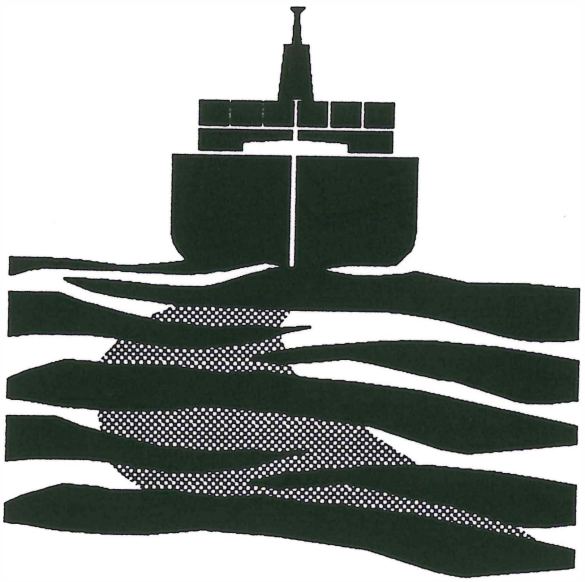
Final Report

FIRDC Grant 89 / 39

Toxic dinoflagellate spores in ships' ballast water :

A danger to aquaculture



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Foreword

The present investigations on "Toxic dinoflagellate spores in ships' ballast water" and "its implications for aquaculture" were funded by FIRDC grant 89 / 39 (Sept 1989 - Sept 1991). This research involved a collaborative effort between CSIRO Division of Fisheries and the Australian Quarantine and Inspection Service (AQIS), and was instigated by the claim by CSIRO that the toxic dinoflagellate *Gymnodinium catenatum* in Tasmanian waters could have been introduced via cyst stages contained in ships' ballast water. In February 1986, contamination of Tasmanian shellfish with dinoflagellate toxins led to the closure of 15 shellfish farms for periods up to 6 months. Subsequently, similar toxic dinoflagellate outbreaks surfaced in the Australian ports of Adelaide (*Alexandrium minutum*) and Melbourne (*Alexandrium catenella*). Genetic evidence (rRNA fingerprints) suggest that these latter species are also ballast water introductions.

The present research received considerable national and international publicity (front page news in the Hobart "Mercury" and "Sydney Morning Herald", national television coverage on the "7.30 report" and "Beyond 2000"). The Australian Quarantine and Inspection Service has responded to this evidence by introducing, as of 1 February 1990, voluntary ballast water guidelines for ships entering Australian ports from overseas. As of 1 November 1991, the International Maritime Organisation (IMO) ratified these guidelines for adoption on an international basis.

The present FIRDC- funded research has functioned as a catalyst for further ballast water research funds (600 K) made available by AQIS and BRR. Regular meetings (Nov. 1989, Feb.1990, June 1991, Oct. 1991, Nov. 1991, Feb. 1992) of a newly established Scientific Working Group on Ballast Water (under direction of Dr.Meryl Williams, BRR) are overseeing the implementation of some of the conclusions of the present research.

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1. Objectives

To survey toxic dinoflagellate spores in the ballast tanks of ships entering Australian ports, and to develop appropriate control action to protect Australia's aquaculture industry.

2. Staff employed on the program

Dr. G. M. Hallegraeff (CSIRO)	Supervisor of project
Mr. C. J. Bolch (FIRDC)	Full-time research assistant
Dr. S. I. Blackburn (CSIRO)	Dinoflagellate culturing

Collaborators

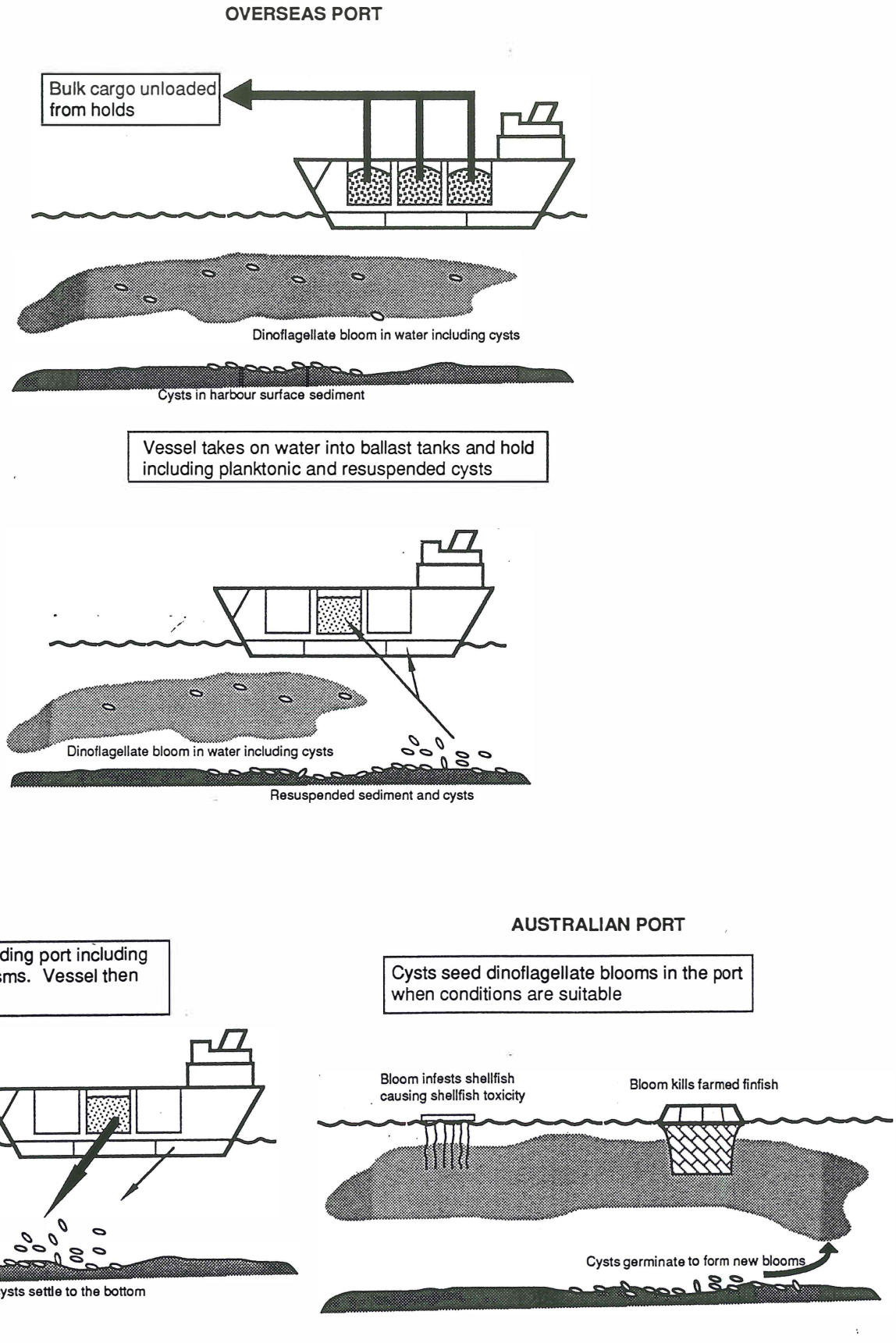
Mr. N. Blain, AGAL, Melbourne	ballast water monitoring
Dr. P. Hutchings, Australian Museum, Sydney	biology of ballast water
Dr. J. Merton, AQIS, Canberra	ballast water monitoring
Mr. R. Murphy, AQIS, Canberra	ballast water monitoring
Dr. Y. Oshima, Tohoku University, Japan	dinoflagellate toxin chemistry
Dr. M. Jones, BRR, Canberra	ballast water guidelines
Dr. G. Rigby, BHP Research Laboratories, Newcastle	ship engineering
Mr. C. Scholin, Woodshole Oceanographic Institution	dinoflagellate genetics
Mr. A. Sherwin, AQIS, Canberra	ballast water monitoring
Dr. S.O.Stanley, Tasmanian Dept. of Sea Fisheries	fish farm diseases
Dr. M. Williams, BRR, Canberra	ballast water guidelines
Mr. R. Williams, NSW Dept. of Fisheries, Cronulla	biology of ballast water

3. Introduction

The Australian economy is heavily dependent on the export of raw materials such as coal, grain, iron ore, wheat and woodchips. Specialised bulk container ships only carry these commodities in one direction (Fig.1), using water as ballast for stability on the return voyage. As a result, some 60 million tonnes of ballast water are discharged in Australian ports every year, with ships sailing from Japan accounting for over a third of this amount (Hutchings *et al* .,1987; Jones, 1991). Cargo vessel ballast water was first suggested as a vector in the dispersal of non-indigenous marine species nearly ninety years ago. The diatom *Odontella (Biddulphia) sinensis*, well known from the tropical and subtropical coasts of the Indo-Pacific, was not reported in European waters until 1903, when it produced dense plankton blooms in the North Sea. Since it was unlikely that this large diatom could have been overlooked previously, and impossible that it could have been carried by currents from distant oceans, Ostenfeld (1908) suggested that this species was introduced by ship, either as part of the fouling community on vessels' hulls or, more likely, discharged with the water or sediment contained in ships' ballast tanks. Since then, ballast water has been suggested as a dispersal mechanism for a large range of marine organisms (see review by Carlton, 1985). In Australia there are at least 15 established marine species known with reasonable certainty to have arrived in ballast water. These include fish (4 spp.), crustaceans (4), polychaete worms (3), molluscs (3) and a seaweed (1) (Pollard & Hutchings, 1990 a,b; see Fig. 2). The potential impact of these species on commercial fisheries, aquaculture and the natural environment is of increasing concern. In Tasmania, the introduced seaweed *Undaria pinnatifida* (current standing crop approximately 400 tonnes) poses a threat to abalone and sea urchin fisheries as well as a proposed marine park zone (Sanderson, 1990). In the Great Lakes of North America, blockage of water inlets by the introduced zebra mussel *Dreissena polymorpha* has been estimated to cost more than US $ 5 billion in control costs over 10 years (Hebert *et al.*, 1989).

If dinoflagellate species that can produce paralytic shellfish toxins (FIRTA grant 86 / 84) are introduced into sensitive aquaculture areas, the results could be disastrous for commercial shellfish farming. The present

Fig.1. Introduction of non-endemic, toxic dinoflagellate cysts via ships' ballast water, and their potential impact on Australian aquaculture.



study was prompted by the recent appearance in Australian waters of toxic dinoflagellate species that appear to have a disjunct global distribution, such as *Gymnodinium catenatum* (confined to Japan, Mexico, southern Europe and Tasmania), *Alexandrium catenella* (confined to North America,Japan, South Africa and southeast Australia) and *Alexandrium minutum* (confined to South Australia and southern Europe) (see Fig. 3a,b). Strong circumstantial evidence provided by examination of historic plankton samples (Hallegraeff *et al.*,1988), cyst surveys in sediment depth cores (Bolch & Hallegraeff, 1990) and genetic studies using enzyme electrophoresis and sexual compatibility experiments (Blackburn et al.,1989) suggested that the toxic dinoflagellate *Gymnodinium catenatum* was introduced to Tasmania at some time after 1980 in the area around the shipping port of Hobart. Benthic cyst beds of this species are now widespread in southern Tasmania, and dense plankton blooms in 1986 and 1991 in southern Tasmanian waters necessitated the closure of 15 shellfish farms for periods of up to 6 months (Hallegraeff & Sumner, 1986). Similarly, rRNA sequencing has shown a remarkable match between Australian (Port Phillip Bay) and Japanese populations of the toxic dinoflagellate *Alexandrium catenella,* and between Australian (Port River, Adelaide) and European populations of the toxic dinoflagellate *A. minutum* (Scholin & Anderson, 1991).

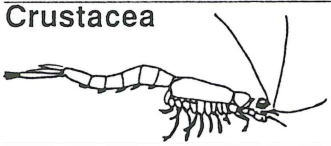
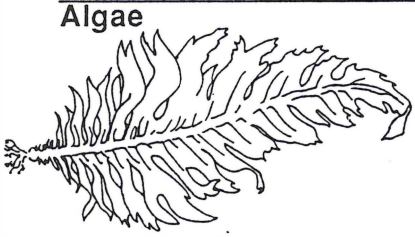
The aims of the present work were (1) to establish unambiguously whether toxic dinoflagellate species can be carried by ships in a viable condition; (2) to elucidate how dinoflagellate cyst abundance in ballast tanks varies according to ship type, ballast-tank configuration, season and port of origin, and the practice of mid-ocean ballast exchange; and (3) to examine the efficacy of various physical and chemical treatments to kill toxic dinoflagellate cysts in ballast water samples.

4. Ballast water survey

4.1 Materials and methods

Officers of the Australian Quarantine and Inspection Service (AQIS) sampled the ballast tanks of 343 cargo vessels which arrived in 18 widely spread Australian ports between November 1987 and March 1990 (Fig. 4). More than half the ships had sailed from Japan; the remainder arrived from east Asia (China and Hong Kong, Indonesia, South Korea, Singapore, Sri

Fig.2. Suspected ballast water introductions of marine organisms into Australia .

Molluscs		*Aeolidiella indica* (Japan) *Musculista senhousia* (Asia) *Theora lubrica* (Japan)
Crustacea		*Eurylana arcuata* (New Zealand) *Neomysis japonica* (Japan) *Pyromaia tuberculata* (?New Zealand) *Tanais dulongi* (Europe)
Annelida		*Boccardia proboscoidea* (?Japan) *Mercierella enigmatica* (India) *Pseudopolydora paucibranchiata* (?Japan)
Pisces		*Acanthogobius flavimanus* (Japan) *Lateobrax japonicus* (Japan) *Sparindentex hasta* (India) *Tridentiger trigonocephalus* (Japan)
Algae		*Alexandrium minutum* (?Mediterranean) *Gymnodinium catenatum* (?Korea,Japan) *Undaria pinnatifida* (Japan)

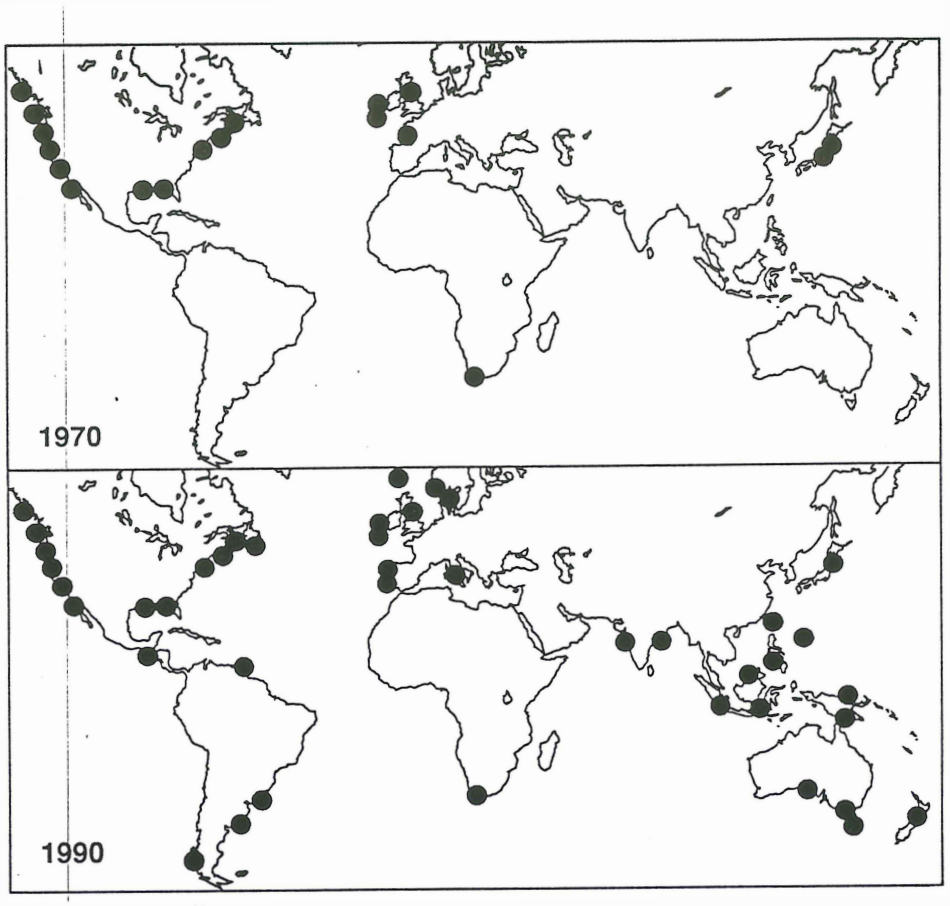


Fig.3a. Global spreading of toxic dinoflagellate blooms (paralytic shellfish poisoning) between 1970 and 1990.

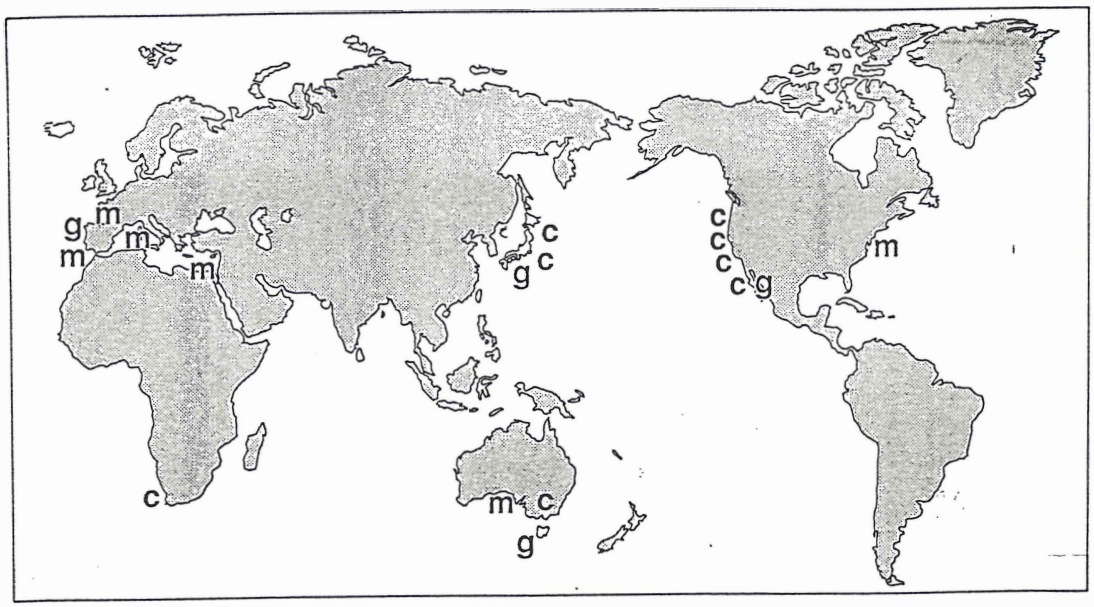


Fig.3b. Toxic dinoflagellate blooms first appeared in Australian estuaries in the late 1980s, caused by *Gymnodinium catenatum* (g) in the port of Hobart, *Alexandrium minutum* (m) in the port of Adelaide, and *Alexandrium catenella* (c) in the port of Melbourne. The disjunct global distribution of these species raised suspicions about ballast water as a possible dispersal mechanism.

Lanka,Taiwan), the Middle East (Turkey, Iran, Yemen), North America (Canada and the United States), Argentina, Mexico and New Zealand. After partial removal of ballast water in Australian ports, access was gained to the ballast tanks or holds of vessels. Replicate 500 mL samples of ballast-tank sediment or, if absent, ballast water samples were collected by hand from different areas of the vessels. Samples were stored in a dark refrigerator (4 ^{o}C) until despatched to CSIRO for analysis.

Ballast-tank samples were sonicated for 2 min (Braun Labsonic homogeniser, 100 W) to dislodge detritus particles. Samples were then fractionated through a 90 um sieve onto a 20 um sieve. This concentrated material was examined by light and scanning electron microscopy. Viable dinoflagellate cysts were isolated by micropipette and washed several times in sterile culture medium. The medium consisted of filtered seawater (28 ^{o}/oo salinity) autoclaved in teflon containers, with nutrients added according to Loeblich's (1975) GPM medium modified with selenium (H_2SeO_3 at 10^{-8} M final concentration).Single cysts were placed in sterile polystyrene petri dishes (36 mm diameter) which contained 5 mL medium. The cysts were incubated at 18 ^{o}C (12:12 h light: dark) at an irradiance of 80 uE $m^{-2}s^{-1}$ and were examined daily for germination.

4.2. Results of ballast water survey

Of the total of 343 ballast-tank samples examined, 65% contained fine brown or black sediment (up to an estimated 100 tonnes of sediment per ship). Some of the remaining samples were sediment-free because the bottoms of the ballast tanks were not easily accessible for sampling. One hundred samples of sediment from 18 Australian ports were selected for detailed study. The cellulose thecae of motile dinoflagellates were rarely seen in ballast-tank samples, but resting cysts were common. Dinoflagellate cysts were found in samples from vessels entering all 18 Australian ports studied (Fig. 4). The cysts were found in 50 of the 100 sediment containing samples; 53 species of cysts were identified, of which 20 species were germinated to produce viable cultures (Table 1). Thus the risk of foreign micro-organisms being introduced into Australian waters via ships' ballast water is widespread and the inoculum represents a great diversity of species.

The identification of cysts of the toxic dinoflagellates *Alexandrium catenella* and *A. tamarense* in 5 of the 100 sediment samples was of

Fig. 4. Map of Australia showing the location of the 18 ports from which ballast-tank samples were collected. Samples from 9 ports contained toxic dinoflagellate cysts (filled squares).

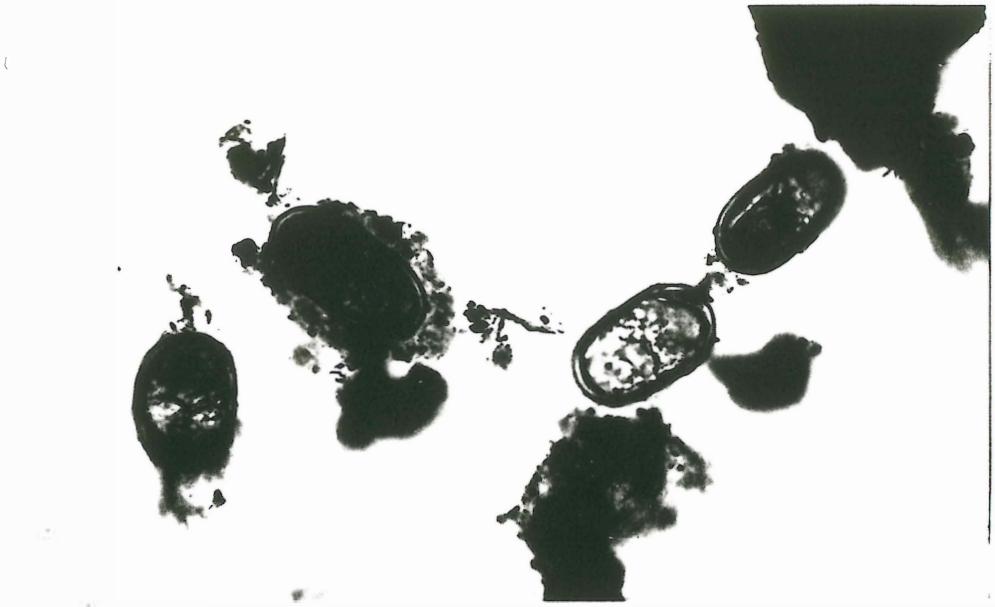


Fig.5a. Live cysts of the toxic dinoflagellate *Alexandrium tamarense* from a vessel arriving in Eden from Muroran, Japan.

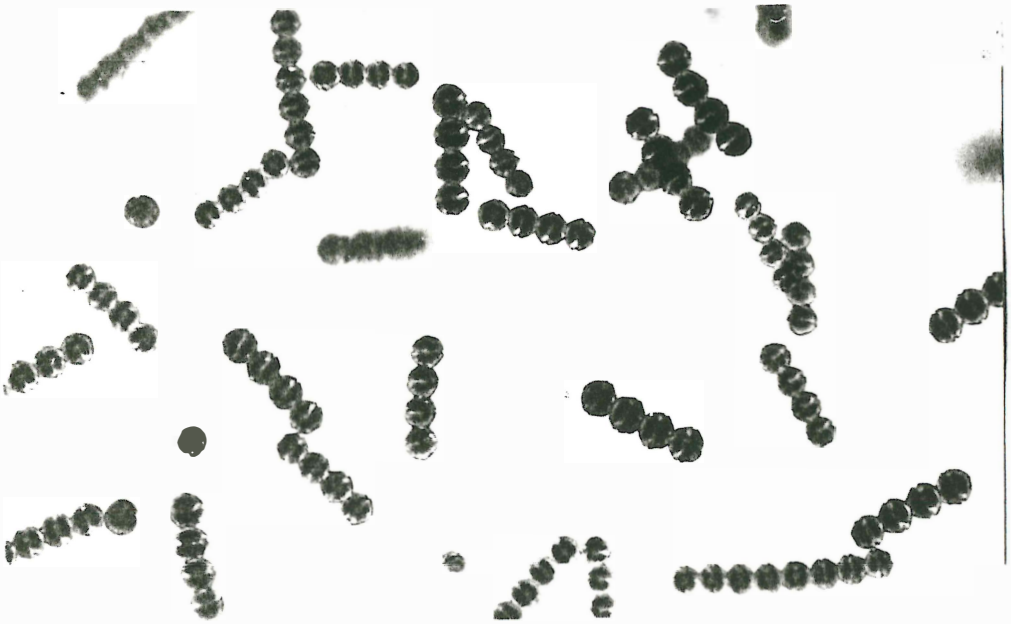


Fig.5b. Motile cells of the toxic dinoflagellate *Alexandrium catenella*, cultured by germinating ballast water cysts from a vessel arriving in Port Hedland from Kashima, Japan.

Table 1. Dinoflagellate cyst species in ballast tank samples (53 species identified; 20 spp. cultured).

Alexandrium catenella/tamarense
Diplopelta
Diplopsalis
Diplopsalopsis
Gonyaulax (7 spp)
Gymnodinium catenatum
Lingulodinium
Peridinium
Pheopolykrikos
Polykrikos
Protoceratium
Protoperidinium (22 spp)
Pyrocystis
Scrippsiella (6 spp)
Zygabikodinium

Table 2. Origin of 20 ballast tank samples containing toxic dinoflagellate cysts.

JAPAN	KOREA
Chiba (*Alexandrium sp.*)	
Fukuyama *(G.catenatum)*	Inchon *(G.catenatum)*
Kashima *(A.catenella)*	Kohong *(G.catenatum)*
Kure (*Alexandrium sp.*)	Kwangyang (*Alexandrium*)
Kushiro (*Alexandrium sp.*)	Pohang (*Alexandrium sp.*)
Muroran *(A.tamarense)*	Samchonpo*(A.tamarense)*
Shimizu *(A.tamarense)*	
Ube *(G.catenatum)*	

considerable concern (Fig. 5a, b). After this CSIRO survey was completed, the Australian Quarantine and Inspection Service (AQIS) contracted the Australian Government Analytical Laboratories (AGAL) to continue monitoring the ballast tanks of ships entering Australian ports. This monitoring detected 9 further positive *Alexandrium* ballast-tank samples. In 5 cases, these *Alexandrium* cysts have been successfully germinated into viable dinoflagellate cultures and their toxicity has been confirmed by high-performance liquid chromatography (Oshima, Bolch & Hallegraeff, in press). Four samples containing toxic *Gymnodinium catenatum* cysts were also identified. Toxic dinoflagellate cysts were found in woodchip, gas and ore vessels, which sailed from either Japan or South Korea and entered 9 Australian ports (Table 2). One ballast tank was filled during a toxic dinoflagellate bloom in the port of Muroran, Japan, during July 1989. A sample taken in Eden, Australia, was estimated to contain more than 300 million *Alexandrium* cysts. One month later, on its next voyage from Shimizu,Japan, to Eden, this ship still contained the same cyst material, albeit in lower concentration. In both cases these cysts failed to germinate until bout 6 months later, which suggests that they were newly formed cysts undergoing a mandatory dormancy period (Anderson, 1980) rather than mature cysts resuspended from harbour sediments during ships' ballasting. Analysis of vessels' departure dates (more live dinoflagellate cysts from May to October) supports the view that the water column during seasonal (spring to early autumn) dinolagellate blooms is the predominant origin of ballast-water cysts.

4.3. Conclusions of ballast water survey

The present work established unambiguously that toxic dinoflagellates , especially those species that produce resistant resting cysts, can be transported in a viable form in the ballast water of cargo vessels. The Australian Quarantine and Inspection Service has responded to the preceding evidence by declaring the disposal of ballast water to be a quarantine issue of national significance. As of 1 February 1990, Australia introduced voluntary guidelines for ships entering its ports from overseas (Fig. 6).These measures include the ships providing certification that water and sediment in the ballasting port are free from harmful organisms (now regularly used by some ships originating from New Zealand), re-ballasting in mid-ocean (now regularly used by woodchip cargo vessels servicing

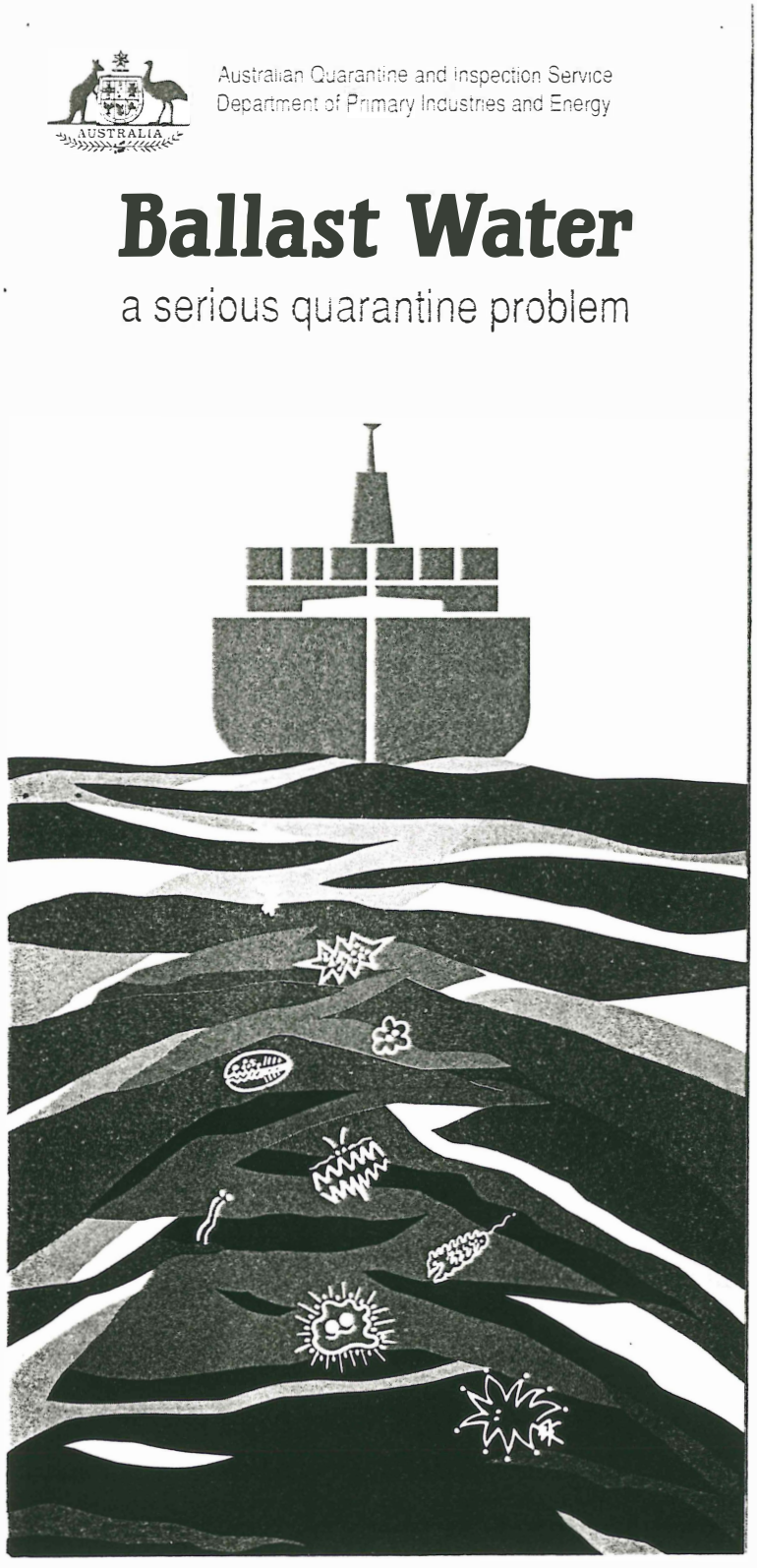


Fig.6. Ballast water leaflet issued by the Australian Quarantine and Inspection Service on 1 February 1990.

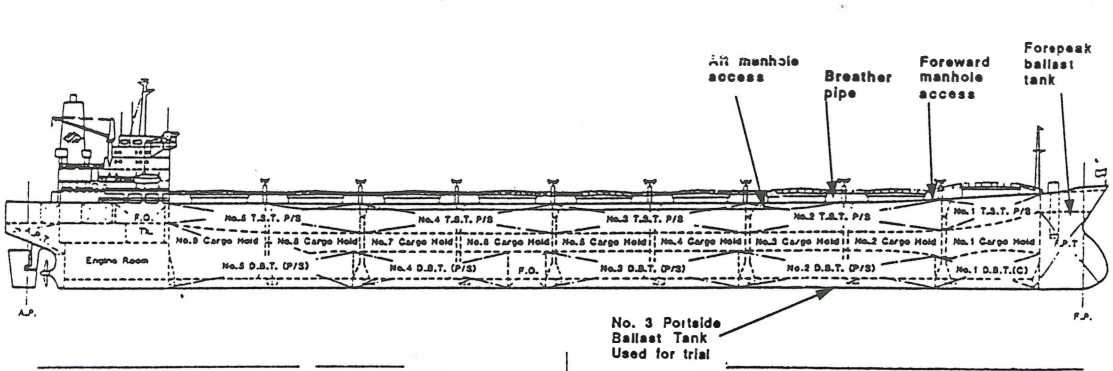
Triabunna,Tasmania), making a commitment not to discharge ballast or to keep ballast tanks as clean as possible. As of 1 November 1991, the Marine Environment Pollution Committee (MEPC) of the International Maritime Organisation (IMO) has ratified the above guidelines for adoption on an international basis.

A major problem with midocean exchange of ballast water is that it can only be carried out by vessels up to 40,000 dead weight tonnage (such as woodchip cargo vessels), and not by the majority of the large ore-cargo vessels visiting the Australian ports of Port Hedland, Dampier and Gladstone. Furthermore, while midocean exchange of ballast waters may be effective in removing organisms in the water column of ballast tanks, it is only partially effective in removing dinoflagellate cysts which have settled to the bottom of ballast tanks. Among 32 vessels which explicitly claimed to have exchanged ballast water in mid-ocean, 14 were still found to contain significant amounts of sediments, including dinoflagellate cysts. A more effective measure would be to avoid ballasting during toxic dinoflagellate blooms in foreign ports.

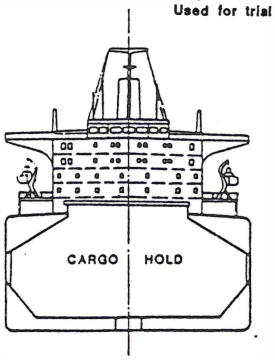
5. Ballast water exchange study on M.V."Iron Whyalla"

The effectiveness of reballasting operations in mid-ocean was studied in detail on board the BHP-owned vessel "Iron Whyalla", en route from Singapore to Port Hedland. The "Iron Whyalla is a nine cargo hold single-deck bulk carrier with a total ballast capacity of 76,330 tonnes (Fig. 7a). Following a complete clean-out of ballast tanks in dry dock, the ship took in ballast water in Singapore Harbour on 13 and 14 Sept 1991. Conditions during ballasting were water temperature 30 ^{o}C, salinity 25 ^{o}/oo (both taken at 10m depth) ,the incidence of a dense (10^{7} cells/liter) diatom bloom (mainly *Chaetoceros,Skeletonema*) and sparse non-toxic dinoflagellate plankton (*Ceratium,Dinophysis,Diplopsalis, Protoperidinium*). Resuspended harbour sediments were clearly visible when the ship left the dock on 15 Sept,10am, but were not evident during the ballasting procedures (harbour depth was 13m). Daily observations were made with a microscope on board ship to study the effect of enclosing a natural community of phyto- and zooplankton in a selected ballast tank (starboard tank no.2, approximately 7,000 tonnes capacity). Water samples were withdrawn by means of a small peristaltic pump using silicon tubing which had been prepositioned at three different levels in the tank during the dry-dock period. Physico-chemical

Fig.7a. Ballast tank configuration on the M.V."Iron Whyalla".



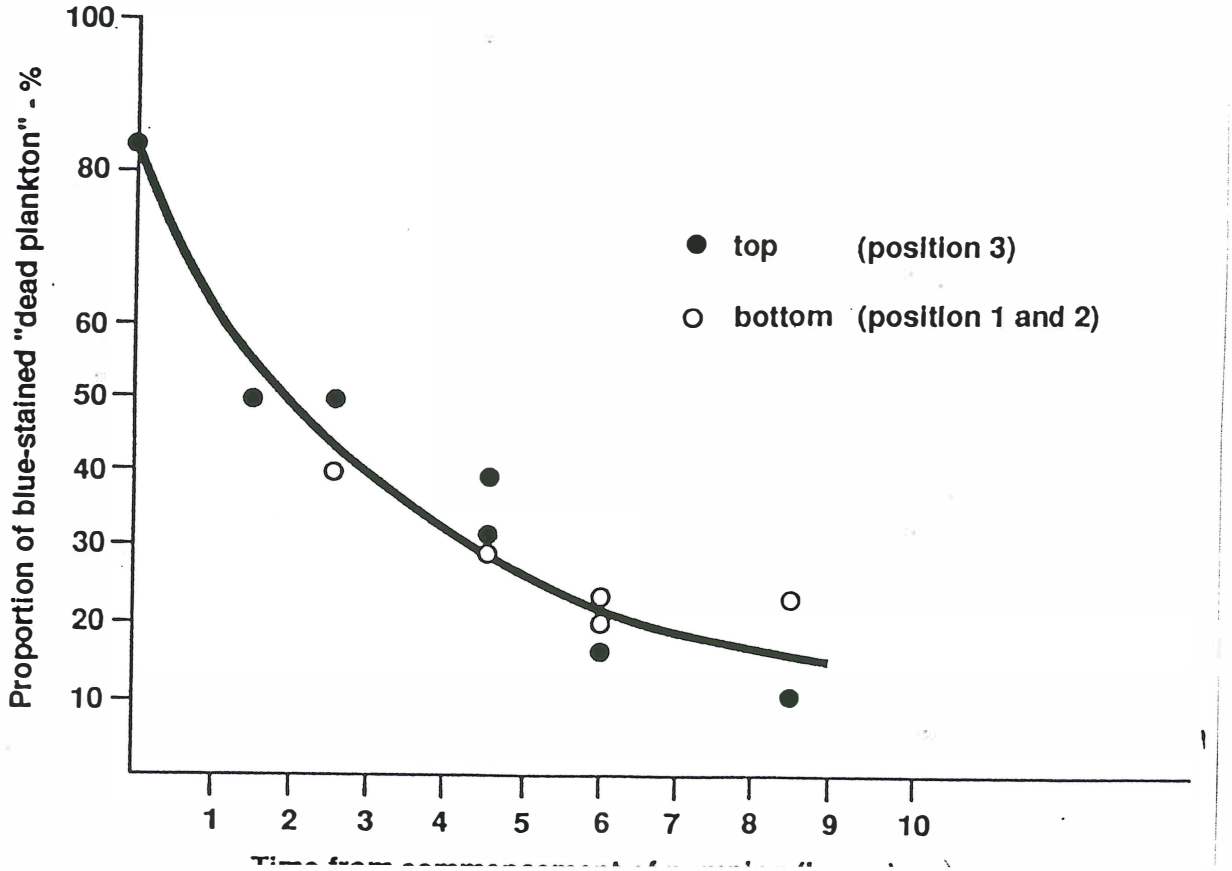
Ballast Tank Capacities (tonnes)		Length of Holds (m)	
No. 1 Top Side and Double Bottom Tks	4275	1	20.8
No. 2 Top Side and Double Bottom Tks	12550	2	20.8
No. 3 Top Side and Double Bottom Tks	12710	3	20.8
No. 4 Top Side and Double Bottom Tks	11270	4	20.8
No. 5 Top Side and Double Bottom Tks	10575	5	20.8
Fore Peak Tk	4380	6	20.8
Aft Peak Tk	565	7	20.8
No. 4 Hold	19555	8	20.8
		9	20.8



MIDSHIP SECTION

Hatchway Details	Length (m)	Breadth (m)	Cargo Capacities	(Grain m³)
1	14.0	18.0	1	16700
2	14.4	23.0	2	18950
3	14.4	23.0	3	18850
4	14.4	23.0	4	18900
5	14.4	23.0	5	19100
6	14.4	23.0	6	19000
7	14.4	23.0	7	19000
8	14.4	23.0	8	18750
9	14.4	18.0	9	15600
			Total	164850

Fig.7b. Ballast tank exchange experiment on M.V. "Iron Whyalla", showing the change in methylene-blue stained plankton sediments over a period of 9 hours of tank flushing.



characteristics such as temperature, salinity and oxygen concentration were also monitored in this way.

After 3 days ,the diatom community had almost completely settled out onto the bottom of the tank and rapidly started to decay as evidenced by increased bacterial and ciliate growth.Increased grazing pressure by zooplankton resulted in a significant accumulation of fecal pellets especially on days 4 and 5. Bottom sediments gradually turned anoxic (down to 0.4 ppm oxygen concentration after 2 days) and started to change colour from brown to black. Temperature and salinity characteristics of ballast tank waters remained constant.Throughout the period of study, dinoflagellate species (both photosynthetic and non-photosynthetic forms) as well as larval crustaceans and molluscs remained fully viable and could be found at different positions in the tank.No evidence was found for resting spore formation by the diatom and dinoflagellate species present.

In order to assess the effectiveness of reballasting, on 19 Sept.,11am , a solution of 10 kg of methylene blue dye dissolved in fresh water was introduced into the tank at three different levels and left to mix overnight. On 20 Sept, 9.15 am , ballast water pumping was started at a capacity of 2,000 tonnes /hr ,taking in new seawater on the bottom and allowing old ballast water to flow out on top. Hourly samples were taken to measure the dilution of the blue dye by colorimetry as well as to examine the proportion of new plankton and blue-stained dead diatom sediments (Fig.7b). A rapid exchange of 50% of ballast tank waters occurred in the first few hours of pumping, but even after 9 hours (equivalent to 3 tank exchanges) up to 25% of original water / sediment had remained on the bottom of the ballast tank . Further details on this work can be found in Rigby & Hallegraeff (1992).

6. Chemical and physical treatment options to kill dinoflagellate cysts

In this part of the study, more than 5,000 culture-produced *Gymnodinium catenatum* cysts were subjected to various chemical (salinity, chlorine, copper sulphate, hydrogen peroxide) and physical (heat) treatments and then incubated to evaluate the effects on cyst germination.Treatment methods that kill resistant dinoflagellate cysts are likely to be equally effective for a range of other marine organisms such as larval zooplankton, copepod eggs and seaweed spores (with the possible exception of bacterial spores and viral particles which may be more difficult to eradicate). The resistance of dinoflagellate cysts has been well-documented (Dale,1983). Normal sediment preparation techniques ,including ultrasonication and subsequent microscopic observation under ultraviolet radiation ,appear to have no effect on their viability.and commonly used electron microscopic fixatives such as osmium tetroxide appear unable to penetrate the cyst walls. Unfortunately, most experimental studies on the viability of dinoflagellate cysts have focused on the factors affecting germination under the normal range of ambient marine environmental conditions (Anderson,1980). However, very little is known about the physical and chemical extremes under which cysts can survive. In the present work, the toxic marine dinoflagellate *Gymnodinium catenatum* was selected as test organism, because (1) the conditions for successful culturing and the complete sexual life cycle are well-documented (Blackburn et al.1989); (2) it has a short cyst dormancy period of only 14 days, compared to 6 months for toxic *Alexandrium* species ,and (3) the cysts require no environmental conditioning (e.g. cold storage) before they can germinate .In addition, (4) the cysts are comparatively large, 45-55 um diameter,and lack an outer mucilaginous covering (as in *Alexandrium*),which makes them easy to manipulate under low power microscopes.

6.1. Material and Methods

6.1.1.Dinoflagellate cysts.

One ml culture suspensions of compatible sexual mating strains of the dinoflagellate *Gymnodinium catenatum* (strain GCDE02 and GCHU10;see Blackburn *et al.* 1989) were inoculated into 55 mm polystyrene petri dishes containing 10 ml of nitrate and phosphate deficient GSe culture medium. The petri dishes were incubated at 17^{o}C and a light intensity of 200uE.m^{-2}.s^{-1} and examined regularly for cyst formation.This procedure produced large numbers of cysts (up to 500 per petri dish) within 7 to 10 days, 90 to 95% of which readily germinated within 14 days to form a swimming planomeiocyte, and 70% of these did divide to produce viable vegetative cultures.In the following experiments, the criterion adopted for cyst survival therefore was the germination of an actively swimming planomeiocyte.

6.1.2. Chemical treatment.

For chemical treatment experiments, cysts were produced as described above .After 10 to 14 days ,the cysts were removed from the petri dishes with a glass microcapillary pipette ,washed twice in sterile GSe medium and immediately used for experiments. Individual cysts were isolated under a Wild M7 stereomicroscope and placed into treatment solutions with varying salinity (as NaCl), pH (prepared by addition of NaOH or HCl), or concentrations of free chlorine (prepared from sodium dichloroisocyanurate), hydrogen peroxide, copper sulphate, or the microbiocide Kathon WT 1.5% (active ingredient 5-chloro--2-methyl-4-isothiazolin-3-one; Rohm and Haas,Philadelphia,USA). Treatment solutions for free chlorine and hydrogen peroxide were prepared in charcoal-filtered seawater to remove interfering organic material. Free chlorine concentrations were measured before and after treatment using a chlorine DPD test kit (Palintest Ltd.,Gateshead ,UK). The treatment solution for copper sulphate was prepared in distilled water to avoid complexation and precipitation in seawater. All treatment solutions were kept for 24 hrs at room temperature (20^{o}C) and in the dark to avoid photodegradation of chemicals. Following treatment, the cysts were removed from the chemical solutions and washed three times in sterile GSe medium. They were then placed into 36mm polystyrene dishes containing 5ml culture medium, incubated as described above and examined regularly for germination and cell division.

Table 3

Effect of different concentrations (ppm) of free chlorine on germination of *G.catenatum* cysts (as % of total number of cysts used).

concentration	germinated/total		Germination (average of expts)
	exp. 1	exp. 2	
2000	0/18	0/25	0%
1000	0/21	0/32	0%
500	1/15	0/23	3%
100	2/37	5/32	10%
50	36/66	10/34	46%
10	9/11	30/31	93%
0	18/20	18/20	90%

Table 4

Effect of different concentrations (ppm) of hydrogen peroxide on germination of *G.catenatum* cysts (as % of total number of cysts used).

concentration	germinated/total		Germination (average of expts)
	exp. 1	exp. 2	
60 000	0/18	0/31	0%
30 000	0/17	0/26	0%
10 000	0/26	0/42	0%
5 000	1/34	0/27	2%
2 500	3/11	6/25	25%
1 000	23/23	14/32	68%
100	21/23	21/24	89%
0 (control)	14/15	17/20	89%

6.1.3. Heat treatment

Two different sets of heat treatment experiments were carried out. The first set of experiments aimed to determine the lethal temperature range. Dinoflagellate cysts were placed in a 100ml test tube containing 30ml of culture medium. The tube was immersed in a 20^{o}C water bath which over a period of 25 min was gently heated to 60^{o}C. Subsamples were withdrawn from the tube at 25, 30, 35, 40, 45, 50 and 60^{o}C, and the cysts incubated for germination as described above. Once the lethal temperature range was determined, the second set of experiments aimed to establish the critical treatment times at the different target temperatures. In this case, dinoflagellate cysts were placed in 36 mm petri dishes (sealed with parafilm) containing only 1ml GSe culture medium and these were immersed in a water bath heated to 38, 40, 43, 45, 47, 49 or 51^{o}C. The petri dishes were allowed to heat up to the target temperature over a period of 60 to 90secs. Temperature within the petri dishes was monitored with a flat (6mm diameter) copper disc thermocouple (Radio Spares,Australia), attached to the inside of an equivalent petri dish and monitored with a digital temperature probe (Digitron 3202K,type K). Replicate dishes were removed after temperature exposures of 30, 60, 90,120 and 150 secs and allowed to cool to 20^{o}C. The dishes were then opened, 3 ml of fresh GSe culture medium added and the dinoflagellate cysts incubated for germination as described above.

6.2. Efficacy of chemical treatment

Dinoflagellate cysts of *Gymnodinium catenatum* exposed for 24 hrs to freshwater showed some disruption to their cell contents, but surprisingly their viability was unaffected. Similarly, dinoflagellate cysts showed no effects after exposures to salinities in the range 15 to 50 ^{o}/oo, and only treatment with extreme salinities as high as 100 ^{o}/oo prevented their successful germination. Cysts exposed to a pH range of 2 to 10 showed the same germination success as control cultures (pH=8.4). Copper sulphate, which readily kills motile dinoflagellate cells at concentrations of 1 mg/l (Taylor 1987), had only minor effects on dinoflagellate cysts (68% germination at concentrations as high as 200 mg/l). The commercial

microbiocide Kathon WT (recommended dosage to kill slime-forming and sulfate reducing bacteria in cooling water towers, 67-332 ppm) had no effect at concentrations as high as 10,000 ppm.

Free chlorine treatment readily caused a bleaching of the brown cyst wall of *G.catenatum* ,but successful germination (10%) was still possible with concentrations up to 100 ppm. However,no germination was observed at concentrations above 500 ppm (Table 3). Hydrogen peroxide was also effective in killing dinoflagellate cysts but only at concentrations of 10,000 ppm or above (Table 4).

6.3. Efficacy of heat treatment

The first set of simple heating experiments showed that germination was unaffected by exposure to temperatures up to 35^{o}C. Germination of *G.catenatum* cysts was reduced to 8% at 40^{o}C , but no germination was observed after heating to 45 ^{o}C or higher (Table 5). This lethal temperature range was confirmed by similar incubations (5-10 mins) of mixed dinoflagellate cyst assemblages contained in natural marine sediments (Derwent River,Tasmania;see Bolch & Hallegraeff 1990) as well as ships' ballast water tank sediments containing *A.tamarense* cysts (Hallegraeff & Bolch 1991). No cyst germination was observed after exposure to 45^{o}C.

The second set of heating experiments (Fig.8) established critical treatment times at the different target temperatures. Exposure of dinoflagellate cysts for 150 secs to temperatures of 36.0 to 38.1^{o}C reduced germination to 65 to 75%. An effective inactivation of *G.catenatum* cysts could be achieved by exposures ranging from 120 secs at temperatures of 38 to 40^{o}C, to 30 secs at temperatures of 44.5 to 46.3^{o}C.

6.4. Evaluation of chemical and physical treatment options

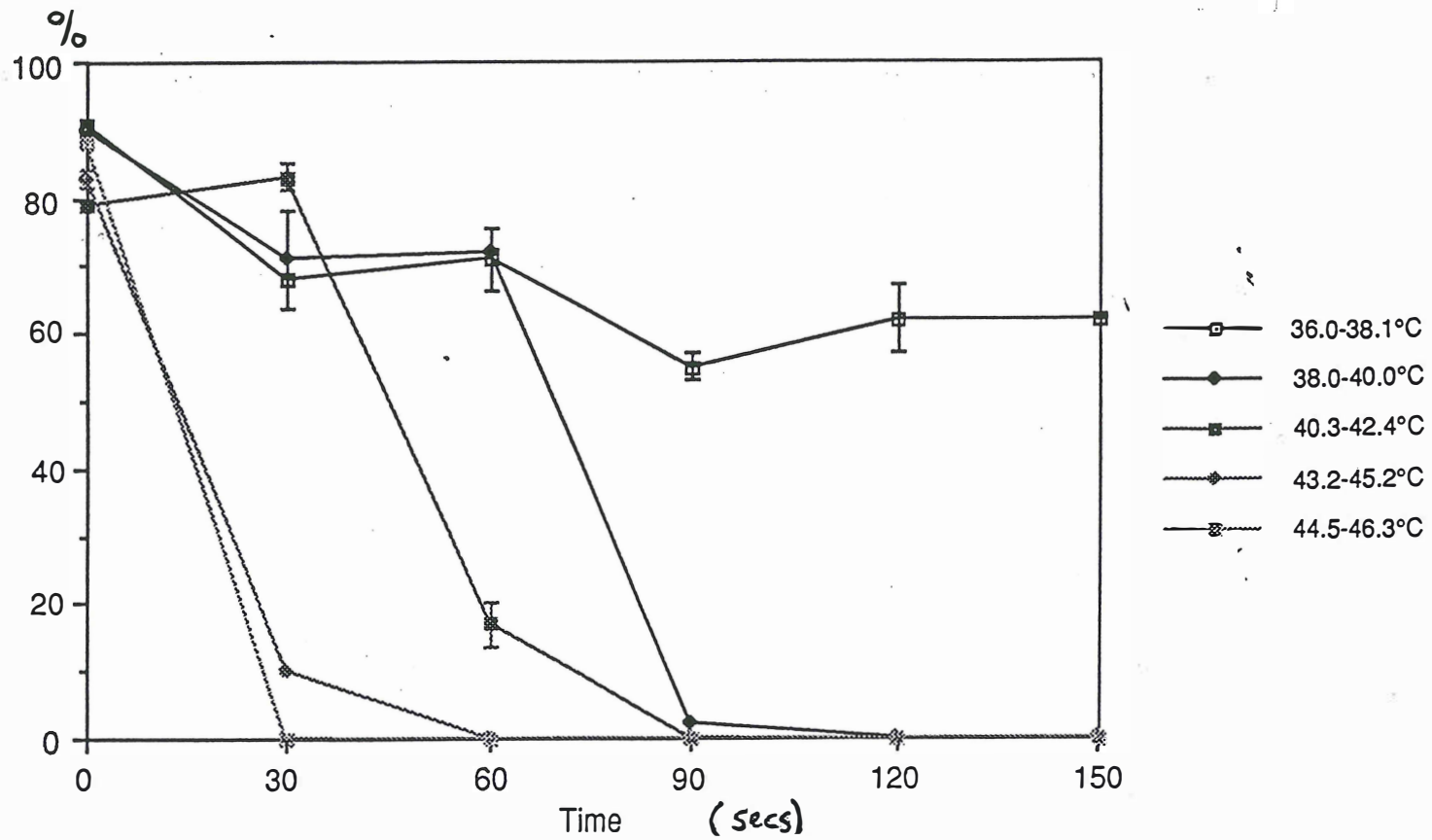
The present work has confirmed the very high chemical resistance of dinoflagellate resting cysts compared to the more fragile motile plankton cells. The microbiocide Kathon WT was completely ineffective even at 30 times the recommended dose rate, most likely because of the limited permeability of the cyst wall to chemicals. However, effective treatment of dinoflagellate cysts could be achieved with high concentrations of free

Table 5

Effect of temperature on germination of *G.catenatum* cysts (as % of total number of cysts used).

temperature (°C)	germinated/total exp. 1	exp. 2	Germination (average of expts)
20	10/12	18/20	88%
30	12/12	17/17	100%
35	12/12	20/21	97%
40	2/10	0/16	8%
45	0/11	0/28	0%
50	0/17	0/31	0%
60	0/17	0/19	0%

Germination of *G.catenatum* cysts (as % of total number of cysts used) after short-term exposures (30 to 150 secs) to five different temperature regimes



chlorine or hydrogen peroxide. Chlorine based chemicals,which rely on the biocidal action of hypochlorous acid, are commonly used to reduce the level of bacteria,viruses,algae and fungi in domestic water supplies,swimming pools,sewage effluent and industrial water systems. Free chlorine levels between 5 and 10 ppm are recommended for ice-water baths in the food industry and up to 40 ppm for seawater-ice baths in fish processing plants (Gardner 1986). Williams *et al* (1982) required free chlorine concentrations (as sodium -or calcium hypochlorite) of 20 ppm over 24 hrs to kill small shrimp and larval fish.in ballast water samples In the present work, free chlorine levels as high as 500 ppm were necessary to kill *G.catenatum* dinoflagellate cysts. This would require 400 tonnes of an industrial solution of 12.5% sodium hypochlorite to treat a 50,000 tonne ballast water tank at a cost of A$200,000 (Rigby *et al.*1992). Apart from being prohibitively expensive and causing environmental problems at the port of discharge, the high sediment load of ships' ballast tanks would considerably reduce available free chlorine levels. Hydrogen peroxide would constitute a more environmentally friendly treatment since this chemical would eventually degrade to water and oxygen. Similarly, Ichikawa *et al.* (1992) recommended the application of 100 mg/l hydrogen peroxide solution for 96 hrs to kill toxic Alexandrium catenella cysts The thick-walled cysts of *Polykrikos schwartzii* were more resistant to hydrogen peroxide than A.catenella, while the motile cells of *Gymnodinium nagasakiense* (3-6 mg/l for 15-30 min) and *Chattonella marina* (15 mg/l for 30 min) were more sensitive.In the present work, much higher levels of hydrogen peroxide up to 5,000 ppm (i.e. five hundred times higher) were needed to kill the more resistant, thick-walled *G.catenatum* cysts.,but admittedly our treatment period was only 24 hrs. To treat a 50,000 tonne ballast water tank thus would require 1000 tonnes of an industrial 50% hydrogen peroxide solution at an indicative cost of A$2,000,000 (Rigby *et al.* 1992).

The present work indicates that a very short (30 to 90 secs) heat treatment of dinoflagellate cysts at temperatures as low as 40 to 45 ^{o}C provides an effective, environmentally friendly solution to the global ballast water problem. Collaborative research with shipping engineers is now underway to develop a ship engineering design in which heat generated by the ship's engines is piped through heating coils in the various ballast water tanks to achieve the above temperature conditions.

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APPENDIX

Publications resulting from FIRDC grant 89 /39 (updated up to April 1993)

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Transport of Toxic Dinoflagellate Cysts via Ships' Ballast Water

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Toxic dinoflagellate species that are not endemic to an area can be inadvertently introduced when their cysts are discharged with the ballast tank sediments of bulk container ships. These species, which can affect fish- and shellfish-farms, pose a serious threat to public health and aquaculture. Among 80 cargo vessels entering Australian ports, 40% contained viable dinoflagellate cysts and 6% carried the cysts of the toxic dinoflagellates *Alexandrium catenella* and *A. tamarense* (up to an estimated 300 million cysts per ship). The introduction of new Australian quarantine measures is discussed; however, the implications of this potential spreading of toxic algae are global.

Dinoflagellates that produce paralytic shellfish poisons (PSP) can proliferate periodically in estuaries in many parts of the world. Their poisons can contaminate shellfish and thus poison humans but occasionally also can kill fish, birds and other mammals. In a strict sense, these are completely natural phenomena which were present well before major human settlement and associated modification of the environment occurred. For example, Captain Vancouver's crew suffered from PSP when they landed in British Columbia in 1793. However, during the past two decades such blooms of PSP-causing dinoflagellates have apparently spread to many new geographical areas where they were previously unknown, e.g. *Alexandrium fundyense* in Massachusetts, 1972; *A. catenella* and *A. minutum* in South Australia, 1986; *Gymnodinium catenatum* in Spain, 1976, and Tasmania, 1986; and *Pyrodinium bahamense* var. *compressum* in Sabah and Brunei, 1976, and the Philippines, 1983 (Anderson *et al.*, 1982; Hallegraeff *et al.*, 1988; Maclean, 1989). Four explanations for this apparent global spreading have been suggested: *1.* increased scientific awareness of toxic species; *2.* increased utilization of coastal waters for aquaculture; *3.* stimulation of plankton blooms by coastal eutrophication and/or unusual climatic conditions; and *4.* transport of dinoflagellates or their resistant benthic resting cysts either in ships' ballast water or associated with movement of shellfish stocks from one area to another (Anderson, 1989; Hallegraeff *et al.*, 1988; Smayda, 1990).

In many estuarine and coastal waters the discharges of industrial, human, agricultural and aquacultural wastes are creating increased nutrient conditions. Phytoplankton species that may always have been present in low concentrations but have previously gone unrecognised ('hidden flora') respond by growing up into bloom proportions and their species composition may shift from diatoms to (dino)flagellates (Smayda, 1990). This stimulation of dinoflagellate blooms enhances the probabilities of dinoflagellates emigrating alongshore. For example, Maclean (1989) presented compelling evidence of a correlation between ENSO climatic events and *Pyrodinium bahamense* blooming and spreading in the tropical Indo-West Pacific. However, the recent appearance of three toxic dinoflagellates in Australian estuaries (*A. catenella*, *A. minutum* and *G. catenatum*), well away from their nearest known distributions in Europe and Japan, may have involved transport in ship's ballast water.

To test this hypothesis, officers of the Australian Quarantine and Inspection Service sampled over 200 ballast tanks of cargo vessels arriving in the Australian ports of Bunbury, Eden, Geelong, Gove, Hobart, Launceston, Newcastle, Port Hedland, Port Kembla, Port Latta and Spring Bay, between 1987 and 1989. Each of these vessels, carrying both organic (wheat, woodchips) and inorganic (coal, iron ore) cargoes, take in between 20 000 and 100 000 t of ballast water in foreign ports. Phyto- and zooplankton species (including larval fish and invertebrates (Williams *et al.*, 1988)) are sucked up with the water as it is being pumped into the ballast tanks of the ships. If weather or tidal conditions stir up the water column during pumping, fine sediment (including spores of diatoms and dinoflagellates, Hallegraeff *et al.*, 1990) will be included in the ballast water. In the present study, over 70% of the ships had sediments on the bottom of their ballast holds. More than half of the ships examined had sailed from Japan; the remainder arrived from Canada, S.E. Asia, the United States, Mexico and Argentina.

Representative 500 ml ballast sediment samples were sonicated to dislodge dinoflagellate cysts from sediment particles and then fractionated through a 90 μm sieve onto a 20 μm sieve. This concentrated cyst material was examined by light and scanning electron microscopy. Any dinoflagellate cysts present were isolated by micropipette and incubated in suitable nutrient medium

for germination and identification (Blackburn *et al.*, 1989; Bolch & Hallegraeff, 1990).

To date, 31 of the 83 samples examined were found to contain non-toxic dinoflagellate cysts (Table 1), and a large number of these (notably *Gonyaulax, Protoperidinium* and *Scrippsiella* species) have been successfully germinated into viable cultures. More seriously, the ballast waters of one ship arriving in Triabunna (Tasmania) from Yatsushiro (Japan), one ship (two separate ballast tanks) arriving in Port Hedland (NW Australia) from Kashima (Japan), and one ship arriving in Eden (New South Wales) from Muroran (Japan) and, on a later voyage, from Shimizu (Japan), respectively, proved to contain cysts of the toxic dinoflagellate *Alexandrium*. The ballast tank, filled during a toxic dinoflagellate bloom in the port of Muroran (Oshima, pers. comm.) and sampled in Eden, was estimated to contain more than 300 million *Alexandrium* cysts (Fig. 1). The cysts from Muroran have been successfully germinated into viable cultures of the toxic dinoflagellate *A. tamarense* and the cysts from Kashima germinated into cultures of the toxic dinoflagellate *A. catenella* (Fig. 2).

While the present work shows conclusively that toxic dinoflagellate species can be transferred via ship's ballast water, it is very difficult to assess how often introduced plankton species have actually established themselves in their new environments. A large number of macroscopic organisms introduced into Australian ports in the same way certainly have done so. The list includes the European crab *Carcinus maenas* (Adelaide), Asian mussel *Musculista senhousia* (Perth), Japanese shrimp *Neomysis japonica* (Newcastle), Japanese gobyfish *Acanthogobius flavimanus* (Sydney) and Japanese seaweed *Undaria pinnatifida* (Triabunna) (Hutchings *et al.*, 1987; Sanderson 1990). In none of these cases have studies been carried out to evaluate the damage to commercial fisheries or native flora and fauna. Species of *Alexandrium* are easily overlooked in routine plankton surveys, and as their benthic resting cysts do not fossilize, it is virtually impossible to prove that introduction has occurred. Furthermore, in some cases new accidental introductions may be simply added to a cosmopolitan population which has established itself over geological time through natural processes of species dispersal (Taylor, 1987). *Gymnodium catenatum*, however, is a conspicuous chain-forming dinoflagellate with a disjunct global distribution and a distinctive fossilisable cyst (Nordberg & Bergsten, 1988). Evidence from cyst surveys in dated Tasmanian sediment depth cores points to the distinct possibility that *G. catenatum* was introduced after 1980 into the area around the shipping port of Hobart (Bolch & Hallegraeff, 1990). Preliminary genetic studies using enzyme electrophoresis and sexual compatibility experiments have supported this hypothesis (Blackburn *et al.*, 1989; Blackburn & Ward, unpublished).

The above evidence has been considered sufficiently serious for the Australian Quarantine and Inspection Service to develop special ballast water quarantine measures for implementation from 1 February 1990. Ships entering Australian waters from all overseas ports are asked to agree voluntarily to comply with one of the following options.

TABLE 1

Summary of cargo vessel ballast tank samples containing toxic and non-toxic dinoflagellate cysts (arranged in order of decreasing cyst concentration).

Sample Code	Cargo Type	Ballast Tank	Port of Origin	Australian port of Arrival	Arrival date	Dinoflagellate cysts	*Alexandrium* cysts
72	woodchips	Not stated	Muroran, Japan	Eden, NSW	20 July 89	++	++
73	woodchips	Not stated	Shimizu, Japan	Eden, NSW	28 Aug. 89	++	++
2	woodchips	No. 4	Kushiro, Japan	Spring Bay, Tas.	11 Dec. 87	++	+
36	ore	Starboard wing 7	Kashima, Japan	Port Hedland, WA	27 June 89	++	+
44	ore	Port wing 7	Kashima, Japan	Port Hedland, WA	27 June 89	++	+
185	ore	Not stated	Wakayama, Japan	Port Latta, Tas.	6 Jan. 89	++	−
199	ore	Double bottom 2	Niihama, Japan	Newcastle, NSW	12 Oct. 89	++	−
201	ore	Not stated	Kimitsu, Japan	Newcastle, NSW	31 Aug. 89	++	−
1	woodchips	Not stated	Yatsushiro, Japan	Spring Bay, Tas.	2 Dec. 87	++	−
8	woodchips	Fore deep	Yatsushiro, Japan	Spring Bay, Tas.	25 Mar. 88	++	−
92	ore	Starboard wing 4	Fukuyama, Japan	Port Hedland, WA	18 July 89	++	−
146	woodchips	Hold 4	Yatsushiro, Japan	Geelong, Vic	27 June 89	++	−
5	woodchips	Not stated	Ishinomaki, Japan	Spring Bay, Tas	20 Feb. 88	+	−
6	ore	Not stated	Kushiro, Japan	Spring Bay, Tas	26 Feb. 88	+	−
46	ore	Topside 4	Mexico	Not stated	Not stated	+	−
53	ore	Fore Peak	Nagoya, Japan	Newcastle, NSW	1 Aug. 89	+	−
75	ore	No 1	Osaka, Japan	Port Hedland, WA	17 July 89	+	−
90	ore	Wing 1	Kisarazu, Japan	Port Hedland, WA	18 July 89	+	−
103	ore	Wing 3	Tampa, Florida, USA	Geelong, Vic	7 Apr. 89	+	−
104	ore	Double bottom 3	Los Angeles, USA	Geelong, Vic	29 Mar. 89	+	−
110	ore	Port	Shimizu, Japan	Gove, NT	4 May 89	+	−
118	ore	Hold 4	Not stated	Newcastle, NSW	17 July 89	+	−
152	ore	Fore peak	Iwakuni, Japan	Bunbury, WA	13 July 89	+	−
153	ore	Topside 1	Singapore	Bunbury, WA	14 July 89	+	−
154	ore	No. 2	Singapore	Bunbury, WA	18 July 89	+	−
163	ore	Fore peak	Niigata, Japan	Bunbury, WA	3 Aug. 89	+	−
176	woodchips	No. 4	Mishima, Japan	Launceston, Tas	20 Mar. 89	+	−
183	woodchips	Not stated	Muroran, Japan	Eden, NSW	25 Feb. 89	+	−
95	woodchips	Deep 1	Kaohsiung, Taiwan	Launceston, Tas	22 June 89	+	−
131	woodchips	No. 4	Iyomishima, Japan	Launceston, Tas	23 Apr. 89	+	−
186	woodchips	Not stated	Sendai, Japan	Launceston, Tas	23 Oct. 88	+	−

−Absent; +Present; ++Abundant ($> 10^4$ per 500 ml sample).

1. Provide a certificate from an overseas authority that the port of origin is free from toxic dinoflagellates.
2. Provide evidence that they have re-ballasted at sea.
3. Provide evidence that they have treated ballast water either in-hold during the voyage or in on-shore holding tanks upon arrival.
4. Discharge their ballast tank sediments in designated safe areas.
5. Provide evidence that their management practice is to keep ballast tanks clear of sediment and to load water that is as clean as possible by not ballasting in shallow water or during toxic dinoflagellate blooms.
6. Give an undertaking not to release ballast water or sediments in Australian territorial waters.

Following the recent ballast water introduction of the European zebra mussel *Dreissena polymorpha* into the Canadian Great Lakes (Hebert *et al.*, 1989), the Canadian Coast Guard has also introduced voluntary measures (May 1989) encouraging ships to flush their ballast tanks in the Atlantic before entering the St Lawrence Seaway. Ballast water introduction of exotic marine organisms is not a new problem. However, the increasing size and speed of cargo vessels together with increased eutrophication of many coastal waters are now increasing the likelihood of successful transfer of species across oceanic boundaries. It is hoped that other countries sensitive to introductions of foreign species will soon follow these examples of ballast water quarantine regulations.

We thank Dr S. Blackburn for help with cyst germination experiments, Dr B. Dale for encouragement and Dr Y. Oshima, Mr J. R. Merton, Mr R. Williams and Dr P. Hutchings for valuable discussions. This work was supported by Fishing Industry Research Trust Account grant 1986/84 and Fishing Industry Research and Development Council grant 1989/39.

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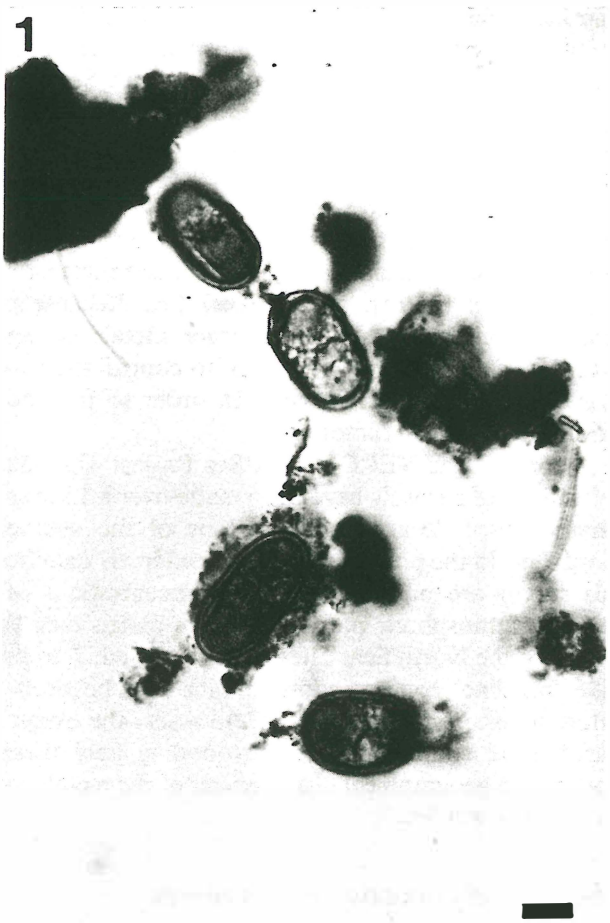

Fig. 1 Benthic resting cysts of the toxic dinoflagellate *Alexandrium tamarense* from a ballast tank sample collected in Eden, Australia, and originating in Muroran, Japan. Scale bar=10 μm.

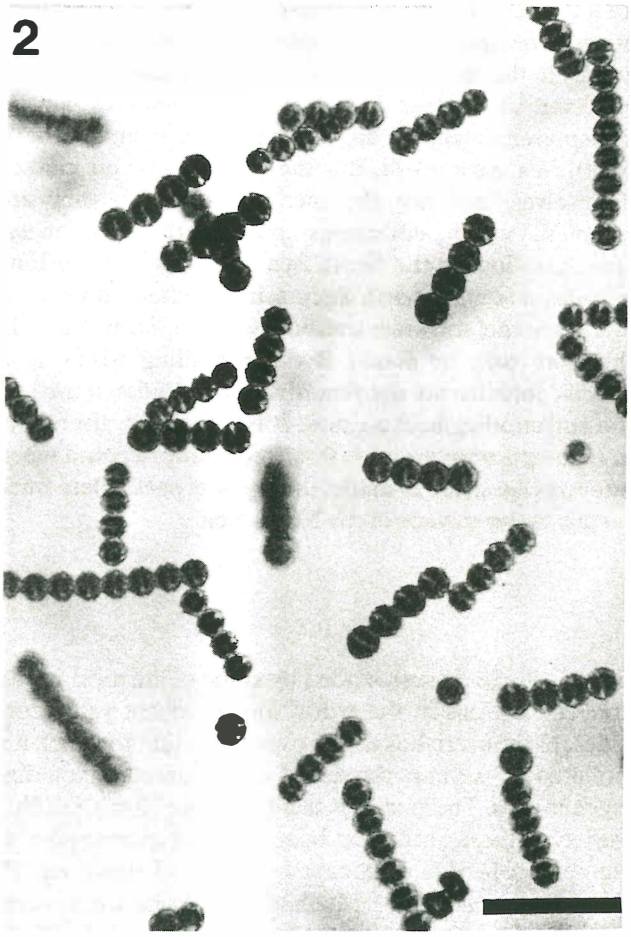

Fig. 2 Culture of the motile stage of the toxic dinoflagellate *Alexandrium catenella*, established through germination of a ballast tank cyst collected in Port Hedland, Australia, and originating in Kashima, Japan. Scale bar=100 μm.

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Source Control on the Distribution of Particulate Trace Metals in the North Sea Atmosphere

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Baseline data are presented on the concentrations of particulate atmospheric trace metals over open sea regions of the southern North Sea. It is shown that there is a south → north decreasing gradient in the emission of trace metals from the land masses surrounding the North Sea which imposes a control on the concentrations of the metals in the air masses which cross the source regions. However, air masses from a variety of sources can be transported to almost any region of the open southern North Sea. As a result, it is the origin of the air masses themselves, and not the location at which they are sampled, which constrains particulate trace metal concentrations in the North Sea atmosphere. Over long periods, a south → north decreasing gradient in particulate trace metal concentrations over the North Sea will therefore only be found if the prevailing winds to a specific location are dominantly from a similar source off the surrounding land masses. It is important, therefore, to take long term air mass flow trends into account when attempts are made to model the input of particulate trace metals to the surface of the North Sea.

The North Sea is surrounded by some of the most industrialized nations in the world and in recent years considerable concern has arisen over the extent to which it is polluted by several classes of contaminants, including trace metals. These metals can reach the North Sea by a variety of routes, but it has been shown that transport via the atmosphere is dominant for some of them; e.g. Pb (Cambray *et al.*, 1975). Much of the data on the concentrations of trace metals in the atmosphere over the North Sea has been extrapolated from collections made at stations on the edges of the surrounding land masses, or from fixed platforms at single locations in the North Sea itself (see Table 2). However, model calculations have indicated that fluxes of trace metals to the North Sea based on data collected at coastal stations in continental Europe and the United Kingdom are probably 2–4 times higher than the actual fluxes into the open North Sea (van Jaarsveld *et al.*, 1986). It is important, therefore, to establish the concentrations of atmospheric trace metals over various open sea regions of the North Sea itself. Recently, collections of atmospheric particulates (aerosols) have been made over the open North Sea from an aeroplane (Otten *et al.*, 1989); however, they were confined to a limited sector over the southern North Sea, and relatively few data have been obtained for the concentrations of particulate atmospheric trace metals over large areas of the rest of the North Sea. Because the input of contaminants, such as trace metals, changes with time as precautions are taken to control their use, data of this kind are necessary in order to provide a baseline for future monitoring.

As part of the NERC 'North Sea Project' shipboard collections of aerosols have been made over a 15 month survey period from various regions of the southern North Sea. In the present paper, 'ground truth' data from this survey are presented for the concentrations of a series of atmospheric particulate trace metals over this region of the North Sea. These data are used; *1.* to provide 'baseline' concentrations, which can be used in future monitoring studies, and *2.* to assess the extent to which local sources on the surrounding land masses control the geographical distributions of the metals over the open North Sea.

Methods of collection and analysis

The aerosol samples were collected from on board the NERC research vessel R.R.S. *Challenger*, from selected wind sectors, using a high-volume filter system which

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Transport of diatom and dinoflagellate resting spores in ships' ballast water: implications for plankton biogeography and aquaculture

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Abstract. Diatom and dinoflagellate species that are not endemic to a region can be inadvertently introduced when their resistant resting stages are discharged with the ballast-tank waters and sediments of bulk cargo vessels. A survey of 343 cargo vessels entering 18 Australian ports showed that 65% of ships were carrying significant amounts of sediment on the bottom of their ballast tanks. All of these samples contained diatoms, including species that are not endemic to Australian waters. Diatom resting spores, especially of *Chaetoceros*, were also detected. Dinoflagellate resting spores (cysts) were present in 50% of the sediment samples. Of the 53 cyst species identified, 20 (including *Diplopelta*, *Diplopsalopsis*, *Gonyaulax*, *Polykrikos*, *Protoperidinium*, *Scrippsiella* and *Zygabikodinium* spp.) were successfully germinated to produce viable cultures. Such diversity of diatom and dinoflagellate species in ships' ballast water suggests that the apparent 'cosmopolitanism' of many coastal phytoplankton species may be due partly to the global transport of seawater ballast. Of considerable concern was the detection in 16 ships of cysts of the toxic dinoflagellates *Alexandrium catenella*, *Alexandrium tamarense* and *Gymnodinium catenatum*. One single ballast tank was estimated to contain >300 million viable *A.tamarense* cysts, some of which were successfully germinated in the laboratory to produce toxic cultures. These toxic dinoflagellate species, which can contaminate shellfish with paralytic shellfish poisons, pose a serious threat to human health and the aquaculture industry. Ballast-water quarantine measures recently introduced in Australia are discussed. Mid-ocean exchange of ballast water is only partially effective in removing dinoflagellate cysts which have settled to the bottom of ballast tanks. The present work indicates that the most effective measure to prevent the spreading of toxic dinoflagellate cysts via ships' ballast water would be to avoid taking on ballast water during dinoflagellate blooms in the water column of the world's ports.

Introduction

Cargo-vessel ballast water was first suggested as a vector in the dispersal of non-indigenous marine species nearly 90 years ago. The diatom *Odontella* (*Biddulphia*) *sinensis*, well known from the tropical and subtropical coasts of the Indo-Pacific, was not reported in European waters until 1903, when it produced dense plankton blooms in the North Sea. Since it was unlikely that this large diatom could have been overlooked previously, and impossible that it could have been carried by currents from distant oceans, Ostenfeld (1908) suggested that this species was introduced by ship, either as part of the fouling community on vessels' hulls or, more likely, discharged with the water or sediment contained in ships' ballast tanks. Whereas the introduction of the diatom *O.sinensis* was apparently without harmful effects, the more recent introduction into the North Sea of the diatom *Coscinodiscus wailesii* (Boalch and Harbour, 1977; Rincé and Paulmier, 1986) has caused problems to fisheries by the clogging of fishing nets with extensive mucus. Ballast water has been suggested as a dispersal mechanism for a large range of marine organisms, including algae,

hydromedusae, polychaetes, copepods, mysids, molluscs and fish [see the review by Carlton (1985)]. The first direct examinations of ballast tanks at the end of a voyage were not, however, made until the 1970s (Medcof, 1975; Howarth, 1981; Williams *et al.*, 1988).

If dinoflagellate species that can produce paralytic shellfish toxins are introduced into sensitive aquaculture areas, the results could be disastrous for commercial shellfish farming. The present survey of ballast-water samples was prompted by the recent appearance in Australian waters of toxic dinoflagellate species that appear to have a disjunct global distribution, such as *Gymnodinium catenatum* (confined to Japan, Mexico, southern Europe and Tasmania) and *Alexandrium minutum* (confined to South Australia and southern Europe) (Hallegraeff *et al.*, 1988, 1991).

The Australian economy is heavily dependent on the export of raw materials such as coal, grain, iron ore, wheat and woodchips. Specialized bulk container ships only carry these commodities in one direction, using water as ballast for stability on the return voyage. As a result, some 60 million tonnes of ballast water are discharged in Australian ports every year, with ships sailing from Japan accounting for over a third of this amount (Hutchings *et al.*, 1987). Preliminary reports on the incidence of dinoflagellate cysts in ballast water appear in Hallegraeff *et al.* (1990) and Hallegraeff and Bolch (1991). This paper summarizes the results of a survey of 343 ballast-water samples. Our aims were (i) to establish unambiguously whether toxic dinoflagellate species can be carried by ships in a viable condition and (ii) to elucidate how dinoflagellate cyst abundance in ballast tanks varies according to ship type, ballast-tank configuration, season and port of origin, and the practice of mid-ocean ballast exchange.

Method

Officers of the Australian Quarantine and Inspection Service (AQIS) sampled the ballast tanks of 343 cargo vessels which arrived in 18 widely spread Australian ports between November 1987 and March 1990 (Figure 1). More than half the ships had sailed from Japan; the remainder arrived from east Asia (China and Hong Kong, Indonesia, South Korea, Singapore, Sri Lanka, Taiwan), the Middle East (Turkey, Iran, Yemen), North America (Canada and the United States), Argentina, Mexico and New Zealand. After partial removal of ballast in Australian ports, access was gained to the ballast tanks or holds of vessels. Replicate 500 ml samples of ballast-tank sediment or, if absent, ballast-water samples were collected by hand from different areas of the vessels. Samples were stored in a dark refrigerator (4°C) until despatched to CSIRO for analysis.

Sample preparation

Ballast-tank samples were sonicated for 2 min (Braun Labsonic homogenizer, intermediate probe, 100 W) to dislodge detritus particles. Samples were then fractionated through a 90 μm sieve onto a 20 μm sieve. This concentrated

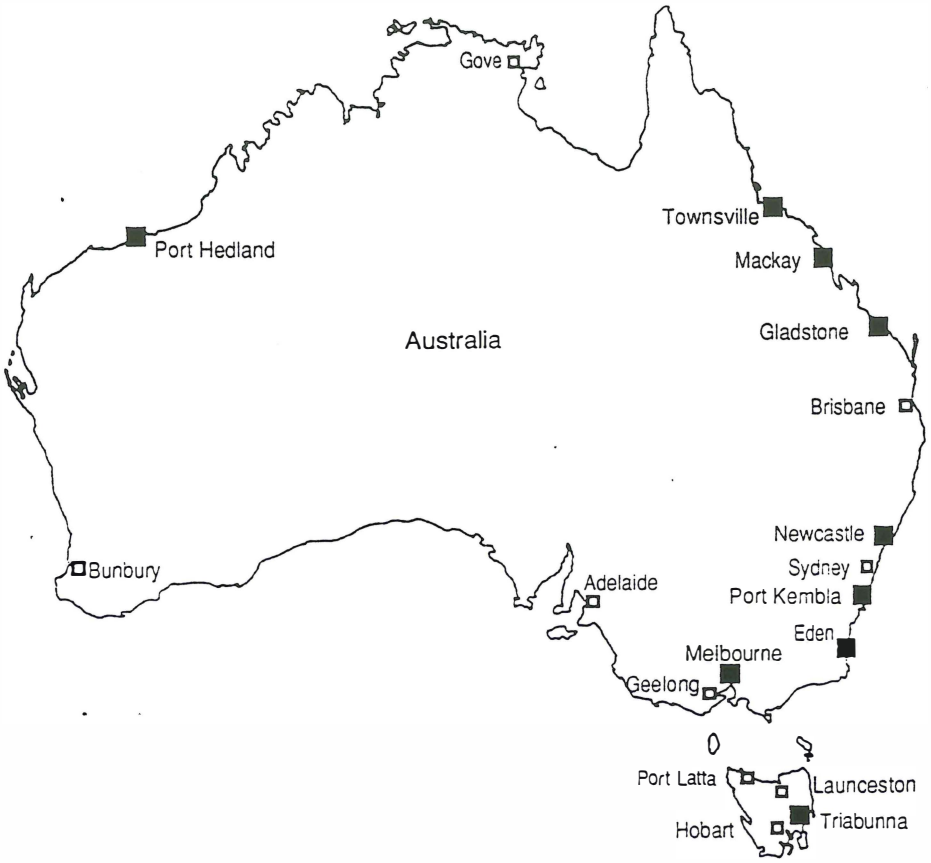


Fig. 1. Map of Australia showing the location of the 18 ports from which ballast-tank samples were collected. Samples from nine ports contained toxic dinoflagellates cysts (filled squares).

material was examined by light and scanning electron microscopy for diatom cells and dinoflagellate cysts. Cyst taxonomy is described by Bolch and Hallegraeff (1990).

Microscopy

Dinoflagellate cysts were photographed with a Zeiss standard, Nikon Diaphot or Zeiss Axioplan light microscope (LM) with bright field, phase contrast or differential interference contrast illumination. Samples with high concentrations of dinoflagellate cysts were counted in a Sedgewick Rafter counting chamber (Rigosha Co., Japan). Subsamples were collected on Nuclepore filters (2 μm pore size), air dried or critical point dried from liquid CO_2, sputter coated with platinum and examined with a Philips 515 scanning electron microscope (SEM).

Cyst germination experiments

Viable dinoflagellate cysts were isolated by micropipette and washed several

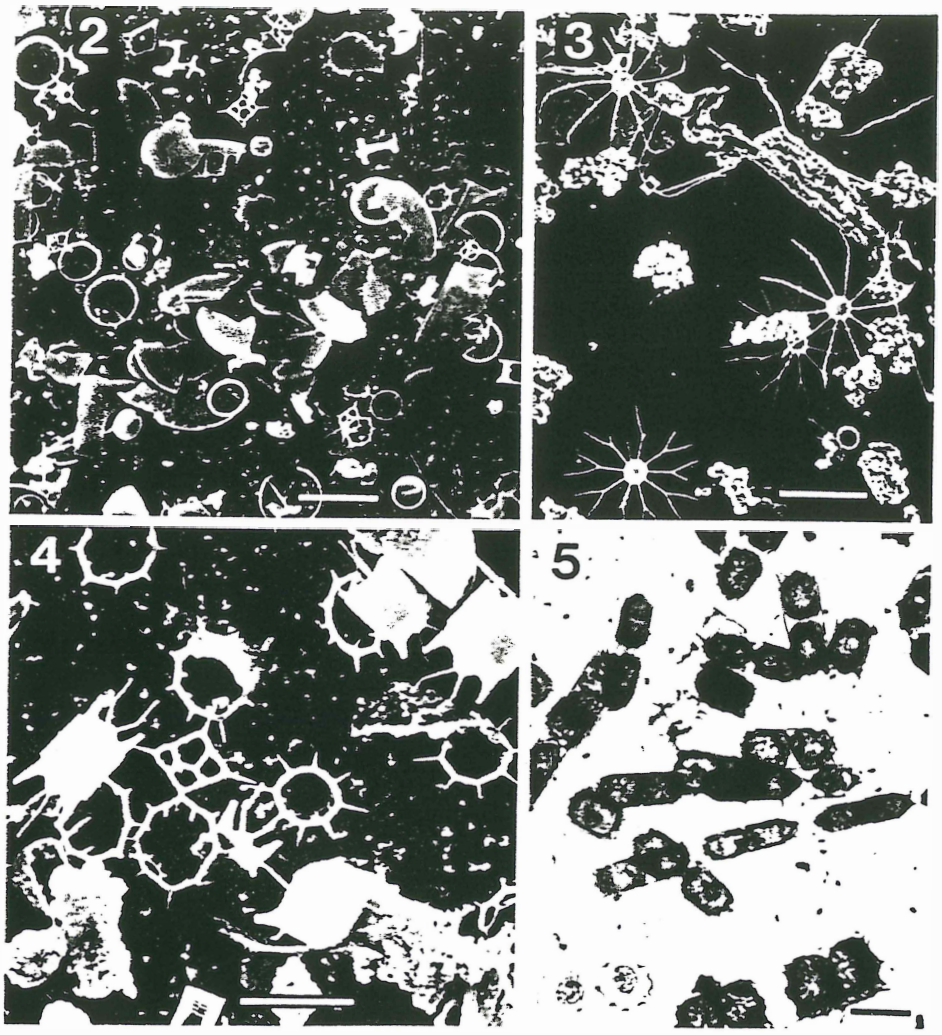
times in sterile culture medium. The medium consisted of filtered seawater (28‰ salinity) autoclaved in teflon containers, with nutrients added according to Loeblich's (1975) GPM medium modified with selenium (H_2SeO_3 at 10^{-8} M final concentration). Single cysts were placed in sterile polystyrene Petri dishes (36 mm diameter) containing 5 ml of medium. The cysts were incubated at 18 ± 0.5°C (12:12 h light:dark) at an irradiance of 80 μE m^{-2} s^{-1} and were examined daily for germination (Bolch *et al.*, 1991).

Results

Of the total of 343 ballast-tank samples examined, 65% contained fine brown or black sediment (up to an estimated 100 tonnes of sediment per ship). Some of the remaining samples were sediment-free because the bottoms of the ballast tanks were not easily accessible for sampling. One hundred samples of sediment from 18 Australian ports (Figure 1) were selected for detailed study. All of these samples contained diatom frustules (Figures 2–8), which were almost always present in mixtures of planktonic (*Bacteriastrum*, *Coscinodiscus*, *Pleurosigma*, *Thalassiosira*) and benthic species (*Paralia*, *Actinoptychus*). A number of ships contained the diatom *Coscinodiscus wailesii* (see Introduction) and diatom species not endemic to Australian waters (e.g. *Thalassiosira anguste-lineata* and *Thalassiosira pacifica*, Figure 6; Hallegraeff, 1984) were also identified. Diatom resting spores, especially of *Chaetoceros* species (Figures 7 and 8), were present in some 20% of sediment-containing samples and viable diatom cultures of *Odontella aurita* (Figure 5) and *Chaetoceros socialis* were produced from ballast-tank sediments (most likely from the germination of resting spores). However, small pennate diatoms that do not have resting stages (e.g. *Navicula*, *Neodelphineis*, *Nitzschia*) were also cultured from the sediments (even after being stored in the dark at 4°C for 6 months).

Dinoflagellate cysts

The cellulose thecae of motile dinoflagellates were rarely seen in the ballast-tank samples, but resting cysts were common. Dinoflagellate cysts were found in samples from vessels entering all 18 Australian ports studied (Figure 1). The cysts were found in 50 of the 100 sediment-containing samples: 53 species of cysts were identified, of which 20 species were germinated to produce viable cultures (Table I). These include the peridinioid dinoflagellates *Protoperidinium* (Figures 9, 10, 14, 15, 16 and 17), *Diplopsalopsis*, *Zygabikodinium*, *Diplopelta* (Figures 11–13) and *Scrippsiella* (Figures 18–20), the gymnodinioid dinoflagellates *Polykrikos* (Figure 21) and *Gymnodinium catenatum* (Figure 32), and the gonyaulacoid dinoflagellates *Protoceratium* (Figures 22 and 23), *Gonyaulax* (Figures 24–27) and *Alexandrium* (Figures 28–31). The non-cyst forming species *Gymnodinium simplex* and *Katodinium rotundatum* were also cultured from ballast-tank sediments. Quantitative cyst counts on selected samples rich in dinoflagellate cysts (up to 22 500 cysts cm^{-3} sediment) are summarized in Table II. In six of the nine samples a single dinoflagellate species (*Alexandrium*, *Diplopelta*, *Polykrikos* or *Scrippsiella*) accounted for 40–70% of total cysts.



Figs 2–8. Diatom frustules, silicoflagellates and diatom resting spores from ships' ballast tanks.

Fig. 2. SEM. Planktonic diatoms *Thalassiosira* and *Coscinodiscus* spp. from an ore vessel arriving in Port Hedland, Australia, from Fukuyama, Japan.

Fig. 3. SEM. Warm-water diatom *Bacteriastrum furcatum* from a woodchip cargo vessel arriving in Triabunna, Australia, from Yura, Japan.

Fig. 4. Diatom *Odontella aurita* and silicoflagellate *Dictyocha speculum* from a woodchip cargo vessel arriving in Triabunna from Yatsushiro, Japan.

Fig. 5. Diatom *Odontella aurita* cultured from a ballast-tank sediment sample from Yatsushiro, Japan.

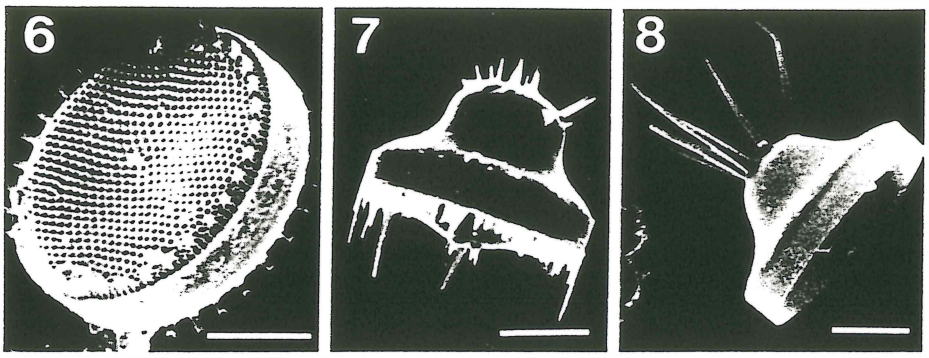


Fig. 6. Diatom *Thalassiosira pacifica*, non-endemic to the Australian region, from a ballast-tank sample from Niihama, Japan.

Fig. 7. Resting spore of the diatom *Chaetoceros* sp. from a ballast-tank sediment sample from Yura, Japan.

Fig. 8. Resting spore of the diatom *Chaetoceros affine* from a ballast-tank sediment sample from Yura, Japan. Scale bars 100 μm (Figure 2); 50 μm (Figures 3, 4 and 5); 10 μm (Figures 6, 7 and 8).

Logistic regression was used to investigate the relationship between the presence or absence of live dinoflagellate cysts and variables such as port of origin, type of vessel, departure season and tank type. The following statistically significant associations were observed. Of the samples with dinoflagellate cysts, ships (n = 45) that departed between May and October (late spring to early autumn in the northern hemisphere) were more likely to contain a high proportion of live cysts (chi-square statistic: χ^2 = 7.65, df = 1, P = 0.007) than ships departing between November and April (n = 53). Furthermore, samples from the bulk cargo holds of ships (n = 28) were more likely to contain a high proportion of live cysts than double-bottom, wing or topside ballast tanks [n = 50: χ^2 = 5.04, df = 1, P = 0.025; see Rigby *et al.* (1991) for details on ballast-tank configurations].

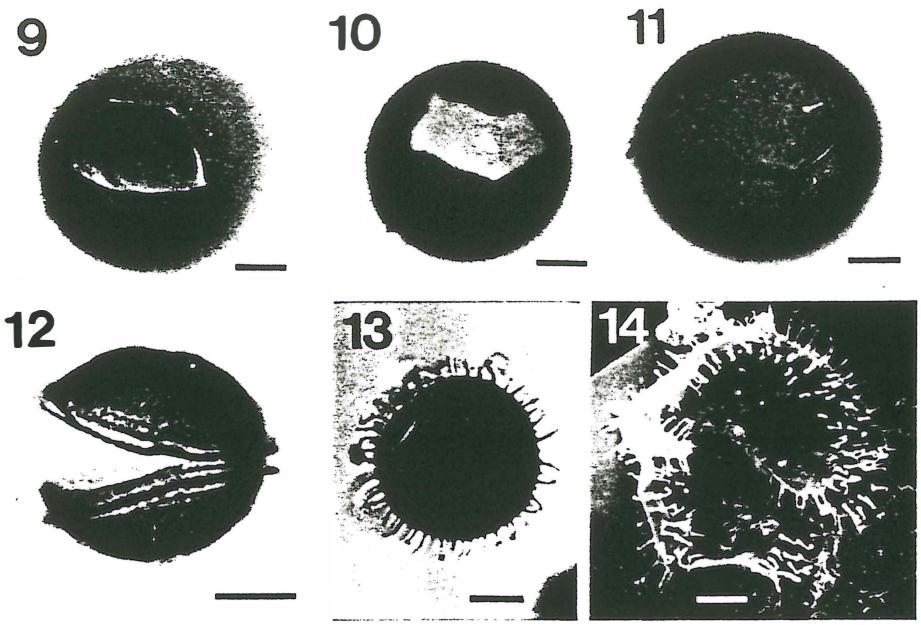
Toxic dinoflagellates

The identification of cysts of the toxic dinoflagellates *Alexandrium catenella* (Figures 29 and 31) and *Alexandrium tamarense* (Figures 28 and 30) in 5 of the 100 sediment samples was of considerable concern. After this CSIRO survey was completed, the AQIS contracted the Australian Government Analytical Laboratories (AGAL) to continue monitoring the ballast tanks of ships entering Australian ports. This monitoring detected nine further positive *Alexandrium* ballast-tank samples (N.Blain, Australian Government Analytical Laboratories, Melbourne, personal communication). In five cases, these *Alexandrium* cysts have been successfully germinated into viable dinoflagellate cultures and their toxicity has been confirmed by high-performance liquid chromatography (unpublished data). Four samples containing toxic *Gymnodinium catenatum* cysts were also identified (Table III). Toxic dinoflagellate cysts were found in

Table I. Dinoflagellate cyst species in 100 selected ballast-tank sediments

Species	Number of samples	Cysts with cell contents	Successful germination
Alexandrium catenella	2	+	+
Alexandrium tamarense	2	+	+
Alexandrium sp.	1	+	−
Diplopelta parva	12	+	+
Diplopsalis lebourae	1	+	+
Diplopsalis lenticula	4	−	−
Diplopsalis sp.	3	−	−
Diplopsalopsis orbicularis	2	+	+
Gonyaulax digitale	6	+	−
Gonyaulax scrippsae	7	+	−
Gonyaulax spinifera:			
Cyst form *Spiniferites ramosus*	3	+	+
Cyst form *Spiniferites mirabilis*	9	+	−
Cyst form *Spiniferites membranaceus*	2	+	−
Gonyaulax sp.	10	+	−
Gymnodinium sp.	1	+	−
Lingulodinium polyedra	7	−	−
Peridinium faeroense	1	−	−
Pheopolykrikos hartmanni	2	−	−
Polykrikos kofoidii	4	+	+
Polykrikos schwartzii	6	+	+
Protoceratium reticulatum	11	−	−
Protoperidinium americanum	5	+	+
Protoperidinium avellana	3	−	−
Protoperidinium conicoides	9	−	−
Protoperidinium conicum	9	+	+
Protoperidinium denticulatum	4	−	−
Protoperidinium cf. *divergens*	3	−	−
Protoperidinium cf. *expansum*	6	+	+
Protoperidinium excentricum	2	+	+
Protoperidinium leonis	12	+	+
Protoperidinium cf. *nudum*	1	−	−
Protoperidinium oblongum	13	+	+
Protoperidinium pentagonum	5	+	+
Protoperidinium punctulatum	3	−	−
Protoperidinium subinerme	4	+	−
Protoperidinium spp. (8 spp.)	23	−	−
Pyrocystis cf. *lunula*	2	+	−
Scrippsiella precaria	1	+	+
Scrippsiella trochoidea	15	+	+
Scrippsiella spp. (4 spp.)	17	+ (4)	+ (2)
Zygabikodinium lenticulatum	7	+	+

woodchip, gas and ore vessels, which sailed from either Japan or South Korea and entered nine Australian ports (Table III; Figure 1). One ballast tank was filled during a toxic dinoflagellate bloom in the port of Muroran, Japan, during July 1989. A sample taken in Eden, Australia, was estimated to contain >300



Figs 9–20. Cysts and motile cells of peridinioid dinoflagellates from ships' ballast tanks.

Fig. 9. LM. Round brown cysts of *Protoperidinium* cf. *expansum*, from a vessel arriving in Launceston from Mishima, Japan, showing distinctive archeopyle opening.

Fig. 10. LM. Cyst of *Protoperidinium* sp., from a vessel arriving in Newcastle from Niihama, Japan, showing archeopyle with detached operculum.

Fig. 11. LM. Cyst of *Diplopsalopsis orbicularis* from a vessel arriving in Port Latta, Tasmania, from Wakayama, Japan.

Fig. 12. LM. Cyst of *Zygabikodinium lenticulatum* from a vessel arriving in Newcastle from Niihama, Japan.

Fig. 13. LM. Spinose round cyst of *Diplopelta parva* from a vessel arriving in Port Latta from Wakayama, Japan.

Fig. 14. SEM. Spinose peridinioid-shaped cyst of *Protoperidinium pentagonum* from a vessel arriving in Port Latta from Wakayama, Japan.

million *Alexandrium* cysts (Table III, sample no. 72). One month later, on its next voyage from Shimizu, Japan, to Eden, this ship still contained the same cyst material, albeit in lower concentration (Table III, sample no. 73). In both cases these cysts failed to germinate until ~6 months later, which suggests that they were newly formed cysts undergoing a mandatory dormancy period (Anderson, 1980).

Discussion

The present survey established that microscopic plankton organisms such as

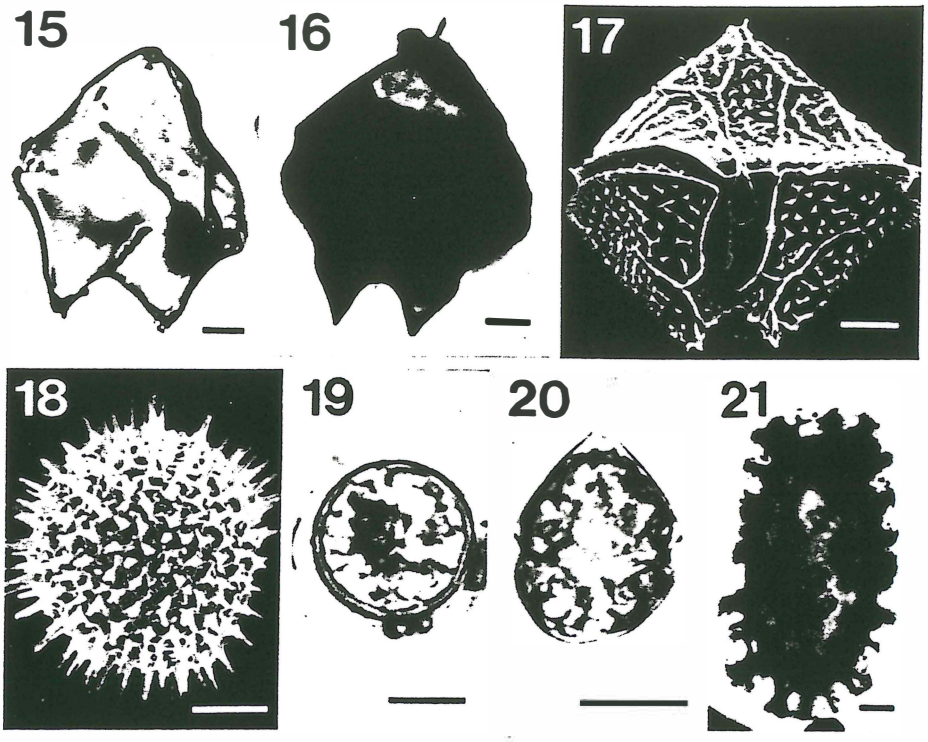


Fig. 15. LM. Cyst of *Protoperidinium* sp. from a vessel arriving in Port Latta from Wakayama, Japan.

Figs 16–17. Cyst and motile cell of *Protoperidinium leonis*.

Fig. 16. LM. Cyst of *P.leonis* from a vessel arriving in Triabunna from Yatsushiro, Japan.

Fig. 17. SEM. Ventral view of the motile cell germinated from the cyst shown in Figure 16.

Fig. 18. SEM. Calcareous spinose cyst of *Scrippsiella trochoidea* from a vessel arriving in Australia from Mexico.

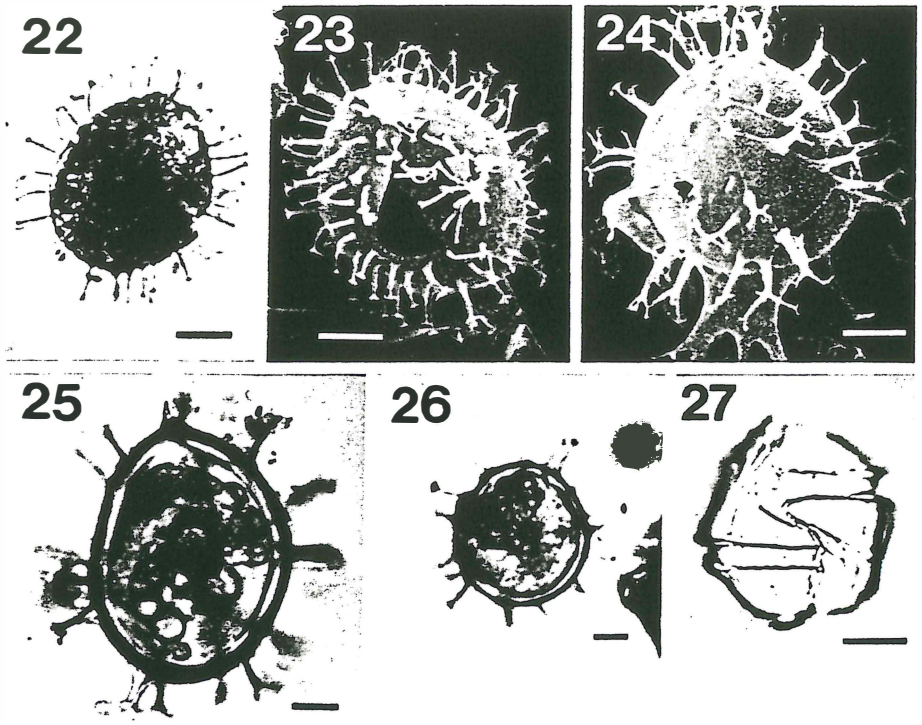
Figs 19–20. Cyst and motile cell of *Scrippsiella* sp.

Fig. 19. LM. Spherical, clear-walled cyst from a vessel entering Port Hedland from Kawasaki, Japan.

Fig. 20. LM. Cultured cell germinated from the cyst shown in Figure 19.

Fig. 21. Cyst of the gymnodinioid dinoflagellate *Polykrikos kofoidii* from a vessel arriving in Eden from Muroran, Japan. (All scale bars = 10 μm).

diatoms and dinoflagellates, especially those species that produce resistant resting stages, can be transported in a viable form in the ballast water of cargo vessels. Because toxic dinoflagellates pose a threat to human health and aquaculture, this investigation has focused on the incidence of dinoflagellate cyst stages in ships' ballast tanks. Twenty species of coastal dinoflagellates were



Figs 22–31. Cyst and motile cells of gonyaulacoid dinoflagellates from ships' ballast tanks.

Figs 22 and 23. Spinose cysts of *Protoceratium reticulatum*.

Fig. 22. LM. Cyst from a vessel arriving in Mackay from Kashima, Japan.

Fig. 23. SEM. Cyst from a vessel arriving in Newcastle from Niihama, Japan.

Figs 24–27. Spinose cysts of *Gonyaulax* spp.

Fig. 24. Cyst of *Gonyaulax spinifera* from a vessel arriving in Mackay from Niihama, Japan.

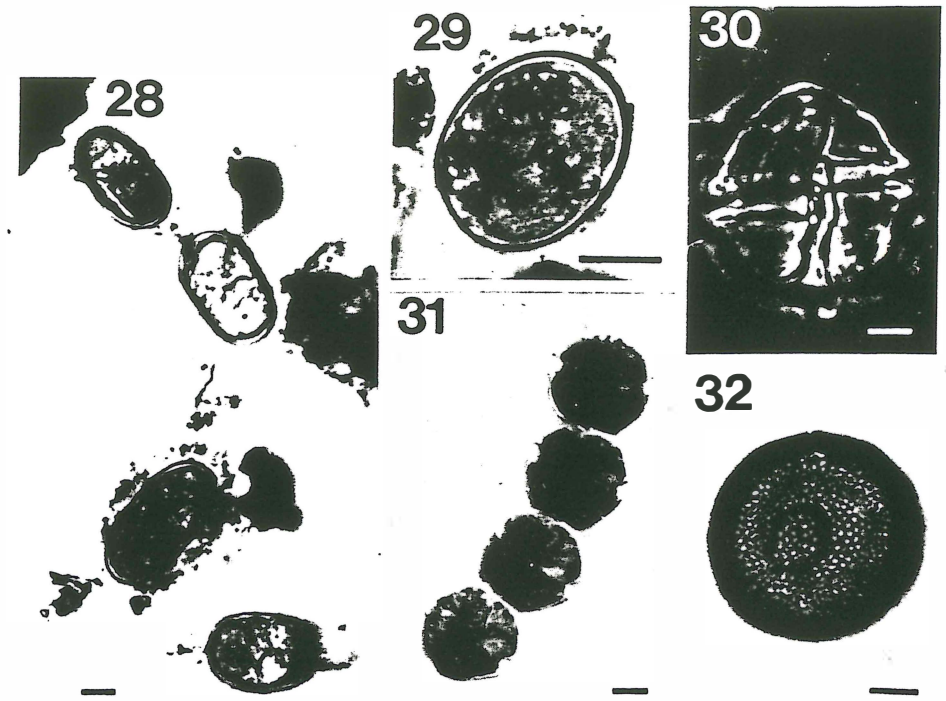
Fig. 25. LM. Live cyst of *Gonyaulax digitale* showing cell contents, from a vessel arriving in Port Hedland from Pohang, Korea.

Figs 26–27. Cyst and motile cell of *Gonyaulax spinifera*.

Fig. 26. LM. Live cyst from a vessel arriving in Port Hedland from Kashima, Japan.

Fig. 27. LM. Motile cell germinated from the cyst shown in Figure 26.

cultured from ballast-tank samples (Table I). Of these, two were species that produce paralytic shellfish poisons (Table III). Cysts were found in some 35% of the 343 samples, which came from a wide range of vessel types and overseas ports, and entered all Australian ports studied (Figure 1). However, toxic dinoflagellate cysts were only found in vessels sailing from Japan or South



Figs 28–31. Mucoid cysts and motile cells of *Alexandrium* spp.

Fig. 28. LM. Live cysts of *Alexandrium tamarense* from a vessel arriving in Eden from Muroran, Japan.

Fig. 29. LM. Cyst of *A.catenella* from a vessel arriving in Port Hedland from Kashima, Japan.

Fig. 30. LM. Motile cell of *A.tamarense* cultured from the cyst shown in Figure 28.

Fig. 31. LM. Four-celled chain of *A.catenella* cultured from the cyst shown in Figure 29.

Fig. 32. LM. Round, brown cyst of *Gymnodinium catenatum* with microreticulate ornamentation and characteristic apical groove; from a vessel arriving in Newcastle from Kohong, South Korea. (All scale bars = 10 μm).

Korea. Significantly, of the 80 known extant dinoflagellate cyst species (Matsuoka *et al.*, 1989, unpublished data), 53 taxa were detected in ballast-tank samples (Table I). Thus the risk of foreign micro-organisms being introduced into Australian waters via ships' ballast water is widespread (Figure 1) and the inoculum represents a great diversity of species (Figures 2–32).

Water column or benthic origin of ballast-water cysts

It must be established whether cysts originate from plankton blooms in the water column or from harbour sediments resuspended during the ships' ballasting

Table II. Species abundance of dinoflagellates in selected ballast-tank samples with high cyst concentrations (arranged in order of decreasing cyst abundance)

Sample code	Port of origin	Departure date	Cargo type	Cyst concentration (cm^{-3})	Dominant species (%)		Cyst type			
							Gonyaulacoid cysts (%)	Peridinioid cysts (excluding *Scrippsiella*) (%)	*Scrippsiella* (calcified and non-calcified cysts) (%)	Others (%)
No. 72	Muroran, Japan	July 1989	woodchips	22500	*Alexandrium tamarense*	67	68	2	12	18
No. 14	Kimitsu, Japan	July 1989	ore	9400	*Polykrikos schwartzii*	75	1	18	4	77
No. 185	Wakayama, Japan	Dec. 1988	ore	5100	*Diplopelta parva*	61	15	78	4	3
No. 340	Kashima, Japan	Feb. 1990	ore	4100	*Protoceratium reticulatum*	22	33	36	25	6
No. 201	Shimizu, Japan	Aug. 1989	ore	820	*Scrippsiella trochoidea*	65	6	6	81	7
No. 81	Pohang, Korea	July 1989	ore	45	*Scrippsiella* sp.	42	8	45	42	5
No. 73	Shimizu, Japan	Aug. 1989	woodchips	40	*Alexandrium tamarense*	38	43	14	19	24
No. 41	Kawasaki, Japan	May 1989	ore	40	*Protoperidinium* sp.	23	9	66	23	2
No. 199	Niihama, Japan	Sept. 1989	ore	50	*Diplopsalis lebourae*	21	6	68	21	5

Table III. Summary of ballast-tank samples containing toxic dinoflagellates cysts

Sample code	Port of origin; departure date	Australian port of arrival; arrival date	Cargo type	Ballast tank	Dinoflagellate species	Cyst abundance	Culture established	Toxicity
No. 2	Kushiro, Japan; 21/11/87	Triabunna, Tas; 11/12/87	woodchips	Hold 4	*Alexandrium* sp.	1 cyst seen	–	not determined
No. 36	Kashima, Japan; 5/6/89	Port Hedland, WA; 27/6/89	ore	Starboard wing 4	*Alexandrium catenella*	2 cysts seen	+	+
No. 44	Kashima, Japan; 5/6/89	Port Hedland, WA; 27/6/89	ore	Port wing 4	*Alexandrium* sp.	1 cyst seen	–	not determined
No. 72	Muroran, Japan; 2/7/89	Eden, NSW; 20/7/89	woodchips	Hold 4	*Alexandrium tamarense*	15 160 cysts cm^{-3}	+	+
No. 73	Shimizu, Japan; 10/8/89	Eden, NSW; 28/8/89	woodchips	Hold 4	*Alexandrium tamarense*	15 cysts cm^{-3}	+	+
No. 18279*	Pohang, Korea; 6/9/90	Gladstone, Qld; 19/9/90	ore	Fore peak	*Alexandrium* sp.	3 cysts seen	–	not determined
No. 003610*	Singapore?	Port Phillip Bay, Vic?; 27/12/89	gas	unknown	*Alexandrium catenella*	4 cysts seen	+	+
No. 19425*	Samchonpo, Korea; 2/10/90	Port Kembla, NSW; 16/10/90	ore	Topside 2	*Alexandrium tamarense*	400 cysts cm^{-3}	+	+
No. 006803*	Kohong, Korea; 8/3/91	Newcastle; 28/3/91	coal	unknown	*Gymnodinium catenatum*; *Alexandrium* sp.	15 cysts; 2 cysts seen	–	not determined
No. 008412*	Fukuyama, Japan	Mackay; 24/3/91	ore	Wing 2	*Alexandrium* sp.	1 cyst seen	–	not determined
No. 007842*	Inchon, Korea	Townsville; 1/3/91	ore	unknown	*Gymnodinium catenatum*; *Alexandrium* sp.	3 cysts; 5 cysts seen	–	not determined
No. 007846*	unknown	Port Kembla; 27/4/91	ore	Topside 3	*Alexandrium* sp.	2 cysts seen	–	not determined
No. 010128*	Fukuyama, Japan 29/4/90	Gladstone; 11/5/90	–	–	*Gymnodinium catenatum*	1 cyst seen	–	not determined
No. 018040*	Kure, Japan	–	–	–	*Alexandrium* sp.	2 cysts seen	–	not determined
No. 009962*	Ube, Japan 27/4/90	Dalrymple Bay; 9/5/90	–	–	*Gymnodinium catenatum*	1 cyst seen	–	not determined
No. 009975*	Chiba, Japan	–	–	–	*Alexandrium* sp.	1 cyst seen	–	not determined

* Samples detected by Neil Blain, Australian Government Analytical Laboratories and forwarded to CSIRO for germination experiments and confirmation of taxonomic identification.

procedures before counter measures can be formulated to reduce the incidence of (toxic) dinoflagellate cysts in ships' ballast-water tanks. The species composition of ballast-water diatoms (Figures 2–4) suggests that the material on the bottom of ships' ballast tanks is a mixture of both planktonic and benthic forms. However, when high concentrations of dinoflagellate cysts were present in ballast tanks (Table II) a single species, not normally common in sediments, dominated on each occasion, which suggests that these samples derived from blooms in the water column during the ships' ballasting. If cysts had been derived from resuspended harbour sediment, one would have expected a much greater species diversity [see Matsuoka (1985) for Japanese ports; Bolch and Hallegraeff (1990) and McMinn (1991) for Australian ports]. Analysis of vessels' departure dates (more live dinoflagellate cysts from May to October) supports the view that the water column during seasonal (spring to early autumn) dinoflagellate blooms is the predominant origin of ballast-water cysts.

Finally, germination experiments with ballast-water *Alexandrium* cysts originating from Muroran, Japan (Table III), were unsuccessful until ~6 months later. This suggests that these were newly formed cysts undergoing a mandatory dormancy period, rather than mature cysts resuspended from bottom sediments. All samples which contained *Alexandrium* cysts could be traced to ports known to be affected by toxic dinoflagellate blooms. On three occasions, the incidence of *Alexandrium* cysts in ballast-water samples could be correlated with known dinoflagellate blooms present in the water column of foreign ports at the time of ballasting [Muroran, Japan, July 1989; Kashima, Japan, June 1989 (Dr Y.Oshima, personal communication); Samchonpo, South Korea, September 1990 (Dr J.-S.Park, personal communication)].

Implications for plankton biogeography

Wind and migratory water birds (Proctor, 1966; Schlichting, 1969) are the main vectors in the long-range dispersal of species of freshwater algae over entire continents. Ocean currents are the main vector of dispersal for marine phytoplankton algae and, because of the apparent continuity of the world's oceans, similar hydrological environments in different oceans tend to have apparently similar phytoplankton assemblages. Taylor (1987) claims that marine phytoplankton species have had ample evolutionary time to reach and inhabit all suitable environments. While this may be true for oceanic diatoms and dinoflagellates, or for some widespread ecologically tolerant coastal diatoms, estuarine dinoflagellates tend to have fastidious nutritional requirements, especially with regard to humic substances from land runoff (Prakash, 1975). For cyst-producing estuarine dinoflagellates that cannot cross oceanic boundaries by means of ocean currents, there is every reason to believe that transport in ballast water has played an important role in species dispersal in the twentieth century. Cargo ships have used water as ballast since the 1850s (Carlton, 1985). However, in the past two decades, the increased size and speed of cargo vessels, together with increased eutrophication of many coastal waters, have intensified the likelihood of successful transfer of species across oceanic boundaries. Thus it is

possible that the 'cosmopolitanism' of many coastal plankton taxa may be the result of extensive blending of coastal waters by the transport of seawater ballast.

Molecular taxonomic techniques, such as enzyme-electrophoresis or RNA sequencing, are now available to examine the genetic relationships of globally disjunct populations of neritic dinoflagellates. For example, rRNA sequencing has shown a remarkable match between Australian and Japanese populations of the toxic dinoflagellate *A.catenella*, and between Australian and European populations of the toxic dinoflagellate *A.minutum* (Scholin and Anderson, 1991). Similarly, enzyme-electrophoretic studies of disjunct global populations of the estuarine dinoflagellate *Gymnodinium catenatum* indicate that they are genetically uniform (S.Blackburn, unpublished data), suggesting a recent dispersal from a single genetic stock.

Implications for aquaculture

The majority of toxic dinoflagellates that affect shellfish aquaculture (e.g. *Alexandrium* spp., *Gymnodinium catenatum*, *Pyrodinium bahamense*) produce resistant resting cysts that can remain fully viable under unfavourable environmental conditions for 10–20 years. If cyst-producing species that are not endemic to an area are inadvertently introduced via ships' ballast water, their cyst stages may be buried below the sediment surface from which they are gradually resuspended into the water column. This may result in years of recurrent germination attempts by the cysts which, when successful, will result in dinoflagellate blooms in the overlying water column. Once the species produces new cyst stages, it will have effectively colonized a new water body from which it cannot be eradicated. Local shipping and coastal currents can then spread the species beyond the initial point of introduction. The impacts on shellfish aquaculture include the need to establish costly paralytic shellfish poisoning toxin monitoring programs and, on the basis of the results, seasonal closures of shellfish farms may need to be enforced. Strong circumstantial evidence, provided by examination of historic plankton samples (Hallegraeff *et al.*, 1988), cyst surveys in sediment depth cores (Bolch and Hallegraeff, 1990; A.McMinn, G.Hallegraeff and C.Bolch, work in progress) and genetic studies using enzyme electrophoresis and sexual compatibility experiments (Blackburn *et al.*, 1989; unpublished data), suggests that the toxic dinoflagellate *Gymnodinium catenatum* was introduced to Tasmania at some time after 1980 in the area around the shipping port of Hobart. The present work (Table III) confirms the role of ballast water as a vector for the dispersal of *Gymnodinium catenatum* cysts. Benthic cyst beds of this species are now widespread in southern Tasmania (Bolch and Hallegraeff, 1990) and dense plankton blooms in 1986 and 1991 in southern Tasmanian waters necessitated the closure of 15 shellfish farms for periods of up to 6 months (Hallegraeff and Sumner, 1986).

Similarly, the toxic dinoflagellate *A.catenella*, which has caused the closure of shellfish farms in Port Phillip Bay, Melbourne, was not known in the area before 1986 (Hallegraeff *et al.*, 1988, 1991). The present work has confirmed that viable

cysts of this species are being introduced into this port via ships' ballast water (Table III, sample no. 003610).

A large range of other micro-organisms that are potentially harmful to human health and aquaculture are also of concern. These include bacteria, viruses and protozoans. In a companion study, the toxic bacterium *Clostridium botulinum* was detected in 1 out of 281 ballast-water samples tested (A.Gibson, unpublished). It is very difficult to assess how often introduced marine micro-organisms have actually established themselves in their new Australian environments and this question is beyond the scope of the present study. There are at least 15 established macroscopic marine organisms known with reasonable certainty to have arrived in Australia in ballast water. These include fish (4 species), crustaceans (4), polychaete worms (3), molluscs (3) and a seaweed (1) (Pollard and Hutchings, 1990a,b). The potential impact of these species on commercial fisheries, aquaculture and the natural environment is of increasing concern. In Tasmania, the introduced seaweed *Undaria pinnatifida* (current standing crop ~400 tonnes) poses a threat to abalone and sea urchin fisheries, as well as to a proposed marine park zone (Sanderson, 1990). In the Great Lakes of North America, blockage of water inlets by the introduced zebra mussel *Dreissena polymorpha* has been estimated to cost more than US$5 billion in control measures over 10 years (Hebert *et al.*, 1989).

Conclusions

The Australian Government has responded to the preceding evidence about toxic dinoflagellate cysts by declaring the disposal of ballast water to be a quarantine issue of national significance. As of February 1, 1990, Australia introduced voluntary guidelines for ships entering its ports from overseas. These guidelines aim to reduce the risk of harmful introductions by encouraging a range of practices, such as reballasting at sea (only feasible for vessels up to 40 000 dead weight tonnage), ballasting in deep water, disposal of ballast-tank sediments outside Australian waters, non-discharge of ballast water in Australian ports, or participation in other compliance arrangements (see Hallegraeff and Bolch, 1991). As of November 1, 1990, the Marine Environment Pollution Committee (MEPC) of the International Maritime Organization (IMO) has ratified the above guidelines for adoption on an international basis. In the present survey, 32 vessels claimed to have exchanged ballast water in mid-ocean, but 14 of these were still found to contain significant amounts of sediment, including dinoflagellate cysts [see also Rigby and Hallegraeff (1992)]. A more effective measure to prevent the spreading of dinoflagellate cysts via ships' ballast water would be to avoid ballasting during toxic dinoflagellate blooms in the water columns of the world's ports. Other options using temperature or chemical treatment (chlorine, hydrogen peroxide) of ballast water, either in hold or in onshore facilities, are also being investigated (Rigby *et al.*, 1991). Clearly, bulk cargo shipping and aquaculture or marine parks are incompatible operations, and should never be planned in the same area.

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CHEMICAL AND PHYSICAL TREATMENT OPTIONS TO KILL TOXIC DINOFLAGELLATE CYSTS IN SHIPS' BALLAST WATER

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More than 5000 culture-produced resting cysts of the toxic marine dinoflagellate *Gymnodinium catenatum* were subjected to a wide range of chemical (chlorine, copper sulphate, hydrogen peroxide, pH, salinity and a commercial microbiocide) as well as physical (heat) treatments. Effective treatment to prevent germination of dinoflagellate cysts in seawater samples could be achieved with high concentrations of free chlorine (500 ppm) or hydrogen peroxide (5000 ppm). However, the high costs involved and environmental as well as ship's safety considerations render these options impracticable as a routine treatment method for ships' ballast water (25 000 to 100 000 t capacity). In contrast, the heating of ballast water (30–90 s at 40–45°C) may provide an effective, environmentally friendly solution to the global problem of ballast water transport of unwanted marine organisms.

KEY WORDS: toxic dinoflagellate cysts, ships' ballast water, chemical treatment, physical treatment.

1. INTRODUCTION

The transfer of unwanted marine organisms via cargo vessel ballast water can pose a major threat to aquaculture developments, public health and endemic marine flora and fauna (Carlton, 1985; Hallegraeff and Bolch, 1991). The recent invasion in the North American Great Lakes of the zebra mussel *Dreissena polymorpha*, which is blocking intake pipes of water cooling systems, has been estimated to cost more than US $5 billion in control costs over 10 years. A survey of cargo vessels entering Australian ports showed that 6% carried the resistant resting cyst stages of the toxic dinoflagellates *Alexandrium catenella*, *A. tamarense* and *Gymnodinium catenatum* which can contaminate shellfish with paralytic shellfish poisons (Hallegraeff and Bolch, 1992). The growing international recognition of this problem has led to the introduction of special ballast water quarantine guidelines by the International Maritime Organisation, from 1 November 1991. These measures aim to improve vessel ballasting procedures such as: avoiding taking in ballast water during toxic dinoflagellate blooms or in shallow ports where sediment uptake is likely; encouraging the practice of mid-ocean exchange of ballast water (only feasible for ships up to 40 000 dead weight tonne); and to discourage ballast water discharge in sensitive aquaculture or marine park areas.

The present work explores the possibility of more rigorous chemical and physical treatment options of ships' ballast water, using toxic dinoflagellate cysts as a model organism. Treatment methods that kill resistant dinoflagellate cysts are likely to be equally effective for a wide range of other marine organisms such as larval zooplankton, copepod eggs and seaweed spores (with the possible exception of bacterial spores and viral particles which may be more difficult to eradicate). The resistance of dinoflagellate cysts has been well documented (Dale, 1983). Normal sediment preparation techniques, including

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ultrasonication and subsequent microscopic observation under ultraviolet radiation, appear to have no effect on their viability and commonly used electron microscopic fixatives such as osmium tetroxide appear unable to penetrate the cyst walls. Unfortunately, most experimental studies on the viability of dinoflagellate cysts have focused on the factors affecting germination under the normal range of ambient marine environmental conditions (Anderson, 1980). However, very little is known about the physical and chemical extremes under which cysts can survive. In the present work, the toxic marine dinoflagellate *Gymnodinium catenatum* was selected as test organism, because:

1) The conditions for successful culturing and the complete sexual life cycle are well documented (Blackburn *et al.*, 1989).
2) It has a short cyst dormancy period of only 14 days, compared to 6 months for toxic *Alexandrium* species.
3) The cysts require no environmental conditioning (e.g. cold storage) before they can germinate.
4) The cysts are comparatively large, 45–55 μm diameter, and lack an outer mucilaginous covering (as in *Alexandrium*), which makes them easy to manipulate under low power microscopes.

In the present work, more than 5000 culture-produced *Gymnodinium catenatum* cysts were subjected to various chemical (salinity, chlorine, copper sulphate, hydrogen peroxide) and physical (heat) treatments and then incubated to evaluate the effects on cyst germination.

2. MATERIAL AND METHODS

Dinoflagellate cysts

One ml culture suspensions of compatible sexual mating strains of the dinoflagellate *Gymnodinium catenatum* (strain GCDE02 and GCHU10; see Blackburn *et al.*, 1989) were inoculated into 55 mm polystyrene petri dishes containing 10 ml of nitrate and phosphate deficient GSe culture medium. The petri dishes were incubated at 17°C and a light intensity of 200 $\mu E\ m^{-2}\ s^{-1}$ and examined regularly for cyst formation. This procedure produced large numbers of cysts (up to 500 per petri dish) within 7 to 10 days, 90% to 95% of which readily germinated within 14 days to form a swimming planomeiocyte, 70% of these divided to produce viable vegetative cultures. In the following experiments, the criterion adopted for cyst survival was therefore the germination of an actively swimming planomeiocyte.

Chemical treatment

For chemical treatment experiments, cysts were produced as described above. After 10 to 14 days, the cysts were removed from the petri dishes with a glass microcapillary pipette, washed twice in sterile GSe medium and immediately used for experiments. Individual cysts were isolated under a Wild M7 stereomicroscope and placed into treatment solutions with varying salinity (as NaCl), pH (prepared by addition of NaOH or HCl), or concentrations of free chlorine (prepared from sodium dichloroisocyanurate), hydrogen peroxide, copper sulphate, or the microbiocide Kathon WT 1.5% (active ingredient 5-chloro-2-methyl-4-isothiazolin-3-one; Rohm and Haas, Philadelphia, USA). Treatment solutions for free chlorine and hydrogen peroxide were prepared in charcoal-filtered

seawater to remove interfering organic material. Free chlorine concentrations were measured before and after treatment using a chlorine DPD test kit (Palintest Ltd., Gateshead, UK). The treatment solution for copper sulphate was prepared in distilled water to avoid complexation and precipitation in seawater. All treatment solutions were kept for 24 h at room temperature (20°C) and in the dark to avoid photodegradation of chemicals. Following treatment, the cysts were removed from the chemical solutions and washed three times in sterile GSe medium. They were then placed into 36 mm polystyrene dishes containing 5 ml culture medium, incubated as described above and examined regularly for germination and cell division.

Heat treatment

Two different sets of heat treatment experiments were carried out. The first set of experiments aimed to determine the lethal temperature range. Dinoflagellate cysts were placed in a 100 ml test tube containing 30 ml of culture medium. The tube was immersed in a 20°C water bath which over a period of 25 min was gently heated to 60°C. Subsamples were withdrawn from the tube at 25, 30, 35, 40, 45, 50 and 60°C, and the cysts incubated for germination as described above. Once the lethal temperature range was determined, the second set of experiments (Figure 1) aimed to establish the critical treatment times at the different target temperatures. In this case, a total of 3500 dinoflagellate cysts were placed in 36 mm petri dishes (sealed with parafilm) containing only 1 ml GSe culture medium and these were immersed in a water bath heated to 38, 40, 43, 45, 47, 49 or 51°C (approximately 500 cysts at each temperature). The petri dishes were allowed to heat up to the target temperature over a period of 60 to 90 s. Temperature within the petri dishes was monitored with a flat (6 mm diameter) copper disc thermocouple (Radio Spares, Australia), attached to the inside of an equivalent petri dish

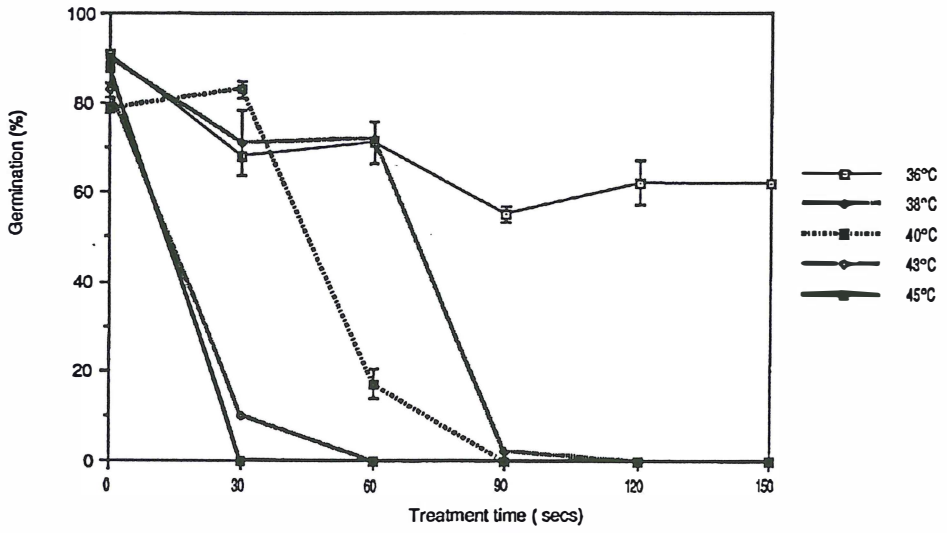

FIGURE 1 Germination of *G. catenatum* cysts (as % of total number of cysts used) after short-term exposures (30 to 150 s) to five different temperature regimes. Error bars indicate average and range of values from two experiments.

and monitored with a digital temperature probe (Digitron 3202K, type K). Replicate dishes were removed after temperature exposures of 30, 60, 90, 120 and 150 s and allowed to cool to 20°C. The dishes were then opened, 3 ml of fresh GSe culture medium added and the dinoflagellate cysts incubated for germination as described above.

3. RESULTS

Chemical treatment

Dinoflagellate cysts of *Gymnodinium catenatum* exposed for 24 h to freshwater showed some disruption to their cell contents, but surprisingly their viability was unaffected. Similarly, dinoflagellate cysts showed no effects after exposures to salinities in the range 15 to 50‰, and only treatment with extreme salinities as high as 100‰ prevented their successful germination. Cysts exposed to a pH range of 2 to 10 showed the same germination success as control cultures (pH = 8.4). Copper sulphate, which readily kills motile dinoflagellate cells at concentrations of 1 mg l^{-1} (Taylor, 1987), had only minor effects on dinoflagellate cysts (68% germination at concentrations as high as 200 mg l^{-1}). The commercial microbiocide Kathon WT 1.5% (recommended dosage to kill slime-forming and sulfate-reducing bacteria in cooling water towers, 67–332 ppm) had no effect at concentrations as high as 10 000 ppm.

Free chlorine treatment readily caused a bleaching of the brown cyst wall of *G. catenatum*, but successful germination (10%) was still possible with concentrations up to 100 ppm. However, no germination was observed at concentrations above 500 ppm (Table I). Hydrogen peroxide was also effective at killing dinoflagellate cysts but only at concentrations of 10 000 ppm or above (Table II).

TABLE I

Effect of different concentrations (ppm) of free chlorine on germination of *G. catenatum* cysts (as % of total number of cysts used). Average of two experiments.

Concentration	Germinated/total		Germination (%)
2000	0/18	0/25	0
1000	0/21	0/32	0
500	1/15	0/23	3
100	2/37	5/32	10
50	36/66	10/34	46
10	9/11	30/31	93
0	18/20	18/20	90

TABLE II

Effect of different concentrations (ppm) of hydrogen peroxide on germination of *G. catenatum* cysts (as % of total number of cysts used). Average of two experiments.

Concentration	Germinated/total		Germination (%)
60 000	0/18	0/31	0
30 000	0/17	0/26	0
10 000	0/26	0/42	0
5 000	1/34	0/27	2
2 500	3/11	6/25	25
1 000	23/21	4/32	68
100	21/23	21/24	89
0	14/15	17/20	89

Heat treatment

The first set of simple heating experiments showed that germination was unaffected by exposure to temperatures up to 35°C. Germination of *G. catenatum* cysts was reduced to 8% at 40°C, but no germination was observed after heating to 45°C or higher (Table III). This lethal temperature range was confirmed by similar incubations (5–10 min) of mixed dinoflagellate cyst assemblages (*Gonyaulax grindleyi*, *Gonyaulax spinifera*, *Protoperidinium* spp., *Scrippsiella* sp.) contained in natural marine sediments (Derwent River, Tasmania) as well as ships' ballast water tank sediments containing *Alexandrium tamarense* cysts (see Bolch and Hallegraeff, 1990; Hallegraeff and Bolch, 1991 for details of these materials). No cyst germination was observed after exposure to temperatures of 45°C or higher.

TABLE III
Effect of temperature on germination of *G. catenatum* cysts (as % of total number of cysts used). Average of two experiments.

Temperature	Germinated/total		Germination (%)
20°C	10/12	18/20	88
30°C	12/12	17/17	100
35°C	12/12	20/21	97
40°C	2/10	0/16	8
45°C	0/11	0/28	0
50°C	0/17	0/31	0
60°C	0/17	0/19	0

The second set of heating experiments (Figure 1) established critical treatment times at the different target temperatures. Exposure of dinoflagellate cysts for 150 s to temperatures of 36.0 to 38.1°C reduced germination to 65–75%. An effective inactivation of *G. catenatum* cysts could be achieved by exposures ranging from 120 s at temperatures of 38 to 40°C, to 30 s at temperatures of 44.5–46.3°C.

4. DISCUSSION AND CONCLUSIONS

The present work has confirmed the very high chemical resistance of dinoflagellate resting cysts compared to the more fragile motile plankton cells. The microbiocide Kathon WT 1.5% (Krzeminski *et al.*, 1975) was completely ineffective even at 30 times the recommended dose rate, most likely because of the limited permeability of the cyst wall to chemicals. However, effective treatment of dinoflagellate cysts could be achieved with high concentrations of free chlorine or hydrogen peroxide. Chlorine-based chemicals, which rely on the biocidal action of hypochlorous acid, are commonly used to reduce the level of bacteria, viruses, algae and fungi in domestic water supplies, swimming pools, sewage effluent and industrial water systems. Free chlorine levels between 0.2 and 1.0 ppm are sufficient to control phytoplankton growth, higher levels between 5 and 10 ppm are recommended for ice-water baths in the food industry and up to 40 ppm for seawater–ice baths in fish processing plants (Gardner, 1986). Williams *et al.* (1982) required free chlorine concentrations (as sodium -or calcium hypochlorite) of 20 ppm over 24 h to kill small shrimp and larval fish in ballast water samples. In the present work, free

chlorine levels as high as 500 ppm were necessary to kill *G. catenatum* dinoflagellate cysts. This would require 400 t of an industrial solution of 12.5% sodium hypochlorite to treat a 50 000 t ballast water tank at a cost of A$200 000 (Rigby *et al.*, 1992). Apart from being prohibitively expensive (except when produced from seawater by a ship-board generator) and causing environmental problems at the port of discharge, the high sediment load of ships' ballast tanks would considerably reduce available free chlorine levels. Hydrogen peroxide would constitute a more environmentally friendly treatment since this chemical would eventually degrade to water and oxygen. Similarly, Ichikawa *et al.* (1992) recommended the application of 100 mg l^{-1} hydrogen peroxide solution for 96 h to kill toxic *Alexandrium catenella* cysts. The thick-walled cysts of *Polykrikos schwartzii* were more resistant to hydrogen peroxide than *A. catenella*, while the motile cells of *Gymnodinium nagasakiense* (3–6 mg l^{-1} for 15–30 min) and *Chattonella marina* (15 mg l^{-1} for 30 min) were more sensitive. In the present work, much higher levels of hydrogen peroxide up to 5000 ppm (i.e. five hundred times higher) were needed to kill the more resistant, thick-walled *G. catenatum* cysts, but admittedly our treatment period was only 24 h. To treat a 50 000 t ballast water tank thus would require 1000 t of an industrial 50% hydrogen peroxide solution at an indicative cost of A$2 000 000 (Rigby *et al.*, 1992).

The present work indicates that a very short (30–90 s) heat treatment of dinoflagellate cysts at temperatures as low as 40–45°C may provide an effective, environmentally friendly solution to the global ballast water problem. The precise physiological mechanism underlying the effectiveness of this comparatively mild heat treatment is not yet known, and dinoflagellate cyst species other than *Gymnodinium catenatum* also need to be examined in more detail. Experiments by Ontario Hydro, Canada, have indicated that elevating the water temperature for 2–6 h to 36–38°C was also sufficient to kill zebra mussels in an infested pipeline (F. Spencer, unpublished data). Collaborative research with shipping engineers is now underway to develop a ship engineering design in which heat generated by the ship's engines is piped through heating coils in the various ballast water tanks to achieve the above temperature conditions. Preliminary calculations indicate that to raise the temperature of 45 000 t of ballast water from 20° to 40°C over a period of 24 h would require additional heat generation power of 45 MW after allowing for the utilisation of 20 MW of waste heat from the ship's main engine. This requirement is more than twice the power of the main engine on such size of bulk carrier (Gutteridge Haskins & Davey consulting engineers, Melbourne, Australia, personal communication). Simultaneous heat treatment of the entire volume of ballast water is therefore not considered to be a practicable option, but heat may need to be applied sequentially to the individual ballast tanks and/or applied on a smaller localised scale. One possibility would be to pump ballast water, during both loading and unloading, through an on-deck heat exchanger and a smaller well-insulated tank. Such system could be designed to require only about 5–10% of the heat energy necessary for direct heating of all of the ballast water (Dr. D. A. Vaccari, Stevens Institute of Technology, New Jersey, USA, personal communication). Heat treatment would also be the preferred option to treat ballast water after it has been pumped into onshore holding tanks.

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