FACTORS AFFECTING THE TOXICITY OF THE DINOFLAGELLATE *GAMBIERDISCUS TOXICUS* AND THE DEVELOPMENT OF CIGUATERA OUTBREAKS

FIRDTF PROJECT

86/10 FINAL REPORT

MICHAEL J. HOLMES, RICHARD J. LEWIS & NOEL C. GILLESPIE

QUEENSLAND DEPARTMENT OF PRIMARY INDUSTRIES
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1. SUMMARY

We have confirmed that the benthic dinoflagellate *Gambierdiscus toxicus* is an origin of putative ciguatoxin precursors called gambiertoxins. It was recently found in French Polynesia that gambiertoxins could be extracted from biodetritus containing wild *G. toxicus* and that gambiertoxins were structural analogs of ciguatoxin. Ciguatoxin is the toxin found in the flesh of ciguateric fishes. We have demonstrated for the first time production of gambiertoxins in strains of *G. toxicus* grown in culture. The production of gambiertoxins appears to be limited to certain genetic strains of *G. toxicus*, with the majority of strains not producing these toxins. We propose that ciguatera occurs when blooms of *G. toxicus* strains genetically capable of producing ciguatoxin precursors (gambiertoxins) enter the marine food chain. The type of maitotoxin produced by *G. toxicus* was also shown to be a stable characteristic within culture strains, but variable between strains.

Disturbance to coral reef environments has been suggested as a factor in promotion of ciguatera outbreaks. We have been unable to find direct evidence to support this hypothesis. Translocation of ciguateric strains of *G. toxicus* (gambiertoxin producers) may explain the sudden appearance of ciguatera in areas previously free of the disease. The ability of living *G. toxicus* cells to be transported, either in ship ballast water or epiphytically upon the hulls of ships, has yet to be demonstrated. *G. toxicus* can survive rafting upon drift algae. Translocation by ships may be a mechanism for introduction of the causative organism to sites where reef damage occurs (eg. harbour works, anchorages and shipwrecks).

G. toxicus is a common component of reef benthos In Queensland. We have shown that *G. toxicus* populations in Queensland generally do not produce ciguatoxin or ciguatoxin precursors. In addition, we have demonstrated that maitotoxin or non-toxic precursors in wild *G. toxicus* populations are not bioconverted to ciguatoxin in fishes. The majority of *G. toxicus* populations in Queensland are therefore probably not involved in ciguatera. This may account for the patchy temporal and spatial distribution of ciguatera, even though *G. toxicus* is ubiquitous on all coral reefs so far examined along the Queensland coast.

Platypus Bay is a major source of ciguateric fishes in Queensland. *G. toxicus* populations which produce gambiertoxins have been found in Platypus Bay biodetritus. A monoclonal strain of *G. toxicus* which produces gambiertoxins in laboratory culture has also been isolated from Platypus Bay. This indicates that Platypus Bay is probably the source of toxin in ciguateric fishes caught in Platypus Bay. However, gambiertoxins were not detected in the majority of biodetrital samples collected from Platypus Bay. Gambiertoxins therefore appear to be only infrequently pulsed into Platypus Bay.

Benthic dinoflagellates other than *G. toxicus* have been suggested as potentially capable of producing toxins associated with ciguatera. Ciguatoxin or gambiertoxins were not produced by cultures of the benthic dinoflagellates *Ostreopsis siamensis* and *Coolia monotis* isolated from Queensland. These species produced water soluble and lipid soluble toxins, respectively. However, there is no evidence for the bioaccumulation of these toxins in the flesh of fishes.

The most important aspect of future research into the origin of ciguatera is probably an examination of the bioaccumulation/bioconversion of gambiertoxins in fishes. This would involve feeding fishes strains of *G. toxicus* which produce gambiertoxins. The QDPI is well placed to carry out this research as it has the only known strains of gambiertoxin-producing *G. toxicus* in culture in the world.

2. INTRODUCTION

Ciguatera is a disease caused by eating toxic individuals of many species of normally edible fishes from tropical and subtropical waters. In Queensland, 617 cases of ciguatera were recorded in the period from 1965 to 1987 with 569 cases being recorded since 1976 (Gillespie *et al.*, 1986; Lewis *et al.*, 1988). However, a large number of cases of ciguatera are not reported to health authorities and the true Incidence of clguatera Is almost certainly greater than these figures would indicate (Gillespie *et al.*, 1986). Ciguatera has a major detrimental impact on the fishing industry by reducing consumer confidence in

seafood. There is also the prospect of successful litigation by victims against seafood retailers and wholesalers.

The principle toxin that accumulates in the flesh of fishes to cause ciguatera is called ciguatoxin (Scheuer *et al.*, 1967). Other minor toxins such as scaritoxin (Chungue *et al.*, 1977), palytoxin (Kodama *et al.*, 1989) ciguaterin (Hashimoto *et al.*, 1969) and maitotoxin (Yasumoto *et al.*, 1976) have also been found in ciguateric fishes. However, ciguatoxin is the only toxin found to-date in the flesh of ciguateric Queensland fishes (Lewis and Endean, 1983, 1984; Lewis, 1985).

Randall (1958) suggested that the toxins involved in ciguatera probably originate from a small benthic organism and that these toxins are transmitted through the food chain from herbivorous to carnivorous fishes. Circumstantial evidence from French Polynesia indicated that the benthic dinoflagellate *Gambierdiscus toxicus* was the elaborator of ciguatoxin (Yasumoto *et al.*, 1977). Yasumoto *et al.* (1977) and Bagnis *et al.* (1980) collected biodetritus containing large numbers of *G. toxicus,* from which they extracted small amounts of ciguatoxin and large amounts of the water-soluble toxin, maitotoxin. However, when grown in culture, *G. toxicus* produced considerable quantities of maitotoxin, but little if any ciguatoxin (Yasumoto *et al.*, 1979). It is now thought that all attempts to produce ciguatoxin from *G. toxicus* cultures have been unsuccessful (Yasumoto, 1990). There have been many studies reporting the extraction of ciguatoxin-like toxins from *G. toxicus* cultures, but in all cases inadequate techniques were used to differentiate ciguatoxin from maitotoxin (Anderson and Lobel, 1987; this report). Maitotoxin is unlikely to have a direct involvement in ciguatera in Queensland as there is no evidence for its bioaccumulation in the flesh of fishes. Benthic dinoflagellates other than *G. toxicus* have also been suggested as potentially capable of producing toxins associated with ciguatera (Regalis, 1984; Tindall *et al.*, 1984 Carlson and Tindall, 1985; Tindall *et al.*, 1990). However, there is no evidence for the bioaccumulation of these toxins in the flesh of fishes.

G. toxicus is a common but minor component of coral reef benthos in Queensland (Gillespie *et al.*, 1985a). However, in the first attempt to extract toxins from wild *G. toxicus* outside of French Polynesia Gillespie *et al.* (1985b) found maitotoxin but no ciguatoxin in biodetritus containing *G. toxicus* collected from Flinders Reef, south Queensland. Gillespie *et al.* (1985b) therefore proposed that *G. toxicus* may not always produce ciguatoxin but would require a ciguatoxin-inducing-factor to stimulate its production. It was thought that the ciguatoxin-inducing-factor may be environmental conditions associated with reef disturbance or genetic variation between *G. toxicus* populations (Gillespie *et al.*, 1985b; Gillespie, 1987). Preliminary laboratory experiments indicated that *G. toxicus* cultures challenged with coral extracts produced a toxic fraction which induced bioassay signs in mice similar to ciguatoxin. Funds were therefore sought for a project to examine toxin production by *G. toxicus.* It was suggested that if the processes leading to ciguatera outbreaks could be determined it may allow development of management strategies to minimise the impact of ciguatera. In addition, if ciguatoxin could be extracted from *G. toxicus* cultures it could relieve the critically short supply of toxin available for ciguatera research.

3. RESEARCH OBJECTIVES

(1) To define the factors influencing ciguatoxin production by cultures of *G. toxicus* or other benthic dinoflagellates. The effect of phenomena associated with reef disturbance and the significance of genetic heterogeneity with respect to toxin production are to be examined

(2) The requirements for growth and bloom formation by *G. toxicus* and other benthic dinoflagellates are to be examined.

The major aim of this project was to determine the origin of ciguatoxin in Queensland. Although originally a three year FIRDC funded project, funds were sought and granted for an additional fourth year of research. During the initial three years of the project we determined that a small number of *G. toxicus* strains produce lipid-soluble toxins distinct from maitotoxin. The fourth (additional) year of funding allowed us to characterise the lipid-soluble toxins and determine that they were ciguatoxin-related toxins which we now call gambiertoxins. Unfortunately, this left insufficient time and resources to complete a major part of the second objective, examining requirements for growth and bloom formation of

gambiertoxin producing strains of *G. toxicus*. However, growth in culture of non-gambiertoxin producing strains of *G. toxicus* and *Ostreopsis siamensis* are reported in the appendices 10.1 and 10.3. A copy of the original grant application is attached (appendix 10.6).

4. METHODS

To achieve the project objectives we examined toxin production in strains of *G. toxicus, O. siamensis* and *Coolia monotis* grown in laboratory culture. A smaller field component was also undertaken examining the toxicity of fishes from Flinders Reef (26°59´S, 153°29´E) and wild *G. toxicus* from Platypus Bay (24°58´S, 153°10´E), south Queensland.

4.1 Culture of benthic dinoflagellates and extraction of toxins.

Cultures of the benthic dinoflagellates *G. toxicus*, *O. siamensis* and *C. monotis* were initiated from living cells micropipetted from biodetrital fractions collected from along the Queensland coast. In addition to nine Queensland strains of *G. toxicus*, one French Polynesian, one Virgin Island and two Hawaiian strains of *G. toxicus* were examined. Details of culture conditions and extraction of toxins for *G. toxicus* and *O. siamensis* cultures are presented in appendices 10.1, 10.2 and 10.3.

A non-axenic culture of *C. monotis* was initiated from biodetritus collected in Platypus Bay. *C. monotis* cultures were grown in f_{10} nutrient media modified as per appendix 10.3. All other culture conditions and the initial toxin extraction and liquid:liquid phase separations were as described in appendix 10.1.

4.2 Simulation of reef disturbance factors (coral damage) on toxin production by *Gambierdiscus toxicus*.

G. toxicus cultures were exposed to coral extracts to simulate the effect of reef disturbance on non-ciguateric populations of *G. toxicus*. Coral extracts were added to exponential and stationary growth phase cultures of a non-clguatoxin producing strain of *G. toxicus* (NQ1, see appendix 10.1). Coral extracts were prepared from the tips of staghorn coral (*Acropora* sp.) collected from Flinders Reef. Coral tips were ground in a mortar and pestle and centrifuged at 3000 RPM for 5 minutes. The mucous supernatant was decanted off and added to culture flasks to make a 0.1% (v/v) concentration. *G. toxicus* cultures were harvested and extracted for toxins after one week exposure to the coral extracts.

4.3 Field research

4.3.1 Flinders Reef

The toxicity of four herbivorous and four carnivorous fish species from Flinders Reef were examined. The collection of fishes and extraction of toxins were as described in appendix 10.4.

4.3.2 Platypus Bay

G. toxicus populations were sampled in Platypus Bay between May 1988 and February 1990. Macroalgal (*Cladophora* sp.) samples were collected by scuba diving and the epiphytes sieved into four size fractions. The 45 µm - 250 µm size fractions were examined for *G. toxicus* and assayed for toxins using the methods described in appendix 10.2.

5. RESULTS AND DISCUSSION

5.1 Ciguatoxin and maitotoxin

Detection of any ciguatoxin that may be produced by *G. toxicus* is difficult because of the large amount of maitotoxin that is produced. The initial detection and characterisation of ciguatoxin and maitotoxin is by mouse bioassay of fractions separated by liquid:liquid phase partitions into hexane, diethyl ether, butanol and water fractions. Any ciguatoxin that is produced partitions into the diethyl ether phase. However, large amounts of maitotoxin also partition into the diethyl ether phase. Many previous investigations claiming the extraction of ciguatoxin-like toxins probably confused maitotoxin for ciguatoxin because they relied upon this liquid:liquid partition to separate the two toxins.

Preparative silicic acid chromatographic columns can separate ciguatoxin and maitotoxin. Ciguatoxin is less polar than maitotoxin and therefore elutes earlier on silicic acid columns. Comparison of toxin production of cultures of a north Queensland strain of *G. toxicus* with cultures of a French Polynesian strain revealed that both strains produced maitotoxin but no ciguatoxin (appendix 10.1). Different chromatographic types of maitotoxin were extracted from the two strains of *G. toxicus.* The type of maitotoxin produced by each strain appeared to be a stable characteristic of that strain. This would indicate that stable strain-dependant differences in toxin production can occur in *G. toxicus.* A large number of *G. toxicus* strains were therefore screened to determine if ciguatoxin production is also a strain dependant characteristic of this species.

5.2 Ciguatoxin precursors (gambiertoxins) from cultures of *Gambierdiscus toxicus*

Cultures of nine Queensland, one French Polynesian, one Virgin Island and two Hawaiian strains of *G. toxicus* were grown in culture and extracted for toxins (appendix 10.2). Two Queensland strains produced putative precursors of ciguatoxin called gambiertoxins, strain NQ2/7 isolated from Arlington Reef (10°42´S, 146°4´E) near Cairns and strain WC1/1 from Platypus Bay. These gambiertoxins were less polar than ciguatoxin but induced bioassay signs in mice similar to those produced by ciguatoxin. The gambiertoxins were shown to be ciguatoxin-related toxins by their pharmacological activity and competitive displacement of brevetoxin in a Na^{+} channel binding assay (appendix 10.2). This is the first time ciguatoxin-related toxins have been characterised from cultures of *G. toxicus*. Legrand *et al.* (1990) and Murata *et al.* (1990) isolated gambiertoxins from biodetritus containing wild *G. toxicus* and determined that these toxins were structural analogs of ciguatoxin. They suggested that gambiertoxins are precursors of ciguatoxin which are oxidatively metabolised to ciguatoxin in fishes.

Gambiertoxin production appears to be limited to only certain strains of *G. toxicus* with the majority of strains not producing these toxins. Production of gambiertoxins also appears to be a stable characteristic of strains that can produce these toxins. We have therefore proposed that ciguatera occurs when blooms of *G. toxicus* strains genetically capable of producing these ciguatoxin precursors enter the marine food chain.

Ship anchorages, shipwrecks and harbour works have been suggested as focal points in the chain of events leading to ciguatera outbreaks (Randall, 1958; Cooper, 1964; Bagnis, 1969). Cooper (1964) suggested that vessels could have carried and translocated a causative organism. Translocation of *G. toxicus* has not previously been considered a problem in Australia because of the extensive distribution of *G. toxicus* in tropical waters. However, if only certain strains of *G. toxicus* are capable of producing ciguatoxin precursors then the translocation of these strains by ships may be a vector for introduction of ciguatera into tropical regions currently free of ciguatera. *G. toxicus* is capable of surviving rafting upon drift algae (Bomber *et al.*, 1988), but the translocation of *G. toxicus* in ship ballast water or epiphytically upon seaweeds fouling the hulls of ships has not been investigated. Concern has been expressed at the possible introduction into temperate Australian waters of paralytic shellfish

poison dinoflagellates by ship ballast water (Hallegraeff *et al.*, 1990)

5.3 Reef disturbance and ciguatera

We could find no direct evidence to support the hypothesis of a causal relationship between reef disturbance and ciguatera. Gillespie *et al.* (1985b) suggested that reef disturbance may act as ciguatoxin-inducing-factor that triggers ciguatoxin production in non-toxic *G. toxicus* populations. Damage to coral reefs was investigated as a possible mechanism for inducing ciguatoxin production in non-ciguateric strains of *G. toxicus*. Coral extracts were added to cultures of a non-ciguateric strain of *G. toxicus* to simulate reef disturbance. Coral extracts did not induce ciguatoxin production in *G. toxicus* cultures. Approximately 1.4×10^7 exponential and 2.5×10^7 stationary growth phase cells harvested after one week exposure to 0.1% coral extracts produced maitotoxin but no detectable ciguatoxin or gambiertoxin.

Field studies in Queensland have also not supported a causal link between reef disturbance and ciguatera. The dredging of a fringing coral reef at Hayman Island did not lead to increased *G. toxicus* populations (appendix 10.5). Lewis *et al.* (1986) also found little change in *G.toxicus* populations or the concentration of ciguatoxin in surgeonfish on Sudbury Reef after cyclone Winifred in January and February 1986. An anecdotal link between environmental disturbance and ciguatera was observed in the Gove area of the Northern Territory after construction of a alumina plant (appendix 10.5). However, in this case the toxic reefs were probably too distant from the alumina plant to have been affected by pollution from the plant or by construction of the plant.

5.4 Bioconversion

The existence of apparently non-ciguatoxin producing blooms of *G. toxicus* at Flinders Reef (Gillespie *et al.*, 1985b) provided the opportunity to investigate the possible bioconversion in fishes of maitotoxin or non-toxic metabolites of *G. toxicus*. To test this hypothesis we examined the toxicity of pooled livers of four herbivorous and four carnivorous fish species from Flinders Reef. Pooled livers of each species of fish were assayed because these tissues accumulate the highest concentrations of ciguatoxin.

Maitotoxin or non-toxic metabolites of *G. toxicus* were not bioconverted to ciguatoxin in fishes (appendix 10.4). Ciguatoxin was not detected in the eight fish species examined. A number of other toxins were found including some which induced bioassay signs in mice similar to those produced by ciguatoxin. However, these toxins differed from ciguatoxin in that they did not accumulate in the livers of fishes.

5.5 Platypus Bay

The majority of reported cases of ciguatera in Australia have been caused by pelagic fishes caught in or near Platypus Bay (Gillespie *et al.*, 1986). Narrow barred Spanish mackerel (*Scomberomorus commerson*i) and barracuda (*Sphyraena jello*) have been the major species implicated (Gillespie *et al.*, 1986; Lewis, 1987; Lewis *et al.*, 1988). Recently, blotched javelin (*Pomadasys maculatis*) and happy moments (*Siganus spinus*) caught in Platypus Bay have also caused ciguatera (unpublished results). We therefore initiated a study to try and determine the source of ciguatoxin in ciguateric fishes caught in Platypus Bay.

Large areas of Platypus Bay benthos are dominated by a green macroalgae (*Cladophora* sp). *Cladophora* sp samples were collected between May 1988 and February 1990 and examined for benthic dinoflagellates. Large *G. toxicus* populations were found in Platypus Bay (Figure 1). The *G. toxicus* populations in Platypus Bay were the largest found in Queensland apart from those at Flinders Reef (Gillespie *et al.*, 1985a). The size of the *G. toxicus* populations in Platypus Bay varied significantly over the study period with low populations in summer. This result is similar to that found for *G. toxicus* at Flinders Reef (Gillespie *et al.*, 1985a).

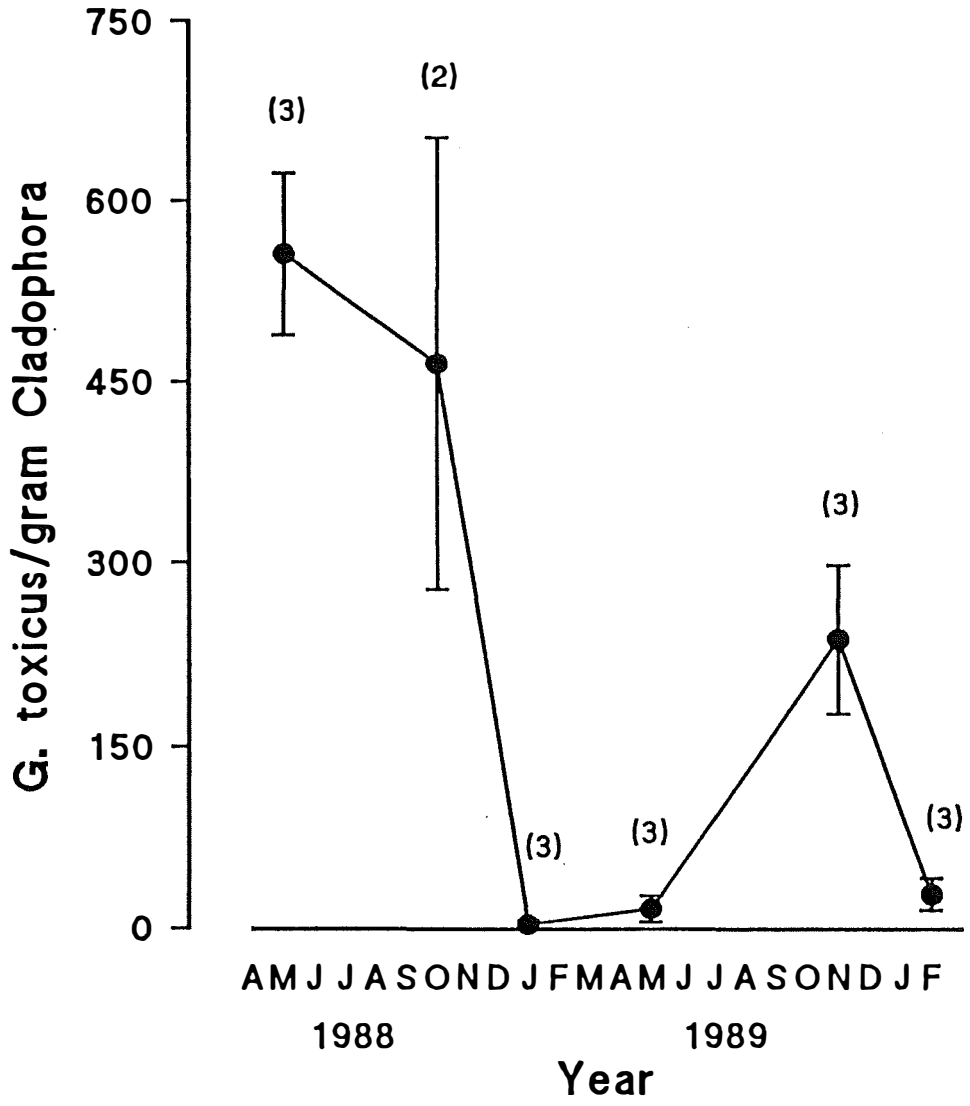

Fig. 1. *Gambierdiscus toxicus* population densities in Platypus Bay. (mean ± SEM, n=2–3 as indicated by numbers in parenthesis). Biodetrital fractions were assayed for toxins for each sample.

Ciguatoxin as found in fishes was not detected in any of the six biodetrital samples examined (Figure 1). Putative gambiertoxins were extracted from a biodetrital sample collected in May 1988 but not from the other five samples. The May 1988 sample also contained the largest *G. toxicus* populations (5.1×10^6 cells extracted). In contrast, no gambiertoxins could be detected from 4.0×10^6 cells extracted in October 1988.

The isolation of gambiertoxins from biodetritus containing wild *G. toxicus* and from a Platypus Bay strain of *G. toxicus* grown in culture (appendix 10.2) indicates that a source of ciguatoxin found in toxic fish could be *G. toxicus* in Platypus Bay. However, the majority of biodetrital samples containing wild *G. toxicus* collected from Platypus Bay did not contain gambiertoxins. The majority of cultured strains of *G. toxicus* isolated from Platypus Bay also did not produce gambiertoxins (appendix 10.2). Ciguatoxin precursors therefore appear to be only infrequently pulsed into Platypus Bay and at present it is not possible to predict the factors initiating these pulses of toxin.

5.6 Toxicity of cultures of *Ostreopsis siamensis* and *Coolia monotis*.

Benthic dinoflagellates other than *G. toxicus* have been suggested as potentially capable of producing toxins associated with ciguatera (Regalis, 1984; Tindall *et al*., 1984; Carlson and Tindall, 1985; Tindall *et al*., 1990). Cultures of *O. siamensis* isolated from Hoffmans Rocks and *C. monotis* isolated from Platypus Bay were assayed for toxins. A small amount of water-soluble toxin, but no ciguatoxin or gambiertoxin, was detected from 3.8×10^7 *O. siamensis* (appendix 10.3). Similarly, a small amount of lipid-soluble toxin, but no ciguatoxin or gambiertoxin, was extracted from 9.1×10^6 *C. monotis*. There is therefore no evidence to suggest that these species contribute to the biogenesis of ciguatera in Queensland.

6. FUTURE RESEARCH

During this project two areas of future research were identified.

1. An examination of the accumulation of ciguatoxin in the flesh of fishes fed on cultures of gambiertoxin-producing strains of *G. toxicus.* We have shown that the gambiertoxins are ciguatoxin-related toxins but the bioaccumulation of these toxins in fishes is still to be demonstrated. This research could also examine excretion of ciguatoxin from fishes.

2. The potential translocation of *G. toxicus* in ship ballast water or epiphytically upon fouling organisms on the hulls of ships should be examined.

7. ACKNOWLEDGEMENTS

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8. PUBLICATIONS

A number of papers have been published or submitted for publication during the period of this project. A paper on toxin production of *G. toxicus* in Platypus Bay is also being prepared. A list of these publications follow.

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APPENDIX 10.1

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TOXICITY OF AUSTRALIAN AND FRENCH POLYNESIAN STRAINS OF *GAMBIERDISCUS TOXICUS* (DINOPHYCEAE) GROWN IN CULTURE: CHARACTERIZATION OF A NEW TYPE OF MAITOTOXIN

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(*Accepted for publication* 22 *March* 1990)

M. J. HOLMES, R. J. LEWIS and N. C. GILLESPIE. Toxicity of Australian and French Polynesian strains of *Gambierdiscus toxicus* (Dinophyceae) grown in culture: characterization of a new type of maitotoxin. *Toxicon* **28,** 1159–1172, 1990.—*Gambierdiscus toxicus* strains isolated from Australia and French Polynesia were grown in modified f_2 and f_{10} nutrient media and the cells extracted for ciguatoxin and maitotoxin. The high nutrient enrichment of f_2 media induced aberrant cell morphology in both strains whereas the majority of cells grown in f_{10} media had the typical lenticular shape of wild *G. toxicus* cells. The Australian strain grew faster and produced greater cell densities than the French Polynesian strain. Different chromatographic types of maitotoxin were extracted from the two *G. toxicus* strains and purified to homogeneity using reverse-phase high-performance liquid chromatography. The toxins elicited similar bioassay signs in mice, but produced different death-time vs dose relationships. The maitotoxin extracted from the Australian strain eluted later on both straight-phase and reverse-phase chromatographic columns than did the maitotoxin extracted from the French Polynesian strain. The maitotoxin extracted from the French Polynesian strain was chromatographically identical to reference maitotoxin. For each strain no differences were found between maitotoxins extracted from cells grown in f_2 or f_{10} media. Only one toxin was produced by each strain of *G. toxicus*. Ciguatoxin was not produced by either strain.

INTRODUCTION

CIGUATOXIN and maitotoxin are believed to originate from the benthic dinoflagellate *Gambierdiscus toxicus* (YASUMOTO *et al.*, 1977*b*). Ciguatoxin is the principal toxin responsible for ciguatera, a circumtropical food poisoning caused by eating toxic individuals of many species of reef fish. Ciguatoxin is a lipid-soluble toxin that is bio-accumulated in the flesh and viscera of fishes via the marine food web (RANDALL, 1958). Maitotoxin was first extracted from the viscera of surgeon fishes (YASUMOTO *et al.*, 1976). Maitotoxin is now characterized as the water-soluble toxin extracted from cultures of *G. toxicus*. There is no evidence for the bio-accumulation of maitotoxin in the flesh of fishes.

Ciguatoxin and maitotoxin have been extracted from samples of reef benthos containing large numbers of *G. toxicus* cells (YASUMOTO *et al.*, 1977*a,b*; BAGNIS *et al.*, 1980). *G. toxicus* cultures produce considerable quantitites of maitotoxin but only meagre amounts, if any, of ciguatoxin (YASUMOTO *et al.*, 1979; BAGNIS *et al.*, 1980). Lipid soluble ciguatoxin-like toxins have been reported in *G. toxicus* cultures, but the lack of assay methods that clearly differentiate ciguatoxin from maitotoxin may have confounded early attempts to separate these two toxins (WITHERS, 1982; MILLER *et al.*, 1984; DURAND *et al.*, 1985; LECHAT *et al.*, 1985).

Two forms of ciguatoxin have been described (NUKINA *et al.*, 1984), but maitotoxin has been assumed to be a single homogeneous compound, the structure of which has been partially determined (YOKOYAMA *et al.*, 1988). Maitotoxin is a putative Ca^{2+} channel agonist (TAKAHASHI *et al.*, 1982; KOBOYASHI *et al.*, 1987; YOSHII *et al.*, 1987), which also stimulates phosphoinositide breakdown in a wide variety of cell types (GUSOVSKY *et al.*, 1989). The source of purified maitotoxin used for the majority of these studies has been cultures of *G. toxicus* isolated from the Gambier Islands, French Polynesia. Maitotoxin from Caribbean strains of *G. toxicus* apparently has similar pharmacological activity (FREEDMAN *et al.*, 1984).

In this paper we compare the growth and toxicity of a strain of *G. toxicus* isolated from Queensland, Australia with a strain isolated from the Gambier Islands, French Polynesia. Using high-performance liquid chromatography (HPLC) techniques we have characterized a new type of maitotoxin from the Australian strain. We name this toxin maitotoxin-2 (MTX-2), to distinguish it from the maitotoxin (MTX-1) extracted from French Polynesian strains of *G. toxicus* which was characterized first.

MATERIALS AND METHODS

Isolation and culture of G. toxicus *strains*

A non-axenic monoclonal *G. toxicus* culture (NQ1) was established from a single cell isolated from turf algae collected from Hastings Reef (16°31′S, 146°1′E) in the central section of the Great Barrier Reef, Australia. A starter culture of a non-axenic *G. toxicus* strain (FP) isolated from the Gambier Islands in French Polynesia was a generous gift from Dr R. BAGNIS. Cultures were maintained at 25°C with 50–60 μEin/m^2/sec of light from Phillips Daylight-54 fluorescent tubes with a 12:12 hour light:dark photoperiod. The cultures were grown in f_2 and f_{10} nutrient media modified as per HOLMES *et al.*(1988). Stock cultures and cultures for growth rate experiments were grown in 250 ml Erlenmeyer flasks containing approximately 100 ml of media. Mass cultures for toxin analyses were grown in 2 litre and 4 litre Erlenmeyer flasks and 1.8 litre Fernbach flasks containing approximately 800 ml, 1 litre and 1 litre of media, respectively. NQ1 and FP mass cultures were grown simultaneously under identical culture conditions to facilitate direct comparisons of the two strains.

Growth rates were calculated from successive counts of 1 ml subsamples (GUILLARD, 1973), of cultures grown in f_{10} media. Mass cultures were harvested in either the late exponential or stationary growth phase, after 3–4 weeks growth. Cell numbers of mass cultures were estimated from 1 ml subsamples. The morphology of cells was examined using a Phillips 505 scanning electron microscope. Cells were fixed in 5% formalin, rinsed with distilled water to remove salt, dehydrated in ethanol and sputter coated with platinum.

Harvest, extraction and preparation of diethyl ether and butanol soluble fractions

Cells from mass cultures were filtered on Whatman GF/A and GF/D glass fibre filters, rinsed with 25 ml of distilled water to remove salt and then stored in methanol at −20°C until extracted. Cells were disrupted in methanol using an ultrasonic homogenizer until microscopic observation revealed no remaining intact cells. The methanol extracts were separated from cell residues by centrifugation. Cell residues were extracted a minimum of four times with methanol and the methanol extracts pooled, filtered through Whatman GF/A glass fibre filters and the filters rinsed with methanol. The pooled extracts were dried *in vacuo*, redissolved in 250 ml of 9:1 methanol:water and extracted as outlined in Fig. 1. Samples of filtered culture media and distilled water rinses were also extracted and tested for toxicity.

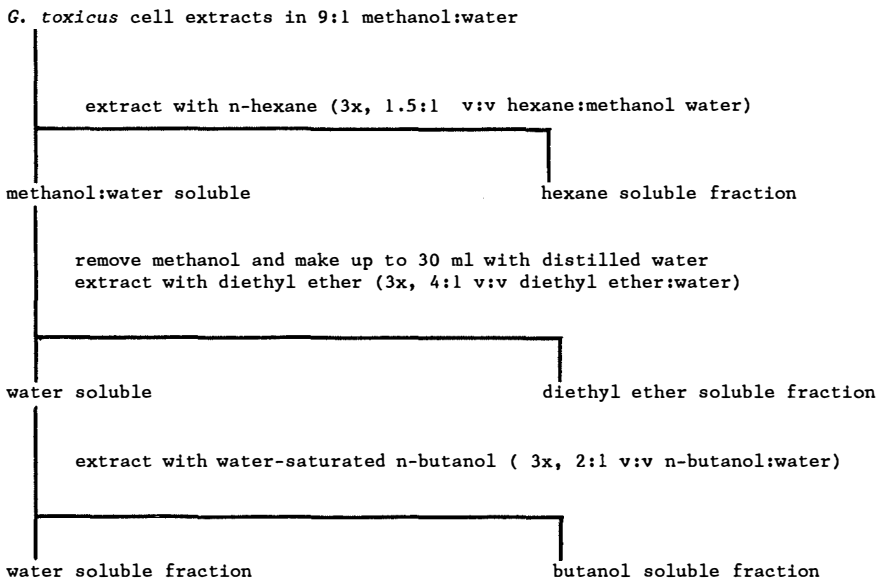

FIG. 1. PREPARATION OF HEXANE, DIETHYL ETHER, BUTANOL AND WATER-SOLUBLE FRACTIONS FROM *G. toxicus* CELL EXTRACTS.

Purification of maitotoxins, MTX-1 and MTX-2

The toxins extracted from diethyl ether and butanol soluble fractions of NQ1 cultures grown in f_2 media and diethyl ether soluble fractions from NQ1 cultures grown in f_{10} media were purified to homogeneity using reverse-phase HPLC. Similarly, diethyl ether and butanol soluble fractions extracted from FP cultures grown in f_2 media and butanol soluble fractions from FP cultures grown in f_{10} media were purified to homogeneity using reverse-phase HPLC.

Initial purification of diethyl ether and butanol soluble fractions was by preparative silicic acid chromatography. Heat activated (120°C, 1 hr) silicic acid columns (Bio-sil A,200-400 mesh, Bio-Rad) were slurry packed in chloroform and washed with 3 bed volumes (Vb) of chloroform. Column fractions were collected from a step-wise solvent gradient of increasing polarity (see Fig. 2) and the fractions bioassayed in mice after solvent removal. (Columns were also run omitting the 1:1 chloroform:methanol fraction). Columns were packed with silicic acid weights greater than 270 and 750 times FP and NQ1 applied sample weights, respectively. Maitotoxin overloaded smaller sized columns. We have observed that if the chloroform fraction moved a green band more than half the length of the column then the column was probably overloaded and maitotoxin would elute in earlier fractions.

The 1:1 chloroform:methanol and methanol fractions from silicic acid columns were pooled and the toxins further purified as shown in Fig. 2. Purified maitotoxins were also characterized on a Resolve silica HPLC column (Waters Associates, 86016, 150 × 3.9 mm, 5 μm) using acetonitrile:water eluents at 1.0 ml/min. The Fractogel (Merck, 14983), PRP-1 (Hamilton, 79444) and Resolve HPLC columns were run isocratically using a Waters Associates M6000A pump and 481 u.v. detector. Eluates were monitored at 230 nm. Toxic zones were located and quantified by lethality to mice as described below.

The capacity factors (k') of the toxins were determined from the PRP-1 and Resolve columns as; $k' = (Tr - Tm)/Tm$, where Tm = time to non retained peak (1.3 min for PRP-1 column) and Tr = solute retention time (POOLE and SCHUETTE, 1984).

Thin-layer chromatography

Silica gel 60 precoated plates (Merck 5553) were activated for 1 hr at 120°C and used for analytical separations. The solvent system pentanol:pyridine:water (4:4:1) was used (YOKOYAMA *et al.*, 1988), and toxins were detected by immediate bioassay. Large losses of toxicity occurred if the plates were dried before being assayed.

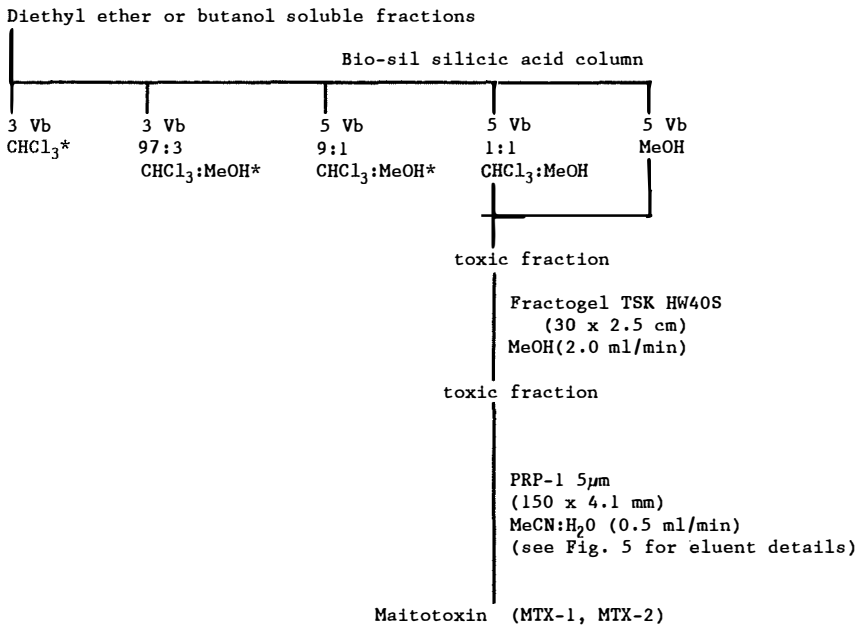

FIG. 2. PURIFICATION OF MAITOTOXINS (MTX-1 AND MTX-2) FROM DIETHYL ETHER AND BUTANOL SOLUBLE FRACTIONS.
Vb, bed volume of column; MeOH, methanol; MeCN, acetonitrile; *, non-toxic to mice at 1 g/kg (fraction weight/mouse body weight).

Mouse bioassay

Fractions to be tested were dried under vacuum and finally freed of solvent under a stream of N_2, resuspended in 0.5 ml of 1% Tween 60 saline and injected i.p. into 18–21 g Quackenbush strain mice at a maximum dose of 1 g of dried fraction weight per kg mouse body weight. Mice were observed over 24 hr and signs and death-times recorded. Rectal body temperatures were measured with a Comark electronic thermometer. Total lethality is expressed in mouse units (MU). One MU is defined as the LD_{50} dose for a 20 g mouse over 24 hr. Fractions were considered non-toxic if injection of a maximal dose was not lethal. For toxic fractions, one mouse was injected per dose and one or two doses of between 3 and 20 MU were injected per fraction.

Death-time vs dose curves were determined for toxins from butanol soluble fractions using a similar method to that used by LEWIS and ENDEAN (1984). The equations derived from these curves were used to quantify the recovery of toxins from each purification step using only one or two mice. The shortest time of survival for mice was calculated using the method of MOLINENGO (1979). Mice were sacrificed in accordance with Australian National Health and Medical Research Council guidelines.

Chemicals used

Reference maitotoxin (from a strain of *G. toxicus* from the Gambier Islands, French Polynesia) was a generous gift from Professor T. YASUMOTO. The solvents methanol, chloroform, isopropanol and acetonitrile (Waters Associates, Lane Cove, Australia) and *n*-hexane (Mallinkrodt, U.S.A.) were HPLC grade. Diethyl ether, *n*-butanol, pyridine and pentanol were A.R. grade. HPLC water was distilled, deionized and filtered through a Norganic cartridge (Millipore, Bedford, U.S.A.). Ciguatoxin was extracted from the viscera of moray-eels collected from the Republic of Kiribati in the central Pacific Ocean. Ciguatoxin was purified by one of us (RJL), using a modification of the method of LEWIS and ENDEAN (1984).

Statistics

Unless otherwise indicated values are expressed as means $\pm$ sample standard deviations. Growth rates and cell densities were compared using Students *t*-tests. Linear regressions and comparison of regression coefficients were as described by SOKAL and ROHLF (1981).

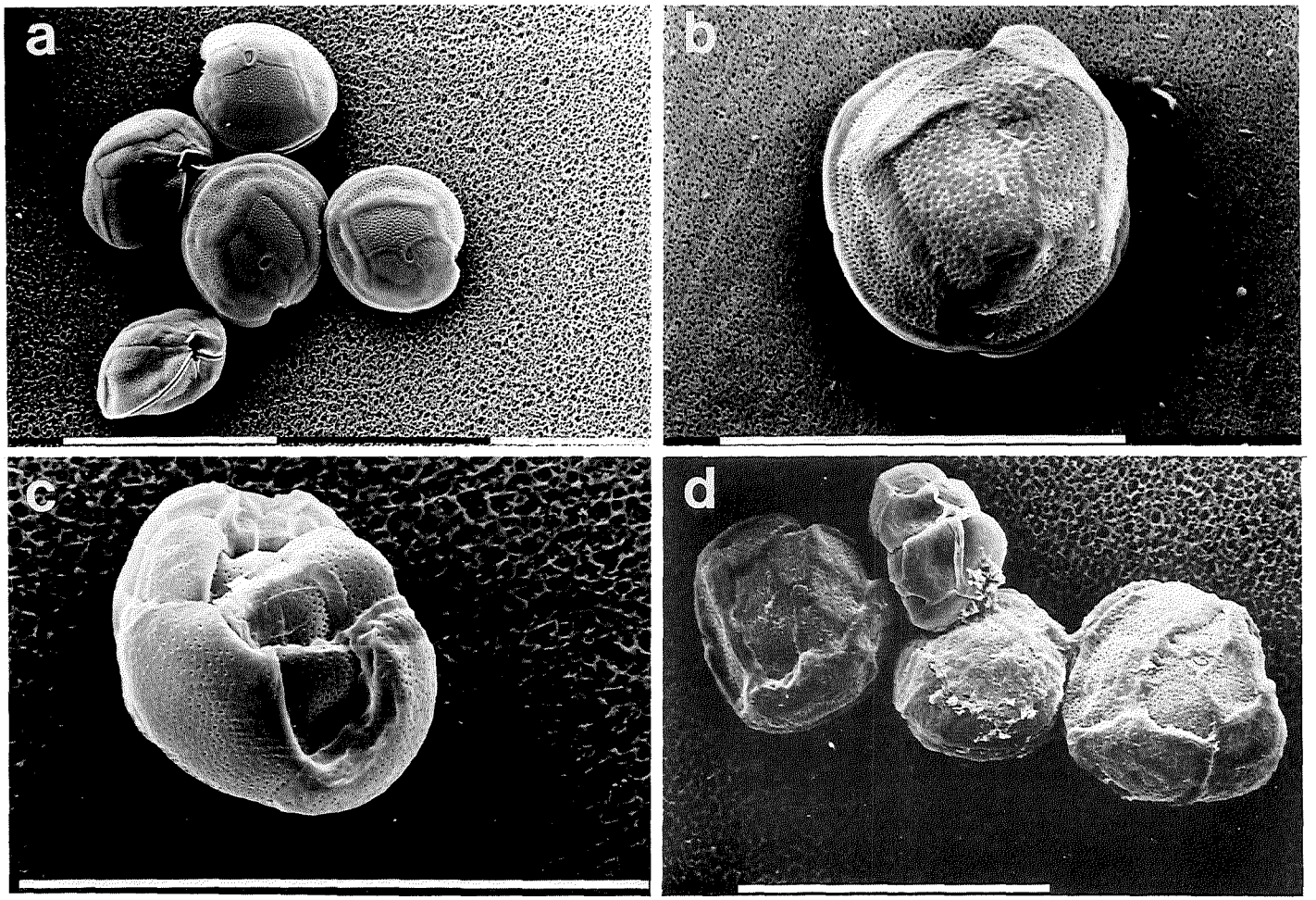

FIG. 3. SCANNING ELECTRON MICROGRAPHS OF FP AND NQ1 *G. toxicus* STRAIN CELLS, BAR = 100 μm.
(a) NQ1 cells grown in f_{10} media. (b) FP cell grown in f_{10} media. (c) NQ1 cell grown in f_2 media. (d) FP cells grown in f_2 media.

RESULTS

Growth and morphology of FP and NQ1 strains of G. toxicus

At least 90% of NQ1 cells grown in f_{10} media had a shape typical of wild *G. toxicus* (Fig. 3a). The shape of the majority of FP cells grown in f_{10} media were also similar to wild cell types but cell shape was less consistent in this strain. Many FP cells retained slight thecal abnormalities when grown in f_{10} media (Fig. 3b). When grown in f_2 media NQ1 and FP strains had a 'lumpy', irregular shape (Fig. 3c,d). Cultures of these teratogenous cells were maintained in culture for 3 and 4 years for FP and NQ1 strains, respectively. Additional sutures were common in epithecal and hypothecal plates of both NQ1 and FP cells. NQ1 cells were a lighter brown colour than FP cells.

NQ1 cultures grew significantly faster than FP cultures ($P < 0.01$). Growth rates of NQ1 and FP cultures grown in f_{10} media were 0.25 ± 0.04 ($n = 5$) and 0.18 ± 0.02 ($n = 5$) divisions per day, respectively. These growth rates were within previously reported ranges but were considerably slower than the maximum growth rates of 0.5–0.6 divisions per day found by CARLSON *et al.* (1984) and BOMBER *et al.* (1988). NQ1 cultures also produced significantly larger maximum cell densities than FP cultures ($P < 0.001$). Maximum cell densities of stationary phase NQ1 and FP cultures were $1{,}480 \pm 139$ ($n = 5$) and 597 ± 223 ($n = 5$) cells per ml, respectively.

Toxicity of FP and NQ1 mass cultures

There was no difference in the toxicity of FP cells grown in f_2 or f_{10} media but NQ1 cells grown in f_{10} media were 1.6 times more toxic than NQ1 cells grown in f_2 media (Table 1). Distilled water rinses of harvested NQ1 cells contained small amounts of maitotoxin (< 0.04% of total toxin). Filtered culture media from NQ1 and FP cultures contained between 0 and 29 MU of maitotoxin per litre of media (< 0.4% of total toxin) indicating that maitotoxin is not excreted extracellularly by *G. toxicus*.

Liquid–liquid partitioning of the methanol:water extracts (Fig. 1), distributed maitotoxin primarily into the diethyl ether and butanol soluble phases. Diethyl ether phases of NQ1 cultures grown in f_2 and f_{10} media contained 65% ($n = 1$) and 88% ($n = 1$) of total maitotoxin, respectively. Similarly, diethyl ether phases of FP cultures grown in f_2 and f_{10} media contained 96% ($n = 1$) and 71% ($n = 1$) of total maitotoxin, respectively. Hexane and water soluble phases contained small amounts of maitotoxin, hexane phases contained less than 0.06% ($n = 4$) of total toxin and water phases contained less than 0.16% ($n = 4$) of total toxin.

The chloroform, 97:3 chloroform:methanol and 9:1 chloroform:methanol fractions from silicic acid columns were non-toxic to mice. The absence of toxin in the 9:1

TABLE 1. TOXIN CONTENT OF NQ1 AND FP *Gambierdiscus toxicus* STRAINS GROWN IN f_2 AND f_{10} NUTRIENT MEDIA

G. toxicus strain	Media	Cell number extracted	Total lethality* (MU)†	Cells/MU
NQ1	f_2	2.83×10^8	1.20×10^6	236
NQ1	f_{10}	4.38×10^7	3.01×10^5	146
FP	f_2	4.62×10^7	1.80×10^5	257
FP	f_{10}	5.74×10^6	2.23×10^4	257

*Bioassay signs consistent with maitotoxin

†MU = mouse unit, one MU = LD_{50} for a 20 g mouse over 24 hr.

TABLE 2. YIELDS OF 9:1 CHLOROFORM:METHANOL FRACTIONS ELUTED FROM SILICIC ACID COLUMNS AND THE LIMITS FOR DETECTION OF CIGUATOXIN FROM THESE FRACTIONS

G. toxicus strain	Media	Weight of 9:1 chloroform:methanol fractions (mg)	Limit for detection of ciguatoxin from 9:1 chloroform:methanol fractions*	
			Ciguatoxin (MU)†	Ciguatoxin per *G. toxicus* cell (MU/cell)
NQ1	f_2	527.1	<13	$<4.6\times10^{-8}$
NQ1	f_{10}	45.0	<1	$<2.3\times10^{-8}$
FP	f_2	178.3	<5	$<1.1\times10^{-7}$
FP	f_{10}	53.9	<1	$<1.7\times10^{-7}$

*The detection limit assumes 0.5 MU can be detected from bioassay signs displayed by 20 g mice injected with maximal doses of 1 g fraction weight per kg mouse body weight.

†MU = mouse unit, one MU = LD_{50} for a 20 g mouse over 24 hr.

chloroform:methanol fraction indicates that ciguatoxin was not produced by FP or NQ1 strains grown in either media (or was produced at concentrations below the detection limit of the assay system). The yields from the 9:1 chloroform:methanol fractions and the limits for detection of ciguatoxin from these fractions are presented in Table 2. Maitotoxin eluted in the 1:1 chloroform:methanol and methanol fractions from silicic acid columns. Only maitotoxin-like signs were observed in mice that had been injected with extracts from FP or NQ1 cultures. No ciguatoxin-like signs were observed from our *G. toxicus* cell extracts.

Purification of maitotoxins, MTX-1 and MTX-2

Maitotoxin from the Queensland strain of *G. toxicus* eluted as a more polar compound than maitotoxin from the Gambier Island strain on straight-phase chromatographic columns, but as a less polar compound on reverse-phase columns. This clear difference in chromatography necessitated the two toxins being named maitotoxin-1 (MTX-1, Gambier Island maitotoxin, from FP cultures) and maitotoxin-2 (MTX-2, Queensland maitotoxin, from NQ1 cultures).

The majority of MTX-1 was recovered from silicic acid columns in the 1:1 chloroform: methanol fraction (83% $\pm$ 10%, $n = 3$), whereas the majority of MTX-2 was recovered in the methanol fraction (60% $\pm$ 21%, $n = 3$). MTX-2 eluted reproducibly from the Fractogel column without loss of toxicity as a single toxic band between 0.7 and 1.1 Vb. MTX-1 was also recovered from the Fractogel column without loss of toxicity, but the volume of methanol required to elute it was inconsistent. MTX-1 generally eluted as a single toxic band after more than 1.8 Vb of methanol.

MTX-1, MTX-2 and moray-eel ciguatoxin were resolved by the PRP-1 reverse-phase HPLC column as discrete and homogeneous u.v. absorbing peaks, corresponding to single toxic bands (Fig. 4a–c). Pooled fractions collected from both sides of toxic peaks were non-toxic. No differences were found on the PRP-1 column between MTX-1 fractions collected from different runs of the Fractogel column. MTX-1 was recovered without loss or toxicity from the PRP-1 column but 30% of MTX-2 toxicity was lost. Acetonitrile and isopropanol washes of the PRP-1 column were non-toxic indicating that MTX-2 toxicity was lost and not separated into different toxins. Small decreases in solvent strength produced exponential increases in the retention time of MTX-1, MTX-2 and ciguatoxin on the PRP-1 column (Fig. 5). MTX-1 had the steepest response to mobile phase changes, ciguatoxin the least. The relationships between k' and mobile phase strength shown in Fig. 5 were approximated by the linear regressions;

$$\text{MTX-1; } \ln k' = 12.56 - 0.30\text{X} \quad (P < 0.05) \tag{1}$$

$$\text{MTX-2; } \ln k' = 9.02 - 0.16\text{X} \quad (P < 0.01) \tag{2}$$

$$\text{Ciguatoxin; } \ln k' = 5.60 - 0.08\text{X} \quad (P < 0.01) \tag{3}$$

where X = % acetonitrile:water. These regressions were significantly different from each other ($P < 0.01$).

MTX-2 extracted from diethyl ether and butanol soluble fractions from NQ1 cultures grown in f_2 or f_{10} media co-eluted on the PRP-1 column. Similarly, MTX-1 extracted from diethyl ether and butanol soluble fractions from FP cultures grown in f_2 or f_{10} media and reference maitotoxin co-eluted on the PRP-1 column. MTX-2 was the only type of maitotoxin produced by NQ1 cultures and this toxin was less polar than MTX-1 on the

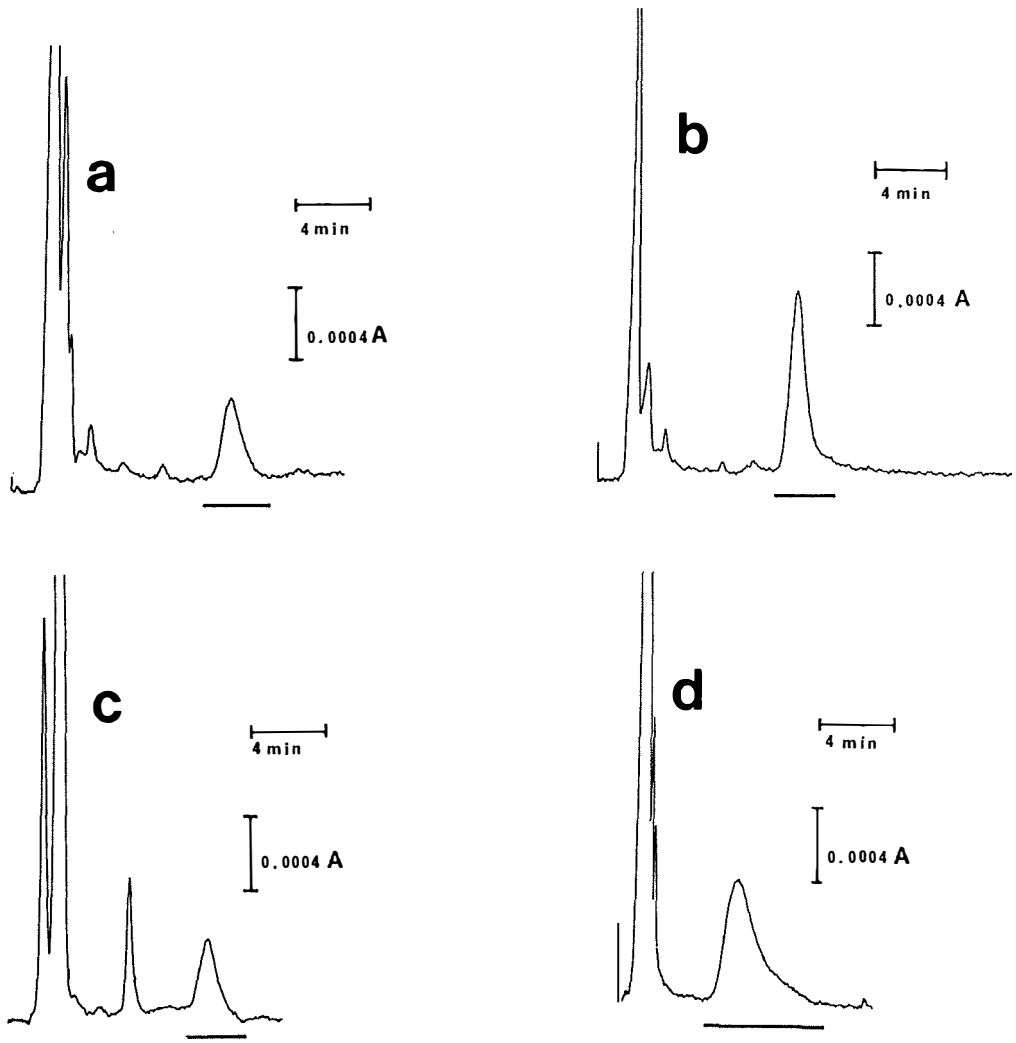

FIG. 4. HPLC OF MTX-1, MTX-2 AND CIGUATOXIN.
MTX-1 and MTX-2 extracted from FP and NQ1 *G. toxicus* strains respectively, ciguatoxin from moray eels. Toxic fractions are indicated by bars and correspond to u.v. absorbing peaks at 230 nm for MTX-1 and MTX-2 and 215nm for ciguatoxin. (a–c) PRP-1 reverse-phase column, 0.5 ml/min. (d) Resolve silica column, 1.0 ml/min.
(a) MTX-1, 14 MU, 35.0% acetonitrile:water. (b) MTX-2, 16 MU, 43.3% acetonitrile:water. (c) ciguatoxin, 5 MU, 46.7% acetonitrile:water. (d) MTX-2, 60 MU, 75.0% acetonitrile:water.

reverse-phase column. MTX-1 was the only type of maitotoxin produced by FP cultures and this toxin was chromatographically identical to reference maitotoxin. Insufficient material was purified to achieve a weight for the toxins for LD_{50} comparisons.

MTX-2 could be eluted as a single toxic peak from the Resolve silica HPLC column (Fig. 4d) using acetonitrile:water mobile phases between 70 and 80% ($1.2 < k' > 26$). MTX-1 eluted between 88% and 93% acetonitrile:water ($1.0 < k' > 26$). MTX-2 was therefore more polar than MTX-1 on straight-phase systems. MTX-1 and MTX-2 were

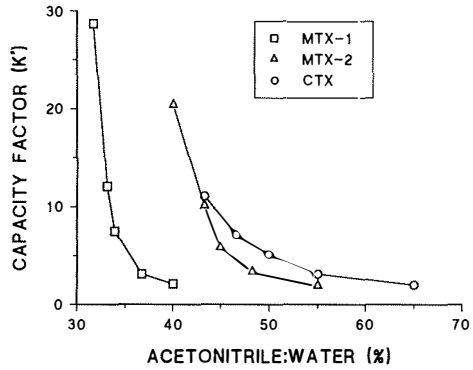

FIG. 5. RELATIONSHIP BETWEEN THE CAPACITY FACTOR (k') AND THE VOLUME FRACTION OF ACETONITRILE IN ACETONITRILE:WATER MOBILE PHASES FOR MTX-1, MTX-2 AND CIGUATOXIN (CTX) ON THE PRP-1 REVERSE-PHASE COLUMN.
$k' = (Tr - Tm)/Tm$; Tm = time to non retained peak (1.3 min); Tr = solute retention time.

recovered without loss of toxicity from the Resolve column. Only one toxin was recovered from each strain after straight-phase HPLC.

Thin-layer chromatography

The *Rf* values of MTX-1 and reference maitotoxin were identical (0.25–0.38), but were lower than the value (0.43) reported previously for maitotoxin extracted from a Gambier Island strain of *G. toxicus* (YOKOYAMA *et al.*, 1988). MTX-2 was more polar than MTX-1 with an *Rf* of (0.0–0.15).

Mouse bioassay

Mice injected i.p. with either MTX-1 or MTX-2 (between 1 and 30 MU) displayed similar signs including piloerection, reduced body temperature, dyspnoea with mostly nasal breathing but occasional gasps, cataracts (not every mouse), progressive paralysis (from hind to fore limbs), slight tremors or convulsions preceding death by at least 30 sec

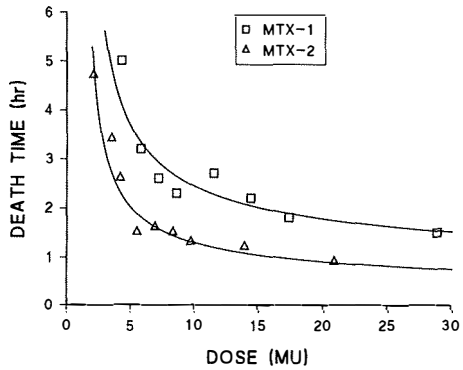

FIG. 6. DEATH-TIME VS DOSE RESPONSE RELATIONSHIPS FOR MICE INJECTED WITH MTX-1 OR MTX-2. One mouse tested at each point.

and long times to death (see Fig. 6). Mice injected with MTX-2 often developed more pronounced gasping and convulsions than mice injected with MTX-1, but these signs were never as pronounced as those displayed by mice injected with ciguatoxin. However, mice injected with excessive doses (> 100 MU of MTX-1 or MTX-2) developed signs including continuous gasping with terminal (hopping) convulsions and short death-times. Signs displayed by mice injected with such high doses may be confused with those produced by ciguatoxin. MTX-1 and reference maitotoxin produced identical bioassay signs in mice.

The death-time vs dose relationships of MTX-1 and MTX-2 (Fig. 6) were approximated by the linear regressions;

$$\text{MTX-1 } \log(\text{dose}) = 6.7 \log(1 + t^{-1}) \ (P < 0.01) \tag{4}$$

$$\text{MTX-2 } \log(\text{dose}) = 4.0 \log(1 + t^{-1}) \ (P < 0.001) \tag{5}$$

where dose = MU and t = time to death in hr.

These regressions were significantly different ($P < 0.01$) and were used to quantify the toxins using the death-times of one or two mice. Bioassay signs displayed by mice injected with either crude or HPLC purified toxins were identical. The shortest times of survival for mice injected with MTX-1 and MTX-2 (from data in Fig. 6) were 72 min and 41 min, respectively.

DISCUSSION

Both the NQ1 and FP strains of *G. toxicus* produced maitotoxin, but no ciguatoxin. Each strain produced chromatographically distinct types of maitotoxin. Culture conditions which induced aberrant cell morphology did not alter the type of maitotoxin each strain produced. Additionally, reference maitotoxin extracted from a Gambier Island strain of *G. toxicus* grown in culture in Japan was identical to MTX-1. The type of maitotoxin produced by each *G. toxicus* strain appears to be a stable characteristic for that strain. Maitotoxin profiles may therefore be of chemotaxonomic value in an analogous way to that suggested for separating *Protogonyaulax* species on the basis of toxin composition (Boyer *et al.*, 1986; Cembella *et al.*, 1987). *G. toxicus* is a common component of the reef benthos along the Great Barrier Reef of Australia (Gillespie *et al.*, 1985*a*). The large north–south cline of the Great Barrier Reef provides an excellent opportunity to investigate further differences in maitotoxin production between geographically distinct *G. toxicus* strains.

The chromatography of MTX-2 indicates that it probably has more polar and non-polar functional groups than MTX-1. MTX-2 is therefore a more amphipathic compound than MTX-1. Yokoyama *et al.* (1988) determined that MTX-1 is a disulphated ester that can be desulphated to form a less toxic compound. The higher polarity of MTX-2 on silica based systems indicates that MTX-2 is unlikely to be a desulphated form of MTX-1. These toxins produce similar bioassay signs in mice and probably belong to a class of toxins that act at the same site to cause an increase of intracellular calcium. This would be analogous to the way the structural analogs of the dinoflagellate toxins saxitoxin and brevetoxin bind specifically to site 1 and site 5 of the sodium channel, respectively (Strichartz, 1986; Baden, 1989). The differences in *Rf* between MTX-1 and maitotoxin purified by Yokoyama *et al.* (1988) probably stem from differences in laboratory conditions and procedures employed for thin-layer chromatography. Different *Rf* values have been reported for ciguatoxin separated in different laboratories using thin-layer chromatography (Chungue *et al.*, 1977; Lewis and Endean, 1984).

NQ1 and FP strains each produced only one form of maitotoxin. Only one water soluble toxin has been reported from Florida (HIGERD *et al.*, 1986) and Gambier Island strains of *G. toxicus* (YASUMOTO *et al.*, 1979; TAKAHASHI *et al.*, 1982; YASUMOTO, 1985; YOKOYAMA *et al.*, 1988). This contrasts with the multiple water-soluble toxins reportedly extracted from Caribbean Sea isolates of *G. toxicus* (MILLER and TINDALL, 1988). The toxins produced by these latter strains were clearly different from MTX-1 and MTX-2. TINDALL and MILLER (1985) and MILLER and TINDALL (1988, 1989) reported purification of maitotoxin using C-18 HPLC columns with isocratic methanol or acetonitrile eluents. These mobile phases would elute MTX-1 and MTX-2 in the non-retained (void) volume. The differences between Pacific Ocean and Caribbean Sea strains of *G. toxicus* may therefore be greater than the differences between Australian and Gambier Island strains.

Diethyl ether–water partitions are used for separating ciguatoxin from more polar impurities present in extracts of ciguateric fish. The majority of MTX-1 and MTX-2 distributed into the diethyl ether phases of diethyl ether–water partitions, therefore these partitions were deemed unsuitable for separating maitotoxin from any potential ciguatoxin in FP or NQ1 *G. toxicus* extracts. YASUMOTO *et al.* (1979) also found that maitotoxin can partition into both diethyl ether and water phases. Bioassay signs in mice can be used to differentiate ciguatoxin from maitotoxin (LEWIS *et al.*, 1988). However, many of the signs displayed by mice injected with ciguatoxin and maitotoxin are similar and care is required to differentiate these toxins. Mice injected with excessive doses of maitotoxin also display additional signs which could be confused with ciguatoxin. However, MTX-1 and MTX-2 produce different death-time vs dose–response curves with considerably longer minimum death-times than those produced by ciguatoxin (see LEWIS and ENDEAN, 1984; NUKINA *et al.*, 1984).

The absence of ciguatoxin from FP and NQ1 cultures of *G. toxicus* is consistent with earlier toxicity studies of *G. toxicus* cultures (YASUMOTO *et al.*, 1979; HIGERD *et al.*, 1986). A possible biosynthetic association between maitotoxin and ciguatoxin has been postulated (WITHERS, 1982). However, maitotoxin or non-toxic metabolites of cultured or wild *G. toxicus* were not bioconverted to ciguatoxin by fishes (SICK *et al.*, 1986; LEWIS *et al.*, 1988). Alternatively, ciguatoxin production may be restricted to certain strains of *G. toxicus* or specific 'ciguatoxin-inducing factors' may be required (GILLESPIE *et al.*, 1985*b*; DURAND-CLEMENT, 1986; GILLESPIE, 1987). The production of different forms of maitotoxin by different strains of *G. toxicus* indicates that strain dependent differences in toxin production can occur in this species.

The atypical cell morphology of FP and NQ1 strains grown in f_2 media appears to be caused by excess nutrient enrichments. This phenomenon has also been observed from cultures of the benthic dinoflagellate *Ostreopsis siamensis* isolated from Queensland, Australia (HOLMES *et al.*, 1988). BAGNIS *et al.* (1980) and DURAND and PUISEUX-DAO (1985) reported that *G. toxicus* cultures isolated from the Gambier Islands contained teratogenous cells. DURAND and PUISEUX-DAO (1985) found these atypical cells occurred in artificial seawater media or when cultures were exposed to excess light. These aberrant cells were larger and produced more toxin per cell than the typical lenticular shaped cells (BAGNIS *et al.*, 1980; DURAND-CLEMENT, 1986). This contrasts with the unchanged toxicity of morphologically aberrant FP strain cells compared to typically shaped cells and the decreased toxicity of aberrantly shaped NQ1 strain cells compared with typically shaped cells. The high toxin content of these typically shaped NQ1 cells makes them an excellent source of maitotoxin for structural studies.

NQ1 and FP strains of *G. toxicus* have different growth characteristics and produce

maitotoxins with different chromatography and bioassay death-time vs dose relationships. Reverse-phase HPLC was found to be a useful tool to characterize maitotoxin production from different strains of *G. toxicus*. The similar bioassay signs in mice indicates that MTX-2 should be considered in the same class of toxins as MTX-1. However, the molecular basis for the biological activity of MTX-2 remains to be determined.

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APPENDIX 10.2

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STRAIN DEPENDENT PRODUCTION OF CIGUATOXIN PRECURSORS (GAMBIERTOXINS) BY *GAMBIERDISCUS TOXICUS* (DINOPHYCEAE) IN CULTURE

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M. J. HOLMES, R. J. LEWIS, M. A. POLI and N. C. GILLESPIE. Strain dependent production of ciguatoxin precursors (gambiertoxins) by *Gambierdiscus toxicus* (Dinophyceae) in culture. *Toxicon* **29**, 761–775, 1991.—Thirteen strains of *Gambierdiscus toxicus* isolated from Queensland (Australia), Hawaii, French Polynesia and the Virgin Islands were mass cultured and extracted for ciguatoxin. A biodetrital sample containing wild *G. toxicus* collected from the Republic of Kiribati was also extracted for ciguatoxin. Ciguatoxin, as characterized from moray eels, was not detected in any of the strains examined. Two Queensland strains and the wild *G. toxicus* produced putative ciguatoxin precursors named gambiertoxins. These gambiertoxins were less polar than ciguatoxin but produced bioassay signs in mice and in-vitro responses in isolated guinea pig atria and vas deferens which were similar (but not identical) to those produced by ciguatoxin. The gambiertoxins from cultured cells were also shown to competitively inhibit the binding of [^{3}H]brevetoxin-3 to rat brain membranes in a dose-dependent manner. The gambiertoxins were more potent than ciguatoxin (on a per mouse unit basis) at stimulating neural elements of guinea pig atria. The two culture strains produced similar amounts of gambiertoxins, even when grown in nutrient media made from different seawater containing different concentrations of nutrients. Changes in nutrient media did not induce the other strains of *G. toxicus* to produce gambiertoxins. The production of these ciguatoxin precursors appears to be limited to only certain genetic strains of *G. toxicus*, with the majority of strains not producing these toxins. We propose that ciguatera occurs when blooms of *G. toxicus* strains genetically capable of producing these ciguatoxin precursors enter the marine food chain. These toxins could then become oxidatively metabolized in fishes to the more polar ciguatoxin. Wild cells produced approximately 100-fold greater quantities of gambiertoxins per cell than did the two culture strains indicating that there is considerable potential for increased production of these ciguatoxin precursors from *G. toxicus* in culture.

INTRODUCTION

CIGUATERA is a form of food poisoning caused by eating toxic individuals of many species of normally edible fishes from tropical and subtropical waters. Ciguatoxin is the name given to the lipid soluble toxin which causes ciguatera. Ciguatera is endemic in

Queensland, Australia with 617 cases being reported between 1965 and 1987 (LEWIS *et al.*, 1988*a*). The majority of these cases were caused by Spanish mackerel (*Scomberomorus commersoni*) caught in Platypus Bay. LEWIS and ENDEAN (1983*a*, *b*; 1984*a*, *b*; 1986) determined that the toxin in the flesh of poisonous Spanish mackerel and barracuda (*Sphyraena jello*) caught in Platypus Bay was chromatographically and pharmacologically comparable to reference ciguatoxin extracted from moray eel viscera. A structure for ciguatoxin extracted from the viscera of moray eels collected from French Polynesia has been reported (MURATA *et al.*, 1989). Ciguatoxin is a cyclic polyether with a structure similar to the brevetoxins (PbTx). Ciguatoxin activates voltage sensitive sodium channels (LEWIS and ENDEAN, 1986) and binds to a specific site on the sodium channel (BIDARD *et al.*, 1984). Binding studies have revealed that ciguatoxin inhibits the binding of [^{3}H]PbTx-3 to rat brain membranes and that ciguatoxin and brevetoxin seem to share a common binding site (LOMBET *et al.*, 1987). A number of less polar forms of ciguatoxin have recently been found in the viscera of moray eels collected from French Polynesia and the Republic of Kiribati (LEGRAND *et al.*, 1989, 1990; LEWIS, unpublished result). LEGRAND *et al.* (1990) suggested that these toxins may be less oxidized forms of ciguatoxin. It is possible that the minor toxin extracted from the viscera of barracuda caught in Platypus Bay (LEWIS and ENDEAN, 1984*a*) was also a less polar form of ciguatoxin.

The benthic dinoflagellate *Gambierdiscus toxicus* Adachi et Fukuyo is the presumed origin of ciguatoxin (YASUMOTO *et al.*, 1977, 1979; BAGNIS *et al.* (1980). YASUMOTO *et al.* (1979) and BAGNIS *et al.* (1980) reported extracting small amounts of ciguatoxin from cultures of *G. toxicus*. However, ciguatoxin production by *G. toxicus* cultures has been apparently unsuccessful (YASUMOTO, 1990; MURATA *et al.*, 1990). In culture, *G. toxicus* produces copious amounts of a water soluble toxin called maitotoxin that may be easily confused with ciguatoxin if detection is by mouse bioassay signs alone (HOLMES *et al.*, 1990). Maitotoxin has no proven direct role in ciguatera. Less oxidized toxic forms of ciguatoxin named gambiertoxins (GTX) have been extracted from biodetritus containing wild *G. toxicus* (MURATA *et al.*, 1989; LEGRAND *et al.*, 1990). However, until ciguatoxin or its precursors are extracted from cultures of *G. toxicus*, the evidence linking *G. toxicus* to the origin of ciguatera remains circumstantial.

G. toxicus is a common but generally minor component of coral reef benthos in Queensland (GILLESPIE *et al.*, 1985*a*). In the only investigation to date of the toxicity of wild *G. toxicus* outside French Polynesia, GILLESPIE *et al.* (1985*b*) found maitotoxin but no ciguatoxin in biodetritus containing *G. toxicus* collected from south Queensland. HOLMES *et al.* (1990) also found maitotoxin but no ciguatoxin in cultures of a strain of *G. toxicus* isolated from north Queensland. In this paper we have examined cultures of *G. toxicus* strains isolated from Queensland, French Polynesia, Hawaii and the Virgin Islands for production of ciguatoxin. In addition, a biodetritus sample containing wild *G. toxicus* was collected from the Republic of Kiribati in the central Pacific Ocean and extracted for ciguatoxin. We describe the extraction of ciguatoxin precursors (GTX) from cultures of particular strains of *G. toxicus*, and also provide the first independent confirmation of these toxins from wild *G. toxicus*. The maitotoxins produced by the *G. toxicus* strains examined in this study are not described and characterization of these toxins will be reported elsewhere.

MATERIALS AND METHODS

Isolation and culture of G. toxicus *strains*

Non-axenic, clonal strains of *G. toxicus* were established from single cells isolated from sites along the coast of Queensland, Australia (Table 1). Strain NQ1 was isolated from Hastings Reef (16°31′S, 146°1′E) in June 1984,

NQ2/7 from Arlington Reef (16°42′S, 146°4′E) in August 1985, HR1 and HR7 from Hoffmans Rocks (24°50′S, 152°28′E)* in July 1986, WC1/1 and WC1/18 from Platypus Bay (24°58′S, 153°10′E) in April 1988, WC3/1 and WC2/1 from Platypus Bay in July 1988 and SQ3/3 from Flinders Reef (26°59′S, 153°29′E) in August 1985. Strain FP (isolated from the Gambier Islands, French Polynesia) was a generous gift from Dr R. BAGNIS. GT178 and GT18/1 are clonal strains isolated from Kahala Bay, Hawaii and were generous gifts from Dr E. MCCAFFREY. A harvested dry pellet of GT350 (from South Sound, Virgin Islands) cells grown in ES medium (MCLACHLAN, 1975) with soil extract (1.5%) was generously provided by Dr D. MILLER. Other cultures were

TABLE 1. YIELDS OF GAMBIERTOXINS (GTX) FROM WILD AND CULTURE STRAINS OF *Gambierdiscus toxicus*

Strain	Origin	Media*	Cells extracted	GTX (MU)†	GTX per cell (MU/cell)	Limit for detection‡ (MU/cell)	
						97:3	9:1
Cultures	**Queensland•**						
NQ1	Hastings Reef	f_2	2.83×10^8	ND		$<5.7 \times 10^{-9}$	$<4.6 \times 10^{-8}$
NQ1	Hastings Reef	f_{10}	4.38×10^7	ND		$<1.1 \times 10^{-8}$	$<2.3 \times 10^{-8}$
NQ1	Hastings Reef	f_{10k}	5.15×10^7	ND		$<9.7 \times 10^{-9}$	$<9.7 \times 10^{-9}$
NQ2/7	Arlington Reef	f_2	4.03×10^8	1.5	3.7×10^{-7}		$<1.2 \times 10^{-7}$
HR1	Hoffmans Rocks	f_2	1.49×10^7	ND		$<3.4 \times 10^{-8}$	$<3.4 \times 10^{-8}$
HR7	Hoffmans Rocks	f_{10}	1.39×10^7	ND		$<3.8 \times 10^{-8}$	$<3.6 \times 10^{-8}$
WC1/1	Platypus Bay Station 1	f_{10}	1.80×10^7	2.7	1.5×10^{-7}		$<2.8 \times 10^{-7}$
WC1/1	Platypus Bay Station 1	f_{10k}	2.99×10^8	117.0	3.9×10^{-7}		$<1.0 \times 10^{-8}$
WC1/18	Platypus Bay Station 1	f_{10}	1.62×10^7	ND		$<3.1 \times 10^{-8}$	$<5.3 \times 10^{-8}$
WC3/1	Platypus Bay Station 1	f_{10k}	2.69×10^7	ND		$<3.2 \times 10^{-8}$	$<6.3 \times 10^{-8}$
WC2/1	Platypus Bay Station 2	f_{10k}	2.79×10^7	ND		$<2.9 \times 10^{-8}$	$<2.9 \times 10^{-8}$
SQ3/3	Flinders Reef	f_2	1.40×10^7	ND		$<3.6 \times 10^{-8}$	$<3.6 \times 10^{-8}$
	Hawaii						
GT178	Kahala Bay	f_{10k}	3.68×10^7	ND		$<1.4 \times 10^{-8}$	$<1.4 \times 10^{-8}$
GT18/1	Kahala Bay	f_{10k}	3.25×10^7	ND		$<1.5 \times 10^{-8}$	$<1.5 \times 10^{-8}$
	French Polynesia						
FP	Gambier Is.	f_2	4.62×10^7	ND		$<3.6 \times 10^{-8}$	$<1.1 \times 10^{-7}$
FP	Gambier Is.	f_{10}	5.74×10^6	ND		$<1.3 \times 10^{-7}$	$<1.7 \times 10^{-7}$
	Virgin Is.						
GT350	South Sound	ES	3.09×10^7	ND		$<4.6 \times 10^{-8}$	$<5.9 \times 10^{-8}$
Wild Cells	**Kiribati**						
-	Marakei Is.	-	1.17×10^6	40.0	3.4×10^{-5}		$<4.3 \times 10^{-7}$

* f_2, f_{10} and f_{10k} culture media modified from GUILLARD and RYTHER (1962) f medium, ES medium modified from McLACHLAN (1975). Details described in MATERIALS AND METHODS.

† MU = mouse unit. One MU = 1 LD_{50} dose for a 20 g mouse determined from time to death (n=2).

‡ Limit of detection of GTX (MU/cell) for non-toxic 97:3 and 9:1 chloroform-methanol eluates from silicic acid columns. This assumes 0.5 MU can be detected from bioassay signs displayed by 20 g mice injected with up to 1 g fraction weight per kg mouse body weight.

• The origin of Queensland strains listed north-south.

ND = not detected, ie. no ciguatoxin or GTX signs induced in mice injected with 97:3 and 9:1 chloroform:methanol eluates from silicic acid columns. Two mice injected for each column eluate.

grown in f_2, f_{10} and f_{10k} nutrient media (see Table 1), modified from GUILLARD and RYTHER (1962) f medium. The f_2 and f_{10} media have 0.5 and 0.1 concentration of f medium nutrients, respectively. The f_2 and f_{10} media were modified by the omission of silicate, the addition of ethylenediaminetetraacetic acid at a chelator: trace metal molar ratio of 2.3:1 and the replacement of ferric sequestrene with equimolar ferric chloride. Vitamins were added to f_2 and f_{10} media to produce the same concentrations in both media for thiamine (2.97×10^{-7} M) biotin (4.1×10^{-9} M) and B_{12} (1.48×10^{-9} M) (HOLMES *et al.*, 1988). Medium f_{10k} was made from modified f_{10} enriched with 10^{-8} M selenium (KELLER and GUILLARD, 1985). Oceanic seawater from Flinders Reef was used to prepare f_2 and f_{10} media. Seawater collected from Platypus Bay was used exclusively for f_{10k} media. Cultures were grown at 25°C with 50–60 μEin/m^2/sec^1 of light from Philips Daylight-54 fluorescent tubes with a 12:12 hour light:dark photoperiod. Mass cultures were grown in 1.8 litre Fernbach flasks and 2 litre and 4 litre Erlenmeyer flasks containing approximately 1 litre, 800 ml and 1 litre of media, respectively.

Harvest, extraction and purification of gambiertoxins (GTX)

Mass cultures were harvested after 12 to 28 days growth (majority harvested between 19 and 25 days). Cells were harvested by vacuum filtration onto glass fibre filters or 45 μm plankton mesh and quickly rinsed with 25 ml of distilled water to remove salt. The cells were disrupted in methanol using an ultrasonic homogenizer and extracted a minimum of four times with methanol as described previously (HOLMES *et al.*, 1990). The cell residues of WC1/1 and GT350 cultures remaining after methanol extraction were further extracted twice with 30 ml of acetone and the acetone extracts pooled, dried and bioassayed for toxicity in mice.

The pooled methanol extracts were dried, redissolved in 250 ml of 9:1 methanol:water and extracted by liquid–liquid partitions to produce hexane, diethyl ether, butanol and water fractions (HOLMES *et al.*, 1990). The hexane and water fractions were non-toxic to mice or contained small amounts of maitotoxin. All butanol fractions contained maitotoxin. The dried diethyl ether fractions were dissolved in chloroform and applied to silicic acid columns (Bio-sil A, 200-400 mesh, Bio-Rad) and fractions collected from a step-wise solvent gradient of increasing polarity (HOLMES *et al.*, 1990). Each fraction (chloroform, 97:3 chloroform:methanol, 9:1 chloroform:methanol and methanol) was collected and bioassayed for toxicity in mice. The 97:3 chloroform:methanol fractions from WC1/1 and NQ2/7 cultures were lethal to mice and the WC1/1 toxin was further purified on a semi-preparative μbondapak C_{18} high-performance liquid chromatography (HPLC)) column (Waters Associates, 84176). The toxin was eluted isocratically at 2 ml/min with 4:1 acetonitrile:water. The toxin was then chromatographed isocratically on a Fractogel TSK HW40-S column (Merck, 14983, 25 × 1.0 cm) with acetonitrile at 1 ml/min. Toxic fractions were detected by mouse bioassay.

Collection and extraction of biodetritus containing wild G. toxicus

A sample of a green macroalgae (*Halimeda* sp.) was collected from Marakei Island, Republic of Kiribati (Gilbert Islands) on the 25th April 1989. A biodetritus sample (37–150 μm) was shaken and sieved from the macroalgae. Cell numbers of benthic dinoflagellates were estimated using the differential sedimentation technique of GILLESPIE *et al.* (1985*a*). The biodetritus contained a nearly monospecific benthic dinoflagellate bloom of *G. toxicus*. One *Prorocentrum* sp. cell was observed in one of three subsamples indicating that approximately 2700 cells of *Prorocentrum* sp. were extracted in the sample. The biodetritus sample was extracted in methanol. The lipid-soluble toxin was purified in a similar manner to the WC1/1 toxin except that the 97:3 chloroform:methanol eluate from the silicic acid column was applied to a Fractogel TSK HW40-S column (30 × 2.5 cm) eluted isocratically with methanol at 2 ml/min.

Mouse bioassay

Fractions to be tested were dried under vacuum and further freed of solvent under a stream of N_2, resuspended in 0.5 ml of 5% Tween 60 saline and injected i.p. into 18–21 g Quackenbush strain mice (either sex) at a maximum dose of 1 g of dried fraction weight per kg mouse body weight. One mouse was injected per dose and one or two doses were injected per fraction (for non-toxic fractions 10% and 90% of fractions were injected where possible). Mice were observed over 24 hr and signs and death-times recorded. Rectal body temperatures were measured with a Comark electronic thermometer. Total lethality is expressed in mouse units (MU). One MU is defined as the LD_{50} dose for a 20 g mouse over 24 hr. Fractions were considered non-toxic if injection of a maximal dose was not lethal. Accurate LD_{50} values were not obtained because of the small amounts of lipid-soluble toxins available. Lethality of extracts containing GTX were estimated using a dose vs death-time equation for ciguatoxin: $\log(\text{dose}) = 2.3 \log(1 + t^{-1})$ (LEWIS, unpublished result) where dose is in MU, and t = time to death in hr for death from 0.8 hr to 3.5 hr. Animals were sacrificed in accordance with Australian National Health and Medical Research Council guidelines.

Mechanical response of guinea pig atria and vas deferens

Male guinea pigs (250–350 g) were killed by a blow to the head and bled. The left and right atria and central portion of the vas deferens were excised and mounted vertically in 5 ml organ baths and the mechanical

responses measured isometrically. Tissues were maintained under a resting tension of 1 g. Ringer solutions were maintained at 32°C and bubbled continuously with 95% O_2: 5%CO_2. The atrial Ringer contained (mM): NaCl, 115.0; $NaHCO_3$, 25.0; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; glucose 10.0 and $CaCl_2 \cdot 2H_2O$, 1.2 (LEWIS and ENDEAN, 1986). Atria were washed several times during an equilibration period (> 1 hr). Left atria were stimulated with rectangular voltage pulses 3 msec in duration and 20% above threshold through a pair of platinum electrodes. Unless otherwise indicated left atria were stimulated at 1 Hz. The vas deferens Ringer contained (mM): NaCl, 120.0; $NaHCO_3$, 25.2; KCl, 4.8; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.3; glucose, 5.8 and $CaCl_2 \cdot 2H_2O$, 1.2 (OHIZUMI *et al.*, 1981). Vas deferens were used for experiments after noradrenaline (3×10^{-6} M) and KCl (4×10^{-2} M) produced consistent responses to consecutive applications of each agonist added at 20 min intervals. Because of the small amounts of toxins available, WC1/1 and biodetrital GTX were applied to only three and two vas deferens each, respectively. Agonists were repeatedly added before and after application of toxins.

Rat brain membrane binding assay

Rat brain membranes were prepared according to the method described by DAVIO and FONTELO (1984). [^{3}H]PbTx-3 (14.4 Ci/mmole) was prepared by Dr D. G. BADEN (University of Miami, FL) by the reduction of PbTx-2 with [^{3}H] sodium borohydride, as previously reported (POLI *et al.*, 1986). Purity was greater than 99%, as determined by HPLC analysis. A stock solution of 1.3 μg/ml in methanol was kept in a freezer at −10°C and dilutions made immediately prior to use. The assay was performed in a binding medium consisting of (mM): HEPES, 50.0 (pH 7.4); choline chloride, 130.0; glucose, 5.5; $MgSO_4$, 0.8 and KCl, 5.4. The binding medium contained 1 mg/ml bovine serum albumen (BSA) and 0.01% Emulphor EL-620. Emulphor EL-620 (GAF Corp., NY) is a non-ionic emulsifier used to aid in solubilizing hydrophobic toxins. Rat brain membranes (125–150 μg) suspended in 0.1 ml binding medium were added to 1.5 ml polypropylene microcentrifuge tubes containing [^{3}H]PbTx-3 (0.5 nM final concentration) and increasing concentrations of cold PbTx-3, GTX or maitotoxin-2 in 0.9 ml binding medium. After incubation for 1 hr at 4°C, samples were centrifuged for 5 min at 15,000 × *g*. The supernatant solutions were aspirated and the pellets rapidly washed twice with several drops of ice-cold wash medium containing 5.0 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 1 mg/ml BSA. The pellets were then transferred to scintillation vials and the bound radioactivity measured with a Beckman liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA). Data points were analysed in triplicate. Non-specific binding was measured in the presence of a saturating concentration of unlabelled PbTx-3 (1 μM) and subtracted from total binding to yield specific binding. Free [^{3}H]PbTx-3 was determined by counting an aliquot of the supernatant prior to aspiration. Competition curves were fitted by the IBM PC Radioimmunoassay Data Reduction System, version 4.1 (RODBARD *et al.*, 1978).

Chemicals and drugs used

The solvents methanol, chloroform, acetonitrile (Waters Associates, Lane Cove, Australia), *n*-hexane and water (Mallinckrodt, Clayton, Australia) were HPLC grade while diethyl ether, *n*-butanol, acetone and KCl were analytical reagent grade. Aqueous solutions of noradrenaline (Winthrop, Sydney, Australia), DL-propranolol HCl and tetrodotoxin (TTX) (Sigma, St Louis, U.S.A.) were directly added to organ baths. GTX was dissolved and added to organ baths in dimethylsulphoxide (DMSO). The total volume of DMSO added to organ baths did not exceed 0.01 ml. Final bath concentration of drugs and toxin are indicated throughout. Maitotoxin-2 was purified to homogeneity from cultures of *G. toxicus* strain NQ1 as described previously (HOLMES *et al.*, 1990).

Statistics

Unless otherwise indicated values are expressed as means ± 1 S.E. and the significance of differences between means determined using the Student's *t*-test. A number of single observations were also compared with means using a special case of the *t*-test as described by SOKAL and ROHLF (1981). The significance of linear regressions were determined as per SOKAL and ROHLF (1981).

RESULTS

Purification of gambiertoxins (GTX)

The diethyl ether fractions extracted from the 13 culture strains and the biodetritus sample from Marakei Island were all lethal to mice. The diethyl ether fractions of all culture strains except WC1/1 and NQ2/7 induced signs in mice similar to those produced by maitotoxin (HOLMES *et al.*, 1990). The signs displayed by mice injected with WC1/1 or NQ2/7 diethyl ether fractions appeared as a composite of maitotoxin and ciguatoxin signs

including copious diarrhoea, gasping without asphyctic convulsions, pronounced cyanosis and progressive paralysis (from hind to fore limbs). The diethyl ether fraction extracted from the biodetritus sample induced only ciguatoxin-like signs. The acetone extracts of WC1/1 cell residues were non-toxic to mice, but the acetone extracts of GT350 contained approximately 2 MU of maitotoxin. This result confirms that methanol is capable of extracting all of the GTX and the maitotoxin and that further extraction of cell residues with less polar solvents is unnecessary.

The chloroform and 9 : 1 chloroform : methanol fractions from silicic acid columns were non-toxic to mice. Ciguatoxin elutes from silicic acid columns in 9 : 1 chloroform : methanol (LEWIS and ENDEAN, 1984*a*), indicating that ciguatoxin (as found in fishes) was not produced by the wild or cultured *G. toxicus*. For all strains the methanol fractions from silicic acid columns were lethal to mice and all induced signs consistent with maitotoxin. The 97 : 3 chloroform : methanol fractions from all culture strains except WC1/1 and NQ2/7 were non-toxic to mice and did not induce any signs of intoxication. The 97 : 3 chloroform : methanol fractions from WC1/1, NQ2/7 and the biodetritus sample contained toxins hereafter referred to as GTX. These fractions were lethal to mice and induced similar signs in mice including copious pungent diarrhoea, piloerection, hypersalivation, reduced body temperature, gasping and convulsions (usually just prior to death). A number of mice also developed pronounced hind limb paralysis. All of the above signs with the exception of hind limb paralysis are similar to those induced by ciguatoxin. GTX were therefore less polar than ciguatoxin and maitotoxin and induced bioassay signs in mice different to those produced by maitotoxin.

The quantities of GTX and the limits for detection of toxins from 97 : 3 chloroform : methanol and 9 : 1 chloroform : methanol fractions from silicic acid columns are presented in Table 1. Only two of the 13 cultured strains of *G. toxicus* produced GTX. Similar levels of GTX were detected in WC1/1 cultures grown in f_{10} and f_{10k} media and NQ2/7 grown in f_2 media. Strain NQ1 did not produce these toxins when grown in f_2, f_{10} or f_{10k} media. Interestingly, WC1/18 which was isolated from the same site and at the same time as WC1/1 did not produce GTX. The limit for detection of GTX per cell from non-GTX producers was typically one order of magnitude lower than the toxin levels detected in WC1/1 and NQ2/7 cells (Table 1). WC1/1 and NQ2/7 cultures produced 100-fold less GTX than that produced by wild cells in the biodetritus sample.

The WC1/1 97 : 3 chloroform : methanol fraction (44 mg, 90 MU) was applied to μbondapak C_{18} HPLC column. The toxin was eluted between 26 min and 36 min. Approximately 46 MU of toxin was recovered from this column in less than 1 mg. The majority of this toxin (40 MU) was then applied to a Fractogel column. The toxin was poorly resolved from this column, being recovered without loss in a broad band between 0.5 bed volume (Vb) and 2.3 Vb of acetonitrile. The toxin remaining after bioassay was pooled and characterized on isolated guinea pig atria and vas deferens. The effect of this GTX fraction on the binding of [^{3}H]PbTx-3 to rat brain membranes was also examined. A portion of the biodetritus sample (wild cell) 97 : 3 chloroform : methanol fraction from the silicic acid column (10.5 mg, 6 MU) was applied to a Fractogel column. The toxin eluted in 0.5 Vb to 1.0 Vb of methanol, was pooled and characterized on isolated guinea pig atria and vas deferens.

Mechanical response of guinea pig left atria to GTX from cultured cells

GTX from cultured cells (WC1/1) caused a positive inotropic increase in force of isolated left atria without increasing resting tension (Fig. 1a). The mean time to onset of

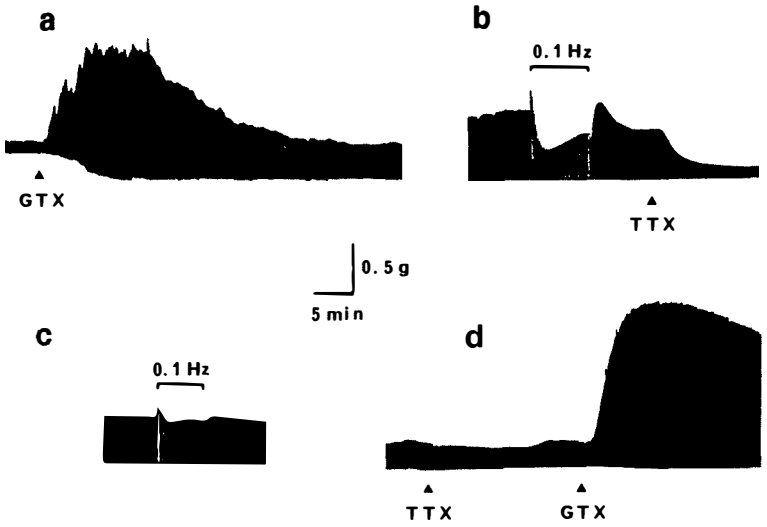

FIG. 1. THE POSITIVE INOTROPIC EFFECTS OF GTX FROM CULTURES OF THE WC1/1 STRAIN *Gambierdiscus toxicus* ON GUINEA PIG LEFT ATRIA.
Atria were stimulated at 1.0 Hz unless otherwise indicated. (a) Transient and sustained positive inotropy produced by GTX (0.1 MU/ml). The sustained positive inotropy was stable for several hours and was resistant to washing with Ringer. (b) One hour after GTX (0.1 MU/ml), reducing stimulation frequency to 0.1 Hz during the sustained positive inotropy reduced twitch responses. Tetrodotoxin (TTX, 10^{-8} M) reduced the response to GTX to control levels. Washing with Ringer completely reversed the inhibitory effects of TTX. (c) Forty-five minutes after GTX (1.0 MU/ml), reducing stimulation frequency to 0.1 Hz had little effect on twitch tension during the sustained positive inotropy. (d) Preapplication of 10^{-8} M TTX did not prevent the development of the transient positive inotropic phase in atria exposed to GTX (0.1 MU/ml). TTX did abolish the irregularity in development of the transient phase (see panel a).

the response (1.2–5.5 min, $n = 2$–5) was inversely related to concentrations of toxin between 10^{-4} MU/ml and 0.1 MU/ml. Cumulative doses between 10^{-4} MU/ml and 10^{-2} MU/ml generally produced transient inotropic responses. Cumulative or single doses equivalent to 0.1 MU/ml produced a biphasic response with an initial large transient increase followed by a smaller sustained response (Fig. 1a). The development of the transient response produced by 0.1 MU/ml of GTX was uneven and "spiky". The time to peak force during this phase was 10.2 ± 1.1 min ($n = 6$) and the duration of the initial phase was between 20 min and 35 min. The peak force produced during the transient phase increased linearly ($P < 0.001$) with order of magnitude increases in the concentration of GTX between 10^{-4} MU/ml and 0.1 MU/ml (Fig. 2). The maximum transient force was produced at 0.1 MU/ml of GTX, with additional toxin producing no significant increase in force (Fig. 2).

The second, sustained phase, seen only at high doses of GTX (0.1 MU/ml), was maintained for the duration of the experiments (3–4 hr), and was resistant to repeated washing with toxin-free Ringer. Additional toxin up to 1.0 MU/ml had no further positive inotropic effect on this phase. Reducing the stimulation frequency to 0.1 Hz caused a large reduction in the twitch response of atria exposed to 0.1 MU/ml of toxin (Fig. 1b). However, reducing the stimulation frequency had little effect on the twitch response of atria exposed to 1.0 MU/ml of toxin (Fig. 1c). Low doses of TTX significantly reduced the sustained response produced by 0.1 MU/ml of GTX (Fig. 1b), with 10^{-8} M TTX reversing

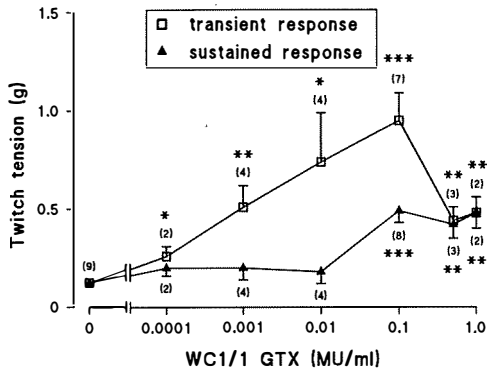

FIG. 2. CUMULATIVE DOSE RESPONSE FOR GTX FROM CULTURES OF THE WC1/1 STRAIN *Gambierdiscus toxicus* ON GUINEA PIG LEFT ATRIA.
Data presented as means ± 1 S.E. (n = 2–9, as indicated by numbers in parenthesis). Significant differences between responses to GTX and control (0 MU/ml GTX) are indicated, *** = $P < 0.001$, ** = $P < 0.01$ and * = $P < 0.05$.

the response to control levels (Fig. 3). The sustained response of atria to 1.0 MU/ml of toxin was little affected by 10^{-8} M TTX, but was reduced to control by 10^{-7} M TTX (n = 1). Washing with toxin free Ringer completely reversed the inhibitory effects of TTX, exposing the previously developed positive inotropic response. Unexpectedly, preapplication of 10^{-8} M TTX 15 min before 0.1 MU/ml of GTX did not significantly alter the time to onset or peak force of the initial transient response, but did abolish the irregular development of positive inotropy (n = 3) (Fig. 1d). The sustained response produced by 0.1 MU/ml of GTX could be abolished to control by 10^{-7} M propranolol (n = 2). Propranolol (10^{-7} M) also reduced the sustained response produced by 1.0 MU/ml of toxin (n = 1) by 54%. Washing completely reversed the inhibitory effects of propranolol.

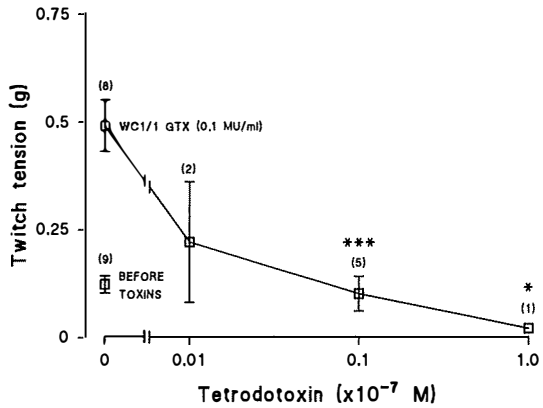

FIG. 3. THE EFFECT OF CUMULATIVE DOSES OF TTX ON THE SUSTAINED POSITIVE INOTROPIC EFFECT OF GTX (0.1 MU/ml) ON GUINEA PIG LEFT ATRIA.
GTX from cultures of the WC1/1 strain of *Gambierdiscus toxicus*. Data presented as means ± 1 S.E. (n = 1–9, as indicated by numbers in parenthesis). Significant differences between responses to GTX (0.1 MU/ml) before and after TTX (tetrodotoxin) are indicated, *** = $P < 0.001$ and * = $P < 0.05$.

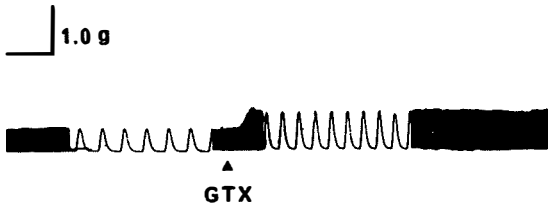

FIG. 4. POSITIVE INOTROPIC AND CHRONOTROPIC EFFECTS OF 0.1 MU/ml OF GTX FROM CULTURES OF THE WC1/1 STRAIN *Gambierdiscus toxicus* ON SPONTANEOUSLY BEATING GUINEA PIG RIGHT ATRIA. Horizontal scale = 5 min and 1 min for slow and fast chart speeds, respectively.

Mechanical response of guinea pig right atria to GTX from cultured cells

WC1/1 GTX produced positive inotropic and chronotropic responses in spontaneously contracting right atria ($n = 2$)(Fig. 4). Washing did not reduce the force or rate of twitch contractions over 2 hr. TTX (10^{-7} M, $n = 1$) or propranolol (10^{-7} M, $n = 1$) reduced the positive inotropy to below that of control and reversed the rate of contraction to that of control. Washing reversed the inhibitory effects of TTX but not those of propranolol.

Mechanical response of guinea pig left atria to GTX from wild cells

Cumulative concentrations of wild cell GTX (1.5×10^{-3}–0.15 MU/ml) caused positive inotropic responses in isolated left atria without increasing resting tension ($n = 2$). The response produced by 1.5×10^{-3} MU/ml was transient. The response produced by 1.5×10^{-2} MU/ml was biphasic and similar to that produced by 0.1 MU/ml of GTX from cultured cells, except that the transient phase was of shorter duration and it developed smoothly. Reducing the frequency of stimulation to 0.1 Hz reduced the force of twitch contractions of an atrium exposed to 1.5×10^{-2} MU/ml but not that of an atrium exposed to 0.15 MU/ml of toxin. The response produced by 0.15 MU/ml of wild cell GTX was reduced to below that of control by 10^{-7} M TTX ($n = 1$). Washing reversed the inhibitory effect of TTX.

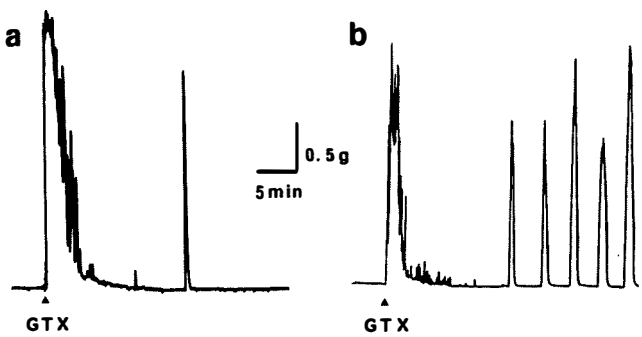

FIG. 5. ACTION OF CULTURED AND WILD GTX ON GUINEA PIG VAS DEFERENS. (a) GTX (0.2 MU/ml) from cultures of the WC1/1 strain *Gambierdiscus toxicus*. (b) GTX (0.1 MU/ml) from wild *G. toxicus*.

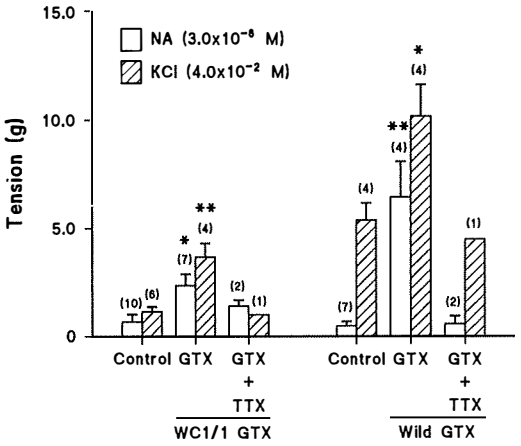

FIG. 6. RESPONSES OF GUINEA PIG VAS DEFERENS TO REPEATED APPLICATIONS OF NORADRENALINE AND KCl BEFORE (CONTROL) AND AFTER THE ADDITION OF GTX.
GTX from cultures of WC1/1 strain *Gambierdiscus toxicus* (0.2 MU/ml) or biodetritus containing wild *G. toxicus* (0.1 MU/ml). Data from replicates using three and two vas deferens for WC1/1 and wild cell GTX experiments, respectively. The number of applications of noradrenaline and KCl are indicated in parenthesis. Noradrenaline (NA) and KCl were added at 20 min intervals. Tetrodotoxin (TTX, 5×10^{-7} M) was added 30 min after GTX and 15 min prior to agonists. Data presented as means + 1 S.E. Significant differences between treatments and corresponding controls are indicated, ** = $P < 0.01$ and * = $P < 0.05$.

Response of guinea pig vas deferens to GTX

GTX from culture (WC1/1, 0.2 MU/ml) caused a large transient contraction (1.36 ± 0.80 g, $n = 3$) followed by occasional spontaneous contractions in isolated guinea pig vas deferens (Fig. 5a). The responses of vas deferens to noradrenaline (3×10^{-6} M) and KCl (4×10^{-2} M) were significantly potentiated by GTX from cultured cells (Fig. 6). The sensitization of vas deferens to these agonists were not reduced by repeated washing for over 1 hr. TTX (5×10^{-7} M) did not significantly reduce these potentiated responses to noradrenaline or KCl (Fig. 6), but this may stem from the small experimental sample size.

GTX from wild cells (0.1 MU/ml) caused a transient contraction followed by regular spontaneous contractions ($n = 2$) (Fig. 5b). The responses to noradrenaline (3×10^{-6} M) and KCl (4×10^{-2} M) were significantly potentiated by wild cell GTX in a similar manner to that of WC1/1 GTX (Fig. 6). The potentiation (and the spontaneous contractions) were resistant to repeated washing for over 3 hr. TTX (5×10^{-7} M) significantly reduced the potentiated noradrenaline but not the KCl response, although the latter was reduced (Fig. 6).

Inhibition of [^{3}H]PbTx-3 to rat brain membranes by GTX from cultured cells

The effect of increasing concentrations of PbTx-3, GTX and maitotoxin-2 on the binding of [^{3}H]PbTx-3 to rat brain membranes is shown in Fig. 7a. GTX and PbTx-3 dose-dependently inhibited the binding of [^{3}H]PbTx-3 with 50% effective concentrations of 1.9×10^{-2} MU/ml and 7.1×10^{-4} MU/ml, respectively. In contrast, maitotoxin-2 appeared to slightly enhance the binding of [^{3}H]PbTx-3 (Fig. 7a). Double reciprocal plots (Fig. 7b) intersected at 1/FREE equal to zero, indicating that GTX competitively inhibits the binding of [^{3}H]PbTx-3 to rat brain membranes.

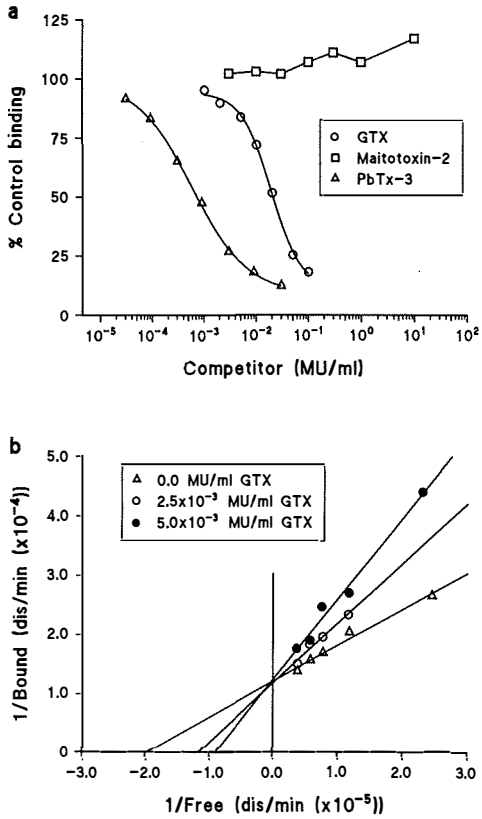

FIG. 7. INHIBITION BY GTX OF [^{3}H]PbTx-3 BINDING TO RAT BRAIN MEMBRANES.
GTX from cultures of the WC1/1 strain *Gambierdiscus toxicus*. (a) GTX and PbTx-3 dose-dependently inhibit the binding of [^{3}H]PbTx-3 (0.5 nM) to rat brain membranes. Maitotoxin-2 does not inhibit [^{3}H]PbTx-3 binding. 100% binding of [^{3}H]PbTx-3 for GTX and PbTx-3 experiments were calculated from best-fit competition curves. Specific binding in the absence of competitor was used as 100% [^{3}H]PbTx-3 binding for maitotoxin-2 data. PbTx-3 concentrations (ng/ml) were converted to MU/ml assuming 1 MU = 3.4 μg (BADEN and MENDE, 1982). (b) Double reciprocal plot of the influence of GTX on the binding to rat brain membranes of varying doses of [^{3}H]PbTx-3. The intersection of the curves at 1/FREE = 0 (infinite [^{3}H]PbTx-3 dose) indicates that GTX competitively inhibits the binding of PbTx-3. The slope in the absence of GTX suggests an apparent dissociation constant (K_D) for PbTx-3 of 1.8 nM.

DISCUSSION

This is the first study confirming the production of putative ciguatoxin precursors (GTX) from *G. toxicus* in culture. Ciguatoxin precursors have only previously been extracted from biodetritus that contained wild *G. toxicus* (LEGRAND *et al.*, 1990). This study also provides the first independent confirmation that ciguatoxin precursors can occur in wild *G. toxicus*. The cultured and wild cell toxins found in this study eluted in 97:3 chloroform:methanol, indicating that they are less polar than ciguatoxin. Several GTX were extracted from biodetritus containing wild *G. toxicus* in French Polynesia and all were also less polar than ciguatoxin (LEGRAND *et al.*, 1990). MURATA *et al.* (1989, 1990) have clearly shown a structural relationship between one of these GTX and ciguatoxin. The WC1/1 and NQ2/7 culture and Kiribati wild cell GTX are therefore probably

ciguatoxin precursors, although confirmation awaits structural elucidation of these toxins. These toxins are probably oxidatively metabolized to ciguatoxin in fishes as suggested by LEGRAND *et al.* (1990), a process that infers an order of magnitude increase in potency (MURATA *et al.*, 1990). The polarity of WC1/1 GTX on preparative silica columns is comparable with the minor toxin extracted from barracuda caught in a similar area of Platypus Bay (LEWIS and ENDEAN, 1984*a*).

GTX competitively inhibits the binding of PbTx-3 to rat brain membranes. Ciguatoxin is the only toxin that has been previously shown to inhibit the binding of PbTx-3 (LOMBET *et al.*, 1987; BADEN, 1989). On a molar basis, ciguatoxin has a greater affinity for the Na^+ channel than brevetoxin (LOMBET *et al.*, 1987). On a MU basis, GTX was 27-fold less potent than PbTx-3 at inhibiting the binding of [^{3}H]PbTx-3 to rat brain membranes. This apparent anomaly is probably explained by pharmacokinetic differences between the two types of toxins. In rats, PbTx-3 partitions primarily into less vital areas, particularly skeletal muscle (POLI *et al.*, 1990). GTX from wild and cultured *G. toxicus* produced in-vivo signs in mice and in-vitro responses in guinea pig atria and vas deferens which were similar, but not identical with those produced by ciguatoxin. The chromatography, binding assay and in-vivo and in-vitro responses clearly differentiate GTX from maitotoxin. The in-vitro studies and binding data show GTX to be a Na^+ channel activator toxin. Maitotoxin was produced by all cultured and wild *G. toxicus* cells, ciguatoxin as found in moray eels was produced by none. Earlier investigations claiming the extraction of ciguatoxin from wild *G. toxicus* (YASUMOTO *et al.*, 1977; BAGNIS *et al.*, 1980) may have extracted less polar forms of ciguatoxin (GTX). This would explain the discrepancy in the *Rf* reported for silica thin layer chromatography of wild *G. toxicus* ciguatoxin (0.5–0.7) and reference ciguatoxin (0.1–0.3) (YASUMOTO *et al.*, 1977, 1980).

There are no previous investigations of the pharmacological action of the less polar forms of ciguatoxin. Ciguatoxin shifts the voltage dependence of Na^+ channel activation in the hyperpolarizing direction which stimulates nerves (LEWIS and ENDEAN, 1984*b*) and can directly increase the force of atrial contractions by increasing internal Ca^{2+} via the Na^+–Ca^{2+}-exchange (LEWIS and ENDEAN, 1986; SEINO *et al.*, 1988). The inotropic and chronotropic increases in atrial twitch contractions caused by WC1/1 and wild cell GTX were similar to those caused by ciguatoxin (MIYAHARA *et al.*, 1979; LEWIS and ENDEAN, 1986; SEINO *et al.*, 1988). The non-competitive block of these responses by TTX (a Na^+ channel blocker) is also consistent with blockade of the effects of ciguatoxin. However, 0.1 MU/ml of WC1/1 GTX produced a maximal sustained positive inotropy in left atria which was entirely mediated indirectly via the neural release of neurotransmitters (noradrenaline) from the nerves associated with atria. This is shown by the block of these responses by low doses of TTX (nerve block) and propranolol (β-receptor block). Ciguatoxin produces a sustained maximal response at 0.15 MU/ml which is approximately equivalent to the sustained response produced by 0.1 MU/ml of WC1/1 GTX, but this ciguatoxin response is predominantly caused by activating myocardial Na^+ channels (LEWIS and ENDEAN, 1986). In contrast to ciguatoxin, WC1/1 GTX produces transient positive inotropy at very low concentrations. This toxin is therefore considerably more potent (per MU) on atrial nerves than ciguatoxin. However, at high concentrations of WC1/1 GTX (> 0.5 MU/ml) a component of positive inotropy probably includes a direct action on the myocardium as propranolol could not abolish this response. Additionally, at high concentrations the toxin overrides the positive staircase effect of increasing force of twitch contractions with increasing stimulation frequency as seen for the direct effects of ciguatoxin (LEWIS and ENDEAN, 1986). It remains to be determined if the less polar forms

of ciguatoxin extracted from moray eel viscera (LEGRAND *et al.*, 1990; LEWIS, unpublished result) are also more neurally active on mammalian tissues than ciguatoxin.

Ciguatoxin causes a contraction in guinea pig vas deferens indirectly by stimulating release of noradrenaline (OHIZUMI *et al.*, 1981). The toxin also produces a non-specific potentiation of noradrenaline and KCl responses (OHIZUMI *et al.*, 1982; LEWIS and ENDEAN, 1984*b*). WC1/1 and wild cell GTX produced similar responses on the guinea pig vas deferens. However, wild cell GTX appeared more potent than WC1/1 *in vitro* (on both vas deferens and left atria) compared to the in-vivo responses (per MU). The presence of several GTX may explain the differences observed between the responses produced by WC1/1 and wild cell toxins. LEGRAND *et al.* (1990) detected four GTX from wild cells.

Only two out of 13 *G. toxicus* strains produced GTX. The origin of ciguatoxin and the aetiology of ciguatera is therefore probably restricted to genetic strains of *G. toxicus* which produce these putative ciguatoxin precursors. The low frequency of GTX producing strains of *G. toxicus* may explain why previous culture studies on the toxicity of *G. toxicus* failed to find GTX. Strain-dependent differences in the composition of toxins produced by *G. toxicus* have been described. The type of maitotoxins produced by *G. toxicus* strains have been found to vary between but not within strains (HOLMES *et al.*, 1990). Non-ciguatoxic wild *G. toxicus* have also been reported (GILLESPIE *et al.*, 1985*b*; LEWIS *et al.*, 1988*b*). It is possible that culture strains could lose the ability to produce GTX over time. However, mass cultures of NQ2/7 were not harvested for toxin analysis until more than two years after this strain was first isolated into culture. Production of GTX therefore appears to be a stable temporal characteristic of at least some *G. toxicus* strains. The origin of the seawater used for culturing *G. toxicus* is also unlikely to effect the ability of viable cultures to produce GTX as cultures of the WC1/1 strain produced comparable GTX, whether grown in media made from Flinders Reef or Platypus Bay seawater. Flinders Reef is an area of low ciguatera risk (LEWIS *et al.*, 1988*b*) which produces high numbers of non-ciguatoxic *G. toxicus* (GILLESPIE *et al.*, 1985*b*), whereas Platypus Bay has a relatively high incidence of ciguatera (GILLESPIE *et al.*, 1986).

The 100-fold greater production of GTX (MU/cell) by wild cells compared to WC1/1 and NQ2/7 cultured cells, indicates that there is considerable scope for increased production of ciguatoxin precursors from *G. toxicus* in culture. The different seawater and nutrient media used for growing WC1/1 and NQ2/7 strains did not produce major differences in the quantities of toxin produced per cell. There is much still to be learnt about the relationship between culture conditions and toxin production by *G. toxicus*. However, because of the small amounts of toxin produced, these studies would be facilitated by a more sensitive assay than the mouse bioassay. The pharmacological action of GTX on guinea pig left atria and the inhibition by GTX of brevetoxin binding to rat brain membranes could form the basis of sensitive assays for detecting GTX in crude fractions as both assays can detect $< 10^{-2}$ MU/ml of these toxins. It is possible that many of the *G. toxicus* strains examined in this study produce GTX (or ciguatoxin) at levels below the detection limit of the mouse bioassay.

Concern has recently been expressed at the possible introduction into temperate Australian waters of paralytic shellfish poison producing dinoflagellates by ship ballast water (HALLEGRAEFF *et al.*, 1990). Translocation of *G. toxicus* has not been considered a problem in Australia because of the extensive distribution of *G. toxicus* in tropical waters. However, if only certain strains of *G. toxicus* are capable of producing ciguatoxin precursors then the translocation of these strains by ship ballast water or as epiphytes upon seaweeds fouling the hulls of ships may be vectors for introduction of ciguatera into

tropical regions currently free of ciguatera. *G. toxicus* is capable of surviving rafting upon drift algae (BOMBER *et al.*, 1988). Translocation may explain the appearance of ciguatera for the first time in an area, e.g. at Hao Atoll (BAGNIS, 1969).

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APPENDIX 10.3

Proceedings of the 6th International Coral Reef Symposium, Australia, 1988, Vol. 3

TOXICITY AND MORPHOLOGY OF OSTREOPSIS CF. SIAMENSIS CULTURED FROM A CIGUATERA ENDEMIC REGION OF QUEENSLAND, AUSTRALIA

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ABSTRACT

Benthic dinoflagellates of the genus Ostreopsis have been suggested as having a minor role in the production of toxins associated with ciguatera. The toxicity of a laboratory culture isolated from a ciguatera endemic region of Queensland, Hervey Bay, was examined. A small amount of water-soluble toxin but no ciguatoxin was detected from 3.8×10^7 cells, indicating that this Ostreopsis species is unlikely to contribute to the aetiology of ciguatera in Queensland. The taxonomic characteristics of these cells were inconsistent with typical O. siamensis or O. ovata. Cultured cells were significantly smaller than has been reported for wild O. siamensis. However, on the basis of the broadly ovate cell shape, and the production of a water-soluble toxin, the dinoflagellate cultured in this study was assigned to O. siamensis Schmidt.

INTRODUCTION

Ciguatera is a circumtropical disease caused by eating fish which contain ciguatoxin. This lipid-soluble toxin is thought to originate from the benthic dinoflagellate Gambierdiscus toxicus (Yasumoto et al. 1979). However, we have been unable to detect ciguatoxin from wild or cultured G. toxicus from Queensland (Gillespie et al. 1985, Gillespie 1987). Other benthic dinoflagellates, including Ostreopsis spp. have been suggested as potentially capable of producing toxins associated with ciguatera (Regalis 1984, Tindall et al. 1984, Carlson & Tindall 1985).

Four species of Ostreopsis have been described: O. siamensis Schmidt, O. lenticularis Fukuyo, O. ovata Fukuyo and O. heptagona Norris, Bomber & Balech, all of which have been shown to produce toxins in culture (Nakajima et al. 1981, Norris et al. 1985, Tosteson et al. 1986). The number and arrangement of the main thecal plates are identical for the four species (Fukuyo 1981, Norris et al. 1985). Therefore, species differentiation between these thecate dinoflagellates is based primarily upon morphological characteristics, such as cell shape and size (Fukuyo 1981, Steidinger 1983).

Ciguatera is endemic to Queensland and is prevalent in the Hervey Bay region (Gillespie et al. 1986). This paper examines the toxicity and morphology of O. siamensis cultured from the Hervey Bay region and discusses its role in the aetiology of ciguatera in Queensland.

MATERIALS AND METHODS

Collection, isolation and culture of O. siamensis

Ostreopsis cells were collected from biodetritus samples sieved from macroalgae at Heron Island, Lady Elliot Island and Hoffmans Rocks (figure 1) using the method of Yasumoto et al. (1980a), excepting that 212 µm, 150 µm and 38 µm sieves were used and the residue from the 38 µm sieve was diluted to 100 ml. Heron Island and Lady Elliot Island biodetrital samples were collected in May 1983 and December 1983, respectively and were preserved in 5% formalin. Ostreopsis cultures were initiated from living cells micropipetted from Hoffmans Rocks biodetrital samples collected on 15 July 1986. Cultures were maintained at 25°C under a 12:12 hour light-dark photoperiod. Light was provided from Phillips Daylight-54 fluorescent tubes. Cultures were grown in modified Guillard & Ryther (1962) f_2 and f_{10} nutrient media. The media were modified by the omission of silicate, the addition of EDTA at a chelator-trace metal molar ratio of 2.3:1 (Harrison et al. 1980) and the replacement of ferric sequestrene with equimolar ferric chloride. Vitamins were added to f_2 and f_{10} media to produce the same final concentrations in both media for thiamine (2.97×10^{-7} M), biotin (4.10×10^{-9} M) and B_{12} (1.48×10^{-9} M).

Mass cultures for toxin assays were grown in f_2 media in 1.8 litre Fernbach flasks or 2 litre

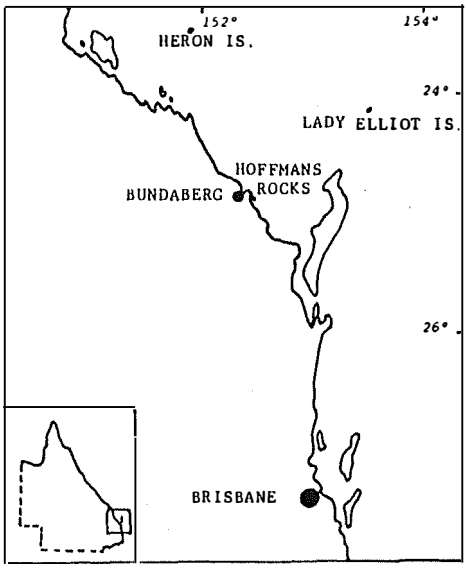

Figure 1 : Map of southeast Queensland. Collection sites at Hoffmans Rocks (a fringing rock reef in Hervey Bay) and Lady Elliot and Heron Islands are indicated.

Erlenmeyer flasks under a photosynthetic photon flux density (PPFD) of 54 $\mu E\ m^{-2}s^{-1}$. Cultures were harvested after 3 to 4 weeks growth after addition of 100 ml innocula. Morphological comparisons were made on cells grown in 250 ml Erlenmeyer flasks in f_2 and f_{10} media under a PPFD of 54 $\mu E\ m^{-2}s^{-1}$ and in f_{10} media under a PPFD of 90 $\mu E\ m^{-2}s^{-1}$. Subsamples of cells for cell counts and morphological studies were preserved in 1% formalin.

Harvest, extraction and assay of toxins

Cells from mass cultures were collected onto Whatman GF/A glass fibre filters by vacuum filtration, rinsed with 25 ml of distilled water to remove salt and then stored in methanol at -20°C. Cells were suspended in methanol and ruptured using an ultrasonic homogeniser, and the methanol extract separated from cell residues by centrifugation. Cell residues were extracted a minimum of 3 times with methanol and the methanol extracts pooled, dried and assayed for toxicity using mice.

The majority of the methanol extract was soluble or could be suspended in chloroform. A portion of this material (411.4 mg) was applied to a silicic acid column (Bio Rad Biosil A 200-400 mesh) slurry packed with chloroform. Fractions were collected from chloroform, 97:3 chloroform-methanol and 9:1 chloroform-methanol eluents and assayed for toxicity using mice (figure 2).

A small amount of the methanol extract (4.3 mg) was insoluble in chloroform and 1.4 mg of this material was dissolved in 20 ml distilled water and the aqueous phase extracted 3 times with 40 ml of water-saturated n-butanol. The butanol fractions were pooled and the butanol and water fractions dried and assayed for toxicity using mice.

Fractions were tested for toxicity using 18 to 21 g Quackenbush strain mice. Fractions to be tested were dried, resuspended in 0.5 ml of 1% Tween 60 saline and injected intraperitoneally at a maximum dose of 1 g/kg (fraction weight/mouse body weight). Mice were observed for 48 hours and signs recorded. Rectal body temperatures were measured with a Comark electronic thermometer. One mouse unit (MU) was defined as the minimum dose of toxin required to kill a mouse within 48 hours. Solvents used were A.R. grade.

Dinoflagellate cell morphology

Dinoflagellate cells and their thecal plates were measured and photographed using a Zeiss Jena Amplival microscope with planachromat objectives. Thecal plates were stained using 0.3% trypan blue (Taylor 1978). Interpretation of thecal plate series, and morphological characteristics for species identification were as described by Fukuyo (1981).

Dorsoventral diameters, transdiameters and lengths of cells were measured from exponentially growing cultures (figure 3). Cell lengths were measured from the apex to antapex of cells in girdle view. Ratios of cell dorsoventral diameters to transdiameters were calculated and used to characterise cell shape. Antapical plate (1'''') dorsoventral diameters and transdiameters were measured (figure 3), and the ratios between these diameters calculated. Cell dimensions were compared using one-way and two-way analysis of variance and student t-tests as described by Sokal & Rohlf (1981).

Cultured cells were fixed in 3% gluteraldehyde, dehydrated in an ethanol series, critically point dried, sputter coated with platinum and examined in a Phillips 505 scanning electron microscope.

RESULTS

Toxicity

The 29 dinoflagellate cultures used for toxin analysis had a mean cell density ($\pm$ standard deviation) of 1440 $\pm$ 460 cells ml^{-1}. A total of 3.8×10^7 cells were homogenised which yielded 488.3 mg of methanol extract containing 70 MU of toxin.

To determine if any ciguatoxin was produced a portion (411.4 mg) of the methanol extract was suspended in chloroform and applied to a silicic acid column. Ciguatoxin elutes with the 9:1 chloroform-methanol fraction (Yasumoto et al. 1979). The yields of fractions eluted from the column are shown in figure 2. The chloroform, 97:3 chloroform-methanol and 9:1 chloroform-methanol fractions were non-toxic to mice at 1 g/kg, indicating that ciguatoxin was not produced by these cultures.

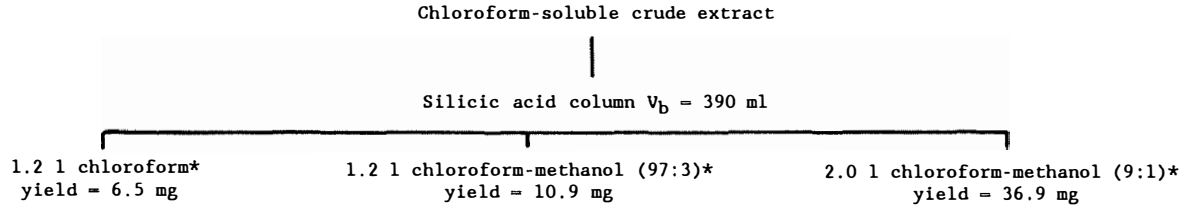

Figure 2. Purification of the chloroform-soluble crude extract (411.4 mg) with indications of elution conditions and yields of each fraction. Fractions marked with an asterisk (*) were assayed with mice. V_b, bed volume of column.

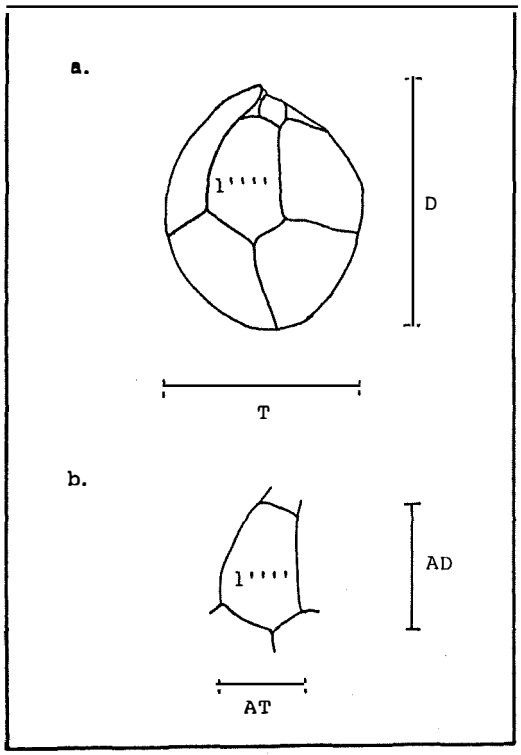

Figure 3a-b. Ostreopsis a. Antapical view of hypotheca, D = dorsoventral diameter, T = transdiameter. Dorsoventral-transdiameter ratio = D:T ratio. b. Antapical plate, AD = antapical dorsoventral diameter, AT = antapical transdiameter. Antapical plate dorsoventral-transdiameter ratio = AD:AT ratio.

Approximately 40 MU of the original 70 MU of toxin was recovered from 4.3 mg of methanol extract which was insoluble in chloroform. A portion of this material (1.4 mg, 14 MU) was dissolved in distilled water and extracted 3 times with butanol. The butanol fraction was non-toxic and all toxin was recovered from the aqueous phase.

Signs displayed by mice following injection of the toxic fractions included; progressive paralysis (from hind to fore limbs), diarrhoea (occasional), tremors, piloerection, reduced body temperature and mild convulsions preceding death by 4 to 7 minutes.

Dinoflagellate morphology

Cultured cells were compared with wild O. siamensis cells from Heron (figure 4a) and Lady Elliot Islands. The only morphological characteristic in which Heron and Lady Elliot Islands cells differed from those reported by Fukuyo (1981) for O. siamensis from the Ryukyu Islands, Japan was the larger size of Queensland cells (table 1).

Cultures grown using f_2 nutrient medium produced morphologically aberrant cells with "lumpy" irregular shapes (figure 4b). These teratogenous cells were grown in cultures for over a year without apparent loss of culture viability. When grown in the lower nutrient f_{10} medium, the cultures produced broadly ovate cell shapes typical of Ostreopsis (figures 4c-f). These latter cells were used for species identification. A few teratogenous cells still occurred in the f_{10} cultures.

Table 1. Size and shape of wild O. siamensis and cultured Ostreopsis cells.

Origin of Cells	Dorsoventral Diameter (µm)	Transdiameter (µm)	Length (µm)	DTR[1]	ADTR[2]
Culture 54 µE $m^{-2}s^{-1}$	41 ± 3.0[3] (35 - 48)[4] n = 65[5]	31 ± 3.6 (25 - 44) n = 65	27 ± 3.8 (22 - 36) n = 19	1.3 ± 0.2 (1.1 - 1.7) n = 65	1.5 ± 0.2 (1.2 - 1.9) n = 28
Culture 90 µE $m^{-2}s^{-1}$	46 ± 4.6 (36 - 60) n = 65	35 ± 4.8 (26 - 51) n = 65	nd[6]	1.3 ± 0.1 (1.0 - 1.8) n = 65	1.6 ± 0.2 (1.3 - 2.0) n = 14
Heron Is. O. siamensis	102 ± 7.7 (88 - 115) n = 20	76 ± 9.2 (64 - 93) n = 20	nd	1.4 ± 0.1 (1.2 - 1.6) n = 20	1.6 ± 0.3 (1.3 - 2.1) n = 7
Lady Elliot Is. O. siamensis	103 ± 8.0 (90 - 115) n = 20	92 ± 7.1 (76 - 101) n = 20	nd	1.1 ± 0.1 (0.9 - 1.4) n = 20	1.6 ± 0.2 (1.1 - 2.0) n = 16

1 DTR = dorsoventral-transdiameter ratio
2 ADTR = antapical plate dorsoventral-transdiameter ratio
3 Mean ± standard deviation
4 Range
5 n = sample size
6 nd = no data

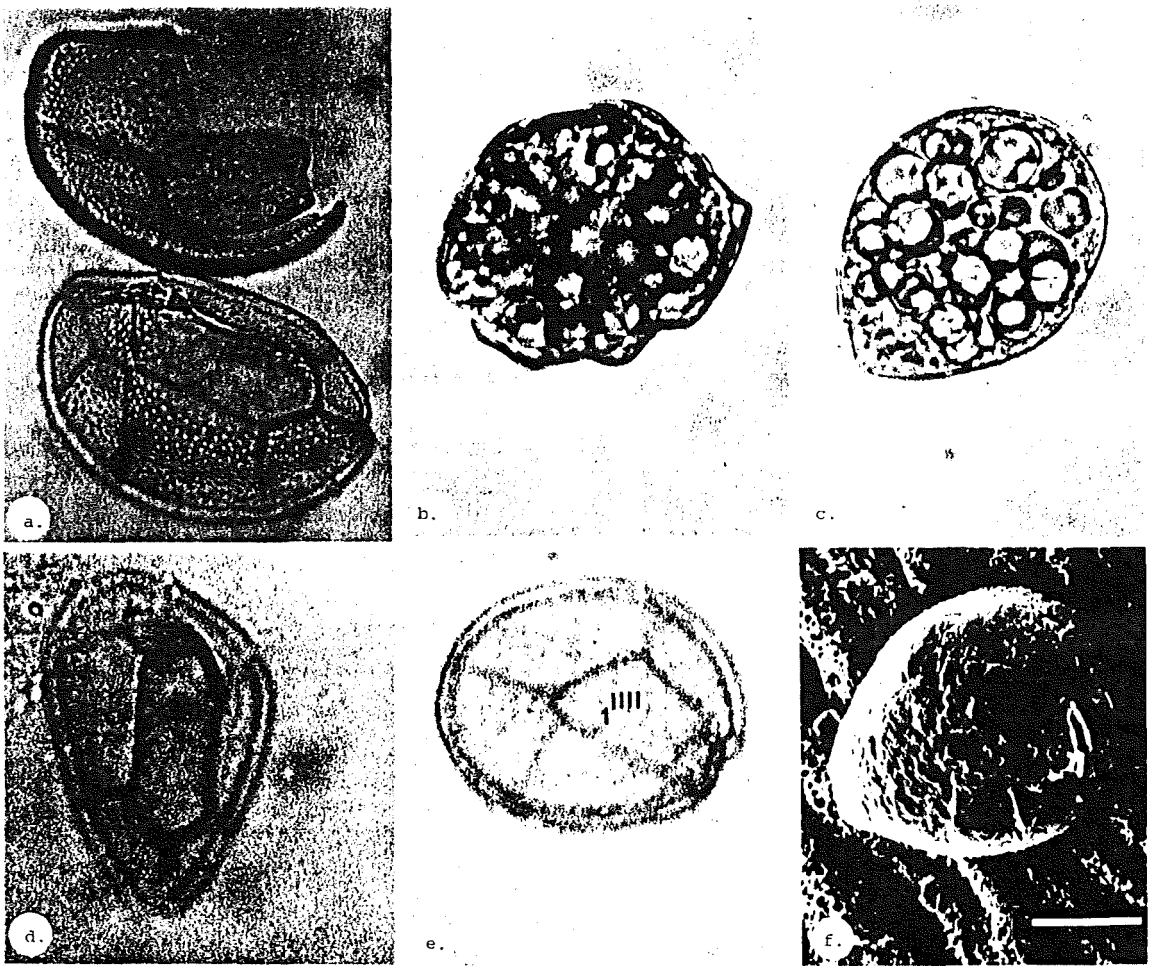

Figure 4a-f. Ostreopsis **a.** Epitheca and partial hypotheca of O. siamensis from Heron Island, 1^{1} = first apical plate, 1^{1111} = antapical plate. **b.** Teratogenous cell from f_2 culture. **c-f.** Cells from f_{10} culture **c.** Whole cell **d.** Epitheca, 1^{1} = first apical plate **e.** Hypotheca, 1^{1111} = antapical plate **f.** Scanning electron micrograph, scale = 10 µm.

Cells grown in f_{10} culture medium had a thecal plate formula identical to that described by Fukuyo (1981) for Ostreopsis. The absence of minute thecal pores between the trichocyst pores and the presence of hexagonally shaped first apical plates (1') differentiated these cultured cells from O. lenticularis and O. heptagona (Fukuyo 1981, Norris et al. 1985). The trichocyst pores of cultured cells were visible at a magnification of 400X but not at 200X, unlike those from wild O. siamensis which were visible at 200X.

The sizes and ratios of cell diameters and antapical plate diameters of wild O. siamensis and cultured cells are presented in table 1. Cultured cells were significantly smaller than Heron Island and Lady Elliot Island cells ($P < 0.001$). Cultured cells were significantly larger when grown under a PPFD of 90 $\mu Em^{-2}s^{-1}$ compared with 54 $\mu Em^{-2}s^{-1}$ ($P < 0.001$). There were no significant differences between the dorsoventral-transdiameter ratios of cells grown under either light regime. The dorsoventral-transdiameter ratios of cultured cells and wild O. siamensis ranged from 1.0 to 1.8 and 0.9 to 1.6, respectively. There were no significant differences between the antapical plate dorsoventral-transdiameter ratios of wild and cultured cells. The range of this latter ratio was between 1.1 and 2.1.

DISCUSSION

The absence of ciguatoxin, or ciguatoxin-like toxins from cultures of the Ostreopsis species examined in this study indicate that this species is unlikely to be directly involved in the aetiology of ciguatera in the Hervey Bay region. The only toxin so far found from the flesh of fishes which produce ciguatera in Queensland is ciguatoxin, although a less polar, ciguatoxin-like toxin was extracted from the viscera of toxic barracuda (Sphyraena jello) from Hervey Bay (Lewis & Endean 1983, 1984). Water-soluble toxins, primarily maitotoxin, have been found in the gut contents of herbivorous fishes (Yasumoto et al. 1971, Bagnis et al. 1985). However, there is no

evidence for the bioaccumulation of these water-soluble toxins in the flesh of fishes.

Water-soluble toxins have also been extracted from O. siamensis and O. lenticularis but not from O. ovata (Nakajima et al. 1981, Besada et al. 1982, Bagnis et al. 1985). The water-soluble toxin found in the present study was of similiar polarity to the water-soluble toxin extracted from cultures of O. siamensis from Okinawa (Nakajima et al. 1981). However, the Queensland cultures were at least an order of magnitude less toxic per cell. The higher polarity of these water-soluble toxins clearly distinguishes them from maitotoxin.

The morphological characteristics of cultured Queensland cells were inconsistent with either typical O. siamensis or O. ovata. These latter taxa are similiar except for cell shape and size (Fukuyo 1981). The Ostreopsis cells grown in culture had a broadly ovate cell shape, similiar to O. siamensis and a relatively small cell size, similiar to O. ovata. In culture, cell shape (as reflected by the dorsoventral-transdiameter ratio) was a stable characteristic unaltered by changes in light regime. The dorsoventral-transdiameter ratio ranges of O. siamensis and O. ovata are 1.0 to 1.5 and 1.5 to 2.0, respectively (Yasumoto et al. 1980b, Fukuyo 1981). Therefore, the dorsoventral-transdiameter ratios of cultured and wild cells from Queensland overlapped the reported range for O. siamensis. The size of dinoflagellates in culture can vary with light (present study, Swift & Meunier 1976, Prezelin & Matlick 1983, Yentsch et al. 1985), temperature (Meeson & Sweeney 1982), nutrients and growth phase (Anderson & Lindquist 1985). Norris et al. (1985) found that the dorsoventral diameters of O. heptagona grown in culture were about 70% of those of wild cells. If a similiar reduction in size had occurred in culture for Queensland cells then the original cell sizes would have been similiar to those reported for O. siamensis. The small trichocyst pores observed for cultured cells were typical of O. ovata. However, the small size could have been a function of small cell and thecal plate sizes.

Norris et al. (1985) reported differences in the relative dimensions of antapical plates of Ostreopsis from the Caribbean. They found antapical plate dorsoventral-transdiameter ratio ranges for O. ovata and O. siamensis of 1.2 to 2.0 and 2.5 to 3.0, respectively. The similar ratios of wild O. siamensis and cultured Ostreopsis from Queensland support assignment of these cells to one species. However, the antapical plate dorsoventral-transdiameter ratios of Queensland cells overlapped the reported range for O. ovata, even though Heron Island and Lady Elliot Island cells were clearly O. siamensis. Therefore, the reported antapical plate ratios of Ostreopsis species from the Caribbean are unlikely to be useful for separation of Ostreopsis species from the Pacific basin.

Dinoflagellate toxin profiles may be useful as chemotaxonomic indicators (Boyer et al. 1986). Nakajima et al. (1981) suggested that while the morphological variation between O. siamensis and O. ovata is not great, the distinctive differences in toxin composition support species separation. Therefore, on the basis of cell shape and the production of a water-soluble toxin, the dinoflagellate cultured in this study was identified as O. siamensis.

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APPENDIX 10.4

Proceedings of the 6th International Coral Reef Symposium, Australia, 1988, Vol. 3

TOXICITY OF LIPID-SOLUBLE EXTRACTS FROM DEMERSAL FISHES AT FLINDERS REEF, SOUTHERN QUEENSLAND

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ABSTRACT

In a previous study, biodetritus from Flinders Reef contained large numbers of G. toxicus but did not contain detectable levels of ciguatoxin. The existence of apparently non-ciguatoxin-producing blooms of G. toxicus at Flinders Reef provided an opportunity to examine whether any precursors produced by the dinoflagellate would be converted to ciguatoxin by fishes. The livers from four herbivorous and four carnivorous species were pooled separately and assayed using mice. No detectable levels of ciguatoxin were found in these fish species. Visceral samples (including gut and visceral content) of two of the herbivorous species showed the presence of a low polarity toxin which also appeared in the liver of these species. Other toxins, some resembling ciguatoxin, were also found in the viscera of several of the herbivorous species. These toxins differed from ciguatoxin in that they did not accumulate in the livers of these fishes. These results indicate that G. toxicus from Flinders Reef does not produce a precursor that can be converted in fish to ciguatoxin.

INTRODUCTION

Ciguatera is a disease that can result from consumption of many species of tropical and subtropical fish species (Gillespie et al. 1986). These otherwise edible fishes become toxic following accumulation of lipid-soluble toxins, principally ciguatoxin (Scheuer et al. 1967, Lewis and Endean 1983, 1984), through the marine food web (Randall 1958, Yasumoto et al. 1971, Banner 1974, Gillespie 1987). Early studies showed that ciguatoxin could be accumulated in fish unaltered (Helfrich and Banner 1963) and that once accumulated, ciguatoxin would persist in fish for at least 2.5 years (Banner et al. 1966).

The origin of ciguatoxin remained a mystery until a report by Yasumoto et al. (1977a) suggested the involvement of a dinoflagellate found attached to macroscopic algae in French Polynesian waters. The dinoflagellate was later identified as Gambierdiscus toxicus (Adachi and Fukuyo 1979) and has subsequently been reported from all ciguatera-endemic regions, including Australia (Gillespie et al. 1985a). Bagnis et al. (1980) reported the production of ciguatoxin or ciguatoxin-like compounds by cultures of G. toxicus. However, other attempts to obtain ciguatoxin from culture have been unsuccessful (Yasumoto et al. 1980, Holmes et al. 1988). In addition, one study has failed to detect ciguatoxin from a sample of reef biodetritus collected during a bloom of G. toxicus (Gillespie et al. 1985b). These results cast doubt on G. toxicus being the producer of ciguatoxin. Alternatively, G. toxicus may require a special set of circumstances before elaborating ciguatoxin. Apparently these circumstances do not always prevail in the wild and have not been mimicked in the laboratory. A hypothesis for the induction of a ciguatera outbreak was proposed by Gillespie et al. (1985b) to take account of the findings described above. These authors postulated the involvement of an as yet unidentified "ciguatoxin-inducing factor".

In this paper we investigate the possibility that precursors of ciguatoxin are produced by G. toxicus and that these precursors are converted to ciguatoxin as they pass through fishes. The existence of apparently non-ciguatoxin-producing blooms of G. toxicus at Flinders Reef (Gillespie et al. 1985b) provided the opportunity to test this hypothesis. Pooled livers of each species of fish were assayed for ciguatoxin as this tissue accumulates the highest concentrations of ciguatoxin (Yasumoto et al. 1971, Lewis and Endean 1984, Vernoux et al. 1985). The viscera of the herbivorous species were also assayed to determine the composition of lipid-soluble toxins entering through their diet.

MATERIALS AND METHODS

Fish collection

Specimens were spearfished from Flinders Reef, southern Queensland (latitude 26.6°S, longitude 153.5°E) in the winter of 1986. Larger specimens of each species were selected out. Sufficient numbers were collected to obtain approximately 50 g of liver from each species. Liver and viscera (including the visceral content) were dissected free, pooled for each species, and the weighed samples stored at -20°C prior to extraction.

Extraction

Liver and viscera were extracted twice with acetone (3:1, v:w) in a blender and the ether fraction obtained as outlined in figure 1. Ether extracts contain the ciguatoxin and a large proportion of the maitotoxin present in the original sample (Holmes et al. 1988). To separate these different polarity toxins the ether fractions were further fractionated on silicic acid columns (Bio-sil A 200-400 mesh, Bio-Rad, California) using 30 g of silicic acid for each 1 g of extract. Chloroform, chloroform-methanol (9:1) and methanol fractions were eluted (5 bed

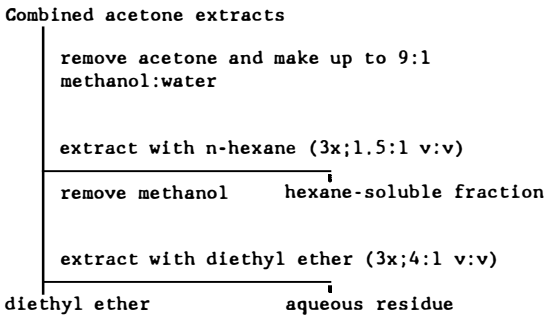

Figure 1. Scheme used to isolate the ether fraction from liver and viscera.

volume of each eluant), dried under vacuum and a stream of N_2, weighed and activity assayed with mice. A.R. grade reagents were used throughout.

Bioassay

Fractions suspended in 0.5 ml 5% Tween 60 saline were assayed by i.p. injection into 20 ± 3 g Quackenbush mice of either sex. Doses < 1 g/kg (i.e. < 20 mg of extract per 20 g mouse) only were administered because doses > 1 g/kg can produce a non-specific toxic reaction (Lewis, unpublished observations). Duplicate assays of each fraction were made unless a fraction contained < 20 mg, in which case the total fraction was injected into a mouse. The response of the mouse and the time to death were recorded for each fraction. For comparison, the response of mice to authentic ciguatoxin and maitotoxin in our laboratory is given in table 1. Degrees of paralysis in mice were not differentiated in this study.

Table 1. Response of mice to ciguatoxin and maitotoxin[1].

Ciguatoxin	Maitotoxin
reduced activity and body temperature	reduced activity and body temperature
copious diarrhoea[2]	no copious diarrhoea
dyspneoa	dyspneoa
lachrymation[2]	slight lachrymation[2]
slight hind-limb paralysis	hind-limb progressing to fore-limb paralysis
hypersalivation[2]	no hypersalivation
death-times from 36 min to 24 hr[3]	death-times from 65 min to 72 hr[3]
cause of death is respiratory failure	cause of death is respiratory failure

1 Responses obtained after i.p. injection of authentic ciguatoxin and maitotoxin.
2 These signs not displayed by every mouse.
3 Death-times are inversely related to dosage.

RESULTS

The fishes collected included herbivorous, omnivorous and carnivorous species (table 2). Table 3 shows the numbers, total weight and weight of pooled livers of each species, as well as the yield and toxicity of ether fractions and subfractions. Toxicity was not detected in the crude ether fractions from any of the species. Further purification by silicic acid chromatography yielded toxin activity in fractions of Siganus spinus and Prionurus microlepidotus which eluted with chloroform. Mice injected with these fractions displayed signs reminiscent of maitotoxin activity rather than ciguatoxin activity (see table 1). Ciguatoxin is typically eluted from silicic acid with chloroform-methanol 9:1 (Lewis and Endean 1983, 1984). The toxic fraction from Orectolobus maculatus that eluted with chloroform-methanol 9:1 did not contain a lethal dose. Consequently, this toxin could not be definitively characterised as having either ciguatoxin- or maitotoxin-type activity. The sensitivity of the mouse assay was such that the livers of all species tested could have contained at most one mouse unit (one mouse unit is the dose of extract required to kill a 20 g mouse) of ciguatoxin per 30 g of extracted tissue.

Table 2. Fish species collected from Flinders Reef and their presumed diet.[1]

Species	Diet
Siganus spinus	herbivorous/omnivorous
Prionurus microlepidotus	herbivorous
Orectolobus maculatus	carnivorous
Acanthurus nigrofuscus	herbivorous
Melychthys vidua	carnivorous
Abudefduf melas	herbivorous/omnivorous
Priacanthus macracanthus	carnivorous
Cheilodactylus gibbosus	carnivorous

1 Based on observations of gut contents.

The viscera of the herbivorous fish species (S. spinus, P. microlepidotus, Acanthurus nigrofuscus and Abudefduf melas) were extracted and assayed for the presence of lipid-soluble toxins. As indicated in table 4, toxic activity reminiscent of maitotoxin was detected at 1 g/kg dose in the ether fractions of S. spinus and A. melas.

Further purification of these ether fractions (except A. melas) by silicic acid chromatography yielded detectable amounts of maitotoxin-type activity at 1 g/kg dose in (a) the chloroform fraction from S. spinus and P. microlepidotus, (b) the chloroform-methanol 9:1 fraction from A. nigrofuscus and (c) the methanol fraction from S. spinus (table 5). The chloroform-methanol 9:1 fraction from P. microlepidotus induced a ciguatoxin-like response in mice (table 5). However, this fraction was not sufficiently pure to be lethal to mice at 1 g/kg making characterisation difficult. This fraction contained approximately 70 mouse units of toxin assuming that a toxic, non-lethal response in mice follows injection of 0.5 mouse units of toxin. The lethal fractions with maitotoxin-type activity from the viscera of S. spinus, P. microlepidotus

Table 3. Yield and toxicity[1] of lipid-soluble extracts from livers of fishes from Flinders Reef.

Species	Number	Fish (kg)	Liver (g)	Ether fraction[1] (mg)	Fractions (mg)[1,2] c	c-m	m
S. spinus	18	4.12	50	236	3.5[3]	42	11
P. microlepidotus	4	11.23	70	436	87[3]	68	174
O. maculatus	2	11.86	340	1470	500	21[3]	303
A. nigrofuscus	42	3.65	60	280	31	43	88
M. vidua	9	1.72	80	295	48	22	186
A. melas	6	2.29	41	322	0.1[4]	44	212
P. macracanthus	9	2.41	54	221	23	30	5
C. gibbosus	25	7.33	78	782	166	102	440

1 Fractions assayed for toxicity in mice. Fractions non-toxic at 1 g/kg unless otherwise indicated.
2 Ether fraction separated on silicic acid columns into a chloroform (c), chloroform-methanol 9:1 (c-m) and methanol (m) fractions.
3 Fractions toxic to mice at 1 g/kg.
4 Not tested for lethality to mice.

and A. nigrofuscus contained approximately 100, 60 and 36 mouse units of toxin, respectively.

DISCUSSION

The livers of herbivorous, omnivorous and carnivorous fishes from Flinders Reef did not contain detectable levels of ciguatoxin. This result supports our previous study which did not detect ciguatoxin in biodetritus containing G. toxicus from Flinders Reef (Gillespie et al. 1985a). Absence of ciguatoxin in fishes from a reef where annual blooms of G. toxicus occur (Gillespie et al. 1985b) indicates that G. toxicus on this reef does not produce precursors convertible to ciguatoxin by the fish species studied. G. toxicus from Flinders Reef does produce large amounts of the water-soluble toxin, maitotoxin (Gillespie et al. 1985a). It would appear that fishes do not utilise maitotoxin as a precursor for ciguatoxin.

Low polarity toxins producing maitotoxin-type effects in mice were found in the livers of the herbivorous fish species S. spinus and P. microlepidotus. This type of toxic material appeared in larger quantity in the viscera of these species. The viscera of A. nigrofuscus contained maitotoxin-type material that was eluted with chloroform-methanol 9:1. It is possible that this toxic material represents metabolised forms of maitotoxin which retain toxicity but are converted to less polar forms. Less polar forms of maitotoxin have not been detected in biodetritus containing G. toxicus (Yasumoto et al. 1977a). S. spinus contained low levels of a toxin whose polarity and effects on mice coincided with maitotoxin.

The viscera of P. microlepidotus contained moderate levels of toxic material that resembled ciguatoxin in both polarity and effects on mice. However, no detectable ciguatoxin appeared in the liver of this species suggesting that the toxin in the viscera of P. microlepidotus was not

Table 4. Yield (g) and response of mice to ether extracts of fish viscera.

Species	Viscera (g)	Ether Extract (g)	Response[1]
S. spinus	600	4.39	MTX
P. microlepidotus	800	4.31	---
A. nigrofuscus	130	2.96	NT
A. melas	160	0.31	MTX

1 Maitotoxin (MTX) type responses and no response (NT) were recorded after injection of 1 g/kg of extract.

Table 5. Yield (mg) and response of mice to ether extracts of viscera fractionated by silicic acid chromatography.[1]

Species	Response[2] c (mg)	c-m (mg)	m (mg)
S. spinus	MTX[3] (2039)	NT (2151)	MTX (113)
P. microlepidotus	MTX[3] (1184)	CTX (2702)	NT (288)
A. nigrofuscus	NT (283)	MTX[3] (724)	NT (277)

1 See table 3, footnote 2 for details.
2 Maitotoxin (MTX) and ciguatoxin (CTX) type response and no response (NT) at 1 g/kg.
3 Lethal at 1 g/kg, other toxic fractions not lethal at 1 g/kg.

ciguatoxin. Ciguatoxin is well known for its ability to accumulate in the liver of fishes (Yasumoto et al. 1971, Lewis and Endean 1984, Vernoux et al. 1985). The presence of toxic material of similar polarity to ciguatoxin in the viscera of fishes that does not accumulate as ciguatoxin adds further complexity to studies on the origin(s) of ciguatoxin.

In contrast to the results of this study, ciguatoxin is easily detectable in the livers of herbivorous, omnivorous and carnivorous fishes from ciguatera-endemic regions (Yasumoto et al. 1971, Yasumoto et al. 1977b). Absence of ciguatoxin in the livers of fishes from Flinders Reef correlates with the absence of ciguatera in the area (Lewis et al. 1987) despite numbers of potentially ciguateric table-fish being caught from Flinders Reef each year. Previous studies in the Pacific have correlated G. toxicus population densities with ciguatera endemicity (Yasumoto et al. 1979, Bagnis et al. 1985). However, the toxin content of dinoflagellates producing paralytic shellfish toxins varies with time, location and latitude (Oshima et al. 1982, Maranda et al. 1985, White 1986). Flinders Reef is the most southerly report for G. toxicus (Gillespie et al. 1985b). The absence of ciguatoxin production by this population of G. toxicus may indicate that toxin production by this species also varies with latitude or location. Alternatively, organisms other than G. toxicus may have a role in the production of ciguatoxin.

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POSSIBLE ORIGINS OF CIGUATERA

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◄ Scanning electron micrograph of *Gambierdiscus toxicus* in biodetritus. This dinoflagellate may elaborate ciguatoxin in fishes.

Ciguatera is a type of poisoning associated with eating coral reef fish. In areas where it occurs, only occasional fish cause human intoxication, even amongst those fish species regarded as being especially dangerous, such as the Red Bass, *Lutjanus bohar*. The unpredictability of ciguatera, together with the lack of a simple reliable method for detecting toxic fish, has made it difficult to correlate ciguatera outbreaks with observed physical or biological changes on a reef. Nevertheless, in the islands of the Pacific, where people live in close association with the sea and coral reefs, specific events, such as the wreckage of a ship, the dumping of rubbish and war damage (Cooper 1964) have been connected with the appearance of toxic fish. However, it is more difficult to correlate the impact of reef changes with ciguatera in Australia, as the main reef fishing areas and coral reefs occur some distance offshore and fishing activities are more diffuse.

Ciguatera in Australia has largely resulted from eating fish caught near Gove and Groote Eylandt areas in the Northern Territory, on sections of the Great Barrier Reef between Bowen and Port Douglas in north Queensland, as well as Hervey Bay in southern Queensland (Gillespie, this publication). In these areas, a number of theories on the cause of ciguatera have been developed by members of the public. For example, commercial fishermen argue that the toxin permeates into the flesh from decomposing viscera when the processing of fish is delayed. In support of this view, they point to the number of cases involving fish caught by recreational fishermen who use 'inferior' handling practices. In the Cairns area, the removal of the section of the fillet comprising the intestinal wall is a common precaution used to minimise the risk of poisoning. However, there is no evidence linking handling practices with toxicity and there have been many instances where fish frozen immediately after being caught have been found to have toxic flesh.

Another common view is that the toxin originates in 'reef scum', or 'coral spawn', terms applied to massive red to brown floating rafts of a bluegreen algae, *Trichodesmium*, which appear along the Queensland coast during spring and summer. However, this phenomenon is so

widespread that it is unlikely to be the cause of ciguatera, which occurs only sporadically.

In southern Queensland, game fishermen have occasionally reported catching Narrow-barred Spanish Mackerel (*Scomberomorus commersoni*) which had been feeding extensively on puffer fishes. Consequently, a view commonly expressed in these circles has been that the toxin involved in cases of intoxication resulting from consumption of this species was tetrodotoxin, a common toxin of puffer fishes (family *Tetrodontidae*). However, Lewis and Endean (1983) have shown that the toxin of *S. commersoni* involved in cases of human intoxication in southern Queensland was identical to ciguatoxin, the principal toxin responsible for the symptoms of ciguatera (Scheuer *et al.* 1967). While the toxins present in coral reef fish that have been responsible for cases of presumed ciguatera in other areas of Australia have not been studied in detail, the reported symptoms (Tonge *et al.* 1967; Broadbent 1968; Gillespie, this publication) indicate that the syndrome as it occurs in Australia, is almost identical to ciguatera as described elsewhere in the Pacific area (Bagnis *et al.* 1980; Narayan 1980; Anderson *et al.* 1983).

Over the past 25 years, considerable advances have been made towards a full understanding of the source of ciguatoxin in coral reef ecosystems. It has been confirmed that ciguatoxin is elaborated at the base of the food chain and transmitted via herbivorous fishes to higher order carnivores (Randall 1958; Bagnis 1969; Yasumoto *et al.* 1971). There is evidence that a dinoflagellate, *Gambierdiscus toxicus*, may be an elaborator of ciguatoxin (Yasumoto *et al.* 1977) and that variations in the toxicity of fishes from different areas correlate with population densities of this organism (Bagnis *et al.* 1985).

This paper briefly reviews the evidence for current theories on the mechanism of ciguatera and examines the results of recent studies on *G. toxicus* in Australia with a view to understanding some of the factors which may lead to the occurrence of ciguatoxin in fish in these waters.

THE FOOD CHAIN THEORY

Numerous hypotheses have been proposed in the past to explain how fishes become toxic, but that which is now widely accepted is that ciguatoxin arises at the bottom of the food chain on coral reefs and is transferred to higher carnivores via their food. Captain Cook was one of the first to adopt this idea and in the journal of his second voyage to the Pacific in 1772–1775, suggested that the fish which poisoned his crew may have become toxic after eating 'poisonous plants'. Dawson *et al.* (1953) favoured benthic algae as a source of toxin after finding algae in the stomach contents of many poisonous fishes.

Randall (1958) examined the food habitats of a wide range of toxic fish and found that they were all tied to benthic life of coral reefs through the food chain. By observing the food habits of commonly toxic herbivores, in particular the Bristle-toothed Surgeonfish, *Ctenochaetus striatus*, a benthic detritivore, he concluded that the originator of the toxin must be a fine benthic algae. In addition, Randall suggested that larger carnivores obtain the toxin by feeding on herbivorous and detritivorous fish species and that they may actually accumulate the toxin.

Evidence to support this theory soon became available from both field and laboratory investigations. A sudden rise in fish toxicity on Hao Atoll in the Tuamotu Archipelago following the commencement of harbour and other works in 1965, was documented by Bagnis (1969). The first fishes to become toxic were herbivorous and detrital feeders and it was not until two years after the beginning of the outbreak that the first case involving a carnivorous fish was recorded. In addition, it has been shown that a normally non-toxic omnivore could be rendered lethally toxic by daily feeding of small amounts of toxic flesh (Helfrich and Banner 1963). Banner *et al.* (1966) also demonstrated that ciguatoxin was not dissimilated by *Lutjanus bohar* even after 30 months in captivity, suggesting that the toxin could accumulate if ingested regularly by this species.

Randall's observations on the role played by the Surgeonfish, *Ctenochaetus striatus*, in the accumulation of ciguatoxin in the food chain in the Society Islands were confirmed by Yasumoto *et al.* (1971), who isolated ciguatoxin from the flesh, liver and stomach contents of this species. An additional water soluble toxin was also found in the liver and stomach contents which was later named maitotoxin after the Tahitian

name for surgeonfish (Yasumoto *et al.* 1976). Because of the limitations of the feeding apparatus of *C. striatus*, which feeds primarily on fine detrital matter, the causative organism was thought to be associated with detritus.

THE ROLE OF DINOFLAGELLATES

Dinoflagellates are photosynthetic organisms which often produce blooms which may discolour the sea. These blooms are then known as red tides. Some species of dinoflagellates produce potent toxins which find their way through the food chain to man. Included amongst these are *Protogonyaulax* spp. which cause outbreaks of paralytic shellfish poisoning as well as *Dinophysis* spp. which are responsible for diarrhetic shellfish poisoning. It is now believed that a dinoflagellate may also be responsible for producing ciguatoxin.

Yasumoto *et al.* (1977a) found large numbers of a species of dinoflagellate in a toxic sample of detritus collected in the Gambier Islands during a massive ciguatera outbreak in that area. This organism did not swim free in the water column but remained attached to surfaces and was subsequently named *Gambierdiscus toxicus* Adachi and Fukuyo (Adachi and Fukuyo 1979). Because these detrital samples contained both ciguatoxin and maitotoxin and they consisted primarily of *G. toxicus* cells it was presumed that this organism was the primary source of ciguatoxin.

The evidence linking *G. toxicus* to ciguatoxin production is powerful but not totally conclusive. A number of studies have been reported in which both ciguatoxin and maitotoxin have been found in samples of detritus containing *G. toxicus* in areas where ciguatoxin is endemic. Moreover, the population density of *G. toxicus* has been found to correlate well with the pattern of ciguatoxin endemicity (Yasumoto *et al.* 1979) and fish toxicity in French Polynesia (Bagnis *et al.* 1985). However, when the organism is grown in laboratory culture, it does not produce detectable ciguatoxin (Tindall *et al.* 1984; Yasumoto *et al.* 1979; Gillespie, unpublished observations), suggesting that ciguatoxin production is either a genetically unstable character or is a response to specific environmental conditions which are not easily duplicated in artificial culture.

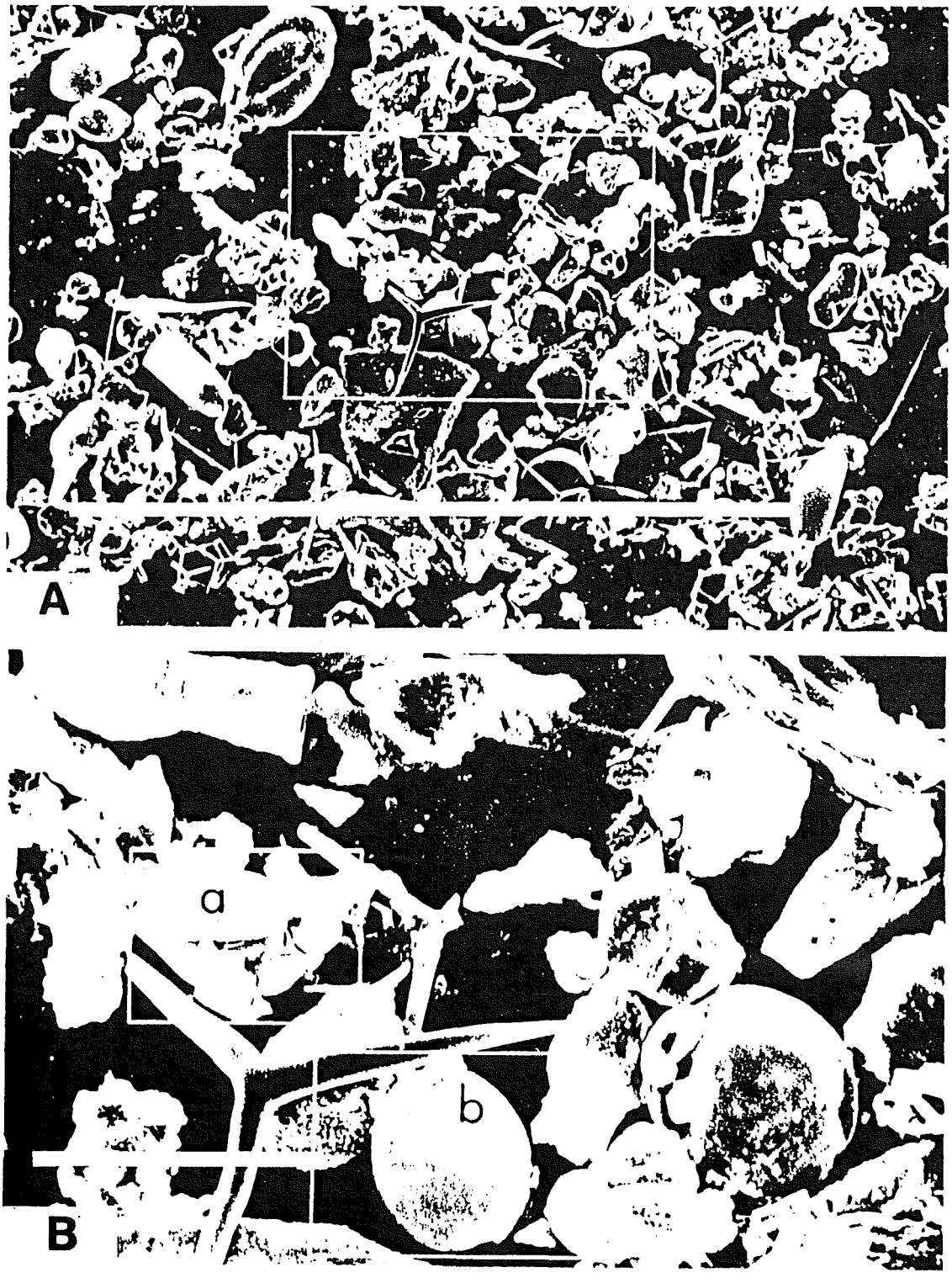
Other species of dinoflatellates found in detrital and algal samples from coral reefs may also play a role in contributing toxin to the food chain. *Ostreopsis siamensis, Prorocentrum lima* and *P. concavum* have all been found to produce ether-soluble toxins active against mice (Nakajima *et al.* 1981; Yasumoto *et al.* 1980). *P. lima* is now known to produce okadaic acid, a polyether compound very similar to ciguatoxin in chromatographic properties but with much less toxicity (Murakami *et al.* 1982). *Ostreopsis lenticularis* has also been reported to produce potent toxins (Ballantine *et al.* 1985).

In the Pacific Ocean, however, *G. toxicus* is generally the predominant bethic dinoflagellate found on coral reefs. *G. toxicus* is a large (dorsoventral diameter 45–150 μm) photosynthetic organism with a flattened lenticular shape (Adachi and Fukuyo 1979). It has a cellulose theca consisting of plates arranged in a pattern typical of the family *Heteraulaceae* (Fukuyo 1981). *G. toxicus* seems primarly an epiphyte of tropical and temperate benthic macroalgae but is also found on dead coral. It attaches by means by a mucous thread to benthic surfaces (Taylor 1979) and has only rarely been found floating in the plankton (Fukuyo 1981). Although vitamin B_{12} is essential for growth and laboratory cultures are stimulated by phosphate enrichment (Yasumoto *et al.* 1984), field studies have not revealed environmental or nutritional factors affecting population of *G. toxicus* (Yasumoto *et al.* 1980). However, strong light and low salinities act as deterrent factors (Yasumoto *et al.* 1980).

G. toxicus is known to have a wide distribution in coral reef areas. Its occurrence, in varying population densities, has been demonstrated in French Polynesia, New Caledonia, Guam, Hawaii, Okinawa, Fiji (Yasumoto *et al.* 1984), Philippines (Gillespie, unpublished data), Kirabati (Tebano 1984) and Australia (Gillespie *et al.* 1985a) in the Pacific Ocean. It has also been found in the Atlantic coastal waters of southeastern Florida (Besada *et al.* 1982) and the eastern Caribbean region (Carlson 1984; Taylor 1985; Bagnis 1979; Ballantine *et al.* 1985).

G. TOXICUS IN AUSTRALIA

Gillespie *et al.* (1985a) have examined the population density of *G. toxicus* and other benthic dinoflagellates occurring on reefs of the Great Barrier Reef (particularly those in the


A
a
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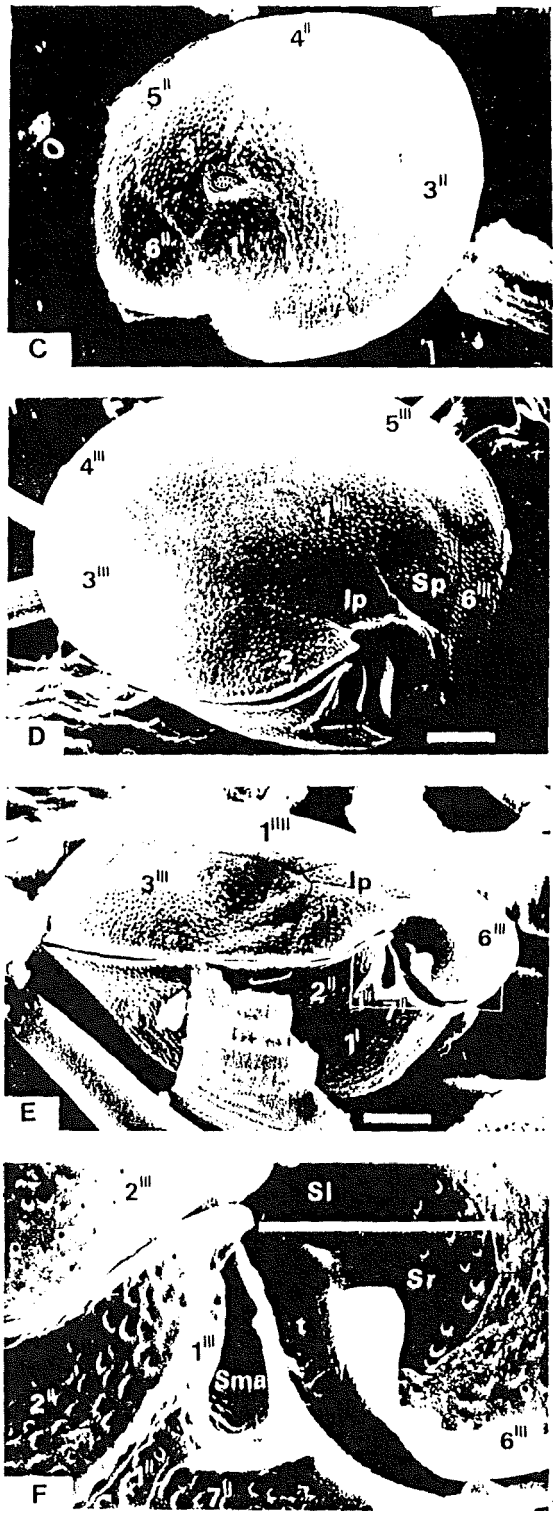
B

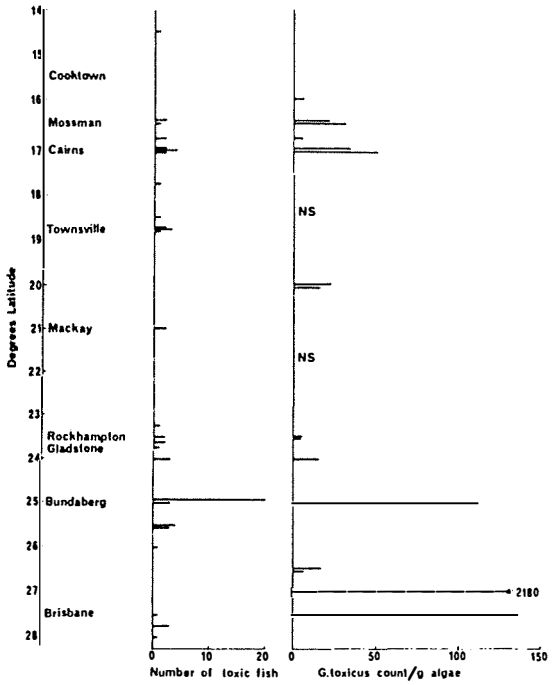
Cairns area and the Capricorn-Bunker Group) as well as inshore and fringing reefs in northern and southern Queensland. *G. toxicus* was found to have a wide general distribution in almost all coral situations examined along the Queensland coast. However, population densities were usually quite low. Greater than 80% of all samples taken had a population density of less than 5 cells/g of macroalgal substrate. *G. toxicus* was the most common benthic dinoflagellate associated with macroalgae on coral reefs in Queensland except on intertidal reef flats where *Ostreopsis* spp. tended to co-occur with it.

Several authors have implicated certain macroalgae as favoured substrates, including *Turbinaria*, *Jania* (Yasumoto *et al.* 1980), *Spyridia* (Taylor 1986) and *Chaetomorpha* (Carlson *et al.* 1984). However, Gillespie *et al.* (1985a) found occasional high population densities on almost all macroalgal species sampled along the coast of Queensland and regarded the organism to be opportunistic in regard to macroalgal substrate.

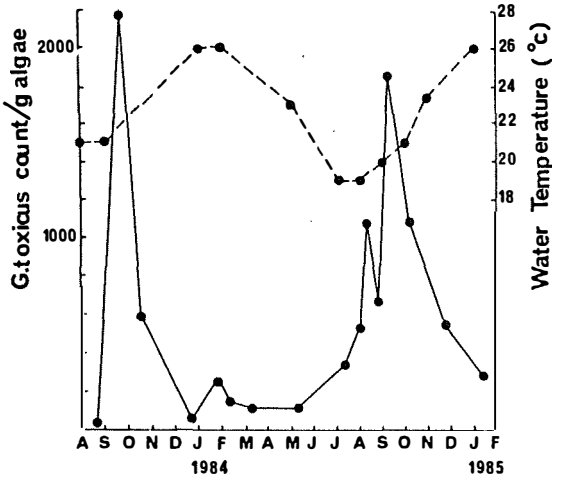
It is useful to examine whether population densities of *G. toxicus* in Queensland reflect the occurrence of toxic fish, in areas for which this data is available. In this case it is preferable to use the 'highest values' of *G. toxicus* population rather than 'mean values', principally because the sampling procedure, which involves the sampling of only foliose macroalgae, is rather biased. The associated figure shows that there is some correlation between highest *G. toxicus* populations and the number of toxic fish reported at various locations along the Queensland coast. Greatest population densities of *G. toxicus* were found in southern Queensland, reflecting the high ciguatera incidence in the Hervey Bay area (25° latitude). It is possible that reefs south of Hervey Bay also contribute to the problem as most cases reported in this area involve pelagic fish species, particularly *Scomberomorus commersoni*, which undertakes a northward spawning migration.



Scanning electron micrographs of *G. toxicus* in biodetritus from Flinders Reef in southern Queensland taken by R. Lewis from material supplied by N. Gillespie. Thecal plates are marked according to Adachi and Fukuyo (1979). **A**. Biodetritus sample (Bar length 1 mm); **B**. *G. toxicus* cells (a & b) mixed with detrital material (Bar length 100 μm); **C**. Epitheca of *G. toxicus* showing fish hook shaped apical pore (Bar length 10 μm); **D**. Hypotheca of *G. toxicus* (Bar length 10 μm); **E**. Ventral view (Bar length 10 μm); **F**. Sulcal region (Bar length 10 μm)



Numbers of reports of toxic fish caught and highest population densities of *G. toxicus* found at various locations along the Queensland coast (N.S. — not sampled).



Seasonal changes in highest population densities of *G. toxicus* (solid line), and water temperature (broken line) at Flinders Reef, southern Queensland.

One location of particular significance in this regard was Flinders Reef (27° latitude), a small exposed littoral rock platform fringed by coral reef, where *G. toxicus* populations up to 2180/g were located. A great deal of seasonal periodicity in *G. toxicus* numbers has been found on Flinders Reef with peak populations occurring each September.

The occurrence of reasonably high populations of *G. toxicus* on Flinders Reef afforded Gillespie *et al.* (1985b) the opportunity to examine whether ciguatoxin was present in a wild population of *G. toxicus* in Australian waters. However it was found that ciguatoxin was not present in detectable quantities upon extraction of 1.7×10^6 cells, although an extremely high concentration of the water soluble toxin, maitotoxin, was present. It is possible that this might indicate that there is considerable intraspecies variation with respect to toxicity, as has been reported previously for toxic dinoflagellates of the genus *Protogonyaulax* (Hall and Reichard 1984). Alternatively, as Gillespie *et al.* (1985b) hypothesised, it could mean that a specific stimulus might be required for ciguatoxin formation.

THE EFFECT OF REEF DISTURBANCE ON CIGUATERA

Man-made or natural reef disturbance in the form of dredging and blasting of channels, storms, and the anchoring of ships often precedes the occurrence of ciguatera outbreaks. Randall (1958) suggested that this could be explained by the colonisation of new surfaces by the organisms producing ciguatoxin, which he assumed to be a bluegreen algae. Both Randall (1958) and Cooper (1964) noted a strong connection between shipping and ships' anchorages and the occurrence of toxic fish. Also, Bagnis (1969) was able to link an eruption of ciguatera at Hao Atoll with harbour works and other construction associated with the establishment of a base station for staging French Atomic Energy Commission activities.

After the discovery of *Gambierdiscus toxicus* as a potential ciguatoxin elaborator in the Gambier Islands, Yasumoto *et al.* (1980) modified the 'new surface' theory of Randall (1958) to explain the link with reef disturbance. He suggested that the death of corals created new surfaces suitable for certain species of algae which in turn attracted epiphytic micro-organisms such as *G. toxicus*. Yasumoto *et al.* (1980) had earlier found that large outbreaks of ciguatera in the Gambier Islands were preceded

by mass mortalities of coral caused by construction activities in the lagoon.

In 1984 Gillespie and co-workers (unpublished data) tested Yasumoto's hypothesis by monitoring the effects of dredging a small boat channel in the fringing reef of Hayman Island on the macroalgae and associated populations of epiphytic dinoflagellates over a period of 12 months. No significant change or increase in foliose macroalgal species present could be demonstrated. Furthermore, *G. toxicus* populations were lower after the dredging than they had been previously. However, it is possible that reef perturbations may need to be on a larger scale and occur over a longer time period than this to disrupt the coral reef ecosystem sufficiently to cause the development of high populations of *G. toxicus*, if, in fact this is the basis for the connection between reef disturbance and ciguatera.

There has been only one known instance in Australia where ciguatera could be linked to man-made or natural reef perturbations. An increase in ciguatera incidence in the Gove area was noted after the construction of an alumina plant and accompanying settlement in 1970–72. A local fishing identity, Mr Noel Whitehead, in a letter to Dr R. Lewis in 1978, observed that ciguatera was unknown around Gove before 1972, even though fishing had previously been a popular recreational activity. In 1972 increasing numbers of people reported becoming ill after eating fish from the eastern side of Bremer Island, which lies to the northeast of Gove peninsula. In ensuing years, the number of fishing spots around Gove producing toxic fish increased and in some cases, particularly in Melville Bay, coral mortalities had been reported prior to the appearance of toxic fish. While it is convenient to implicate the alumina plant in these outbreaks, it is interesting to note that Bremer Island, the area where fish first become toxic would not necessarily have been subjected to pollution from the plant or be affected by it's construction. Rather it would seem that as a popular fishing spot it might have suffered damage as a result of the action of anchors. In addition, most of the reefs implicated in ciguatera in the Gove area are many miles distant from one another and it is unlikely that they were all directly affected by the alumina plant.

Natural causes of reef perturbation include cyclones and sedimentation following flood rains. Of course, great damage to coral reefs along the Queensland coast has resulted from infestations of the crown-of-thorns starfish, *Acanthaster planci*, which can create large areas of dead coral surfaces. However, reports of ciguatera outbreaks in Queensland have not been found to increase in parallel with those of *A. planci* or other natural causes of damage.

REEF ECOLOGY AND CIGUATERA

It has been established that ciguatoxin enters the food chain via those fish which feed on the algae and detritus of coral reefs, especially the surgeonfish (Acanthuridae) and parrotfish (Scaridae) (Banner 1976). In the Great Barrier Reef region it is known that the abundance of algal grazers increases dramatically with distance offshore (Williams 1982). On the mid-shelf and outer shelf reefs these fish feed primarily on fine turf algae, which do not normally harbour high populations of *G. toxicus*. However in the Cairns region, 'patches' of medium population density of *G. toxicus* up to 50/g have been observed on macroalgae such as *Halimeda* (Gillespie *et al.* 1985a). If distributed evenly over the huge reef surface area available in north Queensland, these patches may provide considerable potential for ciguatoxin accumulation.

On inshore or coastal fringing reefs, turf algae do not occur in great abundance and are replaced by a large standing crop of macroalgae, primarily *Sargassum, Turbinaria, Zonaria* (in the south) and other 'leafy' species. Under suitable conditions, these macroalgae can harbour high populations of *G. toxicus*. In areas where there are frequent depressions in salinity and high sedimentation as a result of freshwater run-off, as in coastal areas near Cairns, *G. toxicus* population densities have been found to be low.

However, on sections of drier coastline near Bundaberg in southern Queensland high populations of *G. toxicus* have been found on macroalgae on coastal fringing reefs. Many of the macroalgal species conductive to *G. toxicus* growth appear to be unpalatable to fishes. Consequently, the only route by which toxin from the attached dinoflagellates might enter the food chain could be via the detritus, which may capture the 'fall-out' of cells from these

algae during turbulent conditions. Some evidence that this may be the case is provided by the fact that a benthic detritivore, *Acanthurus dussumieri*, caught on an inshore reef near Bundaberg has been reported to have been responsible for ciguatera. It is possible that inshore reefs in southern Queensland could be a source of the ciguatoxin found in pelagic fish species in Hervey Bay.

CONCLUSION

Some correlation between population densities of *G. toxicus*, fish toxicity and ciguatera endemicity has been shown overseas (Carlson *et al.* 1985; Bagnis *et al.* 1985; Yasumoto *et al.* 1984). In addition, areas exist in Queensland where the occurrence of ciguateric fish appears to correspond with high population densities of *G. toxicus*. However, evidence for a direct link between *G. toxicus* and ciguatoxin formation remains largely circumstantial, principally because of an inability of most workers to demonstrate ciguatoxin formation by laboratory cultures of the organism (Tindall *et al.* 1984; Yasumoto *et al.* 1979b; Gillespie, unpublished observations). Furthermore, some wild populations of *G. toxicus* have been found to contain little or no ciguatoxin, suggeting either genetic variation with respect to ciguatoxin formation or a requirement for a specific stimulus to elicit ciguatoxin production (Gillespie *et al.* 1985b). In this context, physically disruptive forms or reef disturbance may play a key role, creating circumstances which could trigger ciguatoxin formation in an existing population of *G. toxicus*.

ACKNOWLEDGEMENTS

The results of any Queensland-based research described here were the product of a team which, in addition to the author, included Mr John Burke, Michael Holmes and Anita Fifoot, who prepared the illustrations. I would like to thank Dr R. Lewis for making available correspondence with Mr Noel Whitehead, formerly of Gove. Mr John Ellis collected field data and samples for work described on the effects of reef disturbance at Hayman Island. Miss Gail Davidson typed the manuscript. Aspects of Australian research described here received financial assistance from the Fishing Industry Research Trust Account and the Marine Science and Technology Grants Scheme.

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APPENDIX 10.6

FISHING INDUSTRY RESEARCH TRUST ACCOUNT

APPLICATION FOR GRANT 1986/87

1. TITLE OF PROPOSAL

Factors affecting the toxicity of the dinoflagellate, Gambierdiscus toxicus and the development of ciguatera outbreaks.

2. NAME OF APPLICANT

Queensland Department of Primary Industries

3. DIVISION, DEPARTMENT OR SECTION

Fisheries Research Branch

4. PROPOSAL

It is proposed to investigate the mechanism by which ciguatera outbreaks occur through the study of factors influencing growth and toxin production by the toxic dinoflagellate Gambierdiscus toxicus. An earlier project (FIRTA 82/60) "Studies on toxic dinoflagellates responsible for formation of ciguatoxin" found that G. toxicus was widely distributed on coral reefs in Queensland but it was not possible to detect ciguatoxin in wild populations of the organism. This led to scepticism that this organism was the progenitor of ciguatoxin in Australian waters. However, recently it has been found that non-ciguatoxic cultures of G. toxicus after being subjected to environmental conditions resembling those occurring during reef disturbance, will produce a toxic fraction, which on the basis of its chemical properties and symptoms in mice would appear to be similar to ciguatoxin. It is believed that the continuation of this line of investigation will help establish a causative mechanism for ciguatera and appropriate reef management strategies to minimise its incidence.

5. NAME OF PERSON RESPONSIBLE FOR PROGRAM

Mr R.G. Pearson
Director
Fisheries Research Branch
Department of Primary Industries
GPO Box 46
BRISBANE Q 4001
Phone (07) 227 6432

6. QUALIFICATIONS OF PERSONNEL TO BE EMPLOYED ON PROGRAM

Project Leader:

N.C. Gillespie, B.Sc., Ph.D.
Supervising Fisheries Biologist and Officer in Charge
Southern Fisheries Research Centre
PO Box 76
DECEPTION BAY Q 4508
Phone (07) 203 1444

Dr Gillespie has recently completed an ecological study of G. toxicus along the Queensland coast.

Other Staff:

(a) J.B. Burke, Fisheries Technician Division I, Certificate in Biological Laboratory Techniques. Mr Burke has extensive experience in studies of toxic organisms in coral reef areas and with the culture of G. toxicus.

(b) M.J. Holmes, B.App.Sc., Fisheries Technician Division II. Mr Holmes has now a high degree of experience in algal culture and in the culture of benthic dinoflagellates in particular.

7. OBJECTIVES

(i) To define the factors influencing ciguatoxin production by cultures of Gambierdiscus toxicus. The effect of phenomena associated with reef disturbance and the significance of genetic heterogeneity in G. toxicus with respect to toxin production are to be examined.

(ii) To establish the requirements for growth and bloom formation by G. toxicus and other benthic dinoflagellates.

8. JUSTIFICATION

Ciguatera poisoning is caused by eating certain fish from coral reefs and is common along the far northern and eastern coastlines of Australia. The symptoms of the disease involve neurological and gastrointestinal disorders with normal recovery usually requiring several days to several weeks. It has been estimated that ciguatera incidence in some parts of Queensland approaches 3 per 10,000 head of population, which nearly equals the mean rate of ciguatera incidence in the South Pacific. It is now the greatest single factor affecting the marketing of tropical reef fish and such species as narrow-barred Spanish mackerel. The value of the commercial catch for this fishery in the Great Barrier Reef region in 1979-80 was $6 million (Hundloe, 1985). The catch of the small boat fleet comprising the recreational fisheries of the Great Barrier Reef region in 1980 was 6.5 million kilograms of fish. Other fisheries affected but not included in the Great Barrier Reef region are the mackerel fisheries of Hervey Bay and the remainder of southeastern Queensland. It has a considerable effect on consumer confidence in seafood (M. Capra, Final Report FIRTA 82/83) and the prospect of legal action is of considerable concern to the marketing sector.

Derivation of what is known of the mechanism of ciguatera poisoning has been the result of research, hypotheses and inferences over many years. The principal toxin involved is ciguatoxin, a lipid soluble toxin found in the flesh of reef carnivores (Scheuer et al, 1967). A water soluble toxin (maitotoxin) is thought to be responsible for the predominantly gastrointestinal disorders associated with the consumption of herbivorous reef fishes (Yasumoto et al, 1971). Scaritoxin, a lipid soluble toxin found in parrot fishes, is separable from ciguatoxin by thin-layer chromatography and is assumed to be a metabolite of ciguatoxin (Yasumoto et al, 1984).

It was originally speculated by Randall (1958) that blue green algae, or some other small organism associated with the benthos, produce the toxin(s) involved in ciguatera and these were transmitted up the food chain through herbivores to carnivores. In 1977, a benthic dinoflagellate was isolated in large numbers from a toxic area in the Gambier Islands (Yasumoto et al, 1977; Bagnis, 1977) that contained both ciguatoxin and maitotoxin in appreciable amounts. However, cultured cells yielded mainly maitotoxin (Yasumoto et al, 1979). Later named Gambierdiscus toxicus (Adachi & Fukuyo, 1977), there was strong evidence linking this organism with ciguatera.

(i) There was a correlation between the presence and abundance of G. toxicus and ciguateric fish (Yasumoto, 1980).

(ii) The organism, when first isolated, contained both ciguatoxin and maitotoxin.

(iii) The patchy distribution of the organism reflected the sporadic occurrence of ciguatoxic fish.

As mentioned earlier, G. toxicus has not been found to produce ciguatoxin in culture in significant amounts, although large quantities of maitotoxin are produced. In addition Gillespie et al (1985b) found a similar pattern of toxicity in a wild population of G. toxicus sampled in southern Queensland, ie, no detectable ciguatoxin but good amounts of maitotoxin. Accepting the validity of the original work of Yasumoto et al (1977), it was concluded that G. toxicus may not always be toxic but would require a "CTX-inducing factor" to induce toxicity.

It was noted that ciguatera outbreaks often follow disturbance of coral reefs. Bagnis (1969) has demonstrated a causal link between disruptive activities and the appearance of ciguatera in a reef ecosystem. It was therefore proposed (Gillespie et al, 1985b) that the "CTX-inducing factor" may be environmental conditions associated with reef disturbance, which would act directly on an existing population of G. toxicus, triggering ciguatoxin formation.

Since July 1985 field and laboratory experiments exploring the link between reef disturbance and toxicity of G. toxicus have been carried out. The field experiments, which were conducted in September 1985, yielded equivocal results. However, laboratory cultures of G. toxicus when subjected to treatment, were found to produce a toxic fraction, not detected previously, that showed chemical properties and produced symptoms in mice similar to ciguatoxin. Associated with the appearance of this fraction were changes in pigmentation, cell fragility and apparent increases in lipid material associated with cells.

It is suggested that there may be a direct link between reef disturbance of any type that causes coral disruption, and the appearance of ciguatoxin in the reef ecosystem. The effect of disturbance in terms of ciguatera is thought to depend on the population density of benthic dinoflagellates, the genetic capability of associated dinoflagellate strains to produce toxin, and the degree of turbulence normal to a particular location.

Reef oriented tourism is projected to escalate dramatically over the next ten years. Parallel resort development on or near coral reef areas is occurring. It is likely that the Reef-Link Hotel and other planned developments may cause significant localised coral disruption. Depending on the factors mentioned previously, this may have the effect of increasing the incidence of ciguatera fish poisoning from reef fish distributed commercially.

The most effective management measure that could improve public safeguards would be the development of a rapid specific test for ciguatoxin. Despite promising advances in this area it is thought that it will be several years before such a test becomes available.

In the meantime, an improved understanding of the processes leading to ciguatera outbreaks will allow preventative reef management measures to be put in place, particularly in sheltered areas prone to high population densities of G. toxicus.

9. LOCATION OF OPERATION

Most laboratory studies will be carried out at the Southern Fisheries Research Centre, Deception Bay. Field studies will be undertaken around coral reefs in the Capricorn-Bunker Group as well as near isolated offshore coral outcrops. Field studies in northern Queensland will be undertaken on inshore and offshore reefs near Cairns.

10. PROPOSAL IN DETAIL

(a) Plan of Operation

(i) Method of Procedure

Environmental factors influencing toxin production: Mass cultures of G. toxicus and other dinoflagellates, in particular Ostreopsis lenticularis, will be raised in a modification of "f" medium of Guillard, with drastically reduced nutrient enrichment and with chelator:trace metal balance as outlined by Harrison. At present cultures are being subjected to increasing loads of organic materials similar to those which occur during reef disturbance. Cultures of G. toxicus have been responding to this treatment by producing a toxic lipid fraction which produces symptoms in mice similar to those produced by ciguatoxin. In order to maximise cell yields cultures have been treated late in the stationary phase, but yields of "ciguatoxin-like" material have been small. Ongoing experiments are to be carried out earlier in the growth phase, as it is likely that actively growing cells have greater potential for synthesising toxins. Production of the required cell yields will necessitate the use of a much greater surface area of culture vessels than being used at

present. The extraction procedure employed is that employed previously (Gillespie et al, 1985b) with subsequent fractionation of the diethyl-ether soluble material through a silicic acid column. This is necessary to remove maitotoxin that often appears in the diethyl-ether phase. The toxicity of the various fractions are assayed using mice injected i.p.

Influence of genetic variability on ciguatoxin production: Eighteen separate clones of G. toxicus have been obtained from Hastings Reef and Arlington Reef near Cairns and from Flinders Reef in southern Queensland. In addition, cloned and uncloned cultures of a toxic strain of G. toxicus from the Gambier Islands supplied by Dr R. Bagnis in Tahiti are being maintained. Additional strains of G. toxicus from the Atlantic Ocean are also being procured. Toxin production by these individual clones, after a standard treatment, will be assessed as outlined above and correlated with the results of enzyme electrophoresis, performed with the assistance of Dr Alan Cambella from the Canadian Fisheries and Oceans laboratory in Quebec.

Mechanism of bloom formation: Using small tissue culture flasks containing 5ml cultures, large numbers of growth experiments can be carried out, with growth being assessed microscopically. It is planned to utilise these small vessels to assess growth rates in respect to nutrient concentration, pH, light intensity and other parameters.

The growth rate of the organism in coral reef situations will be determined by taking advantage of the phased pattern of cell division in natural populations. This involves the sampling of a fixed location over several hours, with subsequent application of nuclear strains. The growth rate of the population can be calculated approximately from the observed frequency of dividing cells, provided the appropriate constants have previously been determined. Thus the growth rate of the organism in areas of high and low population density can be measured, providing together with laboratory results, an indication of factors involved in bloom formation.

(ii) Facilities Available

The Southern Fisheries Research Centre is a modern laboratory building and has available for this project, constant temperature algal culture facilities, stereomicroscopes and high power microscopes, laminar flow cabinet autoclave, inverted microscope, ultrasonic disintegrater. Within the facilities of the Department of Primary Industries, there are scanning and transmission electron microscopes, HPLC and GLC equipment and a number of high speed and ultracentrifuges and scintillation counting equipment all of which will be used at some stage on this project.

(iii) Supporting Data

The research team involved, Dr Gillespie, John Burke and Michael Holmes, have completed a study concerned with the distribution of benthic dinoflagellates in coral reef areas along the Queensland coast, isolated and cultured the species involved and have assayed both wild populations and laboratory cultures for toxicity.

The group has also completed a study of the effects of reef disturbance on the fringing reef at Hayman Island on the macroalgal

flora and associated benthic dinoflagellate populations, exploring earlier hypotheses on the connection between reef disturbance and ciguatera outbreaks.

In addition an epidemiological study has been carried out, spanning the period 1976-1985 which characterises the symptoms of ciguatera fish poisoning in humans and identifies high risk areas along the Queensland coast as well as fish species.

Dr Gillespie has visited overseas laboratories concerned with ciguatera research in both 1982 and 1985 and has developed close links with French, American, Canadian and Japanese workers active in the field of ciguatera and toxic dinoflagellates. These links have resulted in offers of collaborative research.

11. PROPOSED COMMENCEMENT DATE AND ANTICIPATED COMPLETION DATE

Work on the early stages of this project have already commenced, using present resources, because of the increasing urgency for the data which it will provide.

Support from FIRTA for the project is requested from 1 July 1986 with anticipated completion by 30 June 1989.

12. FUNDS REQUESTED

	Year 1 $	Year 2 $	Year 3 $
(a) Total Salaries and Wages	2 100	8 400	8 400
(b) Total Operating Expenses	8 800	8 800	8 800
(c) Total Capital Items	11 500	1 500	1 500
Gross Total Cost	22 400	18 700	18 700

Funds requested for future years are based on present prices. The replacement vehicle included in the application for 1986/87 replaces a station sedan previously used for travel to sampling areas in the Bundaberg-Gladstone area. While the type of field sampling will change, extensive vehicular travel is still associated with the project. The funds requested include the full initial cost of the vehicle with the sale price of the present vehicle being refunded directly to FIRTA.

13. FUNDS TO BE PROVIDED BY THE APPLICANT

(i)	Salaries	
	J. Burke (70% of time)	23 033
	M. Holmes (100% of time)	28 421
(ii)	Operating Expenses	5 000
(iii)	Capital Items	
	Inverted microscope	8 000
	Cell homogeniser	3 000
	Electrophoresis equipment	5 000
	TOTAL	72 454

14. CO-OPERATING AGENCIES AND THEIR FUNCTION

Collaboration between this group and that of Dr R. Endean at the Zoology Department as well as Dr M. Capra at the Queensland Institute of Technology will take place when in the interests of the overall research effort. Regular communication occurs between these groups.

Dr Gillespie works in close contact with Dr R. Bagnis of Louis Malarde Institute of Medical Research in Tahiti, and Dr P. Scheuer of the Chemistry Department, University of Hawaii and other authorities in this field.

Dr A. Cambella of the Canadian Department of Fisheries and Oceans will assist in the genetic studies by performing enzyme electrophoresis on dinoflagellate extracts.

Dr R. Quinn of the School of Science at Griffith University will consult on chemical problems arising during the program.

15. IS SIMILAR WORK BEING UNDERTAKEN IN AUSTRALIA?

Dr R. Endean of the Zoology Department, University of Queensland has been investigating the action of ciguatoxin on animal preparations. Dr M. Capra at the Queensland Institute of Technology has been researching the effects of ciguatoxin on fish nerves and the nervous systems of human beings.

16. PLANS FOR REPORTING OR PUBLISHING RESULTS

The results of this work will be published in appropriate international journals and articles for publication in "Australian Fisheries" prepared.

FUNDS REQUESTED

1986/87

Project: Factors affecting the toxicity of the dinoflagellate, Gambierdiscus toxicus and the development of ciguatera outbreaks.

	1986/87 $	1987/88 $	1988/89 $
(a) Salaries and Wages			
Technician Division II	2 000	8 000	8 000
On costs	100	400	400
Total Salaries and Wages	2 100	8 400	8 400
(b) Operating Expenses			
(i) Fares	1 000	1 000	1 000
Travelling allowance	1 000	1 000	1 000
Vehicle maintenance	2 500	2 500	2 500
Vessel maintenance	800	800	800
(ii) Culture vessels	500	500	500
Glassware	1 000	1 000	1 000
Chemicals	1 000	1 000	1 000
Solvents	1 000	1 000	1 000
Total Operating Expenses	8 800	8 800	8 800
(c) Capital Items			
Replacement vehicle	10 000	-	-
Miscellaneous	1 500	1 500	1 500
Total Capital Items	11 500	1 500	1 500
GROSS TOTAL COST	22 400	18 700	18 700

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