3 were also analysed for 2,3,7,8-chlorine substituted tetra- to octachlorinated dibenzodioxins and dibenzofurans (the most toxic PCDD/Fs). Lower chlorinated 2,3,7,8-substituted PCDD/Fs including 2,3,7,8-tetrachlorodibenzodioxin, the most toxic of all PCDD/Fs were below the detection limit of 0.1 pg/L in all samples.

Heptachlorinated PCDDs and octachlorodibenzofuran, but in particular octachlorodibenzodioxin were detectable in the samples at low levels. However the concentration in samples which contained *C. raciborskii* or *M. aeruginosa* CFE and which were consequently treated with chlorine were not found to be elevated in comparison

to the control sample (no cyanobacterial material or chlorine added). Interestingly the highest PCDD/F concentrations (i.e. OCDD) were found for the sample of RO water which contained no cyanobacterial material but which was chlorinated (Fig. 4).

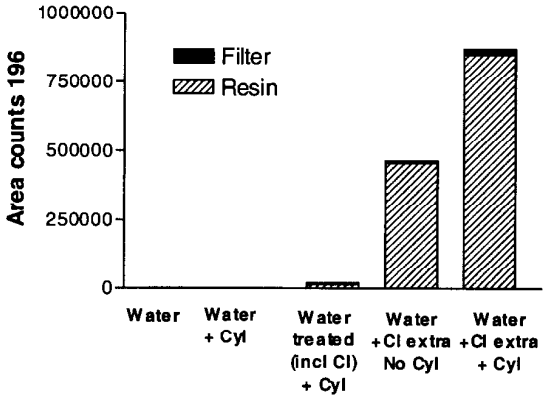

Figure 3 Response of the main ion of trichlorophenol in various treatments of water. Here the water used had background chlorine residual, and extra chlorine represents the addition of more chlorine.

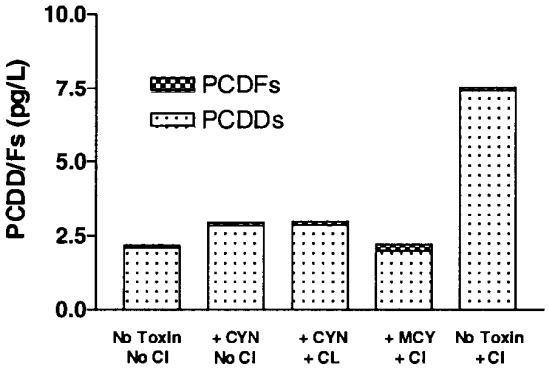

Figure 4 Concentrations of the 2,3,7,8-chlorine substituted dibenzodioxins (PCDDs) and dibenzofurans (PCDFs) in water spiked with CYN as CFE or MCY as *C. raciborskii* or *M. aeruginosa* and/or treated with chlorine. No toxin samples have a background natural OC level of ~ 3mg/L.

In summary the results of this study found formation of relatively low levels of trichloromethane and chlorophenols during treatment of water containing toxic cyanobacterial material. Formation was mainly associated with organic compounds other than the cyanotoxins, and the levels of THMs and chlorophenols formed due to chlorination were low when compared to the drinking water guidelines or even normal drinking water collected from the tap in Brisbane. The levels found in Brisbane tap water are below guideline values. No increased risk which is associated with chlorine treatment of water containing the cyanobacterial material was detected compared to Brisbane drinking water regulartions.

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SUBJECT INDEX