Proceedings of the Eighth Canadian Workshop on Harmful Marine Algae

Stephen S. Bates (Editor)

Fisheries and Oceans Canada
Gulf Fisheries Centre
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8ième Atelier Canadien sur les Algues Marines Nuisibles

28 – 30 mai 2003

Workshop Proceedings

8th Canadian Workshop on Harmful Marine Algae

May 28 – 30, 2003

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ABSTRACT

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The Eighth Canadian Workshop on Harmful Marine Algae, sponsored by Fisheries and Oceans Canada, Gulf Region, was held at the Gulf Fisheries Centre, Moncton, New Brunswick, from May 28-30, 2003. One hundred and five people from Canada and six other countries attended the workshop, with representatives from federal and provincial governments, universities and the private sector. There was good representation from the molluscan aquaculture industry. Twenty five oral and 15 poster presentations encompassed current knowledge and awareness of Harmful Algal Blooms (HABs). The titles of the oral sessions were: Phycotoxin Chemistry and Detection; Effects of HABs and Phycotoxins on Other Organisms; Distribution of HABs and Results of Monitoring Programs; Detection and Distribution of HABs; Dinoflagellate Cyst Distribution and Toxin Production; and International and National HAB Programs. Three Discussion Groups were held: Monitoring Toxins in a Regulatory Setting; Ballast Water and HABs; and The Aquaculture Industry Perspective. These Proceedings contain the abstracts, extended abstracts, and papers of the oral and poster presentations, together with reports from the three Discussion Groups.

RÉSUMÉ

Le Huitième atelier de travail canadien sur les algues marines nuisibles a eut lieu du 28 au 30 mai 2003, sous l'égide de Pêches et Océans Canada, région du Golfe, au Centre des Pêches du Golfe, à Moncton, Nouveau-Brunswick. Cent cinq participants et participantes du Canada et de six autres pays étaient présents, représentatifs de diverses agences de gouvernements fédéraux et provinciaux, des universités et du secteur privé. Il y avait une bonne représentation de l'industrie aquicole. Vingt-cinq présentations orales et quinze affiches ont été présentés, englobant l'état des connaissances sur les floraisons d'algues nuisibles (FAN). Les sessions orales étaient intitulées : Détection et chimie des phycotoxines; Effets des algues nuisibles et des phycotoxines sur d'autres organismes; Distribution des algues nuisibles et résultats des programmes de monitorage; Détection et distribution des algues nuisibles; La distribution des kystes des dinoflagellés et la production de toxines; Programmes internationaux et nationaux. En outre, trois groupes de discussion ont délibéré sur les thèmes suivants: Monitorage des toxines dans un cadre de réglementation; Les algues nuisibles et le délestage de navires; La perspective de l'industrie aquicole. Ce compte rendu comprend les résumés, les résumés allongés et les documents relatifs aux présentations orales et aux affiches, ainsi que les procès-verbaux des trois groupes de discussion.

ACKNOWLEDGEMENTS

The Eighth Canadian Workshop on Harmful Marine Algae was sponsored by the Oceans and Science Branch, Gulf Region, of Fisheries and Oceans Canada. Members of the Phycotoxins Working Group, especially Ian Whyte, Jennifer Martin and Kats Haya who organized the most recent workshops, are thanked for their advice on planning the workshop.

The meeting ran smoothly thanks to the dedicated and professional help of the following people: Steve Chiasson and Nancy Roy (Web support), Phyllis Collette (financial aspects), Marie Daigle (hotel and banquet logistics), Pierre Gautreau and Catherine Vardy (communications, French translations, photography and editorial work), Venitia Joseph (registrations) Andrée LeBlanc (participant list) Claude Léger (French translations, registrations and logistics), Nancy Lewis (logo design), Donna MacDonald (photocopy centre), Don Richard (logistics, registrations, editorial work and general advice) and Dave Robertson (logistics, registrations and audio-visual aspects).

INTRODUCTION

Canada has a long history of dealing with harmful algal blooms (HABs) and with the phycotoxins produced by certain algae. The Canadian Pacific coast has encountered paralytic shellfish poisoning (PSP) toxins as far back as 1793, when a member of Captain George Vancouver's crew died from eating contaminated mussels in British Columbia. The Atlantic coast of Canada has also historically been seriously affected by PSP outbreaks. In the Bay of Fundy, molluscan shellfish toxicity has been monitored since 1943, and is the longest continuous time series of this kind in the world.

Until 1987, PSP was the major shellfish poisoning of concern in Canada. Then an autumn outbreak of a new poisoning, due to eating blue mussels from eastern Prince Edward Island, led to the discovery of amnesic shellfish poisoning (ASP). The neurotoxin domoic acid was found to be the culprit and its source was traced to the pennate diatom *Pseudo-nitzschia multiseries*. After that, diarrhetic shellfish poisoning (DSP) was recognized as a Canadian problem in 1990, in southern Nova Scotia; its source was the dinoflagellate *Prorocentrum lima*, rather than any species of *Dinophysis*. Canadian researchers continued to discover additional toxins. Spirolides, biologically active "fast-acting toxins", were isolated from scallop and mussel viscera in eastern N.S. in 1995; their source was *Alexandrium ostenfeldii*. Pectenotoxins were found in shellfish from N.S., Newfoundland and the Magdalen Islands in June 2000; one source organism of PTX2 is *Dinophysis acuminata*. A strain of *Protoceratium reticulatum* from N.S. was recently confirmed to produce yessotoxin, but this toxin has not yet been found in shellfish. Domoic acid was originally known to be produced only by *P. multiseries*. However, *P. pseudodelicatissima* (now called *P. calliantha*) was soon found to be a toxin producer in the Bay of Fundy. Then, in the spring of 2002, domoic acid caused an unprecedented closing of shellfish harvesting sites in most of the southern Gulf of St. Lawrence. This time, the source was yet another species of *Pseudo-nitzschia*, *P. seriata*. All of the above examples emphasize that new phycotoxins and species of toxic algae can appear, and at any time of the year, in Canadian waters.

The 1987 discovery of domoic acid and ASP raised the awareness that novel phycotoxins could continue to be found. This event therefore led Fisheries and Oceans Canada (DFO) to form the Phycotoxins Working Group (PWG). The PWG's role is to develop advice on the coordination, planning and prioritizing of DFO research on phycotoxins and other harmful aspects of algal blooms and the organisms producing them. It also promotes inter-regional and inter-disciplinary communication on HABs. The mechanism for this is the holding of periodic workshops.

Thus, the series of Canadian Workshops on Harmful Marine Algae (CWHMA) was established to promote the exchange of new scientific information on harmful algae and their effects, to foster the development of cooperative and collaborative scientific programs, and to encourage new research initiatives. The First CWHMA was held in Moncton, New Brunswick, in 1989. Since then, the workshops have been held in six other locations on both coasts of Canada (see the next page). Now, with the Eighth CWHMA, the workshop has returned to its roots. Although these workshops highlight Canadian research on HABs, there has been increasing international participation. This trend shows that the CWHMA structure is conducive for exchanging information and that Canadian HAB research is regarded internationally; indeed, the problem of HABs is international. We have greatly benefited from the participation of our international colleagues. We have also benefited from the participation of industry representatives, who gave their perspective. These Proceedings are a record of the talks, posters and Discussion Group sessions held during the Eighth CWHMA.

Previous Workshop Proceedings in the Series and Related Phycotoxins Working Group Activities

Whyte, J.N.C. (*Editor*). 2001. Proceedings of the Seventh Canadian Workshop on Harmful Marine Algae. Nanaimo, BC, 23-25 May 2001. Can. Tech. Rep. Fish. Aquat. Sci. 2386: xiii + 158 p.

Martin, J.L. and Haya, K. (*Editors*). 1999. Proceedings of the Sixth Canadian Workshop on Harmful Marine Algae. 27-29 May 1998, St. Andrews, NB. Can. Tech. Rep. Fish. Aquat. Sci. 2261: x + 159 p.

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Forbes, J.R. (*Editor*). 1994. Proceedings of the Fourth Canadian Workshop on Harmful Marine Algae. Sidney, BC, 3-5 May 1994. Can. Tech. Rep. Fish. Aquat. Sci. 2016: viii + 92 p.

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Gordon, D.C., Jr. (*Editor*). 1991. Proceedings of the Second Canadian Workshop on Harmful Marine Algae. Dartmouth, NS, 2-4 October 1990. Can. Tech. Rep. Fish. Aquat. Sci. 1799: iv + 66 p.

Forbes, J.R. (*Editor*). 1991. Pacific Coast Research on Toxic Marine Algae. Sidney, BC, 30 April 1991. Can. Tech. Rep. Hydrogr. Ocean Sci. 135: vi + 76 p.

Bates, S.S. and Worms, J. (*Editors*). 1989. Proceedings of the First Canadian Workshop on Harmful Marine Algae. Moncton, NB, 27-28 September 1989. Can. Tech. Rep. Fish. Aquat. Sci. 1712: iv + 57 p.

ORAL SESSION 1:

Phycotoxin Chemistry and Detection

The Role of Analytical Chemistry in the Hunt for Red Tide Toxins

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ABSTRACT

When Captain George Vancouver landed in British Columbia in 1793, tragedy struck when four of his crew became sick and one died due to paralysis and asphyxiation after eating shellfish taken from an area now known as Poison Cove (Vancouver, 1798, quoted in Quayle, 1969). The Captain later noted that the native Indians considered it dangerous to eat shellfish when the seawater was "coloured". This toxic event was one of the earliest recorded incidents of paralytic shellfish poisoning (PSP). Since then, many similar incidents have resulted throughout North America and worldwide. Even today, over 2,000 cases of human poisoning (fatal in 15% of the cases) are reported annually on a global scale. It was not until the 1940s and 1950s that the origins and identities of the toxic substances began to be known (Medcof *et al*., 1947; Burke *et al*., 1960).

The culprits in these poisonings are phytoplankton, microscopic plants that live in the ocean and convert inorganic compounds into complex organic compounds. They are the foundation of the marine food chain. Among the thousands of dinoflagellates and diatom species, there are only a few dozen that produce toxic secondary metabolites called "phycotoxins". Toxic incidents can occur when "blooms" of such toxigenic species, often known as "red tides", appear unexpectedly in shellfish-producing regions. The cell density can be high enough that waters become coloured, sometimes red (hence the term "red tide") but also green and brown. Filter-feeding bivalve molluscs such as mussels and scallops consume the plankton, thereby accumulating phycotoxins in their edible tissues. Herbivorous finfish can also accumulate toxins, which can then become part of the food chain to birds, marine mammals and man. The frequency of occurrence, abundance and geographical range of many phycotoxins appears to be increasing worldwide. Although most plankton blooms are natural phenomena, their proliferation may be partly attributable to agricultural runoff and sewage in coastal waters, redistribution of microalgae via ships' ballast water, and transfer of shellfish stocks. Red tides and phycotoxins present a serious threat to public health and have had a significant economic impact on fish and shellfish farming industries in much of the world.

Phycotoxins hold a particular fascination with chemists because of their unusual structures, toxic nature and history. As shown in Fig. 1, they have some of the most complex structures known in nature, ranging from low to high molecular weights and from very polar to highly lipophilic. They possess multi-functional characters and high degrees of chirality, and cause many different types of toxic effects. Phycotoxins present significant challenges to those interested in structure elucidation, synthesis and analysis.

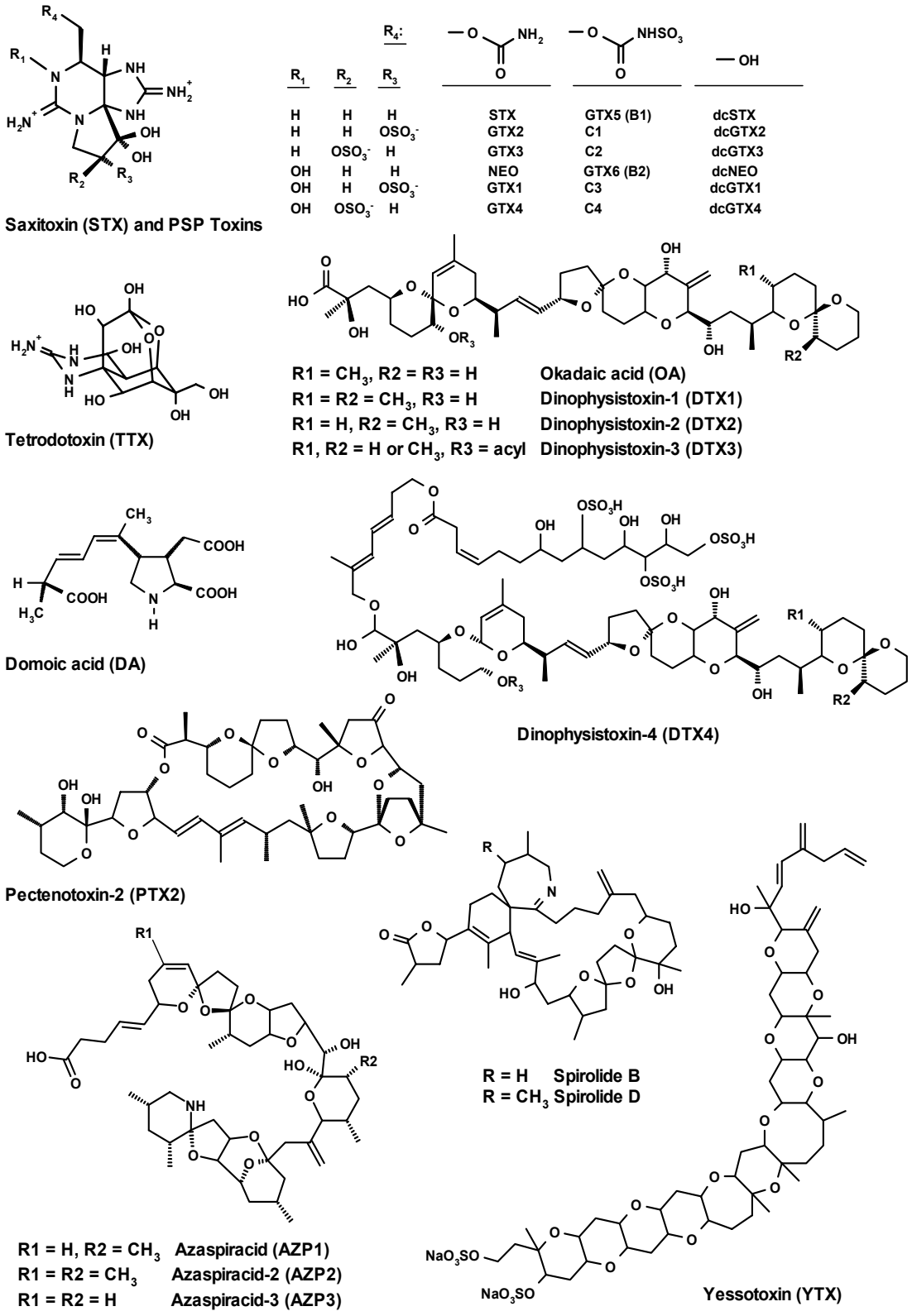

Fig. 1. Structures of various seafood toxins.

The purpose of this overview is to acquaint the reader with some of the different approaches that have been used to identify toxins responsible for seafood poisoning incidents, to investigate the origins of toxins, and to monitor our seafood on a routine basis. It will be shown that advancements in our knowledge of toxins and our ability to protect the public have often followed key developments in separation and analysis technologies. It will not be possible to make this a comprehensive review of all the significant contributions of many other research groups. Instead, specific examples from the author's personal experiences during the last 15 years of research in this field are presented to illustrate the significant role that chromatographic methods play. The presentation is given in an order that reflects the typical sequence of investigations that follow a new toxin episode, *viz*.:

a) identifying the new toxin;
b) developing analytical methods;
c) detecting and confirming similar events;
d) searching for the source of the toxin;
e) searching for its analogues;
f) surveying different geographical areas;
g) implementing routine monitoring; and
h) developing calibration standards and reference materials.

A complete account of this presentation may be found in a recent review (Quilliam 2003).

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Detection and Identification of Spirolides in Norwegian Mussels and Plankton

John Aasen[1], Patricia LeBlanc[2], William Hardstaff[2], Peter Hovgaard[3], Ian W. Burton[2], Shawna L. MacKinnon[2], John A. Walter[2], Tore Aune[1], and Michael A. Quilliam[2]

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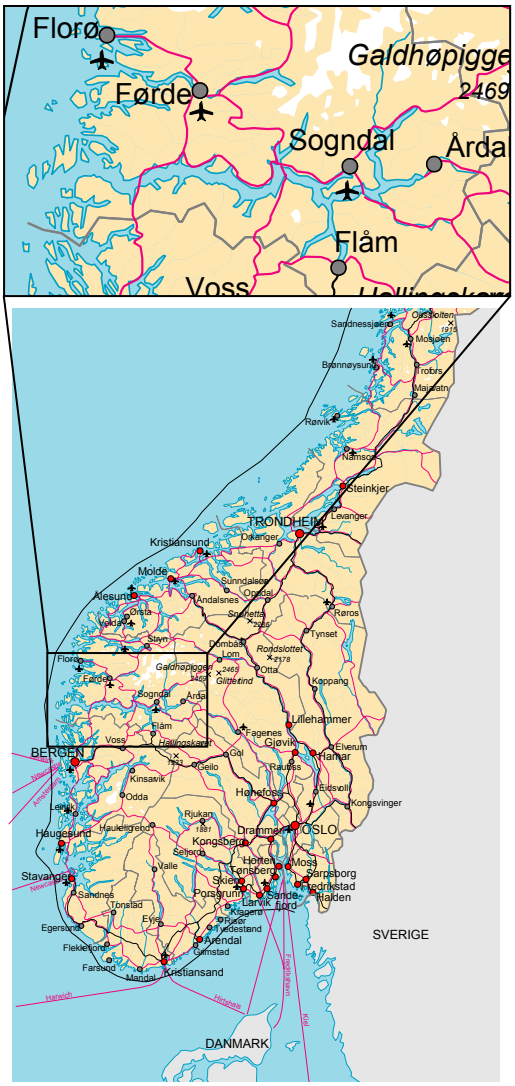
[3]Sogn og Fjordane College, Sogndal, Norway

After the discovery in mid-1990s of some unknown fast-acting toxin(s) in the Sognefjord, location Skjer (Aune *et al*., 1996; Ramstad *et al*., 2001), a routine program was set up to elucidate the problem. Biweekly samples of mussels were taken and analyzed with the mouse bioassay (chloroform extract). Fast-acting toxin(s) did not show up again until April 2002, when the mice showed symptoms, which included cramps, jumping and short survival times (as low as four minutes). These results were repeated in January through April 2003. No people were reported ill, but the location had already been closed due to presence of PSP toxins.



Analysis of lipophilic toxins in shellfish in 2002 displayed okadaic acid (OA), dinophysistoxin-1 (DTX1) and yessotoxin (YTX) present in minor amounts (<50 μg kg^{-1}), whereas azaspiracids (AZA) and pectenotoxins (PTX) were not found. In 2003, DTX1, PTX2 and PTX2sa were present in high amounts, but none of these toxins can explain this rapid onset of symptoms in the bioassay. Furthermore, the toxicity in the lipophilic extract could not be explained by PSP toxins.

A survey of the algae present at the aquaculture site showed that the toxicity correlated with a bloom of *Alexandrium ostenfeldii* in correspondence with previous reports. Up to 2,000 cells L^{-1} were found at the peak of one bloom on 8 April 2003. *Alexandrium ostenfeldii* was dominant among the plankton; other species present were *Dinophysis acuminata*, *D. acuta*, *D. norvegica*, *Protoceratium reticulatum* and *A. tamarense*.

In Canadian waters, *Alexandrium ostenfeldii* is known to be a producer of the cyclic imine toxins, spirolides (Cembella *et al*., 1999; Cembella *et al*., 2000; 2001). Spirolides have also been found previously in plankton samples from Denmark and in the North Sea (Hummert *et al*., 2002; MacKinnon *et al*., in press).

Analysis of the Norwegian mussel extracts using liquid chromatography-tandem mass spectrometry (LC-MS/MS) according to Quilliam *et al*. (2002), revealed the presence of several new spirolides. The toxin pattern was the same in both 2002 and 2003 mussel samples. The same compounds were also found in algal samples dominated by *A. ostenfeldii*, sampled from the Sognefjord in Norway in February 2003. A large-scale preparative extraction of mussel digestive glands (2.7 kg) and chromatographic fractionation of the extracts allowed the isolation and structure elucidation of the main spirolide by means of a combination of nuclear magnetic resonance (NMR) and MS data. The new compound was identified as 20-methyl spirolide G (Fig. 1), a spirolide with a molecular weight of 705.5. It is a methyl homologue of spirolide G, a compound with a 5:6:6 trispiroketal ring system found in plankton from Limfjorden, Denmark (MacKinnon *et al*., in press). This is the first confirmed occurrence of spirolides in mussels and plankton from Norway.

Initial toxicological studies have shown that spirolides are highly toxic in mice through both intraperitoneal and oral administration (Richard *et al*., 2001) and that one member of the spirolide toxin family, 13-desmethyl spirolide C, induces dose-dependent neurotoxicity towards mice and rats upon intraperitoneal injections (Gill *et al*., 2003). Further oral toxicity studies of spirolides are needed urgently to yield information necessary to undertake a risk assessment. The outcome of the risk assessment may form the basis for establishing tolerance levels for spirolides in seafood.

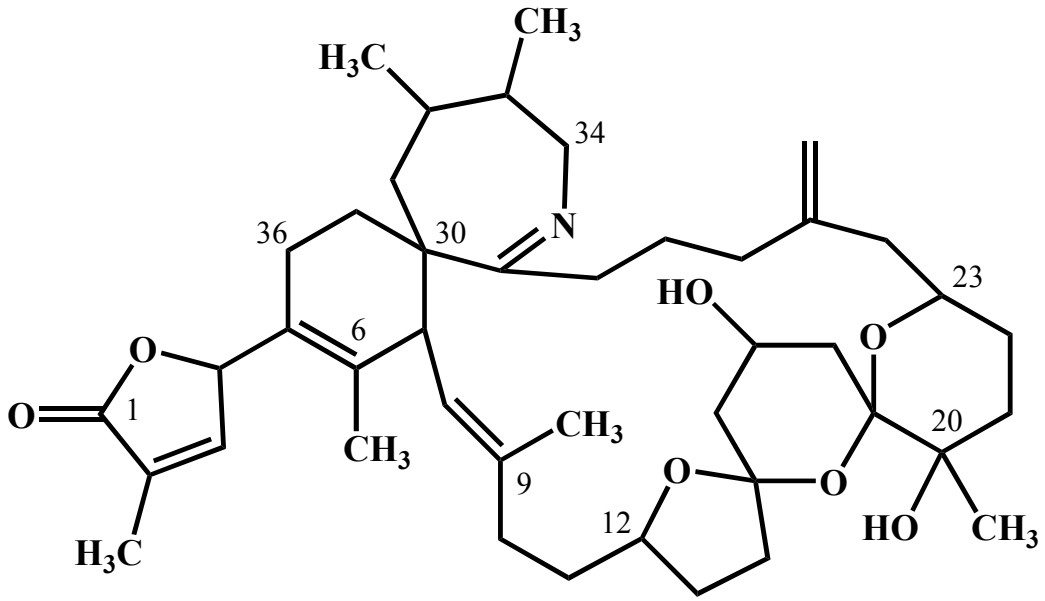


Fig. 1. The structure of 20-methyl spirolide G, the main spirolide found in Norwegian mussels and plankton.

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Detection of Yessotoxin in UK and Canadian Isolates of Phytoplankton and Optimization and Validation of LC-MS Methods

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[2]Phytosciences Research Group, School of Biosciences, University of Westminster, 115, New Cavendish Street, London, W1W 6UW United Kingdom;
[3]Institute for Marine Biosciences, National Research Council, 1411 Oxford St., Halifax, Nova Scotia, B3H 3Z1 Canada

ABSTRACT

Yessotoxin (YTX) and its analogues have been detected worldwide. It was first documented in European waters in Norwegian mussels and mussels from the Adriatic. In this study, we report on the detection of YTX in British and Canadian isolates of *Protoceratium reticulatum*, using liquid chromatography mass spectrometry (LC-MS). An isocratic liquid chromatographic method with mass spectrometric detection was developed and optimized for the analysis of YTX and its analog, 45-hydroxy-YTX, using a short C_8-silica column (50 x 2.1 mm). The range of calibration used was 0.1-1.0 µg YTX mL^{-1}, with a limit of detection of 0.05 µg YTX mL^{-1} using a single quadrupole MS. A solid phase extraction (SPE) method was also developed for the isolation and concentration of YTX from the culture medium. Results showed that a *P. reticulatum* isolate was found to produce YTX in detectable quantities (0.3 pg $cell^{-1}$). Tandem mass spectrometry (LC-MS/MS) was used to confirm the presence of YTX in these cultures. To our knowledge, this was the first recorded evidence of YTX-producing dinoflagellates in UK and Canadian waters.

INTRODUCTION

Yessotoxin (YTX) and its analogues such as 45-hydroxy-YTX (45OH-YTX) have been detected worldwide in shellfish (Murata *et al*., 1987; Satake *et al*., 1997a). YTX was first documented in European waters in mussels from Norway (Ramstad *et al*., 2001) and the Adriatic (Ciminiello *et al*., 1997). It is often associated with diarrhetic shellfish poisoning (DSP) toxins and although the health effects of this lipophilic toxin are unclear, its presence in shellfish complicates the interpretation of DSP events. YTX is known to be produced by *Protoceratium reticulatum* (Satake *et al*., 1997b) and *Lingulodinium polyedrum* (Tubaro *et al*., 1998).

A limited number of chemical techniques exist for the analysis of YTXs, the primary ones being based on liquid chromatography with detection by fluorescence (Yasumoto and Takizawa, 1997) or mass spectrometry (LC-MS) (Draisci *et al*., 1998, 1999; Goto *et al*., 2001; Ito and Tsukada, 2002; Ciminiello *et al*., 2002; Amandi *et al*., 2002). LC-MS is more widely used and is better able to detect modified yessotoxins than the fluorescence technique. In addition, the use of mass

spectrometry offers an unparalleled degree of confidence in the identification of a compound. The detection of characteristic ions in the correct relative ratios to each other at the correct retention time allows the unequivocal confirmation of identity of a compound of interest. Incorporation of an additional MS stage with tandem MS (LC-MS/MS) further increases the degree of confirmation and the possibility of identifying new analogues.

In this study, British isolates of *Lingulodinium polyedrum* (UW32), *Gonyaulax spinifera* (UW321, UW329) and *Prorocentrum micans* (UW46), and British and Canadian isolates of *P. reticulatum* (UW351 and UW409, respectively) were cultured and investigated for the presence of both YTX and 45OH-YTX by both LC-MS and LC-MS/MS.

MATERIALS AND METHODS

Materials

Chemicals, including culture media, were purchased from Sigma-Aldrich Ltd. (Poole, Dorset, UK) and all solvents used were HPLC grade (Rathburns, Walkerburn, UK). YTX and 45OH-YTX standards were a generous gift from Dr. Tore Aune (Oslo, Norway). Isolates were taken from a collection located at the University of Westminster, UK.

Seventy-four dinoflagellate cultures were grown in either Erdschreiber or f/2 culture medium with soil extract (14:10 h light:dark photocycle at 16°C) for 21-28 days. Initially, cultures were stored and transported as centrifuged cultures containing a cell pellet with an aliquot of the supernatant. However, later batches were sent as bulk cultures (700-800 mL) due to concerns over loss of YTXs to the culture medium. All samples were frozen upon harvesting and stored at -20°C during transport and prior to preparation.

Extraction method development

Investigations were made into the percentage of aqueous methanol required for the loading step of the solid phase extraction (SPE) procedure. Different volumes of freshly prepared f/2 culture medium were spiked with 55 µL of toxin stock solution (2.5 µg mL^{-1} each of YTX and 45OH-YTX) and methanol to give 5, 10 and 15% v/v aqueous methanol in the culture medium. The samples (0.9 mL) were loaded onto separate pre-conditioned SPE cartridges (Waters Oasis® HLB, 60 mg, 3 mL), washed with 5, 10 or 15% v/v aqueous methanol (1 mL) and eluted with methanol (1 mL). Fractions were collected from each stage and analyzed by LC-MS.

Subsequent experiments explored the volume of culture medium that could be loaded onto the SPE cartridge. Separate volumes (10, 25 and 100 mL) of culture medium were spiked with 100 µL of toxin stock solution (equivalent to a mass load of 250 ng each of YTX and 45OH-YTX) and methanol to give 2 and 5% v/v aqueous methanol in the sample volume. The samples were then loaded onto individual pre-conditioned SPE cartridges (2% and 5% v/v aqueous methanol). Sample cartridges were washed with either 2 or 5% v/v aqueous methanol (1 mL), followed by distilled water (3 mL). Cartridges were air-dried under vacuum (2 min) and eluted with methanol (5 x 1 mL).

Routine sample preparation

Aliquots of centrifugally concentrated cultures (1 mL) were added to an aliquot of f/2 culture medium (24 mL) before further preparation. Methanol (0.5 mL) was added to all cultures and sonicated for 10 min at ambient temperature. The samples were centrifuged at 3000 rpm for 10 min. Pre-conditioned SPE cartridges (Waters Oasis HLB, 3 mL, 60 mg) were loaded with sample supernatant (25 mL). Cartridges were washed with 2% v/v aqueous methanol (1 mL) and distilled water (3 mL). The cartridges were air-dried under vacuum for 2 min and eluted with methanol (9 mL). The eluted methanol extract was evaporated and reconstituted with methanol (200 µL). The recovery of YTX and 45OH-YTX from the culture media was assessed using spiked cultured media (25 mL, 40 µL of toxin stock solution) taken through the same procedure as the samples.

LC-MS analysis

LC-MS analyses were conducted on an Applied Biosystems API 150EX with TurboIonspray®. Various ions (YTX: *m/z* 1141.5, 1061.5, 570.3; 45OH-YTX: *m/z* 1157.5, 1077.5, 577.3) were used for single ion monitoring (SIM) in negative ion mode, and the total ion count was used for quantification. Chromatographic separation was achieved using 5 mM ammonium acetate (pH 7) in acetonitrile (38:62 v/v) as the isocratic mobile phase (flow rate 0.25 mL min^{-1}) and a Thermo Hypersil BDS-C_8-silica column (50 x 2.1 mm, particle size 3 µm). The sample injection volume was 5 µL and the run time was 10 min. Calibration was performed using standards in the range of 0.1 – 1.0 µg mL^{-1}.

LC-MS/MS analysis

LC-MS/MS analyses were performed on a model 1100 liquid chromatograph (Agilent, Palo Alto, CA, USA) coupled to an API-4000 mass spectrometer equipped with a Turbo IonSpray interface (PE-SCIEX, Thornhill, Ont., Canada). Eluent A was water and B was acetonitrile/water (95:5), both containing 2 mM ammonium formate and 50 mM formic acid. Gradient elution from 20% to 100% B was performed over 10 min and then held at 100% B for 10 min. The flow rate was 0.2 mL min^{-1} and the injection volume was 5 µL. Product ion mass spectra were acquired by colliding the Q1-selected precursor ions with nitrogen in Q2 operated in radio frequency (rf)-only mode and scanning the second quadrupole, Q3, from *m/z* 50 to 1110. A collision energy of 100 eV was used for negative ion MS/MS experiments, while 55 was used in the positive ion mode.

RESULTS AND DISCUSSION

LC-MS method development

Initial studies with flow injection analysis of a YTX standard (1 µg mL^{-1}) with full scan (*m/z* 550-1250) data acquisition showed that it was possible to generate spectra in both the positive and negative ionization modes when using a mobile phase of aqueous acetonitrile buffered with ammonium acetate. In the positive ion mode, an $[M+NH_4]^+$ ion at *m/z* 1160.5 was observed,

whereas in the negative ion mode, two ions at *m/z* 1141.5 and 570.3 were formed, corresponding to $[M-H]^-$ and $[M-2H]^{-2}$. The ratio of the $[M-H]^-$ and $[M-2H]^{-2}$ ions depends upon pH, with the latter being favoured under acidic conditions. For LC-MS analysis, a short 3 µm Hypersil BDS-C_8-silica column (50 x 2.1 mm) was used to achieve rapid analyses. The best chromatography at trace levels was achieved with 5 mM ammonium acetate (pH 7) in acetonitrile (38:62 v/v) at a flow rate 0.25 mL min^{-1}. The retention times for 45OH-YTX and YTX were 1.9 and 5.9 min, respectively. An acidic mobile phase can also be used, but on older columns and with trace levels, the YTXs show more peak tailing. Using selected ion monitoring in the negative ion mode, the detection limit was 0.05 µg mL^{-1} and the limit of quantitation was determined to be 0.1 µg mL^{-1}.

Solid phase extraction development

The results of the investigation into the solvent and volume used in the SPE loading step are summarized in Tables 1 and 2. Loss was observed in 15% v/v aqueous methanol in culture medium solution (Table 1). YTX or 45OH-YTX was not detected in any fraction other than the eluent fraction. Little difference was observed in the recovery for 5 and 10% v/v aqueous methanol. However, 5% v/v aqueous methanol was used in further experiments for increased retention of YTXs on the SPE cartridge.

Table 1. Effect of % methanol added to culture sample on the recovery of YTXs from SPE loaded with 25 mL spiked culture medium.

Methanol (% v/v)	% Recovery	
	YTX	45OH-YTX
5	94	81
10	99	80
15	52	75

Table 2. Effect of sample volume and % methanol on the recovery of YTXs from SPE of spiked culture medium.

Volume of culture medium loaded onto SPE (mL)	% Recovery of YTXs for 2 or 5% v/v methanol in culture medium			
	YTX		45OH-YTX	
	2%	5%	2%	5%
10	79	71	85	76
25	71	71	87	89
100	83	67	89	86

The recovery of spiked YTXs from larger sample volumes taken through the SPE cartridge was observed to be higher using 2% v/v aqueous methanol for the loading step (Table 2). The elution profiles of YTX and 45OH-YTX were tested by collecting 1 mL fractions of methanol and analyses of these fractions indicated that YTX and 45OH-YTX were completely eluted within 5 mL. As a precautionary measure, a higher volume of methanol (9 mL) was used for application to cultures to ensure complete elution of analytes. Overall, YTX recoveries of spiked culture samples were acceptable (73 ± 10%, n = 3).

Application of the method to culture samples

A total of 74 individual cultures were analyzed and results are summarized in Table 3. YTX was confirmed in three of the eight isolates tested (UW32 encysted, UW351 and UW409). 45OH-YTX was not detected in any of the sample cultures. The concentration of YTX found in the samples varied considerably. This study did not investigate the growth stage of the cultures, so the relationship of toxin concentration with culture age has not yet been determined. LC-MS/MS analysis confirmed the presence of YTX in the extracted cultures. The negative product ion spectrum of YTX from the UW409 culture shown in Fig. 1 matches that of authentic YTX.

Table 3. Results from YTX analysis of various culture samples.

Culture number	Species	Source area	Number of cultures		Mean [YTX] ± SD (pg cell^{-1})
			Not detected	Detected	
UW32	*L. polyedrum*	Loch Creran, UK	11	0	na
UW32 [e]	*L. polyedrum*	Loch Creran, UK	4	1	0.02
UW46	*P. micans*	Weymouth Harbour, UK	5	0	na
UW321	*G. spinifera*	Kirkwall Bay, UK	2	0	na
UW391	*G. spinifera*	Kirkwall Bay, UK	0	1	lod?
UW392	*G. spinifera*	Kirkwall Bay, UK	5	0	na
UW393	*G. spinifera*	Kirkwall Bay, UK	2	0	na
UW351	*P. reticulatum*	North Sea, Station 216	1	28	0.3 ± 0.7
UW409	*P. reticulatum*	Bedford Basin, N.S., Canada	1	13	5 ± 3

na = not applicable; lod? = signal at limit of detection; UW32 [e] = encysted

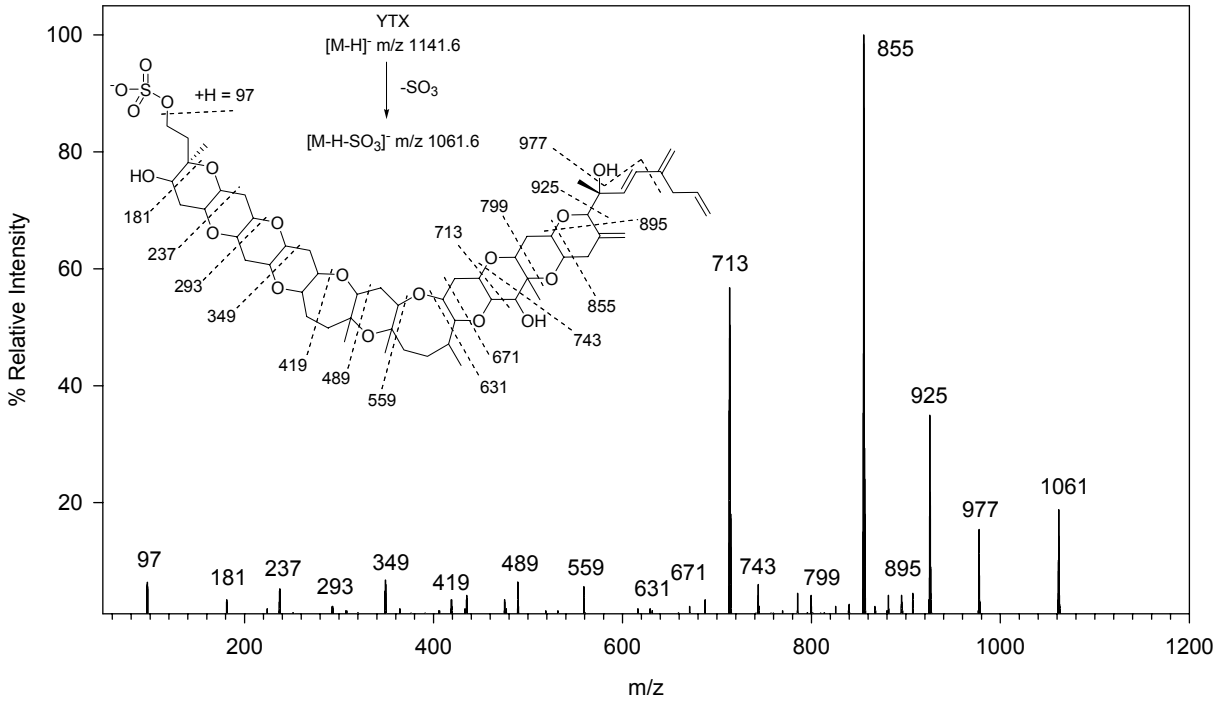

Fig. 1. Confirmatory negative ion MS/MS spectrum of YTX in a Canadian culture extract. Product ions from *m/z* 1141.6(-).

CONCLUSIONS

The developed SPE method has an acceptable recovery (>70%) for the detection of YTX and 45OH-YTX in culture media or seawater. Two *P. reticulatum* isolates and one encysted *L. polyedrum* were found to produce YTX in detectable quantities. YTX was not detected in other *Gonyaulax* and *Prorocentrum* cultures and 45OH-YTX was not detected in any of the cultures. To our knowledge, this is the first recorded evidence of YTX-producing dinoflagellates in UK and Canadian waters.

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An assay for the Brevetoxin Group of Sodium Channel Activators Based on Measurement of Membrane Depolarization in Synaptoneurosomes

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An assay based on CD1 mouse brain synaptoneurosomes and the voltage-sensitive fluorescent probe rhodamine 6G was evaluated for routine quantitation of the brevetoxin (PbTx) group of sodium channel activators in shellfish samples. The assay was optimized to maximize the enhancing effect of the brevetoxin congeners (PbTx-2 and PbTx-3) on depolarization produced by a standard pulse of the sodium channel site 2 activator veratridine. Since greater quantities of the fluoroprobe dissociate from synaptoneurosomes with increasing concentrations of PbTx, PbTx-dependent enhancements can be followed as increases in rhodamine 6G fluorescence. In this assay, preincubation of synaptoneurosomes with PbTx was required, but this treatment did not affect the intensity of fluorescence in synaptoneurosomal suspensions immediately prior to veratridine challenge. The assay is relatively rapid and is capable of detecting brevetoxin activity in the low nanomolar range. For PbTx-2 standard, the calibration curve gave an EC50 of 4.2 ng/assay (1.6 nM) and an EC50 of 6.7 ng/assay (2.5 nM) was established for PbTx-3. The synaptoneurosomal assay was first applied to the analysis of mussel tissue extracts spiked with PbTx-2.

The results demonstrate that the PbTx-2 equivalent activity as measured by the synapto-neurosomal assay is closely related to the amount of toxin added to the mussel extracts (r^2 = 0.97; slope = 1.08). In other experiments, extracts of oysters naturally contaminated with brevetoxins were assayed for PbTx-3 equivalents using the synaptoneurosomal technique, cytotoxicity assay, receptor binding assay and an HPLC-MS technique. Linear regression analysis of these data sets gave rise to r^2 values and slopes of 0.95, 0.06 (cytotoxicity vs. synaptoneurosomal assay), 0.91, 3.60 (receptor binding vs. synaptoneurosomal assay) and 0.75, 0.91 (HPLC-MS vs. synaptoneurosomal assay), respectively. In a parallel series of experiments, the pharmacological effects of various sodium channel toxins on synaptoneurosomal membrane potential (mV) were determined. The resting potential of control (non-depolarized) synaptoneurosomes averaged -71.9 mV and preincubation with 20 ng PbTx-2 alone was without effect. Exposure to veratridine (1.7 μM final concentration) depolarized synaptoneurosomes by 5.3 mV, in contrast to the veratridine plus PbTx-2 combination, which depolarized by 27.6 mV. Saxitoxin and tetrodotoxin had no effect on the resting potential of synaptoneurosomes, but completely prevented the depolarization of synaptoneurosomes produced by veratridine and the veratridine plus PbTx-2 combination. Our data therefore support the validity of the membrane potential assay in terms of brevetoxin sodium channel pharmacology.

Application of Rapid Immunochromatographic Tests to Biotoxin Monitoring: Beyond Shellfish

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Rapid immunochromatographic tests for paralytic shellfish poisoning (PSP) and amnesic shellfish poisoning (ASP) have been used to assess toxicity in shellfish, phytoplankton and fish. This technology was previously marketed under the trade name MIST Alert™, and has been extensively validated on extracts from shellfish tissue in many countries, including Canada, USA, United Kingdom, New Zealand and elsewhere. Applications of the rapid test technology to the detection of toxicity in phytoplankton will be discussed and the merits of this approach compared with other currently available methods. Other applications of the rapid test technology for biotoxin monitoring will also be presented.

Ab Initio Study of Domoic Acid Substructures

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INTRODUCTION

Domoic acid (Fig. 1) has been identified as the neurotoxin responsible for the 1987 outbreak of shellfish poisoning in eastern Prince Edward Island (Wright *et al.*, 1989). A flurry of activity investigating its chemical and spectroscopic properties ensued. The infrared spectrum of the solid produced at varying pH was measured (Falk, 1988). The FAB mass spectrum of domoic acid and its volatile t-butyldimethylsilyl derivatives has been reported (Thibault *et al.*, 1989). Strong $(M+H)^+$ and $(M-H)^-$ peaks are noted. An ultraviolet spectrum of aqueous domoic acid solutions gives an intense absorption band with a pH-dependent λ_{max} of between 240.0-244.7 nm (Falk *et al.*, 1989). A nuclear magnetic resonance (NMR) study gives the pKa values in both light and heavy water (Falk *et al.*, 1991a) and the solubility measured by NMR and UV spectroscopy (Falk *et al.*, 1991b). The X-ray structure gave the solid-state conformation of domoic acid (Nomoto *et al.*, 1992). A combined molecular modelling and NMR study gave some details of the structurally similar kainic acid (Falk *et al.*, 1998). A recent review of the toxicology of domoic acid has appeared (Mos, 2001).

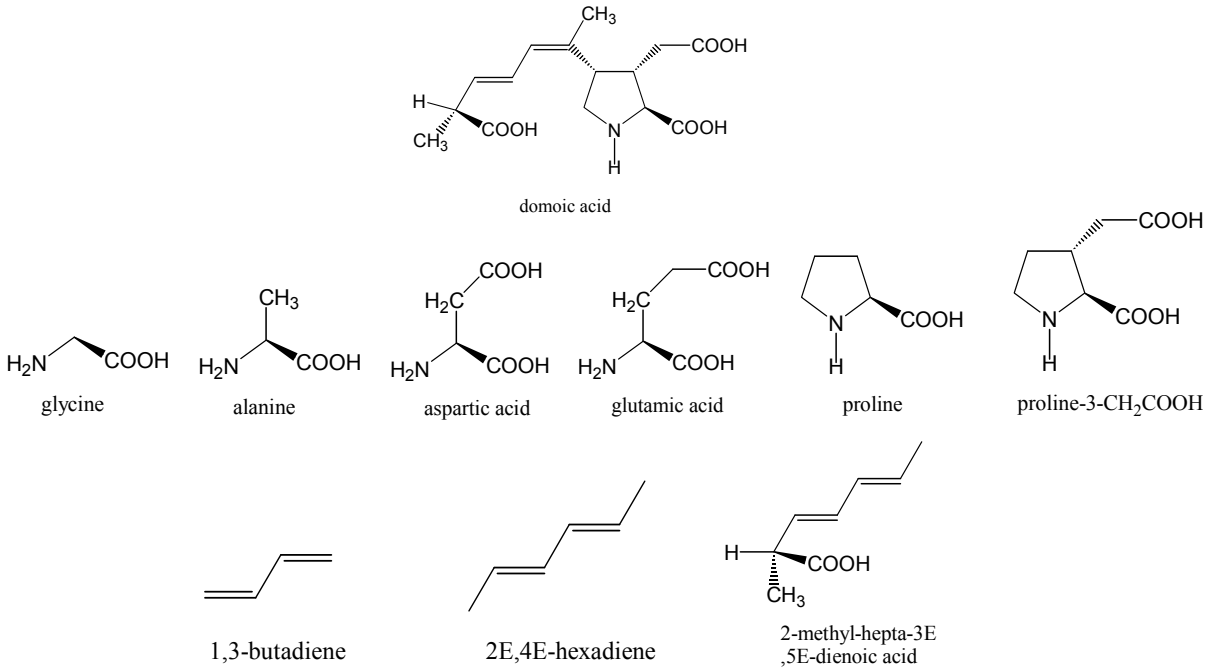

Fig. 1. Substrucures of domoic acid.

The amino acids glycine, alanine, glutamic acid and proline are embedded within the structure of domoic acid. In addition, several more successively complex dienes are also embedded. A computational modelling of these structures, followed by splicing them, should give considerable information as to the conformational energetics of domoic acid itself. We present our results below.

MATERIALS AND METHODS

Calculations were performed using Gaussian 98 (Frisch *et al*., 1998), using standard basis sets. Density functional calculations on glycine were carried out with the standard polarized double-zeta basis set in ADF 2.3 (SCM, 1998) and are an extension of previously published work (Pye and Ziegler, 1999). The MP2 calculations utilize the frozen core approximation. The geometries were optimized using a stepping stone approach, in which the geometries at the levels HF/STO-3G, HF/3-21G, HF/6-31G*, HF/6-31+G*, MP2/6-31G* and MP2/6-31+G* were sequentially optimized. Default optimization specifications (rms/maximum displacement smaller than .0003/.00045 au, rms/maximum force smaller than .0012/.0018 au) were normally used. After each level, where possible, a frequency calculation was performed at the same level and the resulting Hessian used in the following optimization. Z-matrix coordinates constrained to the appropriate symmetry were used to speed up the optimizations. Since frequency calculations are done at each level, any problems with the Z-matrix coordinates would manifest themselves by giving imaginary frequencies corresponding to modes orthogonal to the spanned Z-matrix space. The Hessian was evaluated at the first geometry (opt=CalcFC) for the first level in a series in order to aid geometry convergence. Total electronic energies are tabulated in the Appendix.

RESULTS

Glycine

We first examined structures possessing a mirror plane of symmetry (C_s). In the neutral form of glycine, the conformational degrees of freedom correspond to the single bonds of glycine N-C, C-C, and C-O in each of two orientations corresponding to eight possible conformers. The conformers **C_s #1-#4** and **#8** (Fig. 2) are not minima on the potential energy surface. The first four, which have eclipsed amine hydrogens, have an imaginary vibrational frequency corresponding to rotation about the C-N bond, and conformers **C_s #4** and **#8** have an imaginary vibrational frequency corresponding to rotation about the C-C bond. Conformers **C_s #5-#7** are stable. Conformer **#3** has an internal hydrogen bond and is especially stable. Excluding it, three general conformational trends can be given: (1) only the staggered amine is stable, (2) the hydroxyl hydrogen prefers to be *cis* to the carbonyl, but both are stable, and (3) the carbonyl prefers to be *cis* to the amine. The pattern suggests that there are additional conformers without symmetry (C_1). Five additional conformers were found corresponding to desymmetrization of the unstable C_s conformers along the normal mode corresponding to the imaginary frequency. Conformer **C_s #6**, possessing all three general trends, remains the most stable at all levels investigated, except at the DFT levels, for which **C_s (**or **C_1) #3** is favoured (Table 1).

Experiments show that **C_s #6** is the most favoured conformer (Pye and Ziegler, 1999), in agreement with both the Hartree-Fock and MP2 calculations. Only conformers **C_1 #1-#4** are embedded in the structure of domoic acid because of the conformational restrictions inherent in the proline ring. A recent theoretical study of neutral glycine supports these results (Császár, 1992).

Alanine

Because of the chiral central carbon, the alanine molecule cannot possess any symmetry. It may be derived from glycine by replacing hydrogen with a methyl group. Accordingly, because there are eight stable isomers of glycine, there should be a total of 16 isomers of alanine, with those derived from the symmetric structures being enantiomers. As there are five unsymmetric structures (5x2) and three symmetric structures (3x1), a total of 13 different conformers are possible (Fig. 3). The most stable conformation is **#6** (Table 2), which is derived from glycine **C_s #6**. Only conformers **1a-4a** have the correct orientation and stereochemistry for embedding in domoic acid. The 13 conformers found are the same as those found in a previous exhaustive study (Császár, 1996).

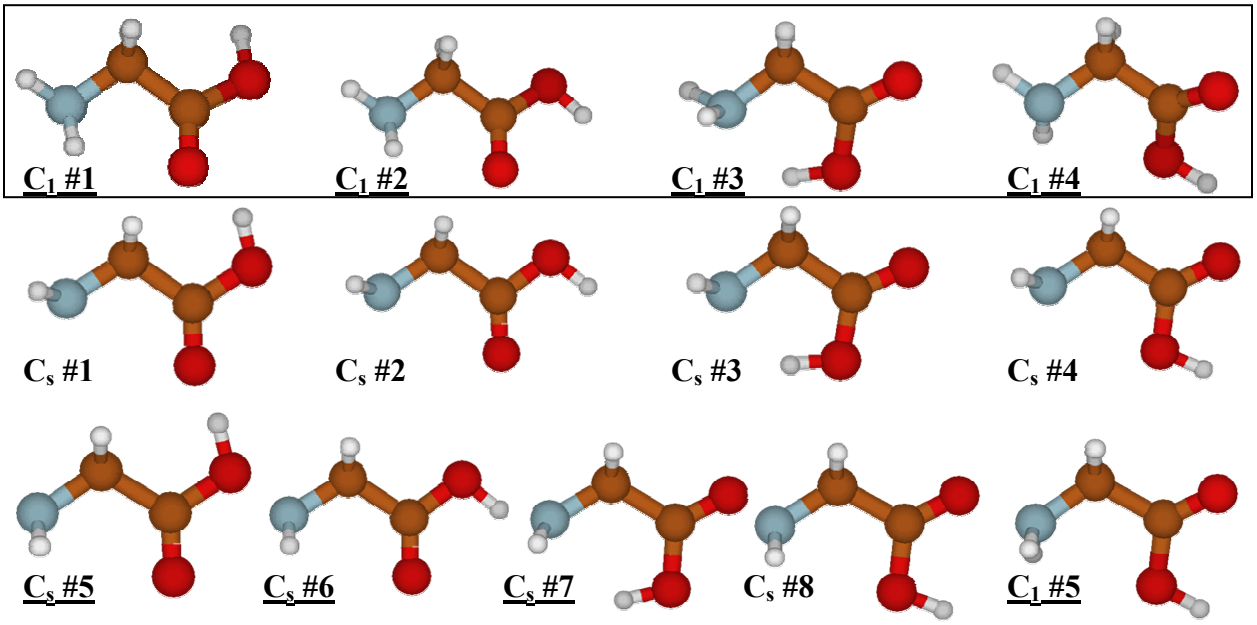

Fig. 2. Conformers of glycine. Underlined = geometrically stable.

Table 1. Energies of glycine structures relative to Cs #6 (kJ/mol).

	HF				MP2		DFT	
	STO-3G	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***	**VWN**	**BP86**
C_s #1	43.75		57.10	55.05	55.73	51.93	41.48	40.70
C_s #2	19.57		23.52	22.10	24.12	21.31	19.95	19.43
C_s #3	7.65	7.52	13.47	11.95	6.05	3.22	-13.92	-4.46
C_s #4	25.30		25.62	23.64	27.06	23.26	20.48	20.48
C_s #5	20.04	34.72	29.28	29.08	27.18	26.78	20.22	19.17
C_s #6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C_s #7	36.92		38.83	36.47	34.75	31.50		19.69
C_s #8	3.60	7.75	7.96	7.45	6.83	6.03	7.35	6.83
C_1 #1	28.34		38.99	37.38	37.25	34.19	21.79	
C_1 #2	6.28	9.36	8.64	7.35	8.93	6.23	2.63	5.78
C_1 #3	5.80	7.31	12.24	11.22	5.11	2.81		-4.73
C_1 #4	5.47	13.41	12.79	10.87	12.99	9.47	12.60	12.86
C_1 #5	2.79	7.74		7.40		5.78		

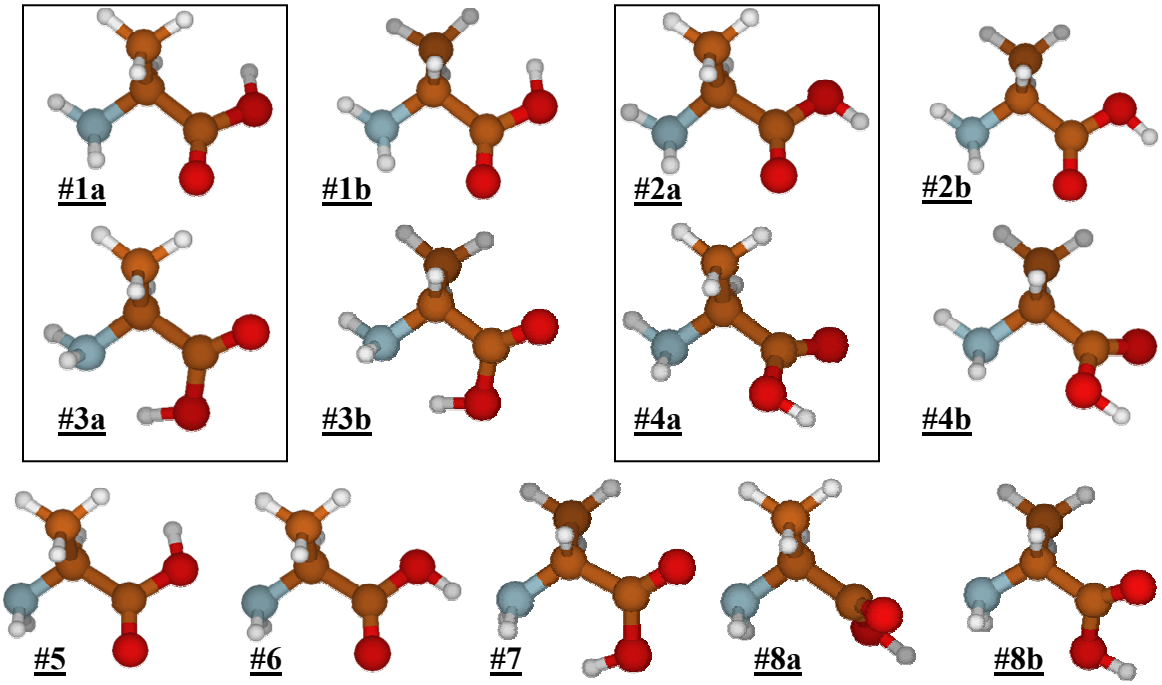

Fig. 3. Conformers of alanine.

Table 2. Energies of alanine structures relative to #6 (kJ/mol).

	HF				MP2	
	STO-3G	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***
#1a	27.12	43.14	37.89	37.09	34.88	33.41
#1b	32.57		39.34	38.34	36.41	34.21
#2a	4.49	7.87	7.81	7.08	7.07	5.84
#2b	7.31	7.91	8.27	7.54	8.08	6.28
#3a	5.46	4.45	9.95	10.34	2.28	2.82
#3b			10.62	9.94	3.15	1.53
#4a	3.59	10.78	10.77	9.40	10.69	8.84
#4b	4.48	8.13	8.66	8.02	8.97	7.37
#5	21.55	36.05	29.88	29.87	27.51	27.45
#6	0.00	0.00	0.00	0.00	0.00	0.00
#7	32.17		37.73	35.48	33.78	31.01
#8a	2.29	6.12	6.87	6.53	6.06	
#8b	2.49	7.68	6.18	5.10	5.64	4.09

Proline

Proline can be formed from alanine by replacing a hydrogen atom from each of the amine and methyl groups with an ethylene bridge. As determined before, only alanine conformers 1a-4a, and the enantiomers of 1b-4b (which have the same C*(CH_3) but opposite amine configuration), may be used. The ring can be either in a half-chair or an envelope configuration, and if the latter, the puckering methylene may be either syn or anti to the methyl group. We examined only six conformers. Proline is the only amino acid in which the amine is part of a ring (strictly speaking, it is an imino acid). This function is very important in proteins, producing a kink in an α helix or β sheet. In spite of their importance, the gas-phase structure of most amino acids remains unknown because of their low volatility and susceptibility to decomposition at higher temperatures. Very recently, the gas-phase structure of proline became known via the combination of laser ablation and high-resolution microwave spectroscopy (Lesarri *et al*., 2002). The observed conformers correspond with **#3** and **#4** below (Fig. 4), which is in agreement with our predictions (Table 3), and with more extensive calculations (Stepanian *et al*., 2001; Czinki and Császár, 2003). Our conformers **#1** and **#2** were also observed by matrix infrared techniques (Stepanian *et al*., 2001). The structure of other amino acids should be amenable to these techniques. One thing to note is the switch in order of the conformers upon progressing to the MP2 levels.

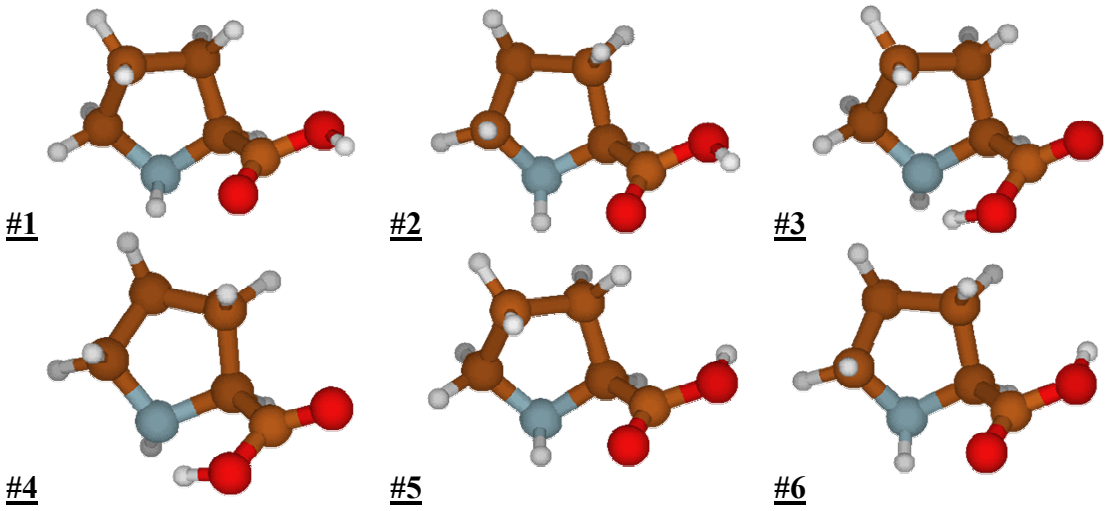

Fig. 4. Selected conformers of proline.

Table 3. Energies of proline structures relative to #3 (kJ/mol).

	HF				MP2	
	STO-3G	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***
#1	1.09	0.26	-1.76	0.05	6.29	9.19
#2	2.68	3.10	-3.38	-2.28	6.08	7.81
#3	0.00	0.00	0.00	0.00	0.00	0.00
#4	0.35	4.51	2.30	1.42	2.54	2.47
#5	20.67	33.76	26.02	27.64	31.23	33.85
#6	22.34	35.92	24.36	25.03	30.85	32.21

1,3-Butadiene

The double bonds in 1,3-butadiene are capable of conjugation. They may arrange themselves so that they form a $\mathbf{C_{2h}}$ structure (s-*trans*) or a $\mathbf{C_{2v}}$ structure (s-*cis*). In the $\mathbf{C_{2v}}$ structure, the terminal hydrogens may experience a steric interaction, and thus the structure twists somewhat to give a nonplanar $\mathbf{C_2}$ **#1** structure (Fig. 5). The s-*trans* structure is consistently predicted to be the most stable (Table 4). The two minima $\mathbf{C_{2h}}$ and $\mathbf{C_2}$ are separated by an energy barrier corresponding to structure $\mathbf{C_2}$ **#2**.

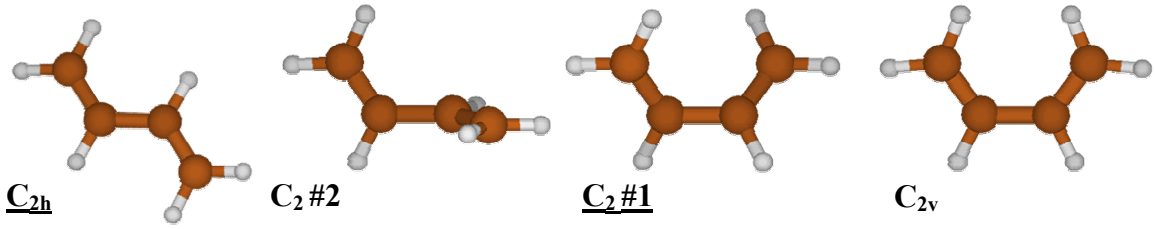

Fig. 5. Conformers of 1,3-butadiene.

Table 4. Energies of 1,3-butadiene structures relative to #C_{2h} (kJ/mol).

	HF				MP2	
	STO-3G	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***
C_{2h}	0.00	0.00	0.00	0.00	0.00	0.00
C_2#2	23.50	23.63	25.40	24.93	24.91	23.03
C_2		11.33	12.71	13.04	11.05	10.57
C_{2v}	7.67	14.48	16.26	16.63	14.93	15.77

Hexa-2,4-diene

This molecule is derived from 1,3-butadiene by placing methyl groups at the ends of the molecule in a *trans* fashion (Fig. 6). If the methyl groups are placed *cis*, then substructures for isodomoic acid D-F are obtained. Only the s-*trans* conformers were examined. The *trans-trans* structure (EE) is lowest in energy (Table 5), followed by the *trans-cis* (EZ) and *cis-cis* (ZZ). The conformation of the methyl groups in which the in-plane hydrogen is *cis* to the double bond is stable.

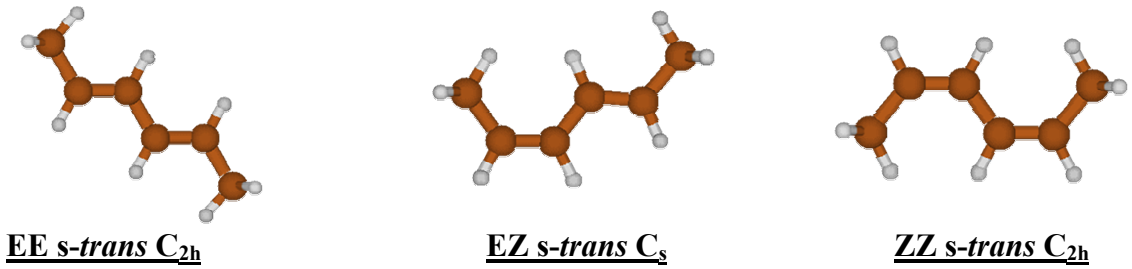

Fig. 6. Conformers of (2E,4E)-hexadiene.

Table 5. Energies of (2E,4E)-hexadiene structures relative to EE s-*trans* (kJ/mol).

	HF				MP2	
	STO-3G	3-21G	6-31G*	6-31+G*	6-31G*	6-31+G*
EE s-*trans*	0.00	0.00	0.00	0.00	0.00	0.00
EZ s-*trans*	6.99	6.42	6.97	6.64	6.09	5.62
ZZ s-*trans*	14.82	13.86	14.82	14.00	13.18	11.92

2-methyl-(3E,5E)-hepta-3,5-dienoic acid

This molecule may be obtained from (2E,4E)-hexadiene by adding a methyl and carboxyl group to one end (Fig. 7). There are a number of conformational degrees of freedom. Firstly, although the hydroxyl can be *trans* to the carbonyl, we have shown before that it prefers to be *cis* in the absence of intramolecular hydrogen bonding. The double bonds have a preference to be *cis* to sigma bonds. The carbonyl can be *cis* to either the skeleton C-C bond, the methyl C-C bond, or the C-H bond. The C-C double bond can be *cis* to either the C-COOH bond, the methyl C-C bond, or the C-H bond. Excluding *trans*-hydroxyl conformers, this gives us nine conformers. Conformers in which the carbonyl is *cis* to the skeletal C-C bond often gave optimization difficulties in which another conformer was obtained very different from the starting one. For the conformer in which C=O is *cis* to the skeleton and C=C is *cis* to the COOH, the only stable conformer that could be found was at the HF/3-21G level (Table 6), and all other levels caused the carbonyl to rotate towards the methyl group to avoid steric interaction with the hydrogen on carbon 4.

Table 6. Energies of 2-methyl-3E,5E-heptadienoic acid structures relative to Me,H (kJ/mol).

		HF				MP2	
C=O *cis*	C=C *cis*	STO-3G	3-21G	6-31G*	6-31+G*	6-31G*	6-31+G*
C-H	C-C:Me	6.03	9.23	8.12	8.23	6.56	7.46
C-H	C-H	-0.42	-3.50	0.59	0.19	0.78	0.10
C-H	C-CO_2H	6.48	4.78	10.63	11.29	7.83	8.54
C-C: sk	C-C:Me	5.97		7.28	8.34	7.10	8.92
C-C: sk	C-H	0.31	-4.98	-0.06	0.11		
C-C: sk	C-CO_2H		1.61				
C-C: Me	C-C:Me	3.93	-1.28	3.55	4.58	1.94	3.86
C-C: Me	C-H	0.00	0.00	0.00	0.00	0.00	0.00
C-C: Me	C-CO_2H	5.28	2.59	6.13	7.90	3.04	5.08

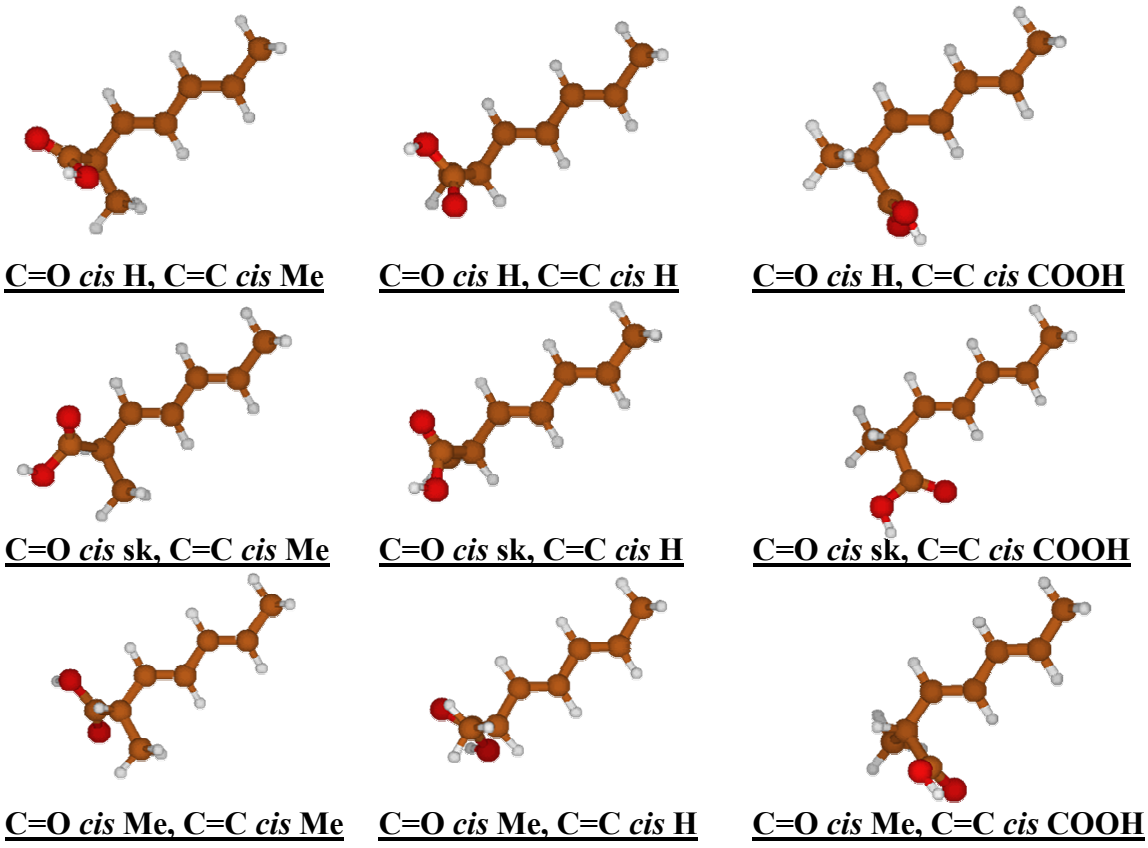

Fig. 7. Conformers of 2-methyl-(3E,5E)-heptadienoic acid.

CONCLUSIONS

By conceptually breaking up domoic acid into substructures and performing theoretical calculations, one can gain much insight into conformational trends that extrapolate to larger substructures of domoic acid itself. Although much work needs to be done, this approach helps to reduce computational cost and offers insight into the potential gas phase structure of domoic acid.

ACKNOWLEDGEMENTS

We would like to thank Fisheries and Oceans Canada for a youth internship (JFG, 2002). We also thank the Department of Astronomy and Physics, Saint Mary's University, for providing access to computing facilities, in particular, to Cygnus, a 10-processor Sun server purchased with assistance from the Canada Foundation for Innovation, Sun Microsystems, the Atlantic Canada Opportunities Agency, and Saint Mary's University.

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I, Domoic

Written by Cory Pye on 19 May 2003, based on Ozzy Osbourne's *I Don't Know*, and sung during the CWHMA Banquet

People look to me and say
Pseudo-nitzschia, fake German philosophy?
Upon these mussels if I dine?
Will they do funny things to my mind?

Chorus
Don't look at me for answers,
What was the question again?
Gone is my short-term memory
Don't ask me, domoic.

Diatoms, a heavy count
Domoic acid lingering about
Run it through the HPLC,
Let's prevent outbreaks of ASP.

Chorus

Bridge
Take a sample, inject intraperitoneally
Is that why they call it a mouse bio-ass-ay?
I'm just an *in silico* chemist,
This *in vivo*'s new to me
You can see, it's all Latin to me,
Ab initio, that's me
In vitro? Not me!

……..

APPENDIX

Table A1. Total energies of glycine structures (a.u.).

	HF				MP2		DFT	
	STO-3G	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***	**VWN**	**BP86**
C_s	-279 +	-281 +	-282 +	-282 +	-283 +	-283 +		
#1	-0.1025104	C_s #5	-0.8093474	-0.8200851	-0.5794027	-0.6035333	-2.2633	-2.0968
#2	-0.1117203	C_s #6	-0.8221392	-0.8326319	-0.5914422	-0.6151956	-2.2715	-2.1049
#3	-0.1162599	-0.2446331	-0.8259653	-0.8364974	-0.5983232	-0.6220862	-2.2844	-2.1140
#4	-0.1095350	C_s #8	-0.8213366	-0.8320471	-0.5903223	-0.6144502	-2.2713	-2.1045
#5	-0.1115387	-0.2342724	-0.8199446	-0.8299752	-0.5902759	-0.6131106	-2.2714	-2.1050
#6	-0.1191725	-0.2474975	-0.8310961	-0.8410507	-0.6006284	-0.6233110	-2.2791	-2.1123
#7	-0.1051093	C_s #3	-0.8163069	-0.8271610	-0.5873918	-0.6113121	C_s #3	-2.1048
#8	-0.1178028	-0.2445446	-0.8280662	-0.8382136	-0.5980261	-0.6210126	-2.2763	-2.1097
C_1								
#1	-0.1083765	Cs #5	-0.8162451	-0.8268143	-0.5864390	-0.6102875	-2.2708	C_s #5
#2	-0.1167803	-0.2439319	-0.8278052	-0.8382495	-0.5972267	-0.6209397	-2.2781	-2.1101
#3	-0.1169617	-0.2447132	-0.8264344	-0.8367775	-0.5986813	-0.6222417	C_s #3	-2.1141
#4	-0.1170880	-0.2423914	-0.8262254	-0.8369109	-0.5956796	-0.6197054	-2.2743	-2.1074
#5	-0.1181080	-0.2445486	Cs #8	-0.8382309	Cs #8	-0.6211109	--------	--------

Table A2. Total energies of alanine structures (a.u.).

	HF				MP2	
	STO-3G	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***
	-317 +	-320 +	-321 +	-321 +	-322 +	-322 +
#1a	-0.6912432	-0.0555373	-0.8535111	-0.8640116	-0.7574689	-0.7826839
#1b	-0.6891655	#5.	-0.8529572	-0.8635370	-0.7568860	-0.7823793
#2a	-0.6998613	-0.0689709	-0.8649667	-0.8754433	-0.7680605	-0.7931847
#2b	-0.6987865	-0.0689566	-0.8647910	-0.8752679	-0.7676752	-0.7930162
#3a	-0.6994948	-0.0702744	-0.8641521	-0.8741992	-0.7698865	-0.7943330
#3b	#3a	#3a	-0.8638961	-0.8743512	-0.7695529	-0.7948247
#4a	-0.7002037	-0.0678614	-0.8638384	-0.8745578	-0.7666812	-0.7920386
#4b	-0.6998659	-0.0688701	-0.8646412	-0.8750851	-0.7673384	-0.7925991
#5	-0.6933663	-0.0582377	-0.8565592	-0.8667613	-0.7602735	-0.7849535
#6	-0.7015726	-0.0719685	-0.8679410	-0.8781381	-0.7707531	-0.7954073
#7	-0.6893191	#3a	-0.8535714	-0.8646227	-0.7578876	-0.7835949
#8a	-0.7007005	-0.0696376	-0.8653235	-0.8756507	-0.7684432	#6
#8b	-0.7006242	-0.0690421	-0.8655888	-0.8761970	-0.7686067	-0.7938494

Table A3. Total energies of proline structures (a.u.).

	HF				MP2	
	STO-3G	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***
	-393 +	-396 +	-398 +	-398 +	-399 +	-399 +
#1	-0.7120121	-0.5451436	-0.7654118	-0.7752538	-0.9239933	-0.9502345
#2	-0.7114062	-0.5440589	-0.7660266	-0.7761415	-0.9240730	-0.9507589
#3	-0.7124282	-0.5452414	-0.7647407	-0.7752742	-0.9263883	-0.9537348
#4	-0.7122944	-0.5435251	-0.7638628	-0.7747334	-0.9254211	-0.9527959
#5	-0.7045563	-0.5323820	-0.7548313	-0.7647484	-0.9144938	-0.9408415
#6	-0.7039193	-0.5315610	-0.7554619	-0.7657419	-0.9146377	-0.9414663

Table A4. Total energies of 1,3-butadiene structures (a.u.).

	HF				MP2	
	STO-3G	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***
	-153 +	-154 +	-154 +	-154 +	-155 +	-155 +
$\underline{C_{2h}}$	-0.0203657	-0.0594566	-0.9196540	-0.9262518	-0.4226556	-0.4338470
C_2	-0.0114152	-0.0504557	-0.9099782	-0.9167560	-0.4131689	-0.4250761
$\underline{C_2}$	C_{2v}	-0.0551402	-0.9148127	-0.9212834	-0.4184472	-0.4298230
C_{2v}	-0.0174439	-0.0539433	-0.9134619	-0.9199176	-0.4169694	-0.4278389

Table A5. Total energies of 2,4-hexadiene structures (a.u.).

	HF				MP2	
	STO-3G	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***
	-230 +	-231 +	-232 +	-232 +	-233 +	-233 +
EE	-0.1931907	-0.7050573	-0.9983747	-1.0042867	-0.7648963	-0.7779200
EZ	-0.1905265	-0.7026109	-0.9957216	-1.0017566	-0.7625756	-0.7757813
ZZ	-0.1875465	-0.6997778	-0.9927303	-0.9989561	-0.7598766	-0.7733815

Table A6. Total energies of (3E,5E)-2-methylhepta-3,5-dienoic acid structures (a.u.).

		HF				MP2	
C=O *cis*	**C=C *cis***	**STO-3G**	**3-21G**	**6-31G***	**6-31+G***	**6-31G***	**6-31+G***
		-453 +	-457 +	-459 +	-459 +	-461 +	-461 +
C-H	C-C:Me	-0.8547436	-0.0838158	-0.6456649	-0.6580017	-0.0206157	-0.0506523
C-H	C-H	-0.8572004	-0.0886633	-0.6485319	-0.6610625	-0.0228179	-0.0534586
C-H	C-CO_2H	-0.8545732	-0.0855092	-0.6447104	-0.6568327	-0.0201313	-0.0502428
C-C: sk	C-C:Me	-0.8547697	CC sk, CH	-0.6459847	-0.6579597	-0.0204096	-0.0500989
C-C: sk	C-H	-0.8569241	-0.0892249	-0.6487788	-0.6610919	Me, H	Me, H
C-C: sk	C-CO_2H	Me, COOH	-0.0867147	Me, COOH			
C-C: Me	C-C:Me	-0.8555443	-0.0878182	-0.6474063	-0.6593907	-0.0223743	-0.0520239
C-C: Me	C-H	-0.8570418	-0.0873295	-0.6487578	-0.6611345	-0.0231139	-0.0534955
C-C: Me	C-CO_2H	-0.8550304	-0.0863420	-0.6464237	-0.6581258	-0.0219550	-0.0515625

Photodegradation of Domoic Acid

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ABSTRACT

The effect of light exposure on domoic acid stability was tested by exposing three water types to different wavelengths of light in a commercially available irradiance chamber. Domoic acid (ca. 75 ng mL^{-1}) was added to deionized water, artificial seawater (amended with iron, or not) and to filtered seawater. The irradiance conditions were: full-spectrum light, and light blocked by filters that cut out wavelengths <320 nm, <370 nm, <385 nm and <400 nm. Control samples were kept in darkness. Domoic acid was not degraded when the samples were kept in darkness. Exposure to full-spectrum light for 22 h resulted in domoic acid degradation in all three water types in the absence of added iron: 36% for deionized water, 44% for artificial seawater and 41% for natural seawater, relative to the time zero value. No detectable domoic acid remained in the deionized water with iron added, after the light exposure. Photodegradation was most rapid within about the first 5 hours of exposure to the full-spectrum light. The greatest decrease in domoic acid occurred when wavelengths <370 nm were present, and in full-spectrum light. These early results show that exposure of domoic acid to light must be considered when carrying out culture experiments and when studying the fate of domoic acid in the ocean.

INTRODUCTION

Domoic acid is a water-soluble neurotoxin produced and released by several species of the diatom *Pseudo-nitzschia* and some red macroalgal species (Bates *et al*., 1998; Bates, 2000), yet there is no evidence that it accumulates in the ocean. Few bacteria are capable of biodegrading domoic acid (Windust, 1992). So far, only those bacteria isolated from within blue mussels (*Mytilus edulis*) and some soft-shell clams (*Mya arenaria*) have exhibited growth with and biodegradation of domoic acid (Stewart *et al*., 1998). What, then, is the fate of domoic acid, once released into the water column?

In a previous study (Bates *et al*., 2003), soluble domoic acid produced by *Pseudo-nitzschia multiseries* unexpectedly declined by ca. 60% during a 10-day period under the irradiance conditions used for the culture (i.e. fluorescent light; ca. 100 µmol m^{-2} s^{-1} continuous irradiance). In a follow-up experiment during that same study, domoic acid added to sterile seawater declined by ca. 70% over a 12-day period under these same light conditions. There was less of a decline when bacteria were added, and even less in darkness. The above results prompted a further investigation into the possible photodegradation of domoic acid by light as a function of wavelength, type of water, and presence or absence of added iron.

MATERIALS AND METHODS

Domoic acid (ca. 75 ng mL^{-1}) was added to 10 mL of deionized water, artificial seawater, or filtered seawater (ca. 31 ppt, from the Damariscotta River, Maine) in duplicate or triplicate 15 mL acid-cleaned Teflon cups covered with saran wrap to minimize evaporation. Artificial seawater was prepared according to Price *et al*. (1989) and passed through Chelex 100 (BioRad) resin to remove contaminant trace metals. The deionized and artificial seawater were used directly, and with iron added in a 1:1 mole ratio with domoic acid (225 nM $FeCl_3$). Trace metal clean procedures were used to minimize contamination during preparation and irradiation of the samples. The water samples were then exposed at 20°C for 22 h or 24 h to simulated solar irradiation in a Suntest Chamber™ (Model CPS+, Atlas Material Testing Technology, Chicago, IL) (Fig. 1). The irradiance conditions were: full-spectrum light, and light blocked by filters that cut out wavelengths <320 nm, <375 nm, <385 nm and <400 nm. Control samples were wrapped in black aluminum foil to block light penetration. Duplicate aliquots of 4 mL were taken and stored frozen in 5 mL cryotubes until the domoic acid was analyzed by the FMOC-HPLC technique (Pocklington *et al*., 1990).

Fig. 1. Suntest tabletop xenon chamber used to expose samples to UV light, showing chamber (left) and Teflon cups (right).

RESULTS

Domoic acid was not degraded when the samples were kept in darkness. However, exposure to full-spectrum light for 22 h resulted in a similar amount of photodegradation in all three water types (Fig. 2). In the absence of added iron, the decrease in domoic acid concentration (relative to the time zero value) was 36% for deionized water, 44% for artificial seawater and 41% for natural seawater. For the deionized water with iron added, the decrease was more dramatic; no detectable domoic acid remained (< ca. 1 ng mL^{-1}) after the light exposure. Photodegradation also occurred when iron was added to the artificial seawater, but there was no substantial difference (48% decrease) relative to the condition with no added iron (44% decrease).

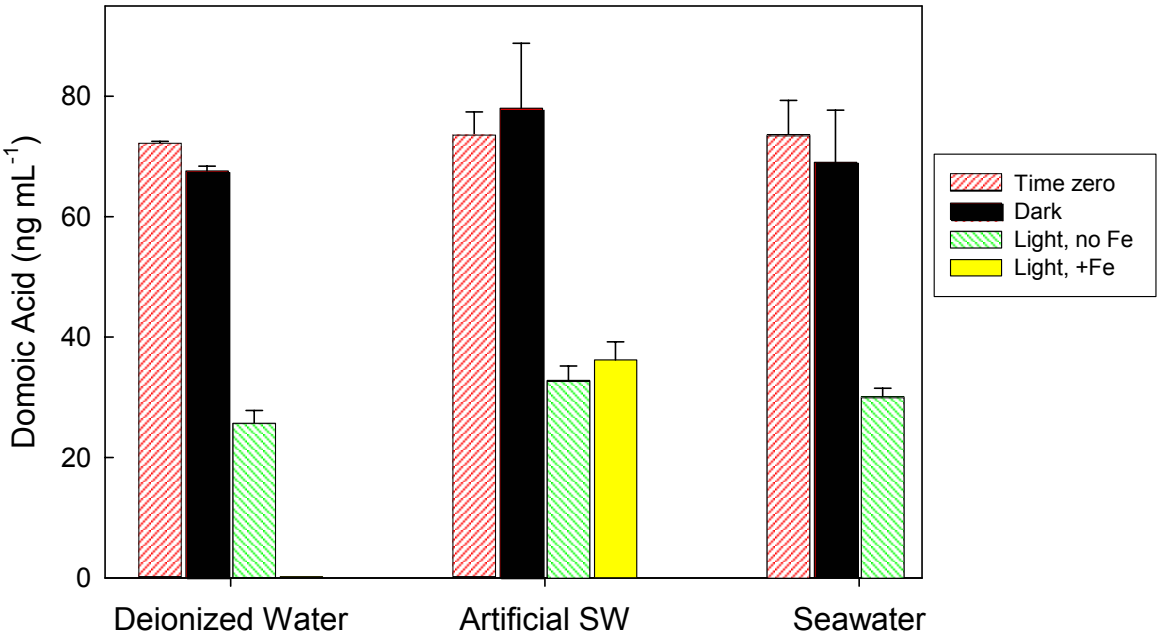

Fig. 2. Change in domoic acid concentration in three types of water and in the presence or absence of added iron, after exposure for 22 h to full-spectrum light; mean of triplicate beakers ± SD. No iron was added to the natural seawater, which already contained natural concentrations of iron.

Domoic acid in seawater was photodegraded at all wavelengths tested relative to the time zero value and the dark control, but the greatest amount of photodegradation occurred when wavelengths <370 nm were present (Fig. 3A). This included the beakers exposed to full-spectrum light.

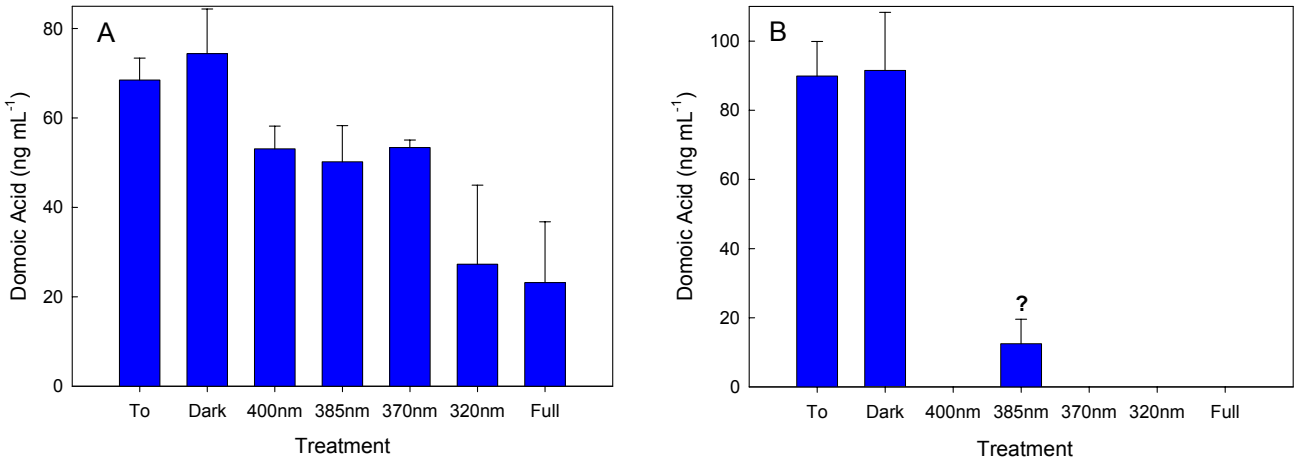

Fig. 3. Photodegradation of domoic acid in (A) seawater and (B) deionized water containing added iron. Samples were exposed for 22 h to full-spectrum light and to light filtered by cutting out wavelengths shorter than the number indicated. To = time zero. The control beakers were kept in darkness. Mean of duplicate beakers, with error bars showing the range.

With the exception of the 385 nm treatment, domoic acid in deionized water containing added iron was completely photodegraded (Fig. 3B); a similar result was shown in Fig. 2. We have no explanation for the ca. 16 ng DA mL^{-1} that remained in the duplicate samples exposed to light filtered by the <385 nm cutoff filter.

The photodegradation was most rapid within about the first five hours of exposure to the light (Fig. 4). As in the above experiment (Fig. 3A), photodegradation was similar in the treatments that cut out wavelengths <400 nm, <385 nm and <370 nm. Also, as in Fig. 3A, photodegradation was greatest in the 320 nm treatment and in the full-spectrum light exposure.

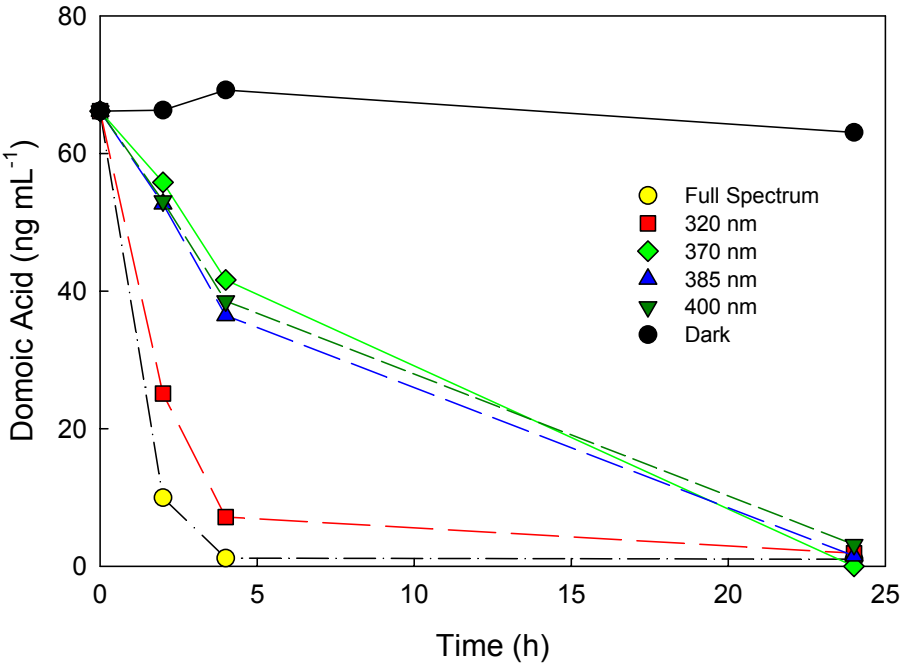

Fig. 4. Time course of domoic acid photodegradation in artificial seawater. Samples were exposed to full-spectrum light and to light that was filtered by cutting out wavelengths shorter than the number indicated. The control beakers were kept in darkness. Mean of duplicate samples normalized to the dark control.

DISCUSSION

Little is known about the fate of domoic acid in the ocean, once it is released by domoic-acid-producing *Pseudo-nitzschia* spp. and certain macroalgae. Aside from possible bacterial degradation (Windust, 1992; Stewart *et al*., 1998), one earlier study did show that domoic acid can undergo photolysis (Wright *et al*., 1990). After a short exposure (15 min) at 30°C to ultraviolet light (250 nm), domoic acid was converted to the geometrical isomers 2, 3 and 4; maximum yield of isomers was reached after 9-12 min (Wright *et al*., 1990). Our study showed that the photolysis of domoic acid was most rapid within the first five h of exposure to light (Fig. 4). We have not yet investigated shorter exposure periods or the photodegradation products.

With the exception of microcystins (Welker and Steinberg, 1999; Feitz and Waite, 2003), few other phycotoxins have been studied with respect to photodegradation. Our results confirm earlier initial findings showing that domoic acid may become photodegraded (Bates *et al*., 2003). In the present study, no degradation of domoic acid was seen in darkness, whether or not added iron was present. Therefore, iron, alone, was not responsible for the disappearance of domoic acid. Similarly, our stock solutions of domoic acid in distilled water have remained stable for up to two years in darkness at temperatures of 3-4°C in a refrigerator. Domoic acid is otherwise known to be unstable if stored at high temperatures (50°C), extremes of pH (2 or 12), or when exposed to oxygen (Quilliam, 2003).

The absorption of sunlight (especially in the UV region of the spectrum) by dissolved organic and inorganic compounds in natural waters leads to the production of a variety of transient reactive species, e.g. hydrogen peroxide, singlet oxygen, hydroxyl radicals and superoxide ions (Hoigné, 1990). These transient intermediates are highly reactive and can promote the photodegradation of different organic molecules (Momzikoff *et al*., 1983).

The presence of iron greatly enhanced the photodegradation of domoic acid in deionized water. Domoic acid may chelate iron (Bates *et al*., 2001; Rue and Bruland, 2001), and the catalytic photooxidation of natural organic matter by iron is well described (Miles *et al*., 1981; Voelker *et al*., 1997). In principle, photooxidation may occur by either: 1) indirect processes, whereby light interaction with other dissolved constituents generates reactive intermediaries that oxidize domoic acid, or 2) by direct photolysis, where a photon absorbed by the iron-domoic-acid complex causes the transfer of an electron from domoic acid to the iron atom. The latter mechanism likely is responsible for the enhanced loss of domoic acid in the pure water solution.

Iron did not appear to enhance photodegradation of domoic acid in artificial seawater, contrary to our expectations. It is possible that salts in some way inhibited the photoreduction of the iron-domoic-acid complex. However, it may also be related to iron solubility in seawater. The maximum inorganic solubility of iron is ca. 0.3 nM in 20°C seawater (Liu and Millero, 2002), so the vast majority of added iron (225 nM) may have precipitated before being complexed to domoic acid. Future study is needed to define the importance of iron for domoic acid photolysis at their ambient concentrations in seawater.

These preliminary findings show that domoic acid is photolabile in both seawater and culture media. Light effects therefore must be taken into account when reporting dissolved domoic acid concentrations. Moreover, significant photolysis occurred at wavelengths of 370-400 nm, indicating that photodestruction of domoic acid may extend well into the water column of coastal waters, and conceivably could be the predominant fate of dissolved domoic acid in the ocean.

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ORAL SESSION 2:

Effects of HABs and Phycotoxins on Other Organisms

Prorocentrum lima is Toxic to Juveniles of the European Sea Bass *Dicentrarchus labrax*

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ABSTRACT

Juveniles of the European sea bass *Dicentrarchus labrax* were forced to feed on the toxic dinoflagellate *Prorocentrum lima*, under laboratory conditions. The fish were placed in tanks containing the toxic cells and fed a commercial fish diet. Before feeding, the tank water was moderately stirred to bring the cells into suspension. The animals ingested the cells along with the introduced fish diet. Fish so treated became inactive, stopped feeding and started dying within three weeks. During this time, only one fish died in the control tanks (tanks with natural seawater without *P. lima*). Histological examinations of the gills and liver tissues of intoxicated fish revealed pathological changes that were absent in similar tissues of the control fish.

INTRODUCTION

The proliferation of toxic harmful algal bloom (HAB) species may not only affect the survival and breeding of adult fish (White, 1984), it may also jeopardize the survival of juvenile fish and, therefore, recruitment to fisheries in coastal waters (Gosselin *et al*., 1989; Robineau *et al*., 1991a,b). In aquaculture operations, toxic HABs have occasioned catastrophic losses to fish farmers (e.g. Dickman, 2001; Hodgkiss and Yang, 2001). Toxic HAB-induced fish kills could result from structural or physiological modifications of tissues in the affected fish. Organs that are commonly affected include the liver, kidneys, gut and gills (Jones *et al*., 1982; Phillips *et al*., 1985; Råbergh *et al*., 1991; Black *et al*., 1991; Andersen *et al*., 1993; Lush *et al*., 1998).

Most toxic fish kills have been associated with microalgal species that produce toxins that cause either paralytic shellfish poisoning (PSP) or neurotoxic shellfish poisoning (NSP) (Shimizu, 1987; Robineau *et al*., 1991a,b; Black *et al*., 1991; Hallegraeff *et al*., 1995; Khan *et al*., 1997, 1998). Although Anderson (1995) hypothesized that several species of fish in US waters might be affected by *Prorocentrum lima*, no microalga that produces diarrhetic shellfish poisoning (DSP) toxins has been associated with fish kills. Most laboratory studies dealing with the effects of toxic algae on fish have, therefore, been carried out with PSP- and NSP-causing algal species.

Prorocentrum lima from Vigo (Spain) killed brine shrimp that ate it (Ajuzie, 2002). It also exhibited an allelopathic effect on *Prorocentrum micans* when the two species were cultured together (Ajuzie and Houvenaghel, 2001). In view of the toxic potentials of *P. lima* to brine shrimp and *P. micans*, we decided to test its potential impact on juvenile fish by employing juveniles of the European sea bass, *Dicentrarchus labrax*.

MATERIALS AND METHODS

Toxic *Prorocentrum lima* cells (strain PL2V) in culture were obtained from Instituto Español de Oceanografía, Vigo, Galicia, Spain. They were, thereafter, cultured and maintained in bacteria-free, K-medium-enriched seawater (Keller *et al*., 1987) in our laboratory in Brussels. One hundred and fifty juveniles (100 days old) of the European sea bass *Dicentrarchus labrax* were obtained from a commercial fish hatchery in Gravelines, France, on 4 July 2000, and transported to our laboratory in an oxygenated enclosure (salinity at 12‰). No fish died during transport from France to Belgium. Also, no mortality was recorded during the period of acclimatization (one week) in our laboratory.

Investigations on the effects of *P. lima* on juveniles of *D. labrax*

A group of sea bass juveniles (referred to as "treated fish") was placed in tanks harbouring whole cells of *P. lima* and fed a commercial fish diet. Ten fish were held separately in two 6 L tanks. Each tank contained 2 L of natural seawater and 2 L of *P. lima* culture (with ca. 4.5×10^3 cells mL^{-1}). Before feed was introduced into the tanks, the tank water was moderately stirred to bring the *P. lima* cells into suspension, since this alga is largely a benthic species. Two control tanks with only natural seawater also housed 10 fish each. The control fish were fed the same commercial fish diet as the treated fish. The medium in which the fish were held was changed once, and the concentration of *P. lima* was adjusted to remain about the same as above. Both treated and control fish were closely monitored for any abnormal behaviours and deaths. All dead fish were removed from the study tanks as soon as they were spotted. Four weeks into the study, specimens of both treated and control fish were prepared for histological examinations of gill, liver, kidney and intestinal tissues. Sampled fish were killed by a quick partial cut at the junction between the head and the trunk, and preserved rapidly so as to prevent the occurrence of any postmortem artifacts (Roberts, 1978; Speare and Ferguson, 1989).

RESULTS

Fish that were brought into contact with *P. lima* (treated fish) exhibited pronounced movements of the opercula. They also attempted to jump out of the medium, particularly during their initial contacts with the medium. During feeding, treated fish ingested *P. lima* cells along with their introduced fish diet (Fig. 1), and gradually became intoxicated. Signs of intoxication started appearing by the third week of the study; treated fish became highly sluggish and performed surface swims with their mouth permanently opened. By this time, they could not aim properly at food particles; every attempt they made to capture food items ended with a miss. Before the start of the fourth week, fish that had not died were no longer feeding. Treated fish started dying during the third week of the study – a period that witnessed 35% mortality among this group. The situation in the control tanks was totally different. Behaviour in control fish was not affected, and fish mortality within this group was minimal – only one control fish died during the study period.

Histological examinations of both treated and control fish revealed pathological changes in the gill and liver tissues of the treated fish. The kidney and intestines, however, showed no

pathologies. Whereas gills of the control fish were normal (Fig. 2), those of the treated fish suffered epithelial lifting (Fig. 3), vacuolation of lamellae tips (Fig. 4), and fusion of lamellae/mucus secretion (Fig. 5). Similarly, whereas liver tissues in control fish were normal and with healthy hepatocytes (Fig. 6), those in the liver of the treated fish were grossly modified – the resultant effect was a total breakdown of tissue architecture, with the hepatocytes failing to pick up the haematoxylin-eosin (H&E) stain (Fig. 7).

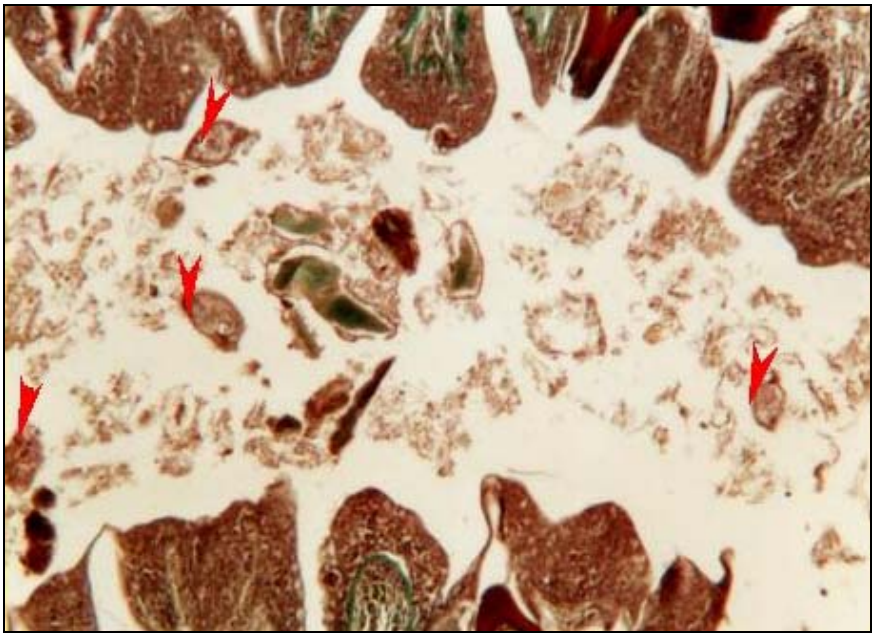

Fig. 1. *Prorocentrum lima* cells (arrows) in the alimentary canal of treated fish.

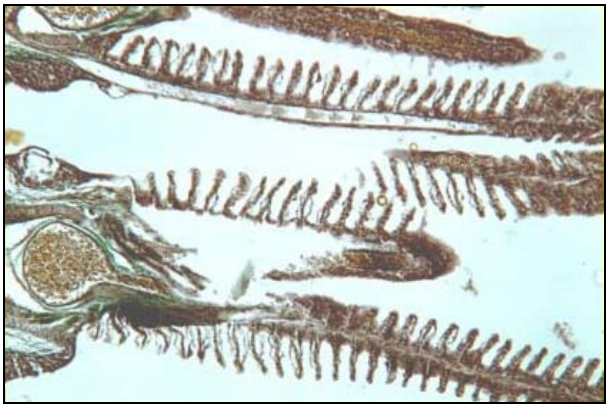

Fig. 3. Gill epithelial lifting in treated fish (LM 125x).

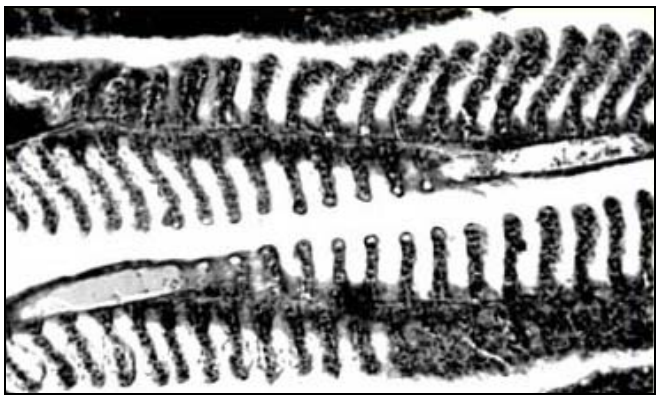

Fig. 4. Gill lamellae of treated fish with vacuolated tips (LM 100x).

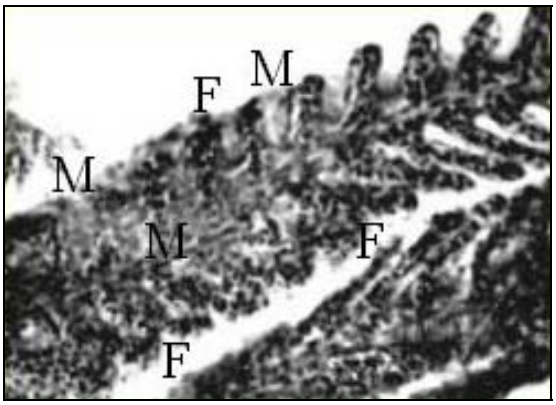

Fig. 5. Fusion (F) of lamellae and mucus (M) in the affected gill of treated fish (LM 200x).

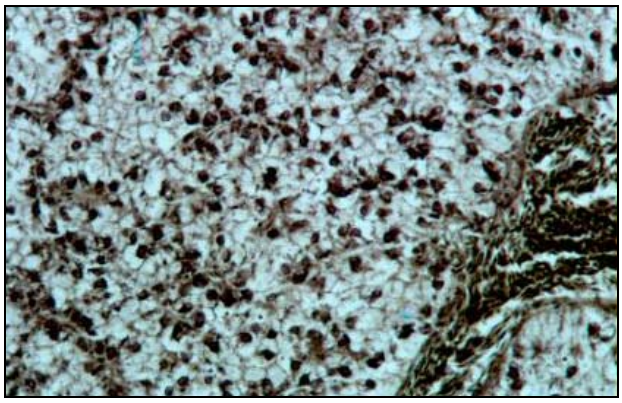

Fig. 6. Unaffected liver of control fish (LM 312.5x).

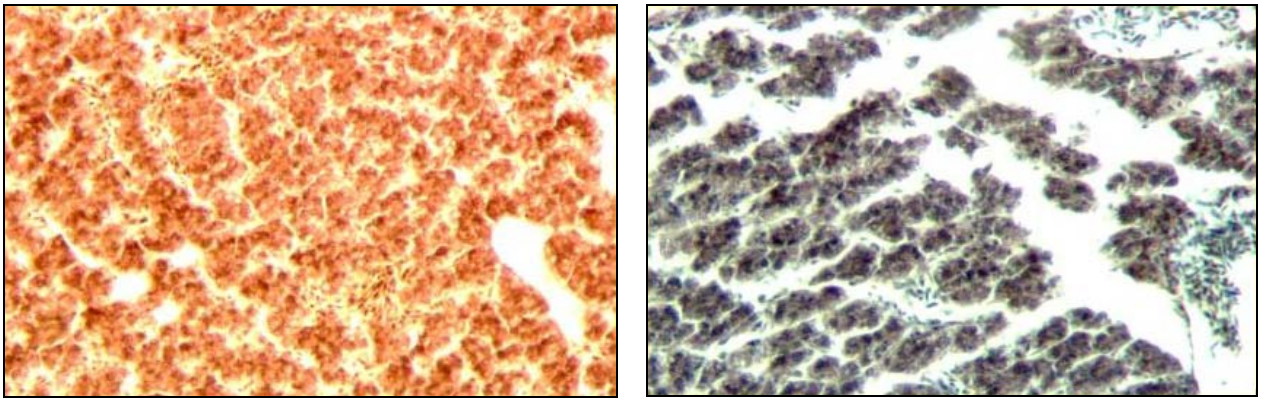

Fig. 7. Severe alteration in tissue architecture of affected liver in treated fish; right and left panels (LM 312.5x).

DISCUSSION

Fish that were exposed to the toxic *P. lima* died within three weeks. A failure in the respiratory and osmoregulatory functions of the gills might have contributed to their death. The structure of the secondary lamellae in the gills of treated fish was grossly modified by this alga. The most readily observable and widespread effect of the toxic cells on the treated fishs' gills was oedematous swellings of gill lamellae, which led to the separation of lamellar epithelia from the lamellar vessels. Consequently, lamellae gaseous exchange (Laurent, 1984) could have been compromised to the extent that death resulted. Lifting of the epithelium in the presence of a toxicant has been regarded as a defense mechanism that increases the distance across which waterborne irritants must diffuse to reach the bloodstream (Morgan and Tovell, 1973; Mallatt, 1985). However, since gill-oxygen transfer is dependent not only on the perfused gill area, but also on the thickness of the blood-water barrier (Pärt *et al*., 1982), lamellar epithelial lifting is injurious to fish. This is because branchial responses that serve to slow the entry of a toxicant

have the undesirable side effect of threatening to suffocate the fish (Skidmore, 1964; Burton *et al*., 1972). In summary, lifting of the gill lamellar epithelium will impair oxygen transfer as a result of the increased distance between water and secondary lamellar capillaries. The secondary lamellar structure is, thus, optimized for exchange with the environmental medium with short diffusion distance (Pärt *et al.,* 1982). In addition, gill swellings will reduce water space between adjacent lamellae, and thus the amount of oxygen available to the gill.

Death of treated fish may have also resulted from hypoxia caused by an over-production of gill mucus. The presence of *P. lima* in the water could have been responsible for the mucous film observed among the gill filaments of treated fish. Several workers, including Lush *et al*. (1998), have observed that fish discharge mucus from the opercular region when brought into contact with toxic algae. The secreted mucus has the disadvantage of overwhelmingly covering the respiratory epithelium of the primary and secondary gill lamellae, such that the aorta blood becomes hypoxic. Hypoxia is capable of inducing a cascade of events that can disrupt the normal metabolic systems of fish and cause their death (Yang and Albright, 1992). Since *P. lima* has no noticeable spine, it is suggested that the toxins of this dinoflagellate caused the damages observed on the gills of treated fish. Support for this assertion is that fish have been reported to absorb biotoxins through the gills (Hughes and Perry, 1976; Colin *et al*., 1979; Pärt *et al*., 1982; Haya *et al*., 1990).

Apart from the problems associated with the disruption of normal gill function, treated fish also suffered severe liver distress that may have resulted in a breakdown in the functioning of the hepatic system. The readily observable pathological change in the liver was dissociated hepatocytes, which placed the integrity and architecture of the parenchyma in total disarray. The liver's inflammatory response to the toxins of *P. lima* leads us to suggest that *P. lima* is capable of producing a hepatotoxic substance(s). Other substances that have proved to be hepatotoxic induced similar pathological changes in the affected liver of fish (Phillips *et al*., 1985; Kent *et al*., 1988; Kent, 1990; Råbergh *et al*., 1991; Andersen *et al*., 1993).

It is highly probable that the liver of treated fish became intoxicated while performing its physiological role connected with detoxifying deleterious materials that find their way into the blood system of the animal (Harder, 1975; Fänge and Grove, 1979; Groman, 1982). During detoxification, hepatocytes are the primary target cells of intoxicants (Runnegar *et al*., 1981). Since the liver is connected to the small intestine (Harder, 1975; Groman, 1982), hepatocytes in the liver of treated fish must have concentrated *P. lima*'s toxins via the bile acid carriers. Uptake of toxins in vertebrates, it should be remembered, is through the multispecific bile acid transport system (Runnegar *et al.,* 1981; Eriksson *et al*., 1990a). This can result in alterations of filaments in the cells' cytoskeleton and the resultant loss of liver architecture (Eriksson *et al*., 1987, 1989; Falconer and Runnegar, 1987; Carmichael and Falconer, 1993).

Some hepatotoxic substances like microcystins have been shown to be potent inhibitors of the phosphoprotein phosphatases type 1 and type 2A (Eriksson *et al*., 1990b; MacKintosh *et al*., 1990; Matsushima *et al*., 1990; Andersen *et al*., 1993). Similarly the DSP toxins, and particularly okadaic acid (OA), are potent inhibitors of phosphatases 1 and 2A (Takai *et al*., 1987; Bialojan and Takai, 1988; Bialojan *et al*., 1988). Since the inhibition of phosphatase activity is accompanied by an increased phosphorylation of, e.g. cytoskeletal proteins, it is

conceivable that the cytoskeletal effects could be a result of phosphorylation of cytoskeletal proteins (Eriksson *et al*., 1990b). Also, since OA is capable of enhancing phosphorylation of cytoskeletal elements (Dho *et al*., 1990), it is possible that OA is hepatotoxic.

CONCLUSIONS

This is the first research to report the fish-killing potential of a DSP-producing microalga. There is a possibility that the DSP toxins of *P. lima* are ichthyotoxic and potentially hepatotoxic. Alternatively, *P. lima* may also produce, in addition to the DSP toxins, some hepatoxic/ichthyotoxic substance(s). Jones *et al*. (1982) suggested a hypothesis involving ichthyotoxins when a bloom of *Gyrodinium aureolum* damaged the gills of farmed salmon and caused their death. Similarly, Lush *et al*. (1998) suggested that cells of the toxic dinoflagellate *Alexandrium minutum* are ichthyotoxic when they caused degenerative and mortal changes in the gills of fish.

The implication of these findings is that, in aquaculture operations, *P. lima* might seriously impact caged fish. Although it is a benthic species, this alga also lives as periphyton on any surface in the water column (Faust, 1993a,b; Lawrence *et al*., 2000). The physical structures of fish cages and the fouling organisms on these structures can provide an ideal substrate for *P. lima*. The suspension of cages in the water column, along with a feeding practice that encourages feed waste by not using demand or automatic feeders (Ajuzie, 1998), can modify nutrient dynamics. Uneaten fish feed particularly, and waste products of metabolism from fish, will contribute micronutrients needed for *P. lima* growth. When *P. lima* blooms under such a condition, it might result in fish kills within the pens and cages.

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Predation on the Rotifer *Colurella* sp. by "*Gymnodinium*" *fungiforme*, an Epibenthic *Pfiesteria*-like Dinoflagellate in British Columbia Waters

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A voracious epibenthic, heterotrophic dinoflagellate that attacks benthic diatoms, other flagellates (including other dinoflagellates) and rotifers in a manner very similar to *Pfiesteria*, was isolated from samples taken at Jericho Beach, English Bay, British Columbia. This dinoflagellate was maintained in an enrichment culture that was initially composed of benthic diatoms (principally *Achnathes*), harpacticoid copepods, hypotrich ciliates and the rotifer *Colurella* sp. It was later grown in single-prey culture using *Dunaliella tertiolecta* as food.

The dinoflagellate, common on the beaches of British Columbia and northern Washington State, has been known in Europe as *Gymnodinium fungiforme* Anassimova (also as *Katodinium fungiforme*). However, we have obtained a cryptic tabulation and a SSU ribosomal DNA sequence, which show that it is neither genus, and are currently describing it for publication. It will retain the specific epithet "fungiforme". It is not a *Pfiesteria* but is similar in size, appearance and mode of peduncle use. No amoeboid stages have been seen in culture.

Usually, it is attracted to dead or injured cells but also to apparently healthy *Colurella* sp., during which swarms of cells attached to the anterior joints of the rotifer and nipped bits of tissue with their pedunclea. After death, the rotifers' bodies were fed upon by increased numbers of the dinoflagellate until displaced by hypotrich ciliates.

It is possible that the dinoflagellate can feed on bacteria by an unknown mechanism, because a diatom culture, heavily invaded by bacteria, was inoculated with the dinoflagellate and most of the bacteria disappeared within 48 hours.

Toxin Dynamics in the Clam *Nuttallia obscurata* when Artificially Contaminated with Paralytic Shellfish Toxins from Cultured *Alexandrium tamarense*

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Cultured cells of *Alexandrium tamarense* contained 43.7, 38.3, 7.7, 7.0, 2.1, 0.6 and 0.6 molar % of STX, C2, GTX 5, NEO, C1, GTX3 and dcGTX3 toxins, respectively, for a composite cellular toxicity of 13.42 pg STXeq $cell^{-1}$. The varnish clam *Nuttallia obscurata* (6.31 g tissue weight), fed in triplicate for 72 h with cultured algae, consumed 1.65×10^6 cells g^{-1} tissue. Clams collected at intervals during intoxication were dissected into foot, gills, siphon, mantle and the viscera, which included the main body of the clam. Toxins were determined by HPLC, using appropriate toxin standards.

Total toxin in the viscera after a 72 h exposure was 27.63 nmol g^{-1}, composed of 51.56% STX, 31.14% C2, 5.83% C1, 4.85% GTX5, 3.64% dcGTX3, 1.72% NEO, 0.41% GTX3, 0.45% GTX2 and 0.40% dcGTX2. From regression analysis, uptake of GTX2, GTX 3 and GTX 5 increased linearly with time, whereas all other toxins increased exponentially throughout the period, except for NEO, which was best represented by a power curve. Total toxicity declined by 46.89% (as STXeq 100 g^{-1}) within 12 d of depuration, and remained relatively constant for a further 16 d of depuration. This rapid exponential decay to a constant level was shown mainly by STX and GTX2. The C2 and GTX3 toxins declined exponentially throughout the depuration period, whereas C1 remained constant and GTX 5 increased with time. Interestingly, GTX5 and C1 formed 38.21 and 17.26 molar % of the low toxicity levels in the initial clams before contamination, suggesting their substantial retention in varnish clam under natural conditions.

After a 28 d depuration period, the viscera contained 13.29 nmol g^{-1} toxin, comprised of 52.78% STX, 12.25% C1, 10.47% GTX5, 7.43% NEO, 6.61% dcGTX3, 6.32% C2, 3.06% dcGTX2, 0.78% GTX2 and 0.30% GTX3. Toxicity clearance results from toxin analyses corroborated a preliminary investigation on PSP loss from clams as assessed by mouse bioassay, which demonstrated 27.5% and 40.6% loss after 5 and 10 d of depuration, respectively.

Transport and Storage Factors Influencing the Concentration of Diarrhetic Shellfish Toxins in Blue Mussels

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Filter-feeding shellfish accumulate toxic substances, which can lead to shellfish poisoning in humans. There are several diarrhetic shellfish poisoning (DSP) toxins, including the acidic compound okadaic acid (OA), dinophysistoxins (DTX1 and DTX2) and acyl derivatives, neutral polyether lactones of the pectenotoxin group (PTXs) and yessotoxins (YTXs). Only the okadaic group has a diarrhetic effect and both OA and DTX1 are potent inhibitors of protein phosphatases and also have tumour-promoting activity. The high incidence of DSP poses a serious problem to both public health and to the shellfish industry and, for that reason, monitoring programs have been set up in many areas. The European Commission has stated that the maximum level of OA, DTXs and PTXs together in bivalve molluscs, echinoderms, tunicates and marine gastropods intended for human consumption shall be 160 μg of OA equivalents kg^{-1}. As part of the DSP monitoring program, for the Food Standards Agency in Scotland, samples are collected and analyzed from numerous sites on a regular basis.

A study was carried out on the effects of different transportation times and storage conditions on DSP toxin levels. This type of information is important in terms of both defining quality control criteria for accepting samples during monitoring programs and for shellfish processing. Blue mussels (*Mytilus edulis* L.) were collected from a site of known DSP activity and analyzed on the day of collection (study control) and at intervals of 1, 3, 5 and 7 days after storage in three different types of container. In addition, the containers were stored under different conditions to simulate the routes samples take prior to analysis at the laboratory. All samples were analyzed by liquid chromatography mass spectrometry (LC-MS), fitted with an atmospheric pressure ionisation (API) source, for the DSP toxins okadaic acid, DTX1 and DTX2. Due to the high variability among homogenates, it was not proven whether the container type had an effect on toxin levels during transportation but it was observed that OA concentrations (and to a lesser extent DTX2) altered over the course of the study.

DNA Microarray Analysis of Gene Expression in Mice Treated with the Cyanobacterial Toxin, Cylindrospermopsin

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Cyanobacteria (blue-green algae) produce toxins which present health hazards to water safety. These toxins include microcystins (MCYSTs), nodularins, saxitoxins, anatoxin-a(s), and cylindrospermopsin (CYN). They are structurally diverse and their effects range from liver damage to neurotoxicity. MCYSTs have been found in aquatic environments in all of the world's continents, and CYN is commonly being detected in many regions of the world. There is considerable information available on the chemistry and toxicology of MCYSTs (Duy *et al.*, 2000). In contrast, relatively little is known about CYN, especially in terms of its toxic mechanisms (Humpage *et al.*, 2000; Shaw *et al.*, 2000; Shen *et al.*, 2002).

CYN is an alkaloid consisting of a tricyclic guanidine moiety combined with hydroxyl-methyluracil. The compound is zwitterionic and highly water soluble (Shaw *et al.*, 2000). CYN was retrospectively implicated in human intoxication reported on Palm Island, Australia in 1979 (Hawkins *et al.*, 1985). In this incident, 138 children and 10 adults suffered symptoms of malaise, anorexia, vomiting, headache, painful liver enlargement, initial constipation followed by bloody diarrhea and various degrees of dehydration. CYN was first isolated and identified from *Cylindrospermopsis raciborskii* (Ohtani *et al.*, 1992). *Cylindrospermopsis raciborskii* has been found in human drinking water sources in many parts of the world, and this has an important public health implication. CYN has now been reported from water supplies within Australia, the USA, Brazil, Europe (France), China and southeast Asia (Thailand and Sri Lanka).

In this research, male Balb/c mice were i.p. injected with a single dose of either 100 μg kg^{-1} of CYN or saline (control). The animals were sacrificed 6 h, 72 h and 96 h post-injection to allow a study of early (6 h) and late (72 h and 96 h) gene expression patterns. Liver RNA was extracted and converted to [α-^{32}P]-dATP-labelled cDNA probe which was hybridized with Clontech Mouse Toxicology Array. Atlas Mouse Toxicology 1.2 Arrays (Clontech, Palo Alto, California, USA), each consisting of 1176 individual mouse genes, were performed according to the manufacturer's instructions. The gene expression signals were analyzed with Quantity One image analysis software (Bio-Rad, California, USA). Regulated genes were categorized into different functional groups. The differentially expressed genes include a number of transcription factors, intracellular kinases, markers of energy metabolism, lipid metabolism, metabolism

enzymes, heat shock proteins, oncogenes and tumor suppressor genes, RNA processing and protein modification genes, as well as genes related to DNA damage/repair and replication.

Results of the microarray screening analysis suggested that tissue transglutaminase (tTGase) mRNA levels were increased in the CYN-treated mice at all time points. The up-regulation of the tTGase gene was confirmed by semi-quantitative RT-PCR. The PCR primer sets for the detection of mouse transglutaminase were 5'GCCAGAGAACTGGGAGTCAG3' and 5'GGGTACAGGTCTGGTGCAGT3' and a 193 bp product was produced. The PCR primer sets for mouse β-actin were 5'CTAAGGCCAACCGTGAAAAGAT3' and 5'CTTCTCTTTGATGTCACGCACG3', and the length of the gene product was 310 bp. The resulting PCR products were subjected to electrophoresis on a 2% agarose gel and stained with ethidium bromide. The intensities of individual bands were analyzed with Quantity One software. The expression of β-actin was used as an external control to normalize the intensities of the tTGase bands. The null hypothesis that CYN did not cause an up-regulation of the tTGase gene was tested by comparing the ratios (obtained by dividing the intensity of the tTGase band of CYN-treated mouse liver by those of the control mouse liver) with unity using Student's *t*-test. Statistical significance was accepted at $p < 0.05$. The increased expression of the tTGase gene 6 h post treatment was confirmed by semi-quantitative RT-PCR (Figs. 1 and 2). It is noteworthy that the expression patterns of tTGase 72 h and 96 h post treatment were consistent with those observed at 6 h, although the ratios at 72 h and 96 h were not significantly different from unity (Fig. 2). This is likely due to the relatively small number of replicates used ($n = 3$) in the analysis.

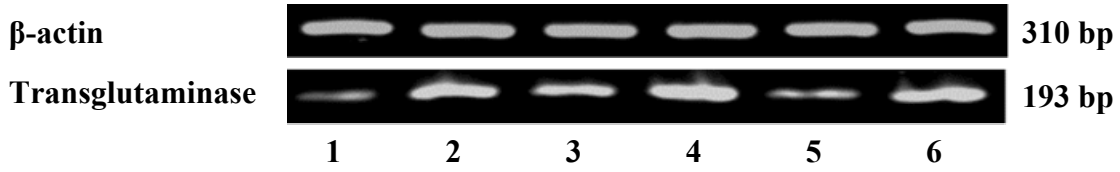

Fig. 1. Confirmation of transglutaminase gene expression with RT-PCR. Gel electrophoresis of the RT-PCR products of transglutaminase.
1 = 6 h control; 2 = 6 h post-CYN treatment;
3 = 72 h control; 4 = 72 h post-CYN treatment;
5 = 96 h control; 6 = 96 h post-CYN treatment.

tTGase is a unique member of the TGase (EC 2.3.2.13) family which catalyzes the post-translational modification of proteins via Ca^{2+}-dependent cross-linking reactions. This enzyme has been implicated in diverse biological processes such as cell death and differentiation, receptor-mediated endocytosis, adhesion and morphological changes of cells, and the induction of apoptosis. The up-regulation of tTGase has also been shown to occur in various pathologies and can lead to severe liver injury.

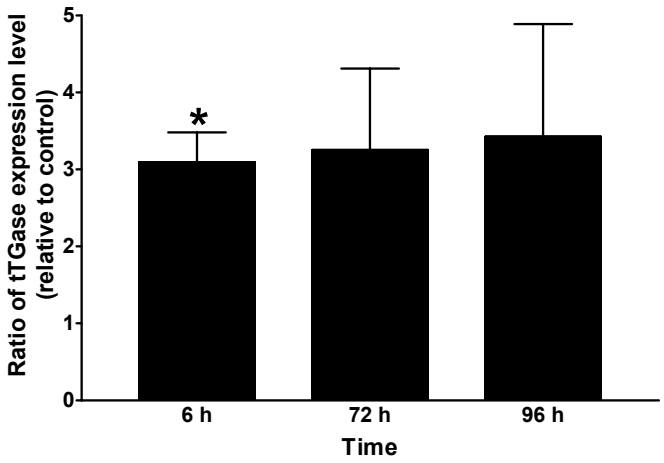

Fig. 2. Ratio of expression levels of tTGase gene (obtained by dividing the intensities of the tTGase band of CYN-treated mouse liver by those of the control mouse liver) in Balb/c mouse liver 6 h, 72 h and 96 h following a single i.p. injection with 100 μg kg^{-1} CYN. Data were obtained from three individual reactions (n = 3) and normalized against the signal of β-actin. Vertical lines are means ± 1 SE. * indicates significant difference from unity at $p < 0.05$.

This study supports the use of gene expression-profiling technology to determine or predict toxic effects. The gene expression profile provides potentially useful information for the elucidation of the possible mechanisms of toxicity of CYN on mice and their health significance.

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ORAL SESSION 3:

Distribution of HABs and Results of Monitoring Programs

Seasonal Dynamics of *Alexandrium fundyense* and PSP Toxins Content in *Mytilus edulis* in an Integrated Aquaculture System

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The management of accumulated phycotoxins must be considered when developing the potential to culture filter-feeding shellfish in marine ecosystems. The dinoflagellate *Alexandrium fundyense* produces toxins which are accumulated by shellfish. Consumption of toxin-contaminated shellfish causes paralytic shellfish poisoning (PSP) in vertebrate and human consumers. This has resulted in annual regulated closures of shellfish harvesting in the Bay of Fundy since the 1940s. Analysis of historical PSP toxicity data, however, indicates that many regions experience only short periods where unsafe levels of PSP toxins are detected in tissues of the blue mussel, *Mytilus edulis*. This suggests that marketing and culturing mussels in the southwest Bay of Fundy may be feasible. The seasonal dynamics of *A. fundyense* and the concentration of PSP toxins in *M. edulis*, the filter-feeding component of an experimental integrated aquaculture system in the southwest Bay of Fundy (Aquanet Project EI#12), were determined during 2001 and 2002.

Alexandrium fundyense cells were observed from May to October in both years, but peak abundances up to 1,500 cells L^{-1} occurred only in mid-June to late July. The highest PSP toxins concentrations in mussels occurred late in June in both years, lagging peak abundance of *A. fundyense* by 7-8 days. The concentration of PSP toxins in mussel tissues exceeded the regulatory level (RL) of 80 µg STXeq 100 g^{-1} wet tissue weight from late May to early July in 2001, and in late June in 2002. The dominant toxins present in mussel tissues were GTX2 and GTX3. During the summer of 2002, mussel tissue PSP toxins profiles had reduced concentrations of STX, GTX3 and GTX4 and enhancement of NEO and GTX2 concentrations, compared with those in *A. fundyense*. Mussels fed cultured *A. fundyense* in the laboratory accumulated a PSP toxins body concentration well in excess of the RL in four days, and had a different PSP toxins profile compared with field mussels. These field and metabolic data will facilitate the development of a mussel culture and harvest management plan in the aquaculture system.

Temporal Pattern of *Alexandrium* spp. in the Strait of Georgia Basin from Time-series Data on Shellfish Toxicity

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Past records of PSP contamination in shellfish can provide historic accounts of seasonal and inter-annual variance in *Alexandrium* blooms at specific locations. As PSP retention is species specific, the use of a sentinel species collected and analyzed at frequent intervals is required to provide comparative and meaningful evaluation of bloom occurrence and intensity. A review of toxicity in *Mytilus californianus*, the sentinel species collected and analyzed by the Canadian Food Inspection Agency from 1990 – 2001, indicated temporal variance within and between geographic sites in the Georgia Basin.

In general, blooms of *Alexandrium* occurred from May to December, but no periodicity in bloom formation was evident at any site during the 11-year period. On the exposed mainland side of the Strait, blooms of *Alexandrium* were rare as judged by toxicity levels, which rarely exceeded the 42 µg STXeq 100 g^{-1} detection level. At sites in mainland inlets, toxicity was substantially higher and became more intense with increasing distance from the open Strait. Sites on the Vancouver Island side of the Strait of Georgia north of Departure Bay were rarely above the detection level. However, from Departure Bay south to Coffin Point, Vesuvius Bay, Burgoyne Bay and Patricia Bay, increasingly high levels of toxicity were evident at two periods: May to August and September to December. Maxima of 4,300 – 8,800 µg STXeq 100 g^{-1} occurred at these sites in October 1997. Ganges Harbour, on the open Strait side of Saltspring Island, exhibited significantly less toxicity than the more protected Vesuvius and Burgoyne Bays on the west side of the island and reflected geographic/environmental impacts on *Alexandrium* growth. The negligible toxicity on the mainland side of the Strait, together with the increased toxicity from north to south on the Vancouver Island side, tended to suggest that the episodic occurrence and intensity of *Alexandrium* in the Georgia Basin may well reflect nutrient enhancement caused by rainfall and fresh water runoff from urban and farming communities.

Harmful Algae Early Warning Program at Newfoundland Aquaculture Sites - an ACRDP Project

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The Newfoundland Aquaculture Industry Association, the Aquaculture Research Section of Fisheries and Oceans Canada and the Institute for Marine Biosciences (NRC, Halifax) have partnered in an Aquaculture Collaborative Research Development Program (ACRDP)-funded project to determine the population dynamics of harmful marine phytoplankton at shellfish aquaculture sites. Information obtained from this research will establish a framework for assessing and managing the risk that harmful algae may pose to the aquaculture industry in Newfoundland and Labrador. A harmful algae early warning system has been designed and implemented on five commercial mussel aquaculture sites in Newfoundland. Sampling has continued monthly from April to November 2002. Phytoplankton samples (whole-water Niskin bottle, 20 µm-mesh net tows) and environmental data (nutrients and SEABIRD CTD casts) were collected at each site. Microscopic analysis using a Zeiss Axiovert 35 inverted microscope and an Hitachi S570 scanning electron microscope were conducted to determine the presence of known harmful algal species. When harmful algal species were detected at a site at concentrations that might affect site management, shellfish growers were immediately notified and additional sampling and testing was done. When suspect species were detected, the following procedures were followed: a) additional plankton sampling was conducted; b) mussels were provided to the Canadian Food Inspection Agency for appropriate tests (e.g. ASP toxin testing in the presence of *Pseudo-nitzschia* spp., PSP toxin testing in the presence of *Alexandrium* spp.); and c) phytoplankton samples were concentrated for chemical analysis at NRC, Halifax. Sampling at selected sites also included the collection of epiphytic algae from the mussel sock or seed collector lines. These samples were examined to determine the presence of *Prorocentrum lima*, a known source of diarrhetic shellfish poisoning (DSP) toxins. A *Pseudo-nitzschia* bloom occurred in Trinity Bay in late July and intensive additional sampling and testing was initiated, but no ASP toxin was found in the mussels. It is unknown whether this bloom is an annual occurrence or a cause for concern, thus the need for additional information on the phytoplankton dynamics at these aquaculture sites is becoming more evident. *Prorocentrum lima*, or a similar species, was found growing epiphytically on mussel seed-collector lines at several locations within one site. Highest cell abundance was found in May, with cell numbers gradually decreasing throughout the summer. Several species of harmful phytoplankton were detected, but abundance was not sufficient to pose a risk to shellfish production. These species included *Prorocentrum lima*, *Dinophysis norvegica*, *Alexandrium fundyense* and several species of *Pseudo-nitzschia*. Providing the shellfish growers with harmful algae information and the resulting husbandry practices that can be taken to ensure the safe transfer or harvest of their product from their site is important to developing a Hazard Analysis Critical Control Point (HACCP) industry standard in Newfoundland.

Overview and Status of the Canadian Food Inspection Agency's Marine Biotoxin Monitoring Program

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The Canadian Shellfish Sanitation Program (CSSP) is a federal program implemented for the primary purpose of controlling the harvest and handling of bivalve molluscs within the tidal waters of Canada in order to provide reasonable assurance that these shellfish are safe for consumption as food. The CSSP is jointly delivered by three federal departments: the Canadian Food Inspection Agency (CFIA), Environment Canada (EC) and Fisheries and Oceans Canada (DFO).

As one component of the CSSP, the CFIA conducts a marine biotoxin monitoring program involving the collection and analysis of shellfish samples from sites representative of all active harvest areas. This monitoring program involves testing shellfish for toxins related to amnesic shellfish poison (ASP), paralytic shellfish poison (PSP) and, in specific circumstances, diarrhetic shellfish poison (DSP). It serves as a preventative management tool in providing the information necessary for making recommendations to DFO for closing and opening shellfish harvest areas, as appropriate.

An overview of the biotoxin monitoring program will be presented. This will include information pertaining to the general structure and implementation of the program, a summary of findings collected in recent years, and future food safety considerations in the control of marine biotoxins.

A Comparison Between Morphological and Molecular Methods for Identifying Phytoplankton

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Phytoplankton have traditionally been identified microscopically based on morphological characteristics. Microscopic identifications can be time-consuming, require expertise in taxonomy, and it is often difficult to distinguish differences among the multitudes of minute, nondescript planktonic organisms without the use of the electron microscope. Molecular techniques, which have revealed new insights into bacterial and picoplankton communities, may also enhance our knowledge of the diversity among communities of the larger plankton. We compared plankton identifications and community assessments based on the two types of techniques (morphological vs. molecular) for surface seawater samples collected on 2 May, 31 July, and 25 September 2000, from several sampling stations in the Bay of Fundy.

Phytoplankton were quantified and identified based on morphology, and DNA was extracted from plankton communities (5 - 100 μm diameter) collected by filtration. 18S rRNA gene fragments were amplified with primers specific for eukaryotes. Denaturing gradient gel electrophoresis (DGGE) was used to develop DNA profiles of eukaryotic phylogenetic diversity and to select cloned 18S rDNA fragments for sequencing. Both methods showed greater temporal than spatial diversity. However, the communities identified with the two different types of techniques were very different. Abundances and taxa diversity determined microscopically were lowest in May, whereas the number of DGGE bands was highest in May and July. Morphological identifications showed a succession of dominant organisms through time. While neither diatoms nor dinoflagellates were dominant in May, diatoms and a few dinoflagellates were dominant in July and September. In contrast, few 18S rDNA sequences were related to rDNA sequences of known identity and few diatoms were identified in the molecular analyses. Molecular phylogenetic analysis indicated the presence of many novel organisms, several of which were most closely related to other unidentified sequences from diverse marine environments representing new lineages.

Our results suggest that we are just beginning to uncover the diversity of eukaryotic marine organisms and that there may be more ubiquitous, heterotrophic plankton than previously realized. Both methods appear to capture a portion of the community. Morphological methods may be more adept at capturing the phototrophic organisms within the community. However, just as for bacteria and picoplankton, molecular techniques may enhance understanding of plankton diversity, particularly by detecting previously unidentified organisms.

Detection and Characterization of the Red Tide Dinoflagellate *Karenia brevis* Using Pigment-based Approaches

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Karenia brevis is a toxic dinoflagellate that blooms annually in the Gulf of Mexico, resulting in extensive fish kills, neurotoxic shellfish poisoning, marine mammal mortalities and human respiratory irritation from the release of aerosolized toxins. We are currently examining methods for detection and characterization of *K. brevis* that are based on analysis of its unique biomarker pigment, gyroxanthin. For detection of *K. brevis* in coastal waters, we have developed a combination filtration and high performance liquid chromatography (HPLC) method that will allow reliable determination of *K. brevis* at concentrations lower than the FDA-mandated limit of 5 cells mL^{-1}. The development of this method involved testing of five different glass-fiber filter types to determine the optimal combination of acceptable retention and fast filtration times for large volumes of water. We found that filtration through GF/C (NPR = 1.2 μm) and 934-AH (NPR = 1.5 μm) filters gave > 98% recovery of cells with filtration times ~ 10-20 min in water with high suspended sediment load. We are currently working on a "barebones" HPLC analytical procedure to optimize for the separation and quantification of gyroxanthin in mixed algal samples; our aim is to achieve an analytical run time of < 15 min. This combined filtration and HPLC method is an alternative or supplement to traditional cell counting approaches and is suitable for use by agencies interested in long-term monitoring programs.

Work in our laboratory also examines the utility of gyroxanthin-based approaches for characterization of the growth dynamics of *Karenia brevis*. Determination of *in situ* growth rates of HAB-forming species is critical to an accurate description of bloom dynamics, but there are currently few reliable methods of directly determining growth rates on natural populations. We are examining the utility of photopigment (chlorophyll *a* and gyroxanthin) radiolabelling, an approach that measures the incorporation of photosynthetically assimilated ^{14}C into photopigment molecules. Results of batch culture experiments show that there is fairly good agreement between growth rates based on time-course measurements of chlorophyll *a* and cell numbers, as compared to those determined by photopigment radiolabelling methods. Growth rates based on chlorophyll *a* were easier to determine than those based on gyroxanthin, because concentrations of gyroxanthin per cell are low, and growth rates are slow (0.3 d^{-1}), resulting in a weak radiolabelling signal in the gyroxanthin pool. Efforts to resolve these issues are continuing.

Potential for Diarrhetic Shellfish Poisoning along the New England Coast of the United States

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Following the occurrence of several unexplained incidents of shellfish-related gastroenteritis, field studies were conducted to determine if diarrhetic shellfish poisoning (DSP) toxins were present in the coastal waters of New England states. The abundance and seasonality of potential toxin producers are followed within the planktonic and epibiotic community. In an effort to evaluate the potential for diarrhetic toxins to contaminate shellfish resources, the digestive glands of wild and cultured shellfish collected at four of the stations are analyzed for okadaic acid content. The seasonal distribution and associated toxin patterns in mussels and oysters will be presented for the first year of this planned two-year study. The ultimate goal of this project is to help evaluate the likelihood of DSP incidents, and the necessity and extent of future monitoring efforts to allay public health concerns.

The Relationship of *Alexandrium fundyense* to the Temporal and Spatial Pattern in Phytoplankton Community Structure Within the Bay of Fundy, Eastern Canada

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The spatial and temporal variation in abundance of *Alexandrium fundyense* in relation to the spatial and temporal variation in phytoplankton community structure at a series of monitoring stations within the Bay of Fundy is examined through a multi-dimensional scaling ordination analysis conducted on data collected in 1991. The results indicate a strong seasonality in both the abundance of *A. fundyense* and in the degree of spatial similarity in the community structure.

Alexandrium fundyense is abundant at only a few of the sampling stations and is most abundant during July. The temporal distribution of *A. fundyense* may therefore be related to the seasonality in the phytoplankton community or its correlates, such as the environmental conditions. Within a season, the spatial distribution of *A. fundyense* appears to be independent of the phytoplankton community structure. Data from an additional year are examined to test the robustness of these findings.

Sediment Profiles of Cysts of *Alexandrium tamarense* and Relationship with Sediment Dynamics

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This study examines potential links between the vertical sediment profiles of cysts of the toxic dinoflagellate *Alexandrium tamarense*, hydrodynamic characteristics near the sea bed and sediment reworking. Profiles of cysts were obtained every cm over the top 10 cm of sediment in two sites: a high-energy site near Baie-Comeau (Lower St. Lawrence Estuary) in 1996, and a low-energy site in Gaspé Bay, Quebec, in 1997. Cores were collected once a month during June, July and August 1996, and twice a month during July, August and September 1997. Sampling also covered hydrodynamic variables (currents and waves) and bed load transport, reworking and resuspension.

In 1996, a major storm hit the Saguenay region in late July, causing major flooding and eroding of at least 10 cm of sediment from the Baie-Comeau site. Maximum cyst concentrations decreased from about 600 cysts cm^{-3} before the storm to less than 200 cysts cm^{-3} after the storm, along with the disappearance of any vertical cyst structure that existed previously. A red tide was observed in the Lower St. Lawrence Estuary about 10 days later, suggesting that cyst resuspension caused bloom initiation. In Gaspé Bay, tidal currents reworked the top 2 cm of sediment, while storms caused an erosion of up to 5 cm of sediment. Cyst concentration was low in the top 3-4 cm (<500 cysts cm^{-3}), increasing with depth. The maximum cyst concentration (generally between 7-10 cm) increased from July to September, reaching 1,500 cysts cm^{-3} in late September. This increase was most notable between late July and early August, likely caused by the arrival of new cysts following the bloom recorded in the region by DFO monitoring services on 22 July 1997.

Influence of Dissolved Organic Matter from Land Origin, Vitamins and Trace Metals on the Growth and PSP Toxin Content of *Alexandrium minutum* AM89BM

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The dinoflagellate *Alexandrium minutum* usually blooms in coastal and estuarine environments characterized by lowered salinity and high levels of terrestrial dissolved organic matter (DOM). *Alexandrium minutum* is capable of producing toxins responsible for paralytic shellfish poisoning (PSP). We investigated the influence of DOM from land origin on growth and PSP toxin content (mostly GTX2+3) of *A. minutum* strain AM89BM. DOM (molecular size > 1000 Daltons) was concentrated by ultra-filtration from estuarine water (salinity 8 PSU) collected in March 1999, from the Charente River estuary (French Atlantic coast). Since vitamins and trace metals are well known growth regulators and are abundant in DOM, we compared the effects of addition of DOM with those of addition of the vitamins + trace metals (VTM) used in the f/2 medium.

The DOM extract was diluted by the original concentration factor (ca. 100 times) in the DOM addition treatments. VTM were added at 1/10 of f/2 concentrations. These additions were combined with three N:P ratios of inorganic nutrients (NO_3 and PO_4), set up at 80, 16 and 5.5 by varying the phosphorus concentration (NO_3 ca. 60 µmol L^{-1}). In all experimental treatments, addition of DOM did not significantly affect growth rates and maximum cell concentrations. In contrast, VTM addition markedly contributed to enhance maximum cell concentrations by 50 to 170%, along with the nearly total consumption of dissolved inorganic nitrogen (DIN). In all treatments with no VTM addition, 30 to 50% of initial DIN was left at stationary phase, mostly as nitrate. In all N:P treatments, DOM addition significantly enhanced the cell PSP toxin content, by up to 8 times at N:P = 80 at stationary phase. VTM addition, however, even more markedly enhanced the cell toxin content. With DOM addition alone, maximum cell toxin content ranged between 16 and 20 fmol $cell^{-1}$ at maximum cell concentration. With VTM addition alone, maximum cell toxin content ranged between 26 and 60 fmol $cell^{-1}$ at maximum cell concentration, increasing with the N:P ratio in the treatments. Highest cell toxin contents, around 100 fmol PST $cell^{-1}$, were obtained at stationary phase in the N:P = 80 treatment and with addition of DOM and VTM. These results suggest that some vitamins or trace metals are necessary in the processes of paralytic toxin synthesis. Whether these micronutrients are required for the toxin synthesis process specifically, or for the cell metabolism such as nitrate uptake and production of precursor metabolites, is an open question. The investigation of specific effects on growth and cell toxin content of each micronutrient is in progress.

Progress on the Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB) Program

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Understanding and prediction of "Harmful Algal Blooms" (HABs) is limited by lack of knowledge regarding the factors controlling the dynamics of individual species, specifically how physiological, behavioural and morphological characteristics interact with environmental conditions in a variety of oceanographic regimes. The mission of the Global Ecology and Oceanography of Harmful Algal Blooms (GEOHAB) Program, initiated by the Scientific Committee on Oceanic Research (SCOR) and the Intergovernmental Oceanographic Commission (IOC), is to foster international co-operative research on HABs in ecosystem types sharing common features, comparing the key species involved and the oceanographic processes that influence their population dynamics. The scientific goal is to improve prediction of HABs by determining the ecological and oceanographic mechanisms underlying the population dynamics of harmful algae, integrating biological, chemical and physical studies supported by enhanced observation and modelling systems.

GEOHAB is not a funding agency; rather, research is paid for by national funding agencies that must respond to national scientific priorities using nationally based facilities, resources and expertise. Within a consistent mission, GEOHAB has been, and will continue to be, an evolving network of scientific programs, with program growth dependent on national and international research priorities, investigator interest, methodological capabilities, availability of funding and other resources and successful accomplishment of stated goals. Within GEOHAB, experimental, observational, and modelling approaches will be integrated, using current and innovative technologies consistent with the multiple scales and oceanographic complexity of HAB phenomena. Through such efforts, the emergence of a truly global synthesis of scientific results should be attained.

In this presentation, the rapporteur will provide information on the GEOHAB Science Plan and the emerging Implementation Plan, which is now under review by SCOR. Several Core Research Projects, representing several different ecosystem types, have been identified and are being implemented under the coordination of the GEOHAB Scientific Steering Committee (SSC). Current issues, including the function and composition of the SSC, the role of the International Programme Office, types of research and framework activities and criteria for participation will be detailed. The Core Research Projects include HABs in: 1) Upwelling Regions, 2) Semi-confined Eutrophic Zones and Estuaries, 3) Fjords and Coastal Embayments, and 4) Stratified Regions. The Core Research Projects will be developed with full and open involvement from the scientific community – GEOHAB is an open network. Scientists are invited to participate by designing research studies in keeping with the goals and objectives of GEOHAB, by applying for endorsement of such research, and by participating in open science meetings and framework activities, including workshops.

GEOHAB-Canada: Preliminary Description of a Proposed Program on the Ecology and Oceanography of Harmful Algal Blooms in Canada

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An international program named GEOHAB (Global Ecology and Oceanography of Harmful Algal Blooms) was set up by the Scientific Committee on Oceanic Research (SCOR) and the Intergovernmental Oceanographic Commission (IOC) in the last few years. National programs have been invited to join in this international effort, which has just started. A group of Canadian oceanographers and specialists on harmful algae has met and proposed a joint research program among several universities, Fisheries and Oceans Canada, and NRC's Institute for Marine Biosciences in Halifax, Nova Scotia. This presentation will outline the proposed research, which revolves around four themes:

1) Sustained field observations of HABs and associated oceanographic conditions along with modelling;
2) Development of tools to improve these sustained observations;
3) Determination of the biological factors affecting population dynamics of harmful algae (with an emphasis on biological loss factors such as microbial-mediated mortality); and
4) Sediment processes affecting the dynamics of these populations.

POSTER SESSION

Pseudo-nitzschia Numbers and Amnesic Shellfish Toxin (AST) in Shellfish at Loch Ewe, a Sealoch in Northern Scotland

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Amnesic shellfish toxins (AST) have had a great economic impact on the Scottish shellfish industry since they were first recorded in 1998. To study the relationship between *Pseudo-nitzschia* numbers in the water column and AST levels in mussels (*Mytilus edulis*) and scallops (*Pecten maximus*), an intensive sampling program was set up from April 2001 at Loch Ewe, a sea loch in northern Scotland. Results showed AST levels in *M. edulis* were related to increases in *Pseudo-nitzschia* spp. numbers during 2002. AST levels in *P. maximus* (whole animal and gonadal tissue) also increased after *Pseudo-nitzschia* numbers greater than 50,000 cells L^{-1} were observed. A prolonged depuration period was observed in the *P. maximus* whole tissue. Transmission electron microscopy analysis was performed on selected phytoplankton samples. The potential toxin producers *P. australis*, *P.* cf. *delicatissima*, *P. fraudulenta*, *P. pungens* and *P.* cf. *pseudodelicatissima* were identified.

Biogeography of Resistance to Paralytic Shellfish Poisoning Toxins in North American Populations of *Mya arenaria*

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This study presents results of an extensive latitudinal survey of North American populations of the softshell clam, *Mya arenaria*, to determine their resistance to paralytic shellfish poisoning (PSP) toxins. Characterization of toxin resistance (proportion of sensitive and resistant individuals) was based on the burrowing response of clams, following 24 h-laboratory exposure to a highly toxic strain of *Alexandrium tamarense*. This burrowing index was validated using the *in vitro* nerve response to saxitoxin. Our previous research demonstrated significant differences in resistance to PSP toxins and thus in the capacity for toxin accumulation between two test populations which correlated with their long-term history of toxin exposure. Resistant clams were prevalent in Lepreau Basin, Bay of Fundy, New Brunswick recurrently affected by PSP toxins, whereas sensitive clams were dominant in a PSP toxin-naïve population from the Lawrencetown River estuary, Nova Scotia, suggesting that populations undergo genetic adaptation to toxins.

These findings are now extended over a wide geographical range. In Atlantic Canada, predominantly resistant populations were identified throughout the Bay of Fundy, on the north and south shores of the St. Lawrence River estuary (Baie de Chevaux and Mont Louis, respectively), and the Baie de Gaspé (Rivière St. Jean estuary), Quebec, regions historically affected by *Alexandrium* blooms. Predominantly sensitive populations occur offshore in the Magdalen Islands, Quebec, and in southeastern Nova Scotia.

In the United States, resistant populations occur in the Gulf of Maine, and extend as far south as Cape Cod. We attribute this occurrence of dominantly resistant populations in southern New England waters, which experience less frequent and intense toxic blooms than northern waters, to larval dispersal and gene flow via the southwestward Western Maine coastal current. Sensitive populations are found in Long Island, New York, an area with no record of PSP toxins. On the Pacific Coast, *M. arenaria* populations showed an increase in the prevalence of resistant clams between Quartermaster Harbor, central Puget Sound, an area only affected by PSP toxins since the 1980s, and Nanoose Bay, British Columbia. Thus, toxin resistance is likely a function of the intensity and frequency of toxic blooms as well as prevailing circulation patterns controlling pelagic larval transport.

Discrimination among Populations of Toxigenic *Alexandrium* Species by Means of rRNA-Targeted Molecular Probes and Toxin Profiling

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A common problem in harmful algal monitoring programs occurs when the "species of interest" is only a minor component of the planktonic assemblage. Many potentially useful measurements are not feasible because of co-occurrence of numerous organisms of other taxa, and interference by detritus. Another constraint arises from difficulties in identifying and distinguishing among morphologically similar species or strains. Such fine levels of discrimination are often not feasible in monitoring programs or studies that generate large numbers of samples for cell enumeration. For example, species of the marine dinoflagellate genus *Alexandrium* (Halim) Balech, such as *A. tamarense*, *A. fundyense* and *A. ostenfeldii*, are difficult to distinguish reliably under conventional microscopy, without detailed critical taxonomic observations of individual cells. The vegetative cells of *A. ostenfeldii* are typically larger and more 'globose' than those of *A. tamarense*, but there is considerable variation in gross morphology among cells of these respective species. Key diagnostic features, such as the size and shape of the ventral pore at the margin of the first apical (1') thecal plate, must be examined individually for each specimen – a tedious procedure. *Alexandrium tamarense* and *A. fundyense* are the primary source of paralytic shellfish poisoning (PSP) toxins in north temperate coastal waters, but populations are often co-mingled with *A. ostenfeldii* (Paulsen) Balech & Tangen, a producer of macrocyclic imine toxins known as spirolides. Such species co-occurrences have been observed in Nova Scotia, the Gulf of Maine, Danish and Norwegian fjords and the Scottish east coast. Species-specific probes, such as those targeted to large subunit (ls) or small subunit (ss) rRNA, are therefore a useful complement to conventional monitoring of harmful phytoplankton.

The studies reported here represent a concerted effort to apply molecular probes for the discrimination of *Alexandrium* species in natural populations from the North Sea and Nova Scotian coastal embayments, combined with instrumental analysis by liquid chromatography with fluorescence- and mass spectrometric-detection of the respective toxin composition of these taxa. Determination of spirolides and PSP toxins by instrumental analysis demonstrated the presence of both toxin groups in plankton assemblages, and confirmed the association of *A. ostenfeldii* with spirolides in northern Europe.

North Coast Water Quality and Biotoxin Program Society

Sandra Cross

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The west coast of British Columbia is known to have seasonal blooms of red tide. Yet, the north coast has been without a shellfish biotoxin monitoring program for almost thirty years, leaving the area closed to any type of shellfish harvest. The First Nations people of the north coast harvest clams, mussels and cockles as part of their traditional heritage. In addition, growing interests in a shellfish aquaculture industry on the north coast has increased awareness of the need for a biotoxin monitoring program. The highest recorded level of paralytic shellfish poisoning (PSP) ever in B.C. was from Work Channel, located on the north coast.

In 1998, through a pilot project created by the Seafood Development Office in Prince Rupert, a program was created to meet the needs of the region and to satisfy federal regulatory testing standards for shellfish biotoxins. Today, the North Coast Water Quality and Biotoxin Program is a non-profit society that has 26 monitoring stations that include traditional harvesting areas of the Nisga'a, Haisla, Haida and Tsimshian peoples. In addition, the society provides monitoring services for the only two shellfish lease sites on the north coast. Through regular monitoring and federal regulatory compliance, the North Coast Water Quality and Biotoxin Program Society is working with Environment Canada (EC), the Canadian Food Inspection Agency (CFIA), and Fisheries and Oceans Canada (DFO) towards opening areas of the north coast to traditional First Nations harvest, and establishing the necessary framework for a future shellfish aquaculture industry.

California mussels (*Mytilus californianus*) are used as an indicator species for PSP toxin levels, as well as amnesic shellfish poisoning (ASP) levels. In addition, diarrhetic shellfish poisoning (DSP) levels are monitored through marine water phytoplankton analysis by a local company, Ocean Ecology. Northern Laboratories Ltd., located in Prince Rupert, is the only non-governmental lab in Canada approved to process both PSP and ASP extracts for the CFIA and fecal coliform tests for EC. Due to the remote nature of most of the monitoring areas on the north coast, local laboratory facilities have become an essential part of the testing process.

Biotoxin sampling occurs weekly from 1 May to 31 October, and every two weeks from 1 November to 30 April. Along with testing services, the North Coast Water Quality and Biotoxin Program Society provides educational opportunities for local First Nations and non-First Nations individuals trying to become involved in a local shellfish industry. The Society, with financial support from Land & Water B.C., has coordinated a series of shellfish business development workshops in Prince Rupert, Lax Kw'alaams, Hartley Bay, Metlakatla and Gitxaala. Last fall, 25 representatives from Prince Rupert and local First Nations communities were invited to attend a field trip to the south coast of B.C. to tour established shellfish farms, hatcheries and research facilities. In addition, the Society is currently running shellfish grow-out projects in elementary schools in four local First Nations' communities.

Computer Assisted Identification of Toxic Algae: Making Taxonomy Approachable

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Identification of a specimen of any species can be challenging. Frequently the process is made even more difficult by classical taxonomic literature and identification keys. The use of overly expert terminology and inclusion of non-quantifiable characteristics can be a significant stumbling block to uninitiated practitioners. Printed dichotomous keys and static tables of diagnostic features are still the primary methods for guiding the investigator to the correct species identification. Organization of information in the classical manner makes it difficult to proceed with confidence through the identification process. Mistakes at one point can lead to blind alleys that are difficult to backtrack through once the error is discovered. This can limit the use of available taxonomic knowledge to experts in the field, even when there is an urgent need for this knowledge to be used practically by a broader audience.

Here, we present a simple example of a software solution that encapsulates the features necessary to identify rapidly the species of *Pseudo-nitzschia* commonly found in the Canadian Maritimes, and of particular interest to toxic algal research and agencies monitoring the safety of shellfish aquaculture. This example uses scanning electron microscopy (SEM)-based morphometrics, but the concept could be adapted for virtually any classification context. Many species of *Pseudo-nitzschia* are well circumscribed and possess quantifiable features (by measurement or counts) that are necessary for their correct identification. These data can be integrated into a dynamic truth table that the user can manipulate as a virtual instrument. The software automatically updates the table as characteristics are entered for the unknown. The investigator is thus freed from manually or mentally keeping this information updated, while making other decisions regarding species identity. Our example application is a stand-alone program with an interactive, visually oriented interface, but the concepts could be easily modified to access dynamic databases or integrated into automated identification systems and more extensive classification packages.

Monitoring Strategies for DSP and Related Toxins in Shellfish using Animal Testing and LC-MS Techniques, and the Example of Shellfish Monitoring in Ireland Since 2001

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ABSTRACT

Until 2002, the surveillance in the EU of all shellfish toxins associated with diarrhetic shellfish poisoning (DSP) symptoms was covered by the directive 91/492/EC. This directive prescribed a method to be used, namely "the customary bioassay". Although the type of test was fixed, no performance criteria or protocols were given for this method. Both rat and mouse bioassays had been used as a consequence of this directive, even though recent data have shown that these tests have a tendency to underestimate the toxicity of some toxins and overestimate the toxicity of others. In particular, the discovery of yessotoxins, which interfere with the mouse test, and azaspiracids, for which the traditional version of the mouse bioassay may not have appropriate detection limits, initiated discussions at the scientific, regulatory and industrial levels. In 2002, these discussions were implemented into legislation (225/2002/EEC), thereby opening the door to alternative tests, including liquid chromatography coupled to mass spectrometry (LC-MS). A list of compounds to be monitored has been specified and limit values have been set. Here, we discuss the advantages and disadvantages of animal tests and LC-MS-based methods. Possible monitoring strategies are reviewed with the aim of international harmonization by defining generic performance characteristics rather than the test methods applied. Performance characteristics evaluated include classical parameters such as limit of detection, specificity and recovery, as well as fitness-for-purpose parameters such as the turnover time, cost and possibility to standardize a test. In Ireland, both mouse bioassays and LC-MS techniques have been used in parallel on a routine basis for the monitoring of DSP toxins in shellfish since May 2001. Comparison of the two methods shows good correlation for the first year (93% agreement) and excellent results for the second year (99% agreement).

INTRODUCTION

Currently, four different groups of lipophilic compounds are classified as diarrhetic shellfish poisoning (DSP) toxins: okadaic acid (OA) and dinophysistoxins (DTXs), azaspiracids (AZAs), pectenotoxins (PTXs) and yessotoxins (YTXs). This classification is likely to be reviewed since only OA, DTXs and AZAs actually show diarrhetic effects. PTXs and YTXs have been initially included in the classification due to their effects in the mouse bioassay, a commonly used technique to detect lipophilic toxins present in shellfish.

Whereas OA and DTXs had been identified in the 1970s as shellfish toxins in Japan and Europe, PTXs and YTXs were discovered in the late 1980s and 1990s in Japan. YTXs have since also been found in Europe, mainly in Italy and Norway. AZAs were found in Ireland, following food alerts in 1995, 1996, 1997 and 1999. However, the toxin has now equally been found in the UK and Norway.

Following the initial food poisoning incidents in Japan, Yasumoto *et al*. (1978) developed a mouse bioassay to detect diarrhetic toxins. This assay has proven useful to date thanks to its sensitivity to a number of lipophilic toxins. However, its usefulness has shown limits due to the co-existence of toxins with compounds that may not be orally toxic and thus interfere with the assay. This paper discusses the experience at the Marine Institute in comparing two techniques (animal testing and instrumental methods), and attempts to review the current options for monitoring lipophilic toxins in a regulatory context.

Legislative Background and Methodology

EU directive 91/492/EEC laid down that the content of diarrhetic shellfish toxins in shellfish should not be detectable by the "customary bioassay". The protocol of analysis was not specified, allowing for a number of different assays, including mouse and rat bioassays.

The recent Commission Decision 2002/225/EC has reinforced that the reference method be the mouse bioassay. The same decision also specifies limits for a number of toxin groups, namely okadaic acid and dinophysistoxins, pectenotoxins, azaspiracids and yessotoxins. The levels set for OA, DTXs and PTXs are expressed in OA-equivalents and should not exceed 160 μg kg^{-1}. Similarly, AZAs are summed as AZA-equivalents and should not exceed 160 μg kg^{-1}. It was also recognized that YTXs have a lesser oral toxicity and the limit was set to 1 mg kg^{-1} YTX-equivalents.

Internationally, limits for OA-equivalents have been set in several countries, including Canada (200 μg kg^{-1}), New Zealand (200 μg kg^{-1}) and Japan (200 μg kg^{-1} within Japan, 160 μg kg^{-1} for export).

Unfortunately, there is no international agreement on the most appropriate methods to monitor these toxins and further harmonization should be achieved to guarantee free markets.

Method Comparison in Ireland, 2001-2002

Following a review by state regulatory agencies, the shellfish industry and scientists at the Marine Institute, chemical testing of lipophilic toxins in shellfish was introduced in Ireland in 2001 on a routine basis.

From July to September 2001, all shellfish samples were analyzed both by the mouse bioassay (Yasumoto *et al*., 1984) and by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) (Fig. 1, left-hand side). From January 2002 onwards, all samples were analyzed

by both methods (Fig. 1, right-hand side). The instrumental method set up at the Marine Institute followed developments by Quilliam *et al.* (2001), and was published by Hess *et al.* (2001) and Hess *et al.* (in press).

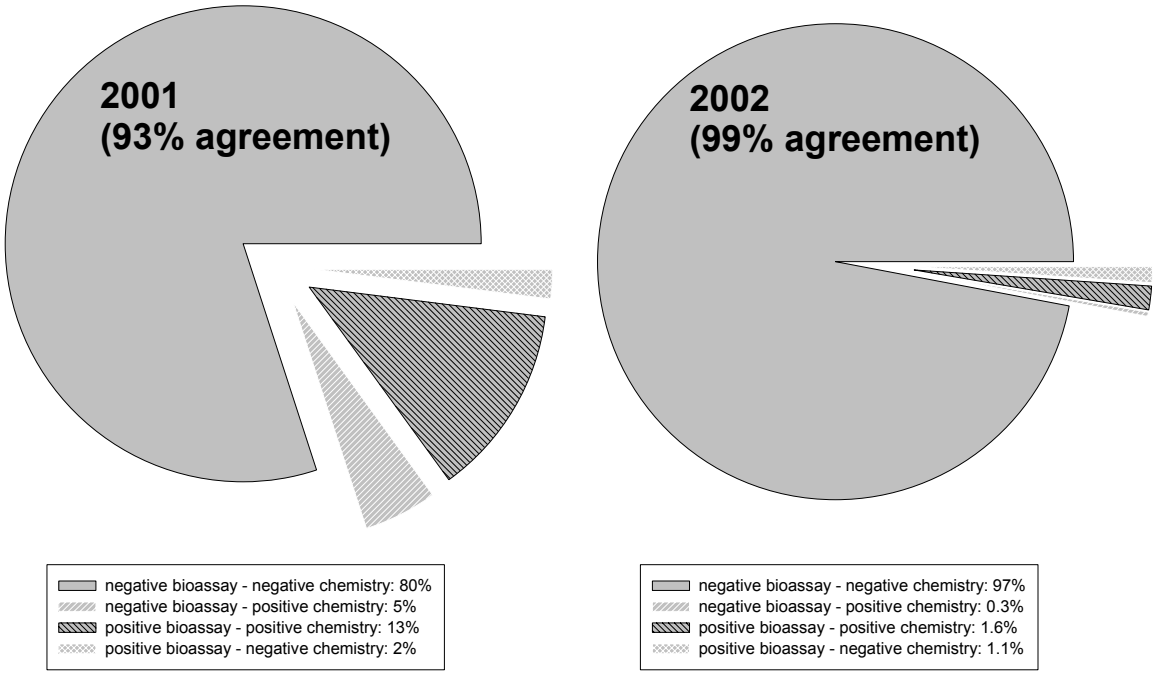

Fig. 1. Comparison of mouse bioassays with LC-MS testing for lipophilic toxins in Ireland from 2001-2002 (415 samples in 2001, 3115 samples in 2002). LC-MS/MS analysis included OA, DTX-2, AZA-1, AZA-2 and AZA-3.

Samples were split into four categories to be able to compare the quantitative LC-MS results with the semi-quantitative results of the mouse bioassay:

1) those samples which were negative by bioassay and showed values below the limits for OA-equivalents (OAEs) and AZA-equivalents (AZEs);
2) those that were negative by bioassay and showed LC-MS results above the limits of OAEs and AZEs;
3) those that were positive by bioassay and had LC-MS values below the limits for OA-equivalents (OAEs) and AZA-equivalents (AZEs); and
4) those that were positive by bioassay and had LC-MS results above the limits of OAEs and AZEs.

Fig. 1 shows that during 2001, 93% agreement was found between both methods, and that during 2002, the agreement between the assays accounted for 99% of all samples.

DISCUSSION

The monitoring strategy taken by any one institute or body will depend on the role of the monitoring body as well as the available techniques. Thus, a laboratory responsible for monitoring live bivalve molluscs (pre-harvest) will potentially choose a different approach than a laboratory responsible for the control of shellfish placed on the market (post-harvest). Furthermore, the sampling strategy and frequency may differ according to these framework conditions or due to local hydrographic conditions. Our paper is particularly aimed at discussing scenarios for the different methodologies available (Table 1).

Commission Decision 2002/225/EEC has set limits for the four toxin groups discussed here and opened the way for using methods other than the previous "customary bioassay". New monitoring strategies may now be put in place, and the strengths and weaknesses of these strategies are presented in Table 1. OA and DTXs can currently be determined using either approach, i.e. mouse bioassay, LC-MS or a functional assay such as PP2a. However, for all the other toxin groups, the choice of strategy is very limited. According to the EU legislation, a whole flesh assay must be used to determine AZAs. From the experience presented above (Method comparison in Ireland, 2001-2002), we concluded that when AZAs and OA/DTXs are present, good correlation can be obtained between LC-MS testing of whole flesh and a mouse assay based on hepatopancreas. A mouse assay according to a 2001 update of Yasumoto *et al*. (1984) may be used when YTXs are present. However, this assay is not confirmatory and if YTXs are present, the customary bioassay, which still serves as the reference method, may fail even though the levels for YTXs are not exceeded. Thus, when we look for international harmonization we should aim not to base any recommendation on a particular technique.

Whereas the procedure for the mouse bioassay is designed to give good recoveries, specificity is very broad and the limit of detection high. In terms of throughput, LC-MS techniques have become competitive compared to mouse bioassays due to the need for a 24 h observation period to detect OA/DTX-esters in the bioassay. Hydrolysis that transforms these esters into the parent compounds can be performed in less than one hour, allowing for overnight analysis of hydrolyzed and unhydrolyzed samples by LC-MS. Although the capital cost of LC-MS as a technique is high, the running costs of LC-MS are comparable to the bioassay since the bioassay requires a lengthy sample preparation, thus increasing the staff component of the cost.

If the strategy proposed to fulfil an international agreement is not based on a particular technique, then performance criteria need to be set for the techniques used in any given strategy. These criteria should include typical method performance criteria such as accuracy, precision, specificity, speed, robustness, confirmatory character, and statistical number of false positives and false negatives. Generically, these performance characteristics are easier to determine for fully quantitative assays. In particular, specificity and confirmatory character can probably not be described for the determination of lipophilic toxins using live animal assays. A recent inter-comparison organized by the Community Reference Laboratory also demonstrated that live animal assays are very difficult to validate according to internationally accepted protocols (Anonymous, 2001b). Therefore, the authors would recommend the use of a strategy of Type 5 (Table 1), which uses available functional or biochemical assays, LC-MS and bioassays. This strategy should allow reducing the number of live animal tests to a minimum.

Table 1. Advantages and disadvantages of different methodological approaches for monitoring lipophilic toxins.

	1. Purely based on small mammal assays	2. Purely based on LC-MS	3. Purely based on functional and biochemical assays	4. Combination of LC-MS and functional / bio-chemical assays	5. Combination of small mammal assays, LC-MS and functional / bio-chemical assays
+	Relatively easy to implement (low tech) Covers potentially all lipophilic toxin groups Gives one answer for overall toxicity May detect new toxins	Confirms presence of specific compounds Fully quantitative Can also be used for early warning and research applications Can be validated step-wise	Rapid technique Relatively low tech Relatively robust	Has a limited capacity to detect toxicity Has a high likelihood to detect all toxins with a low likelihood for false positives	May detect new toxins Number of mice can be reduced gradually Quantitative and semi-quantitative tests can be adapted to each country's toxin profile
-	Semi-quantitative, may not reach adequate limits Interferences possible from salt, FFAs, PSPs, YTXs and PTXs High solvent consumption Labour intensive Difficult to validate	Measures compounds, not toxicity Requires standards Cannot detect new toxins initially Requires adequately trained staff (high tech)	Requires standards for validation Requires large amounts of toxins for development Commercially only available for OA and DTXs	May not be able to detect new toxins Relatively high cost and high tech	Relatively high cost and high tech

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Identification of Some *Pseudo-nitzschia* Species from Western Washington Waters

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ABSTRACT

Domoic acid has been present on the U.S. west coast at least since 1991, when cormorants and pelicans died in California (Work *et al*., 1993) and razor clams and Dungeness crabs were tainted in Oregon and Washington (Horner and Postel, 1993). At that time, the causative *Pseudo-nitzschia* species was not confirmed for Washington, but it was thought to be *P. australis* as it was in California. Since then, investigators have worked to determine which *Pseudo-nitzschia* species are present in western Washington waters and which are the domoic acid producers. The poster summarized the taxonomic information and, where possible, linked species with domoic acid production information obtained from the literature.

INTRODUCTION

The diatom genus *Pseudo-nitzschia* is commonly found in Pacific Northwest/western Washington marine waters, including the open Pacific Ocean coast and inland waters of Puget Sound. It is present every month of the year. At least seven, possibly eight, species occur there. Of these, four are known to produce domoic acid, but they do not always produce it when they are present or only produce small quantities of the toxin.

The genus is fairly easy to recognize in the light microscope because the elongate cells are joined together in stepped colonies by the overlapping of the cell ends. However, distinguishing species is not easy and generally requires either scanning or transmission electron microscopy for positive identification. Characters used include valve outline, valve width, density of striae versus fibulae, shape of the valve ends in girdle and valve views, and length of overlap of the cell ends. Cells must be seen in valve view for identification (Hasle, 1965; Hasle *et al*., 1996; Skov *et al*., 1999).

RESULTS AND DISCUSSION

Pseudo-nitzschia australis Frenguelli is known from Pacific coast beaches, the Strait of Juan de Fuca and inland waters of Puget Sound. It is thought to have been the source of domoic acid in the fall of 1991, when domoic acid was first reported from Oregon and Washington. It was the source of domoic acid in the fall of 2002, when domoic acid in razor clams (*Siliqua patula* Dixon) at beaches on the open Pacific coast reached 188 $\mu g\ g^{-1}$ and prevented the fall

recreational razor clam harvest. It was still above the closure level (20 μg g^{-1}) in the spring of 2003, but was back to low levels at most beaches by the fall of 2003.

Pseudo-nitzschia multiseries (Hasle) Hasle is known from all western Washington marine waters. It has been present at a number of locations in Puget Sound when low concentrations of domoic acid have been reported, but it has not been associated with any major domoic acid events there.

Pseudo-nitzschia pungens (Grunow) Hasle is also known from all western Washington marine waters. It has produced domoic acid in culture (Trainer *et al*., 1998) and in the natural environment (Wekell *et al*., 1998).

Pseudo-nitzschia fraudulenta (Cleve) Hasle has been found on the open Pacific coast. It has not been reported to produce domoic acid in Washington waters.

Pseudo-nitzschia heimii Manguin is also found on the open Pacific coast and has not been reported to produce domoic acid there.

Pseudo-nitzschia delicatissima (Cleve) Heiden has been identified from a number of sites on both the open coast and in Puget Sound. It is not known whether it produces domoic acid there, but it has occurred in low concentrations with known domoic acid producers when domoic acid has been present (Stehr *et al*., 2002).

Pseudo-nitzschia pseudodelicatissima (Hasle) Hasle is widely distributed in western Washington waters. It was the dominant species in September 1998, when domoic acid levels in razor clams were about 295 μg g^{-1}.

CONCLUSIONS

Pseudo-nitzschia spp. are common in Pacific Northwest marine waters and are present in every month of the year. They are rarely the dominant genus in a phytoplankton bloom but may occur as almost mono-specific blooms. The blooms may contain only one species, several species, or there may be species succession as a bloom progresses. Not all species produce domoic acid and those that do, do not always produce it or do not always produce high concentrations. The environmental conditions that promote *Pseudo-nitzschia* spp. growth are not well known. On the open coast, timing and duration of upwelling and the presence of adequate nutrients are believed to be key factors. Stratification and sunlight, followed by periods of rain or other freshwater input, influence blooms both on the coast and in inland waters of Puget Sound.

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Effect of Temperature on Motility of Three Species of the Marine Dinoflagellate *Alexandrium*

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ABSTRACT

We have used Digital In-line Holography (DIH) to track the motility of dinoflagellates. We compared the swimming behaviour, velocity and trajectory of three species of *Alexandrium* at 12, 16 and 20°C. Numerical reconstruction of DIH holograms yields high-contrast 3-D images of the trajectories of many motile cells swimming simultaneously throughout the sample volume. Cells in exponential growth were preconditioned at the experimental temperatures for two days before image acquisition. Preliminary results indicated that acclimated strains of the species *Alexandrium ostenfeldii* and *A. tamarense*, isolated from the cold temperate waters of Nova Scotia, swim faster and less erratically at 16°C than at 20°C, a temperature at which they exhibit stressed or abnormal swimming behaviour. In contrast, cells of *A. minutum,* isolated from the warmer waters of South Australia, swim normally and significantly faster at 16 and 20°C than at 12°C. This holographic technology offers the potential for quantitative and qualitative evaluation of the genetics and physiological acclimation of swimming responses of living cells in a relatively unobtrusive manner.

INTRODUCTION

Very little is known about the details of swimming behaviour in the dinoflagellate genus *Alexandrium*, although vertical migration and sinking are undoubtedly important nutrient acquisition (MacIntyre *et al*., 1997) and life-history (Anderson *et al*., 1983) strategies. There have been many studies of swimming in dinoflagellates, in which behaviour such as phototaxis (Forward, 1974; Hand and Schmidt, 1975), vertical migration (Eppley *et al*., 1968; MacIntyre *et al*., 1997), and the form and function of the transverse flagellum (Leblond and Taylor, 1976; Gaines and Taylor, 1985) have been considered. Yet, remarkably, there is a paucity of information on swimming speeds of these cells and how their swimming may be affected by environmental and genetic factors. Swimming behaviour varies widely among flagellated species, even among those with similar morphology. Among the dinokont species, including those of the genus *Alexandrium*, movement is accomplished using two flagella, a posteriorly directed trailing flagellum associated with the sulcus, a longitudinally oriented groove on the ventral side of the cell, and a transverse or ribbon flagellum located in the cingulum which

encircles the cell. The movement of the ribbon flagellum causes the cell to rotate and move in a forward direction ("propeller"), while the trailing flagellum controls the direction of movement ("rudder") (Gaines and Taylor, 1985). When the trailing flagellum is lost, the cell appears to lose control of its forward momentum and begins swimming erratically (F.J.R. Taylor, pers. comm.).

In addition to the morphologically determined differences in swimming behaviour and velocity among dinoflagellates, motility also appears to be affected by various environmental factors, including the temperature at which cells are acclimated. Of course, sudden thermal shocks to *Alexandrium* cells may result in rapid death at elevated temperatures, whereas a rapid "shift-down" can cause loss of motility and sedimentation of cells because of the formation of pellicular cysts and consequent loss of the flagella and thecae (A.D. Cembella, pers. obs.). Less dramatic temperature shifts can lead to modified swimming behaviour but not necessarily loss of motility. Previous studies of movement in dinoflagellates were accomplished using conventional compound microscopy and video cameras to record the motion of the cell. Although it is possible to track swimming cells in a single plane of focus using video cameras with sophisticated image analysis systems, recording and quantifying the motion of algal cells in three-dimensional space is challenging. Conventional microscopy can capture motion only in two dimensions because of limited depth of field. Calculations of algal swimming speeds are difficult, therefore, due to the movement of the cells through many focal planes. Nevertheless, there have been a few successful attempts. Iken *et al*. (2001) used a CCD video camera attached to an Olympus BX60 microscope to quantify the movement of *Hincksia irregularis* spores. Accurate measurements of velocity have been made using an electronic tracking system to study the effects of changes in temperature and salinity on the dinoflagellates *Lingulodinium polyedrum* (= *Gonyaulax polyedra*) and *Gyrodinium* sp. (Hand *et al*., 1965).

In this study, we use Digital In-line Holography (DIH) to track algal motion in 3-D and in time with sub-micron and sub-second resolution, respectively. Reconstruction yields high-contrast 3-D images of motile cells, which facilitates precise measurement of the velocity of swimming cells. Isolates of three species of *Alexandrium* with different temperature optima for growth were chosen for the investigation. Preliminary evidence showed that swimming behaviour was affected by exposure to different (non-lethal) temperatures following acclimation and that the response was species-specific, if not strain-specific.

MATERIALS AND METHODS

Collection and maintenance of isolates

Alexandrium ostenfeldii (AOSH1) was isolated from Ship Harbour, an estuarine fjord in Nova Scotia, from a size-fractioned pump sample collected at a 3 m depth when the water temperature was 10°C. Surface water temperatures in the estuary ranged from below 0°C in winter to about 20°C in summer. *Alexandrium tamarense* (SB50) was isolated from Shelburne Harbour, on the southeastern shore of Nova Scotia, also from a size-fractioned pump sample collected at a 3 m depth. The water temperature was 12°C at the time of collection. *Alexandrium minutum* (AMAD-06) was germinated from a cyst collected from sediments in Port River, South

Australia, where water temperatures range from 10°C in winter to 25°C in summer (Cannon, 1993a), with higher temperatures occasionally occurring in embayments. In temperate Australian waters, blooms of *A. minutum* generally occur in spring or fall when the surface water temperature is about 16°C. Reference isolates have been maintained in culture at the Institute for Marine Biosciences (NRC), at 16°C, under a photon flux density of 100-120 µmol m^{-2} s^{-1} on a 14:10 h light:dark photocycle (AMAD-06 and SB50) and at 14°C, 50 to 80 µmol m^{-2} s^{-1}, 14:10 h light:dark photocycle (AOSH1) prior to experimentation.

Growth experiments

In the first experiment, *Alexandrium* isolates AMAD-06 and SB50 were grown at 8, 12, 16, 20 and 24°C to determine the temperature for optimum growth under a constant light intensity. Inoculum for both species was incubated at 16°C, at a photon flux density of 100-120 µmol m^{-2} s^{-1} on a 14:10 h light:dark photocycle. After two weeks of growth under these conditions, during exponential growth phase, AMAD-06 reached 6.6×10^4 cells mL^{-1} and SB50 achieved 1.3×10^4 cells mL^{-1}. At this time, borosilicate glass culture tubes (50 mL) containing 20 mL of L1 growth medium were inoculated with 5 mL of exponentially growing cultures. Replicates (n = 3) were incubated under an ambient photon flux density of 125-150 µmol m^{-2} s^{-1} on a 14:10 h light:dark photocycle. Tubes were rotated every second day to account for slight differences in light intensity at each position in the rack. Cell concentrations were estimated by *in vivo* fluorescence, using a Turner Designs fluorometer equipped with a filter set optimized for chlorophyll *a*, according to the method of Parkhill and Cembella (1999). Fluorometer readings were taken every two days at the same time (10:00 – 10:30 h), beginning the day after inoculation. Culture tubes were gently inverted four times to resuspend cells and to create a homogenous distribution before readings were taken. In a second experiment several weeks later, the optimum temperature for AOSH1 was determined using the methods described above. Inoculum for this strain was grown at 14°C under a photon flux density of 50 to 80 µmol m^{-2} s^{-1} on a 14:10 h light:dark photocycle. Cell concentration at the time of inoculation was 6×10^3 cells mL^{-1}. Fluorometry measurements were taken every two days, starting on the day of inoculation.

Principle of Digital In-line Holographic Microscopy

In-line holography with numerical reconstruction, or Digital In-line Holography (DIH), has been perfected to the point at which sub-micron resolution is routinely achieved with light. The method has been used in such diverse areas as cell biology, microparticle imaging and tracking, and polymer crystallization. Details of the method and a thorough discussion of its history and potential have been presented in a number of publications (Xu *et al*., 2001; 2003).

In a DIH microscope, light from a laser is focussed onto a pinhole with a diameter of the order of the wavelength. This acts as the "point source" from which a spherical wave of wavelength λ emanates. The wave illuminates an object and forms a geometrically magnified diffraction pattern on a CCD chip, a few centimeters away. If the scattered wave from the object is small compared with the unscattered reference wave, the interference pattern on the screen constitutes a hologram. The hologram is stored as a digital image in a computer (Fig. 1a). The next step is numerical reconstruction by which the three-dimensional structure of the object is obtained from the two-dimensional hologram on the screen (Fig. 1b). It is achieved with a Kirchhoff-

Helmholtz transform (Gabor, 1949), which has been implemented by a self-contained program called LEEPS **(**Kreuzer and Pawlitzek, 1993), originally applied to electron holography.

Capturing four-dimensional information in a single hologram

High quality contrast images of objects that are in motion can be constructed if a succession of holograms is taken. The sequential position of the objects at successive recording times can be obtained by the following procedure: 1) Record holograms ($h_1 \ldots h_n$) with exposure time τ and at time intervals Δt. 2) Generate new holograms by subtraction of consecutive hologram pairs, i.e. (h_1-h_2), (h_3-h_4), etc. 3) Sum these difference holograms to obtain one final hologram. 4) Re-construct this final hologram to obtain images of the objects in different depth planes in the sample. This approach ensures that unwanted background effects such as interferences from sample containers and other stationary objects are eliminated so that the space and time evolution of object trajectories are clearly captured (Fig. 1c).

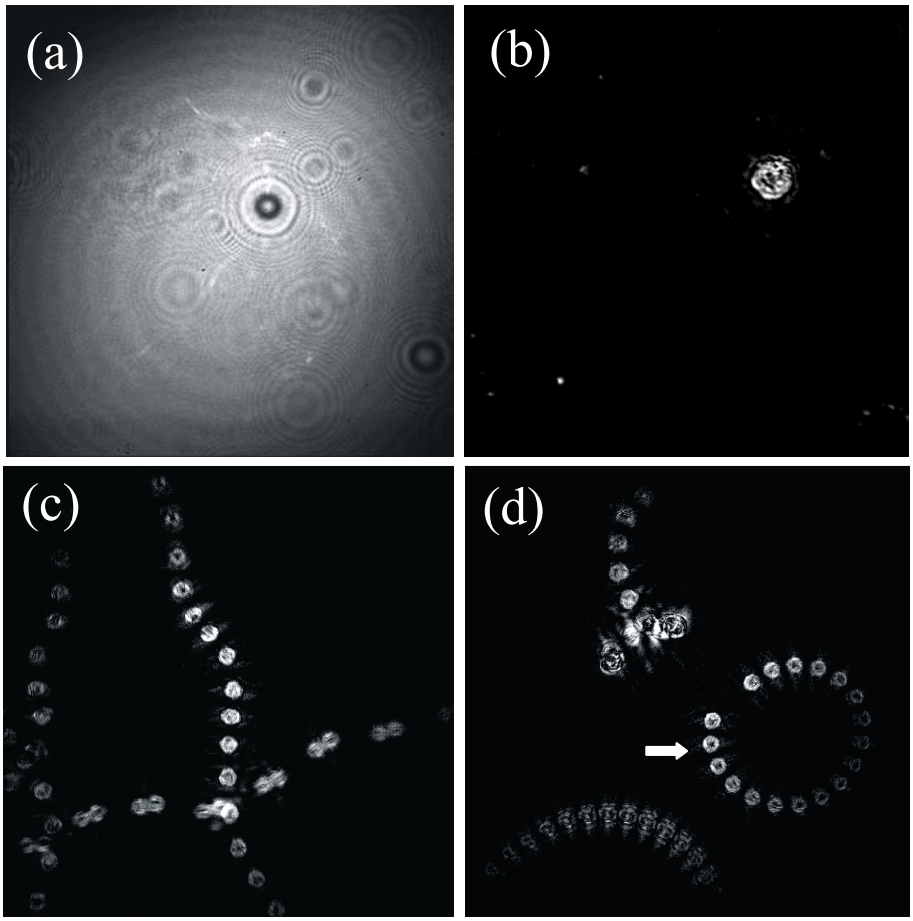

Fig. 1. (a) Hologram of *A. tamarense* (SB50) before reconstruction, and (b) after numerical reconstruction with Kirchhoff-Helmholtz transform. (c) Compound hologram of *A. ostenfeldii* (AOSH1) at 16°C, obtained by adding 20 holograms taken at 0.33 sec intervals, and (d) hologram obtained at 20°C under the same conditions, showing erratic swimming behaviour.

RESULTS

Optimum temperature for cell growth

Growth rate and cell concentration (as measured by *in vivo* fluorescence) in the three species of *Alexandrium* studied were directly affected by temperature. In general, we have observed that isolates of *A. minutum* grow faster and reach higher cell concentrations than do those of *A. tamarense* in culture. Of the three species, *A. ostenfeldii* was consistently the slowest growing under all conditions. The strain of *A. minutum* (AMAD-06), isolated from the warmer waters of Tasmania, exhibited the fastest growth and reached its maximum cell concentration (as measured by *in vivo* fluorescence) at 24°C.

Initially, there was no apparent difference in cell concentration between cultures grown at 16 and 20°C, but those at 20°C maintained exponential growth longer and achieved a higher maximum cell concentration. Growth was inhibited at temperatures below 16°C. Optimum growth of *A. tamarense* (SB50) occurred at 16°C. There was no substantial difference in cell growth at 20 and 12°C, but cultures of this strain grew very slowly at 8°C. All three replicate cultures were dead after two weeks at 24°C. Optimum growth of AOSH1 occurred at 16°C, with little difference in growth rates of cultures at sub-optimum temperatures. Cells of this isolate could not tolerate 24°C and died in less than two weeks under this condition.

Swimming speeds

The data met the requirements of homoscedasticity and normality, permitting an analysis of variance (ANOVA) using SigmaStat, V.2.03 (SPSS Science). Swimming speeds of all three species were significantly higher at 16°C than at 12°C ($p < 0.05$) (Fig. 2). There was no significant difference between the swimming speeds of AOSH1 and AMAD-06 at 12°C, but SB50 swam significantly faster than both these isolates at this temperature ($p < 0.05$).

The strains AOSH1 and SB50 swam significantly faster at 16°C than at 20°C ($p < 0.05$). An unusual circular pattern of swimming was observed for many cells of both these species at the higher temperature. This behaviour was not observed when cells were incubated at lower temperatures, suggesting that cells were experiencing thermal stress at 20°C. There was no significant difference ($p < 0.05$) in the swimming speed of AMAD-06 at the highest temperature compared to 16°C, although much slower movement was observed at 12°C.

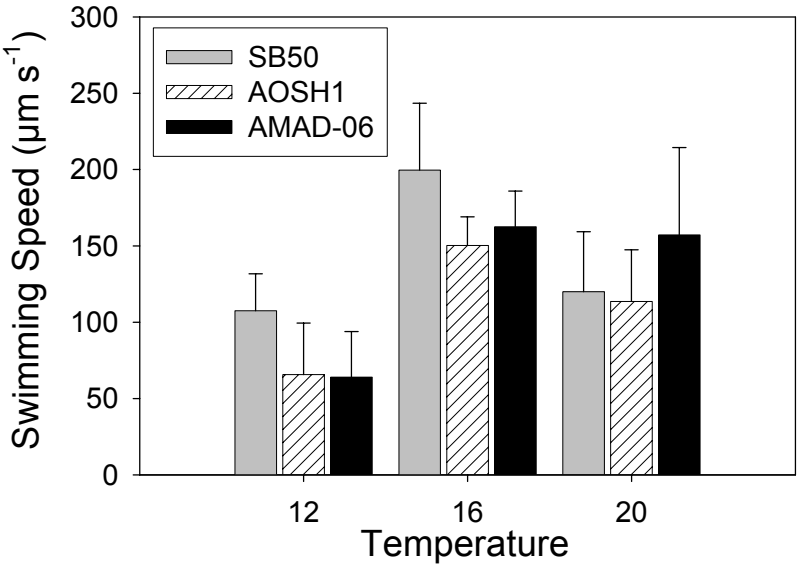

Fig. 2. Swimming speeds of three species of *Alexandrium* at three different temperatures. Each point is a mean of two or three measurements of velocity calculated along the trajectory of one cell at a point when movement is linear; mean ± SD.

DISCUSSION

Direct comparisons of growth among algal species are difficult to make by fluorometry because of variations in the amount of chlorophyll per cell. The temperature dependence of emitted fluorescence also means that comparative growth rates at different temperatures determined by fluorescence must be interpreted cautiously. Nevertheless, *in vivo* chlorophyll *a* fluorescence has been shown to be a valid method for easily measuring phytoplankton cell growth in marine dinoflagellates, including *Alexandrium tamarense* (Parkhill and Cembella, 1999) and *A. ostenfeldii* (Maclean *et al*., 2003), under a variety of environmental conditions. Comparison with cell count data has shown excellent correlation except during extremes of nitrogen deprivation – conditions not encountered in the present experiments.

Cannon (1993b) determined that optimum growth of *A. minutum* (AMAD-06) in culture occurred at 16°C, at a photon flux density of 100 µmol m^{-2} s^{-1}. This strain of *A. minutum* has been in culture for over 10 years and thus growth requirements may have changed. Furthermore, we used a different nutrient medium in our experiments and the light intensity (125-150 µmol m^{-2} s^{-1}) was considerably higher than in the previous study. Nevertheless, this species grew quite well at 16°C and motility was not significantly different from that at 20°C ($p < 0.05$).

We determined that temperature does have an effect on the swimming speeds of isolates of different species of *Alexandrium*. Given the differences in size and morphology among the three species, different maximum swimming speeds would be expected, with *A. minutum* cells (the smallest) being the fastest swimmers. Highest rates of swimming were recorded for each species

at the temperature at which optimum growth was observed. Swimming speeds of all three species have yet to be measured at more extreme temperatures, such as at 8 and 24°C. While much lower speeds were measured for both species of *Alexandrium* isolated from Nova Scotia at 12°C than at higher temperatures, *A. tamarense* (SB50) was significantly faster than *A. ostenfeldii* (AOSH1) ($p < 0.05$). These laboratory observations from cultures are consistent with the swimming behaviour of cells of both species in field samples freshly collected during the early spring in Nova Scotia when water temperatures are between 10-12°C. It is difficult to differentiate *A. tamarense* from *A. ostenfeldii* at low magnification based on morphology alone, but their different swimming behaviour makes it easier to distinguish the more lethargic *A. ostenfeldii* when isolating single cells. Isolates of this latter species, including AOSH1 isolated from Ship Harbour, Nova Scotia, grow well initially at 16°C, but eventually die at this temperature. This species is not found in field samples from Nova Scotia when the ambient surface water temperature exceeds 16°C for a substantial time (a few days).

The swimming rates of *Lingulodinium polyedrum* (= *Gonyaulax polyedra*) and *Gyrodinium* sp. have been shown to be affected by rapid changes in temperature and salinity, with maximum speeds occurring at conditions optimum for each species (Hand *et al*., 1965). The two species studied responded to a rapid temperature decline with a marked decrease in swimming rate. The authors suggested that this might have a marked effect on the distribution of species that vertically migrate through the water column, allowing cells to become concentrated by the action of thermoclines. Species of *Alexandrium* have also been shown to migrate through the water column in search of nutrients (MacIntyre *et al*., 1997). At optimum growth temperatures, the maximum swimming speeds of all three species of *Alexandrium* were between 150-200 $\mu m\ s^{-1}$ or 0.5-0.7 $m\ h^{-1}$, clearly a fast enough swimming rate to allow migrating cells to accomplish this migration. Our data suggest an impaired ability to swim at lower temperatures, which, in addition to the physical gradient imposed at density discontinuities, may account for the tendency of this species, as with many other dinoflagellates, to accumulate near thermoclines.

The technology employed in this study was ideally suited to studying motility in microalgal cells. Such a holographic microscope can yield high-resolution images of a cell through a series of focal planes, making it possible to track movement of motile cells in three-dimensional space. There are many advantages to this system, including its simplicity (consisting of a laser, pinhole and a CCD camera). The LEEPS software allows the reconstruction of a sequence of holographic images in 4-D (X, Y, Z and time). A single hologram contains all of the information about the three-dimensional structure of the object. Optimum resolution of the order of the wavelength of the laser can be obtained easily. Finally, there is no sample preparation necessary and it is possible to study living cells in a non-destructive manner.

These DIH systems also have great potential for studying such ecological questions *in situ*. With minor modifications to the system, it could be used as a tool to study the movement of plankton, organic particles, air bubbles etc., in the water column. With DIH, magnified images of objects (unlike conventional off-axis holography) can be obtained, making it a powerful new tool for the study of biological systems, including investigations of bacterial attachment to surfaces, bacterial biofilm formation, remote sensing and environmental monitoring, and particle velocimetry, i.e. tracking of the motion of particles in liquid or gas flows and many others.

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The Characterization of Two Novel Spirolides Isolated from Danish Strains of *Alexandrium ostenfeldii*

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A class of macrocyclic imines known as spirolides was first identified in extracts of the digestive glands of mussels and scallops from the Atlantic coast of Nova Scotia, Canada, in the early 1990s. The distinguishing feature of these compounds is the presence of a cyclic imine moiety, which has been found elsewhere only in the marine toxins known as pinnatoxins, pteriatoxins, spiro-prorocentrimine and gymnodimine. This unusual cyclic imine feature is the pharmacophore responsible for the "fast-acting" symptomology observed when these compounds are assayed by intraperitoneal administration into mice.

The marine dinoflagellate *Alexandrium ostenfeldii* (Paulsen) Balech & Tangen was identified as the cause of spirolide toxicity in Nova Scotia. This finding was surprising because *A. ostenfeldii* has been previously known as a source of neurotoxins associated with paralytic shellfish poisoning (PSP), an unrelated toxin syndrome. To date, seven compounds belonging to the spirolide class have been isolated and structurally characterized from shellfish extracts and cultured dinoflagellate isolates from Nova Scotia. Using LC-MS methodology, spirolides have recently been detected in cultured isolates of *A. ostenfeldii* obtained from Limfjord, Denmark. When we isolated and elucidated the structures of the two major spirolide compounds present, we discovered the presence of a unique trispiroketal ring system in the second compound which, had not been previously observed in other cyclic imine toxins. The assignment of the structures of these two novel spirolides will be presented.

Production of a Novel Proteinaceous Hemolytic Exotoxin by the Dinoflagellate *Alexandrium taylori*

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It has been well documented that some *Alexandrium* spp. produce potent neurotoxins such as saxitoxin (STX) and its derivatives. Our previous study revealed that *Alexandrium taylori* isolated from Shioya Bay, Okinawa Island, Japan, produced no STX. In this study, we found that *A. taylori* produces a lethal effect on *Artemia* and causes species-specific hemolysis against mammalian erythrocytes. Among the erythrocytes tested, rabbit and guinea-pig erythrocytes were highly sensitive, but human, sheep and cattle erythrocytes were insensitive. The cell-free culture supernatant also showed potent hemolytic activity toward rabbit erythrocytes as seen in whole cell suspensions. The hemolytic activity in the culture medium gradually increased with an increase in cell number during the exponential growth phase, and relatively high activity was maintained even after reaching the death phase.

These results suggest that a hemolytic substance is actively released into the medium from *A. taylori* cells, rather than simple leakage from ruptured or dead cells, and that some of this substance is steadily accumulated in the medium during algal growth. Chemical characterization with ultrafiltration and trypsin-treatment suggested that the hemolytic substance released into the medium is a protein-like compound with a molecular weight of more than 10,000 Daltons. The ammonium sulfate precipitated fraction obtained from the cell-free supernatant of *A. taylori* showed cytotoxic effects on HeLa cells as well as hemolytic activity in a similar concentration range on a protein content basis. Our results suggest that *A. taylori* produces a novel proteinaceous hemolytic exotoxin.

Preparation of a Certified Reference Material for Paralytic Shellfish Poisoning Toxins

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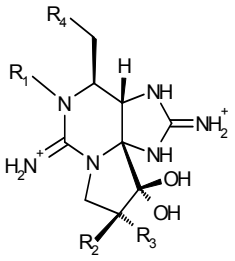
INTRODUCTION

Paralytic shellfish poisoning (PSP) toxins are potent neurotoxins, produced by several marine dinoflagellates. These toxins are tetrahydropurine derivatives based on the parent compound, saxitoxin (STX) and can be divided into three classes: carbamate, N-sulfocarbamoyl and decarbamoyl toxins (Fig. 1). There are 18 principal structural analogs but many more have been identified. They all have different toxicities, with the carbamate toxins being the most toxic and the N-sulfocarbamoyl toxins being the least toxic.



STX = saxitoxin
NEO = neosaxitoxin
GTX = gonyautoxins

			Carbamate toxins	N-Sulfocarbamoyl toxins	Decarbamoyl toxins
R_1	R_2	R_3			
H	H	H	STX	GTX5 (B1)	dcSTX
H	H	OSO_3^-	GTX2	C1	dcGTX2
H	OSO_3^-	H	GTX3	C2	dcGTX3
OH	H	H	NEO	GTX6 (B2)	dcNEO
OH	H	OSO_3^-	GTX1	C3	dcGTX1
OH	OSO_3^-	H	GTX4	C4	dcGTX4

Fig. 1. Structures of principal PSP toxins.

Enforcement of regulatory directives on food contaminants such as PSP toxins requires validated analytical methods and certified reference materials (CRMs) for both calibration standards and tissue homogenates. The most widely used chemical analysis methods are based on liquid chromatography coupled with either post-column oxidation/fluorescence detection (LC-pcr/FLD) (Oshima, 1995) or tandem mass spectrometry (LC-MS/MS) (Quilliam *et al*., 2001). Tissue-based CRMs play an important role in method validation and quality control, as they allow the testing of an entire method from extraction through analysis. Although the European Commission has recently announced a CRM for decarbamoylsaxitoxin in lyophilized mussel tissue (van Egmond *et al*., 2001), it is not being distributed currently. Furthermore, a reference

material (RM) based on liquid slurry of homogenized whole mussel tissue with a wider range of toxins would provide a better match to natural samples. This paper presents our work towards the development of a mussel tissue CRM for PSP toxins, with the testing of a pilot scale product, RM-PSP-Mus-p.

RESULTS AND DISCUSSION

RM design criteria

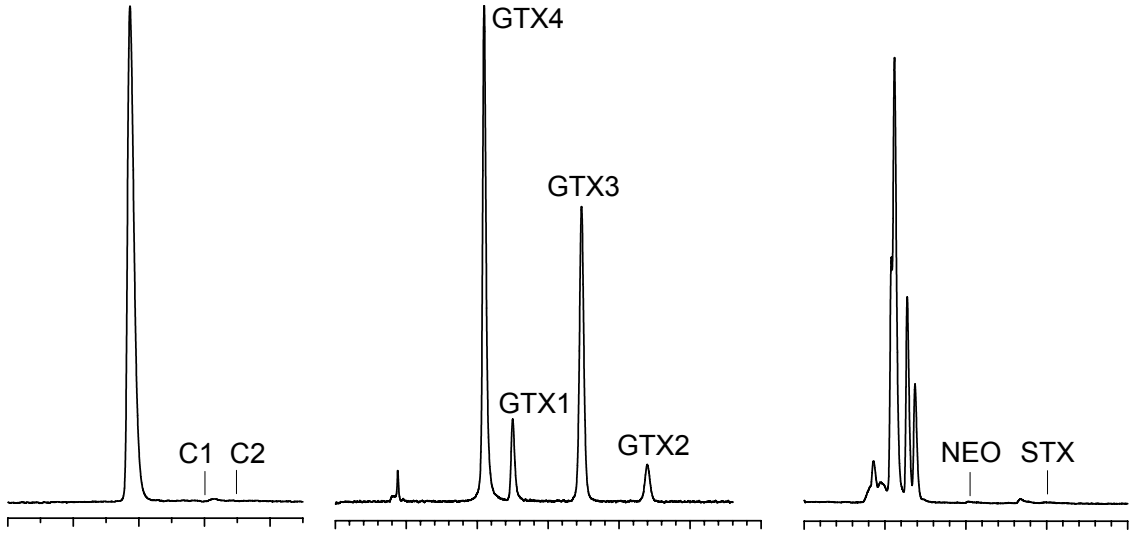
The purpose of this work was to design a wet, thermally sterilized mussel tissue homogenate, similar to our other CRMs for amnesic shellfish poisoning and diarrhetic shellfish poisoning (NRC CRM-ASP-Mus and CRM-DSP-Mus). A blend of mussel tissue and cultured plankton was selected as the best means of producing a suitable toxin concentration and profile. We felt that this is justified, as any natural shellfish sample is in fact a blend of mussel tissue and plankton that the animal has been eating. The target toxin level was to be about five times the regulatory level of 800 µg STX equivalents per kg whole tissue. The toxin profile was to include STX, NEO, GTX2&3, dcGTX2&3 and GTX1&4. It was decided that N-sulfocarbamoyl toxins (Bs and Cs) should not be present in the tissue homogenate, as these decompose under both high and low pHs. Finally, as a possible alternative should there be problems with the liquid slurry approach, we also planned to test the effectiveness of a lyophilized material.

Preparation of plankton

Our algal research group at IMB has produced large quantities of two PSP-producing cultures, *Alexandrium tamarense* (AL18b) and *A. minutum* (AL1V). *Alexandrium tamarense* produces primarily C2, GTX3, NEO and STX, whereas *A. minutum* produces GTX4, a small amount of GTX3 and no C toxins (Fig. 2). There are problems with simply blending such biomass directly with mussel tissue to produce a CRM. Firstly, the GTX4 and GTX3 will gradually epimerize to GTX1 and GTX2, respectively. It is therefore important to stabilize the epimer ratios prior to blending with the mussel tissue. More problematic is the gradual conversion of C toxins to dcGTXs at high pH and to GTXs at low pH, during storage and extraction. The toxin interconversions for the C1 set are illustrated in Fig. 3. Therefore, it is best to eliminate C toxins before blending with the mussel tissue. Finally, it is also important that the final product be adjusted to an acidic pH (3-4) for overall stability of the GTX, STX and NEO toxins.

The role of *A. minutum* was to provide the GTX1 and GTX4 epimers. Our calibration solution CRM for GTX1&4 (CRM-GTX1&4) has a GTX1/GTX4 concentration ratio of three, which was set as our goal for the ratio in mussel tissue RM. The first step, therefore, was to stabilize the epimer ratio prior to blending with the mussel tissues. The plankton used in the procedure was grown in the summer of 2001 and stored at –12°C. Due to this long storage time, the GTXs had already begun to epimerize and the GTX1/4 ratio was already at two. The effect of pH on the toxin epimerization in plankton was investigated. Epimerization is favoured at a high pH (7.6), but under such conditions the GTXs were rapidly destroyed (Fig. 4). At a natural pH (5), an equilibration was achieved in a short period of time (30 min), with relatively little decomposition (Fig. 5).

Alexandrium minutum



Alexandrium tamarense

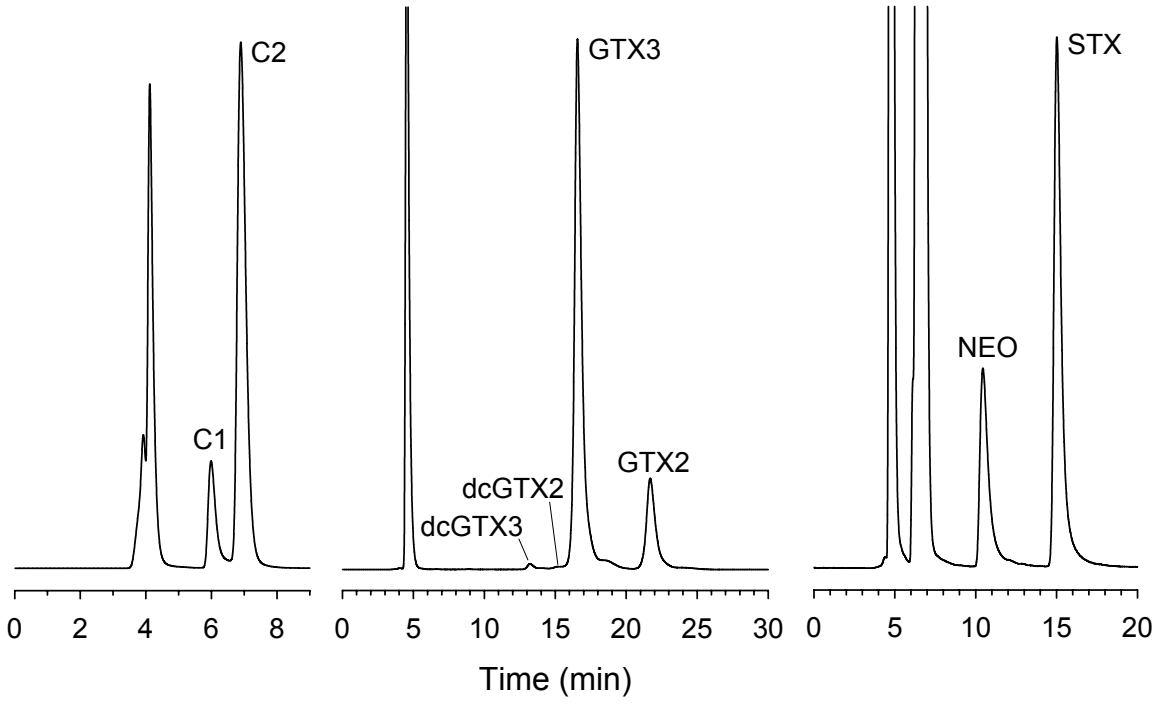


Fig. 2. Analysis of plankton cultures using LC-pcr/FLD (modified Oshima method) (Oshima, 1995).

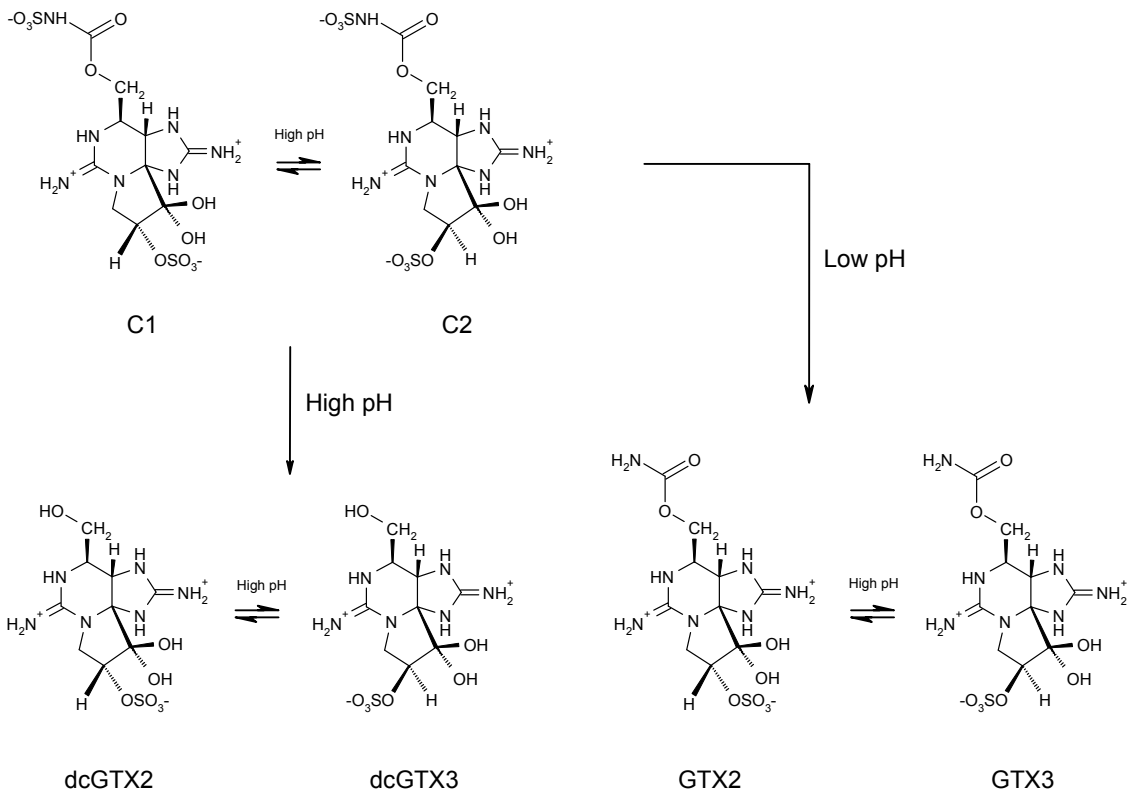


Fig. 3. Schematic of toxin interconversions within *Alexandrium tamarense*.

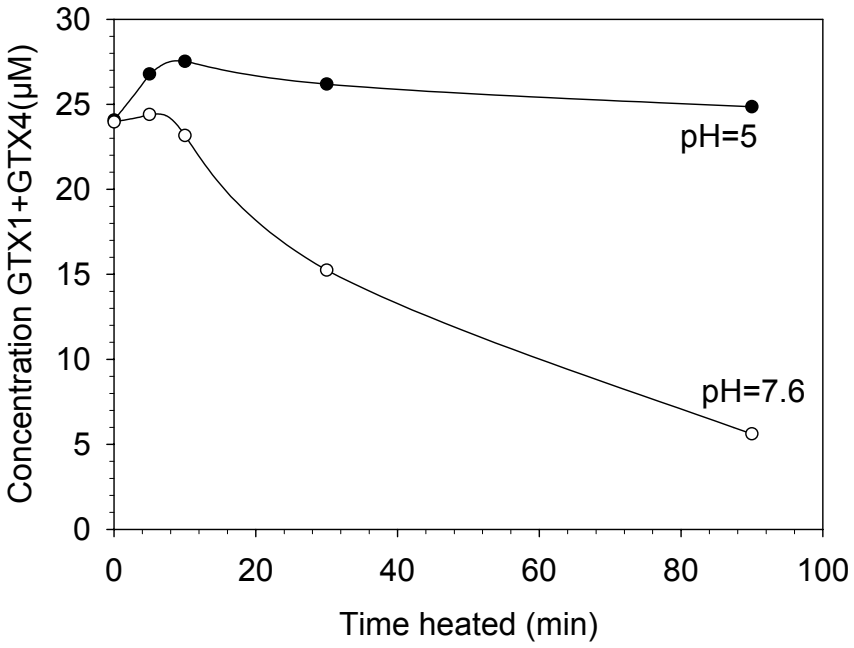


Fig. 4. Destruction of GTX1&4 at natural pH (5) and high pH (7.6) in AL1V plankton heated at 100°C.

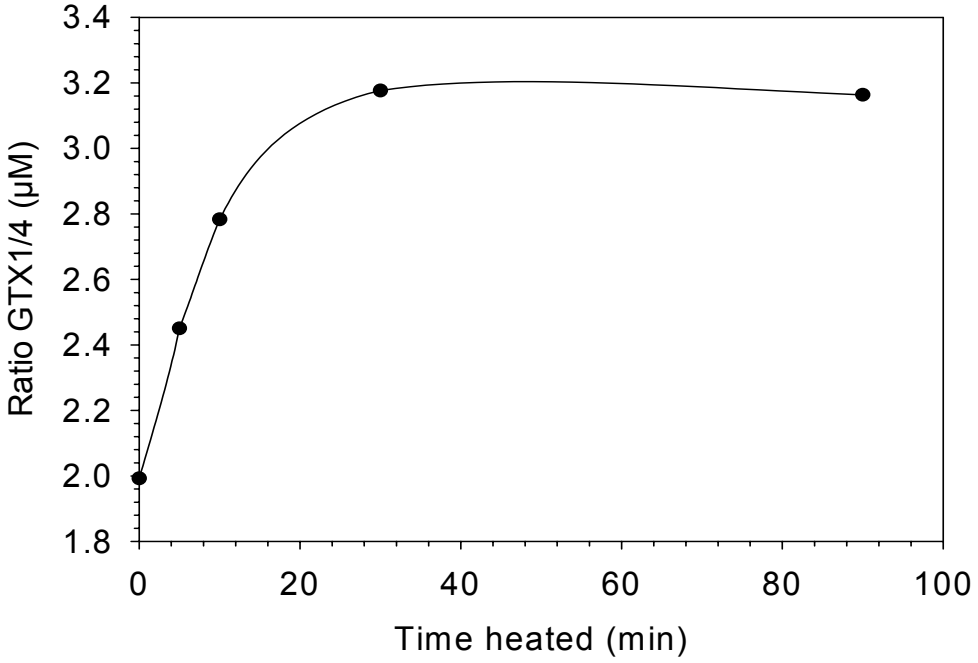


Fig. 5. Epimerization of GTX1&4 at natural pH (5) in AL1V plankton heated at 100°C.

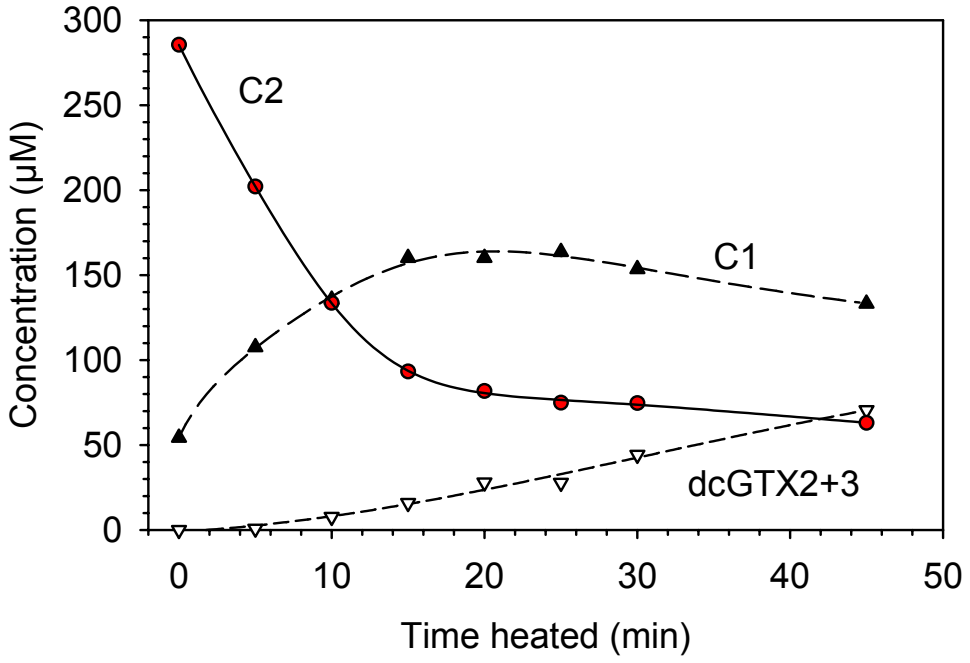


Fig. 6. Epimerization of C toxins and production of dcGTX2&3 in AL18b plankton at natural pH (5), heated at 100°C.

The role of *A. tamarense* in the PSP-Mus-p was to provide GTX2&3, dcGTX2&3, NEO and STX. Our current calibration solution CRMs for the GTXs have stable concentration ratios of 3:1 (CRM-GTX2&3-b) and 3.5:1 (CRM-dcGTX2&3). These values were set as our goals for the ratios in the mussel tissue RM. In order to achieve these ratios, the C toxins were first epimerized to a stable ratio by heating the plankton biomass. The plankton was then acidified and heated (100°C) until the C toxins were all converted to GTXs.

The pH of the plankton has a significant effect on epimerization of the toxins. Initial experiments with heating the AL18b plankton at a high pH of 7.6 resulted in the epimers reaching equilibrium quickly, but this also resulted in a rapid conversion of the C toxins to dcGTXs. Heating at a natural pH of 5, equilibrium was reached with only a partial conversion of C toxins to dcGTXs (Fig. 6). STX and NEO also decomposed partially during the treatments but especially under basic pH, which we concluded should be avoided during the epimerization step.

After epimerization of the C toxins and partial conversion to dcGTXs, the plankton must be hydrolyzed to convert the unstable C toxins to the more stable GTXs. This may be accomplished by heating the plankton at low pH. Different conditions were explored to find one appropriate for our desired result. Various proportions of HCl to plankton and different heating times were examined to determine the optimum conditions. Small-scale reactions were carried out using 0.1, 0.5 and 1 M HCl in different proportions to determine the most appropriate conditions for our application. We determined that an equal volume of 0.5 M HCl added to the plankton was sufficient to convert the C toxins in a reasonable amount of time, without excessive dilution (Fig. 7).

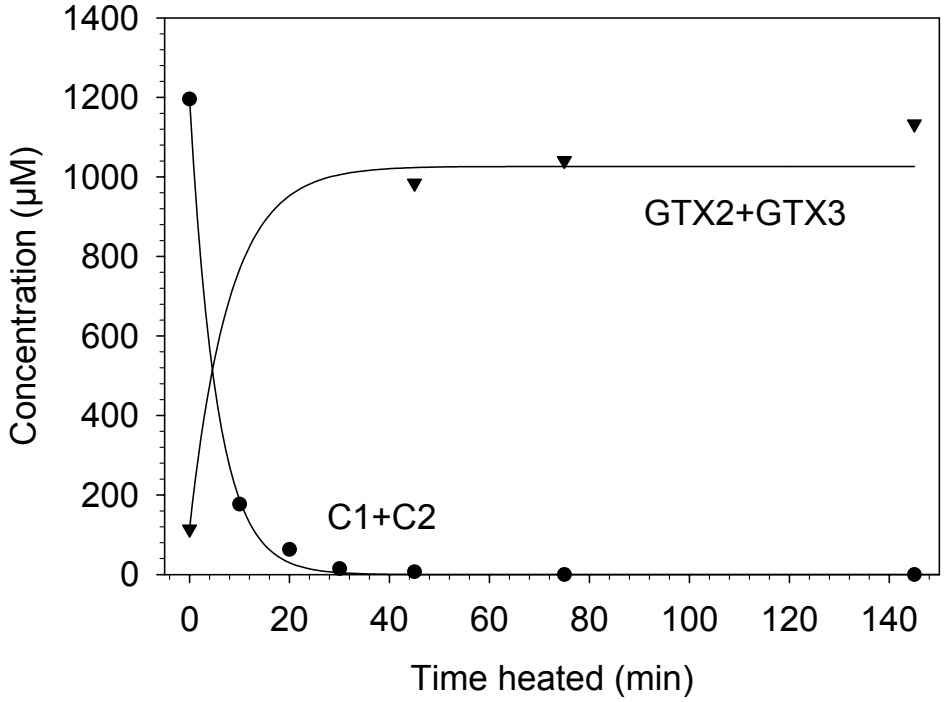


Fig. 7. Hydrolysis of C toxins and production of GTX2&3 in AL18b plankton at natural pH (5), heated at 100°C.

Preparation of RM-PSP-Mus-p

Alexandrium minutum (60 g) was diluted 1:1 (v/v) with deionized H_2O and placed on a heated stir plate. The temperature was monitored and samples were taken at timed intervals. The toxin profile was monitored by LC-pcr/FLD until the GTX1/GTX4 ratio reached a stable equilibrium. A sample of *A. tamarense* (25 g) was diluted 1:1 (v/v) with deionized H_2O and placed on a heated stir plate. The temperature and C toxin area ratios were monitored by LC-pcr/FLD until the C1/C2 ratio reached equilibrium. Throughout this process, water was added to maintain the original volume. After a stable C1/C2 ratio had been reached, 0.5 M HCl was added to the plankton (1:1 v/v). It was returned to the hot plate and monitored by LC-pcr/FLD until C toxins were destroyed. The prepared plankton was stored overnight until the mussel homogenate was ready for plankton addition.

The mussel tissue used for this pilot study was a 9:1 mixture of cooked mussels from the Netherlands and Prince Edward Island, respectively. The former contained okadaic acid and the latter contained domoic acid. The presence of these additional toxins should prove useful for our research projects on "universal" extraction. It was necessary to remove byssus thread fibres from the larger Dutch mussels to prevent tubing obstruction during filling of the bottles. The tissues were then homogenized in a RobotCoupe RSI6.

The moisture content of the tissue was determined by drying an aliquot of the tissue at 100°C. The amount of water required to elevate the moisture content to 85% was calculated. The prepared AL1V and AL18b plankton was added to the mussel homogenate along with deionized water to bring the final moisture content to 85%. The final homogenate contains ca. 3% plankton by weight. The pH of the prepared homogenate was adjusted with HCl from 5.5 to a final pH of 3.9.

The prepared homogenate was dispensed into polypropylene bottles. The aim was to prepare 8 g samples. Each bottle was weighed after filling and if the sample was less than 8 g, it was rejected. Bottles filled to greater than 8.3 g of sample were also rejected. The bottles were sealed and thermally sterilized in a steam retort. The final tally of acceptable bottles was 264.

Extraction and analysis

Different extraction procedures are being developed and tested on this pilot scale RM for the eventual certification of the final CRM. A modification of the AOAC extraction procedure (boiling with 0.1 M HCl) will be employed, but with an increased v/w ratio and volumetric control for accuracy. It is our goal to establish a new "universal" extraction procedure, suitable for extraction of all toxins, both polar and lipophilic.

Initial extraction work on RM-PSP-Mus-p consisted of mixing 0.1 M HCl with the contents of one bottle of the RM-PSP-Mus pilot (ca. 8 g). The weight of the empty bottle is subtracted from the weight of the full bottle to determine the exact amount of tissue used for the extraction. The sample was exhaustively extracted by boiling the HCl/mussel tissue homogenate. The decanted supernatant was brought to a final volume of 25 mL in a volumetric flask. Samples were filtered

and analyzed by LC-pcr/FLD to determine the final toxin concentration. Our prepared pilot RM had a toxin level of ca. 4,600 µg STX equivalents per kg whole tissue.

Table 1. Toxin concentrations measured in RM-PSP-Mus-p.

Toxin	Concentration (µmoles/kg tissue)	Relative Toxicity	µg STX equivalent per kg tissue
GTX-4	0.69	0.7261	186
GTX-1	2.73	0.9940	1009
dcGTX-3	2.19	0.3766	307
dcGTX-2	8.26	0.1538	473
GTX-3	1.64	0.6379	389
GTX-2	5.61	0.3592	750
NEO	1.42	0.9243	488
STX	2.6100	1.0000	971
		total µg STX equivalents/kg tissue:	**4600**

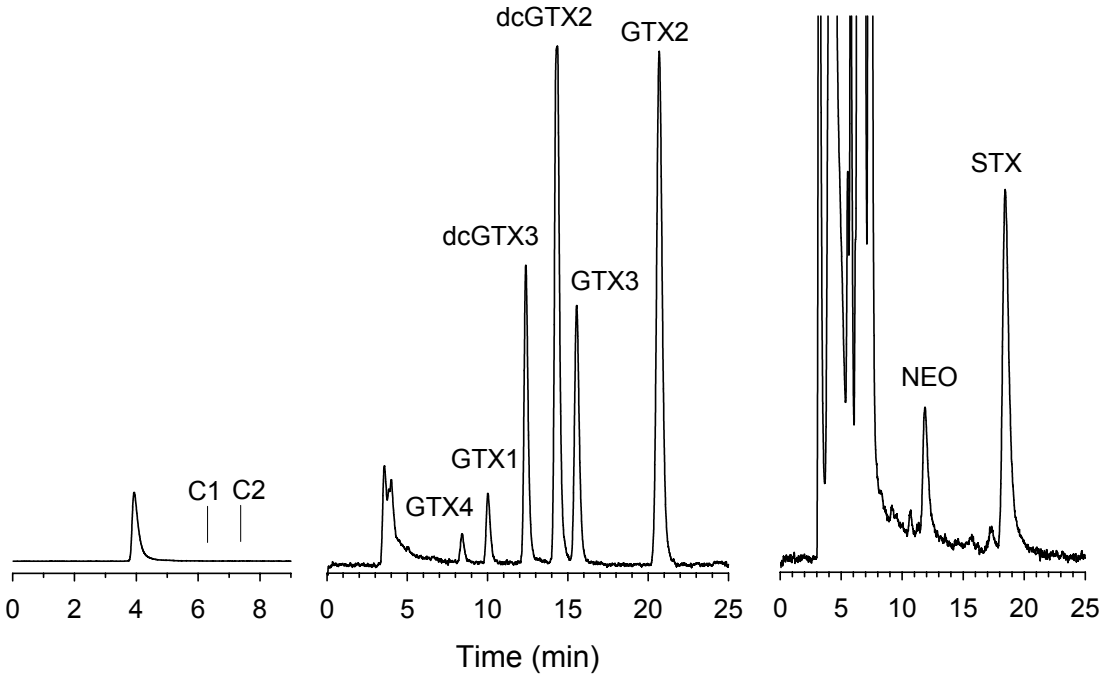


Fig. 8. Analysis of RM-PSP-Mus-p, using a modified AOAC extraction procedure and LC-pcr/FLD (modified Oshima method) (Oshima, 1995).

CONCLUSIONS

Our preliminary research has determined that a mussel tissue CRM for PSP toxins can be produced by blending plankton and mussel tissue, after conversion of toxins in the plankton to a stable mixture. After further method development work and a stability study on both wet and lyophilized material, a large-scale CRM will be prepared, we hope in 2004.

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Bacterial Community Dynamics in the Bay of Fundy: Relationships with Abiotic Factors and the Phytoplankton Community

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Bacteria and phytoplankton dynamics are thought to be closely linked in coastal marine environments, with correlations frequently observed between bacterial and phytoplankton biomass. In contrast, little is known about how these communities interact with each other at the species composition level. The purpose of the current study was to analyze bacterial community dynamics in a productive, coastal ecosystem and to determine whether they were related to phytoplankton community dynamics. Near-surface seawater samples were collected in February, May, July, and September 2000, from several stations in the Bay of Fundy, New Brunswick. Savin and others analyzed the phytoplankton community in simultaneously collected samples. The attached and free-living bacterial communities were collected by successive filtration onto 5 μm and 0.22 μm pore-size filters, respectively. DNA was extracted from filters and bacterial 16S rRNA gene fragments were amplified and analyzed by denaturing gradient gel electrophoresis (DGGE).

DGGE revealed that diversity and temporal variability were lower in the free-living than the attached bacterial community. Both attached and free-living communities were dominated by members of the *Roseobacter* and *Cytophaga* groups. Correspondence analysis (CA) ordination diagrams showed similar patterns for the phytoplankton and attached bacterial communities, indicating that shifts in the species composition of these communities were linked. Similarly, canonical CA revealed that the diversity, abundance and percentage of diatoms in the phytoplankton community accounted for a significant amount of the variability in the attached bacterial community composition. In contrast, ordination analyses did not reveal an association between free-living bacteria and phytoplankton. These results suggest specific associations between phytoplankton and the bacteria attached to them influence the composition of both communities.

Certified Reference Materials for Marine Toxins

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ABSTRACT

Calibration standards are crucial to the development and routine implementation of analytical methods. The National Research Council's Certified Reference Materials Program (CRMP) in Halifax has, as its main focus, the production of certified reference materials (CRMs) for toxins of marine algal origin. These include both calibration solutions and shellfish tissue homogenates. Preparation of calibration standards requires careful attention to purification, stability determination and quantitation. Periodic checks on certified concentrations, a good storage and distribution system, and eventual replenishment of CRMs are also required. An expanded suite of marine toxin CRMs is now in preparation. The procedures involved in the production and certification of CRMs will be presented, along with our plans for future materials.

INTRODUCTION

The lack of accurate calibration standards for algal toxins has been and still is a significant problem in the development and implementation of analytical methods for routine monitoring of seafood. In addition, regulatory labs now face the need to operate under GLP and ISO guidelines, which require validated methods, accurate calibration standards, and CRMs. The National Research Council's Certified Reference Materials Program (CRMP) began producing toxin standards in response to the domoic acid crisis in Canada in 1987. Since then, the program has expanded to include calibration solution CRMs and shellfish tissue CRMs for a variety of toxins of marine algal origin, including those responsible for amnesic, diarrhetic and paralytic shellfish poisoning (Table 1).

An ambitious program was launched in 2001 to expand the suite of marine phycotoxin CRMs (Table 1). This work is being performed in collaboration with a number of other organizations and with funding from the Trade and Investment Liberalisation and Facilitation fund of the Asia-Pacific Economic Cooperation (APEC), Health Canada (HC), the Canadian Food Inspection Agency (CFIA), and the UK Fisheries Research Service (FRS). Our collaborating organizations (FRS [UK], Marine Institute [Ireland], Cawthron Institute [NZ] and AgResearch [NZ]) provide in-kind contributions such as plankton or shellfish tissues, purified toxin or technical expertise. Collaborators also receive part of the final product in return for their contributions.

Preparation of CRMs requires careful attention to toxin purity and stability. Accurate quantitation involves a cross-comparison of results from different procedures, including gravimetry, nuclear magnetic resonance (NMR) spectroscopy, and separation methods, such as liquid chromatography (LC) and capillary electrophoresis (CE) coupled with diverse detection systems such as ultra-violet (UVD), fluorescence (FLD), mass spectrometry (MS) and chemiluminescence (CLND). Periodic checks and eventual replenishment of CRMs are part of our program.

Table 1. Toxin calibration solution CRMs and mussel tissue CRMs from NRC-CRMP.

Available at present:	
Section 1.01	**Amnesic Shellfish Poisoning (ASP) Toxins**
NRC CRM-DA-d	domoic acid calibration solution CRM
NRC CRM-ASP-Mus-b	mussel tissue CRM for ASP toxins
Section 1.02	**Diarrhetic Shellfish Poisoning (DSP) Toxins**
NRC CRM-OA-b	okadaic acid calibration solution CRM
NRC CRM-DSP-Mus	mussel tissue CRM for DSP toxins
Section 1.03	**Paralytic Shellfish Poisoning (PSP) Toxins**
NRC CRM-STXdiAc	saxitoxin calibration solution CRM
NRC CRM-STX-d	saxitoxin dihydrochloride calibration solution CRM
NRC CRM-dcSTX	decarbamoylsaxitoxin calibration solution CRM
NRC CRM-NEO-b	neosaxitoxin calibration solution CRM
NRC CRM-GTX1&4-b	gonyautoxin-1 and -4 calibration solution CRM
NRC CRM-GTX2&3-b	gonyautoxin-2 and -3 calibration solution CRM
NRC CRM-GTX5	gonyautoxin-5 (B1) calibration solution CRM
NRC CRM-dcGTX2&3	decarbamoylgonyautoxin-2 and -3 calibration solution CRM
Section 1.04	**Other Toxins**
NRC CRM-PTX2	pectenotoxin-2 calibration solution CRM
NRC CRM-SPX1	spirolide (13-desmethyl-C) calibration solution CRM
NRC CRM-GYM	gymnodimine calibration solution CRM
Under development:	
Section 1.05	**Paralytic Shellfish Poisoning (PSP) Toxins**
NRC CRM-C1&2	N-sulfocarbamoylgonyautoxin-2 and -3 calibration solution CRM
NRC CRM-dcNEO	decarbamoylneosaxitoxin calibration solution CRM
NRC CRM-C3&4	N-sulfocarbamoylgonyautoxin-1 and -4 calibration solution CRM
NRC CRM-GTX6	gonyautoxin-6 (B2) calibration solution CRM
NRC CRM-dcGTX1&4	decarbamoylgonyautoxin-1 and -4 calibration solution CRM
NRC CRM-PSP-Mus	mussel tissue CRM for PSP toxins
Section 1.06	**Other Toxins**
NRC CRM-PTX2sa	pectenotoxin-2 seco acid calibration solution CRM
NRC CRM-YTX	yessotoxin calibration solution CRM
NRC CRM-AZA	azaspiracid calibration solution CRM

RESULTS AND DISCUSSION

Production and purification of toxins

Toxic strains of algae are grown in large quantities to produce the biomass needed to isolate the toxins for the calibration solutions. In some instances, the required toxin may be isolated from toxic shellfish. Occasionally, toxins are derived from others via semi-synthetic operations. For example, the primary PSP toxin produced by our culture of *Alexandrium tamarense* is C2. The following conversions have been performed: C2 → C1 → GTX2, GTX3 → STX → dcSTX; C2 → dcGTX2, dcGTX3; and C2 → GTX5 (Laycock *et al*., 1995). Toxins are taken through several stages of preparative chromatography in order to achieve a high degree of purity (Laycock *et al*., 1994).

Purity analyses

Isolated toxins are analyzed with a variety of methods to ensure there are no significant impurities that will interfere with the intended use of the CRM. For example, the PSP toxin CRMs are designed mainly for LC-FLD and LC-MS analyses, so it is important that there be no unresolved impurities. The purity of the GTX5 used in the NRC CRM-GTX5 calibration solution was determined using a combination of LC-FLD, CE-UVD, LC-CLND, LC-MS and NMR. Four analytical traces are provided in Fig. 1. Fig. 2 shows the analyses of another CRM, NRC CRM-GTX2&3-b.

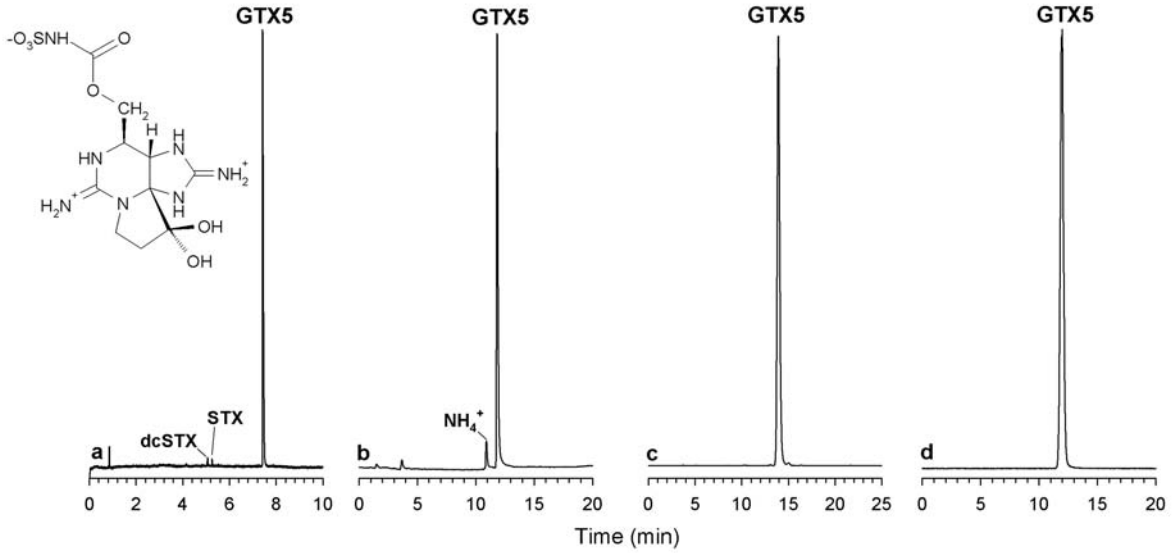

Fig. 1. Purity analyses of GTX5 using: (a) capillary electrophoresis with diode-array detection (Thibault *et al*., 1991); (b) LC with chemiluminescence nitrogen detection (Quilliam *et al*., manuscript in preparation); (c) LC-fluorescence with post-column oxidation (Oshima, 1995); and (d) LC-MS (Quilliam *et al*., 2001).

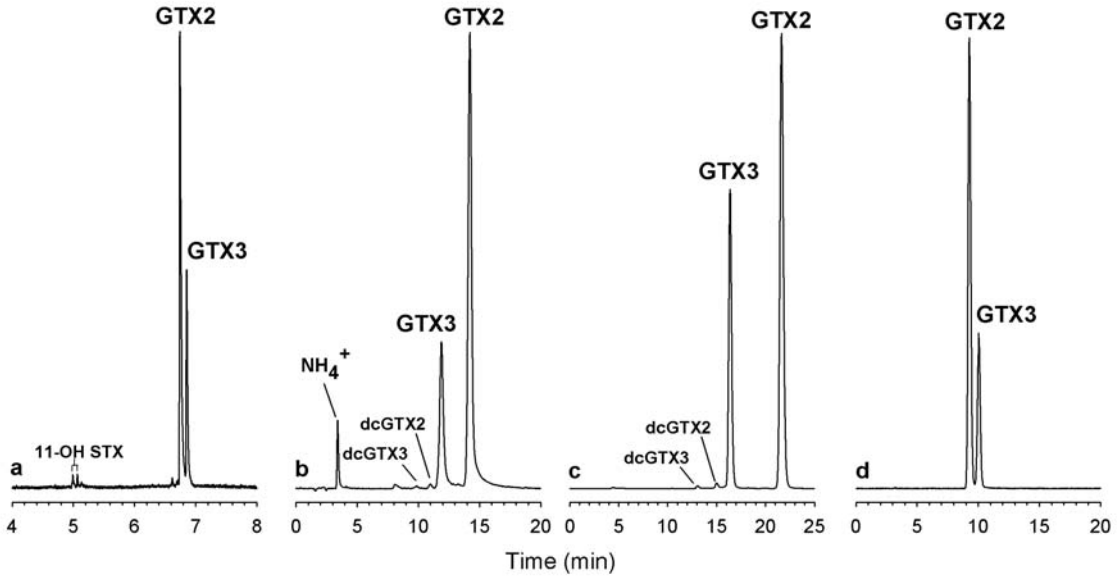

Fig. 2. Analyses of GTX2&3 using: (a) capillary electrophoresis with diode-array detection (Thibault *et al*., 1991); (b) LC with chemiluminescence nitrogen detection (Quilliam *et al.*, manuscript in preparation); (c) LC-fluorescence with post-column oxidation (Oshima, 1995); and (d) LC-MS (Quilliam *et al*., 2001).

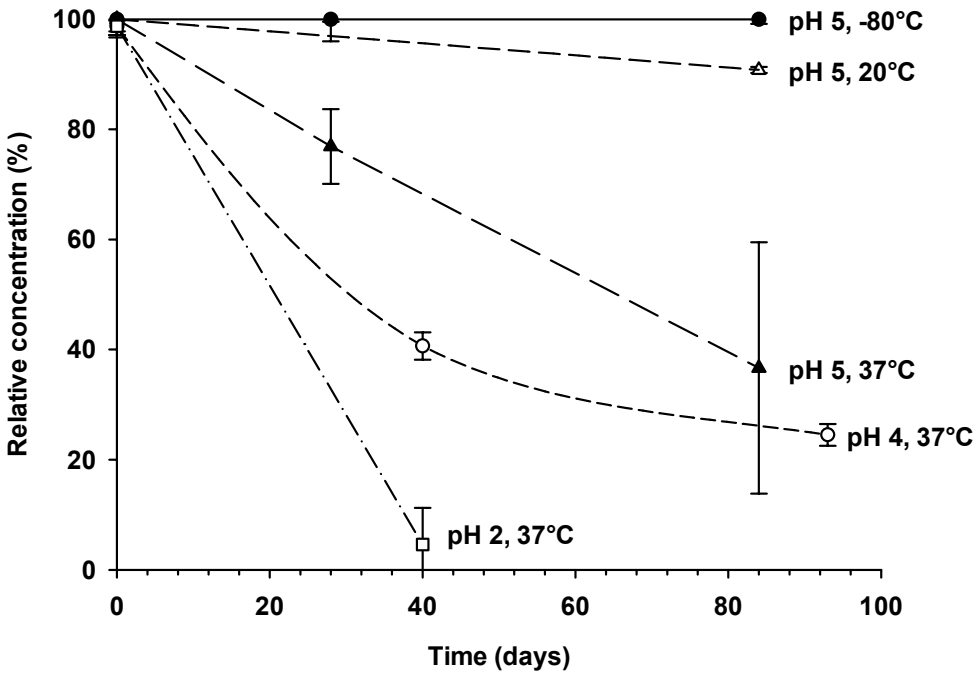

Fig. 3. Stability of GTX5 under various storage conditions.

Stability studies

Stability studies are an important part of CRM preparation. Valuable quantities of toxins are used in the production of a product, and more importantly, we need to be confident of the stability of the final product during storage (which may be five to 10 years). We must also ensure that stability during shipment is maintained. Parameters investigated include type of solvent and sensitivity to oxygen, light, pH, and temperature (Fig. 3). Increased temperatures are used to accelerate decomposition which otherwise may be difficult to detect over relatively short periods of time.

For the PSP toxins, special consideration must be given to toxins that exist as epimeric pairs (e.g. GTX2 and GTX3), which interconvert. It is important to stabilize the pair to an equilibrium ratio and to measure their individual molar concentrations. It should be noted that relative molar responses vary between individual PSP toxins, including epimers, so it is important to have standards for all individual toxins.

Quantitation

For many toxins, which may be isolated in only 10-100 mg quantities, it is difficult to determine the amounts of associated salts, counter-ions or water of hydration. PSP toxins are particularly difficult because they cannot be crystallized easily, are hygroscopic and are in the form of salts. Quantitation of a CRM is done using at least two independent methods. Molar responsive systems are used to determine the concentration of the toxin in a stock solution, which is then diluted accurately. In Table 2, three PSP toxin calibration solution CRMs were quantitated using NMR (Walter *et al*., manuscript submitted) and LC-CLND (Quilliam *et al*., manuscript in preparation). A routine check six months later revealed good agreement with previous measurements.

Table 2. Assignment of concentrations (μM) of some PSP toxin CRMs.

	CRM-STX-d	CRM-NEO-b	CRM-dcSTX
NMR	66 ± 3	63.8 ± 0.9	62.1 ± 0.8
LC-CLND	65 ± 1	65.7 ± 0.5	62.5 ± 0.5
Certified value	65 ± 3	65 ± 2	62 ± 2
6-month check (LC-CLND)	65 ± 1	65 ± 3	62 ± 3

Production of standards

When a solution has been prepared using the purified toxin, it is then placed into ampoules using an automated ampouling machine. Each ampoule is pre-purged with argon, filled with a small amount of solution, then immediately flame-sealed to prevent any evaporation. After the ampouling process is completed, each individual ampoule is inspected for proper volume and labelled with a number that indicates order of filling. A representative number of ampoules from those produced are selected to test for homogeneity of the analyte concentration throughout the whole set. Each product is then stored under specific conditions that are continuously monitored.

Careful planning is needed to ensure that each product is replaced when stocks are depleted. As well, concentrations must be checked on a regular basis to ensure the certified values hold true.

Production of tissue CRMs

For any analytical method, it is important to test the entire method for accuracy with a tissue CRM. To produce such a CRM, large amounts of naturally contaminated tissue homogenates are treated with antioxidant, de-aerated under vacuum, then dispensed into small bottles. These are sealed and taken through a steam retort process for sterilization. After cooling, the seals are inspected and cap closures installed. After receiving a unique number according to the order of filling, each bottle is individually heat-sealed in trilaminate pouches. Toxin concentrations are then measured using at least two independent and complimentary analytical methods.

Distribution

CRMP products are sold around the world. Therefore, shipments must be planned and monitored carefully to ensure the products are held under the appropriate conditions should the package be held up in customs or encounter other unusual circumstances. Once a product is sold and shipped to the customer, the certified values are typically guaranteed for one year, providing the product has not been opened and has been stored under the specified conditions.

CONCLUSIONS

The National Research Council's CRMP is addressing the lack of reference materials for algal toxins by expanding its product line of calibration solution CRMs and shellfish tissue CRMs. Particular care is taken to ensure accuracy of concentrations and to investigate the stability of the CRMs. CRMP is now the world's primary distributor of marine toxin CRMs.

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Occurrence and Abundance of *Ceratium* Species in the Maheshkhali Channel of the Bay of Bengal, Bangladesh

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Editor's note: Dr. Khan was unable to attend the workshop, but her paper is presented, below. She may be reached at: salehamk@bdcom.com.

ABSTRACT

Ceratium species composition and their occurrence and abundance in the Maheshkhali channel of the Bay of Bengal were studied. Plankton and water samples were collected and analyzed monthly from a fixed station in the Maheshkhali channel from June 2000 to May 2001. Seven species of *Ceratium*, namely *C. furca* (Ehrenberg) Claparede and Lachmann, *C. tripos* (O.F. Müller) Nitzsch, *C. fusus* (Ehrenberg) Dujardin, *C. trichoceros* (Ehrenberg) Kofoid, *C. inflatum* (Kofoid) Jørgensen, *C. azoricum* Cleve and *C. hircus* Schröder, were identified. The three most commonly encountered *Ceratium* species were *C. furca, C. tripos* and *C. fusus*. Among these three species, *Ceratium furca* was dominant during the monsoon and pre-monsoon seasons, whereas *C. fusus* was dominant during the post-monsoon season. *Ceratium tripos* exhibited its highest abundance during the pre-monsoon months. Some hydrographic parameters, e.g. surface water temperature, salinity, pH, NO_3-N and PO_4-P, were recorded and their relationship with the occurrence and abundance of *Ceratium* species were studied. The bloom of *Ceratium* spp. was first observed in August, and showed its highest abundance of 40×10^5 cells L^{-1} in September (monsoon month). Cell density increased with increasing water temperature. Numbers of zooplankton and diatoms during the bloom of *Ceratium* were significantly lower, indicating a possible relationship to *Ceratium* blooms. This is the first report of *Ceratium* spp. blooms from Bangladesh waters.

INTRODUCTION

Harmful, noxious, or simply exceptional algal blooms are increasing in frequency, intensity and geographical extent in coastal seas, and novel species are becoming important components of phytoplankton communities in regions where they were previously unknown (Anderson, 1989; Smayda, 1989).

The dinoflagellate *Ceratium* is the most ubiquitous thecate dinoflagellate genus and, although not generally abundant, is a persistent component of tropical open ocean plankton communities (Beers *et al.*, 1982). Phytogeographic studies have shown close agreement between temperature and the distribution of the individual species. Whereas some species are fairly tolerant of wide

ranges in temperature, others have restricted distributions and are limited to tropical or cold waters. Species of *Ceratium* have been referred to as excellent indicators of warm tropical waters (Graham, 1941).

"Red tides" by *Ceratium* species have been reported in tropical, sub-tropical and temperate embayments in Korea, Thailand, Sweden, Ireland and Mexico (Lee and Huh, 1983; Edler, 1984; Pybus, 1984; Guerra-Martinez and Lara-Villa, 1996). Although mass mortalities of fish in the sea caused by toxic blooms are more common, catastrophes are occasionally brought about by the decay of non-toxic phytoplankton populations in coastal waters. Sometimes fish mortality may occur due to anoxia of *Ceratium* blooms (Mahoney and Steimle, 1979).

Many rivers in Bangladesh discharge freshwater runoff to the Bay of Bengal. The volume of runoff water increases several fold during the monsoon months due to heavy rainfall, and thus provides nutrients sufficient to convert the coastal waters to eutrophic conditions. Maheshkhali channel is important as a large fishing ground and a center for recreation. On the other hand, Bakkhali River opens into this channel, which brings much of the domestic, agricultural and industrial wastes into it. The river inflow thus influences the Maheshkhali channel water, resulting in increased nutrients and growth of phytoplankton, which in turn affects other organisms. Therefore, continuous monitoring of plankton organisms as well as environmental factors of this channel is necessary. This paper deals with the species composition and abundance of *Ceratium* species and their relationship with environmental factors at the Maheshkhali channel of the Bay of Bengal.

MATERIALS AND METHODS

Monthly plankton samples were collected using a plankton net (25 μm mesh) at a fixed station near the mouth of the Maheshkhali channel of the Bay of Bengal, Cox's Bazar, Bangladesh. Sampling was done for 12 months, from June 2000 to May 2001. Samples were collected during the daytime at high tide. For qualitative plankton information, the plankton net was towed just under the water surface for one minute at a speed of approximately 1 m s^{-1}. The collected material was drained from the net into a polyethylene bottle and preserved with 5% buffered formalin in seawater. For quantitative data, a known volume (100 L) of sub-surface water was passed through the plankton net, and the concentrate collected from the bucket was preserved in 5% buffered formalin in seawater. The quantitative estimation of phytoplankton was done using a Sedgewick-Rafter counting chamber (S-R cell) under an Olympus phase-contrast microscope.

Surface water temperature and salinity were determined in the field using a Celsius thermometer and a hand refractometer, respectively. Nitrate-nitrogen (NO_3-N) and phosphate-phosphorus (PO_4-P) concentrations were measured in the laboratory using an HACH kit (DR/2010, a direct reading spectrophotometer) and "high-range" chemicals (NO_3-N by NitraVer 5 Nitrate Reagent Powder Pillows for 25 mL samples and PO_4-P by PhosVer 3 Phosphate Reagent Powder Pillows for 25 mL samples).

For species identification, a sample was gently shaken to resuspend all material. After settling for one minute, an aliquot was removed from the middle of the sample and four drops were

placed on a glass slide. A cover slip was placed on the slide and the entire slide was scanned for the species present. Observations were made using an Olympus phase-contrast microscope at 100 to 400 X with brightfield illumination. Taxonomic identification was based on Steidinger and Tangen (1996).

RESULTS

The coastal waters of the Bay of Bengal are influenced by three seasons: monsoon (June through September), post-monsoon (October through January) and pre-monsoon (February through May). At the mouth of the Maheshkhali channel, *Ceratium* spp. occurred throughout the year, with a seasonal high abundance (bloom) in monsoon months and a low abundance in post-monsoon months (Figs. 1 and 2). In the channel, seven species of *Ceratium*, i.e. *C. furca*, *C. tripos*, *C. fusus*, *C. trichoceros*, *C. inflatum*, *C. azoricum* and *C. hircus*, were recorded (Table 1). The cell density of *Ceratium* spp. was highest (40×10^5 cells L^{-1}) in September and lowest (2×10^5 cells L^{-1}) in December (Figs. 1 and 2).

Table 1. Seasonal distribution of *Ceratium* spp. at the mouth of the Maheshkhali channel of the Bay of Bengal from June 2000 to May 2001.

Ceratium species	Jun	Jul	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May
C. furca	x	x	x	x	x	x	x	x	x	x	x	x
C. tripos	x	x	x	x	x	x	x	x	x	x	x	x
C. hircus	-	-	x	x	-	-	x	x	-	x	-	-
C. fusus	x	x	x	x	x	x	x	x	x	x	x	x
C. azoricum	x	x	-	-	-	-	-	x	-	-	-	-
C. trichoceros	x	-	x	x	-	-	x	x	-	x	x	-
C. inflatum	-	x	x	-	-	x	x	x	-	-	-	-

'x' indicates the presence and '-' indicates the absence of a particular species.

During the study period, the surface water temperature fluctuated between 22°C and 33°C; it increased from August to September and thereafter gradually decreased to the lowest value during the month of December. The highest temperature recorded was in September, which coincided with a peak in *Ceratium* spp. cell density (Fig. 1).

Salinity showed an irregular pattern, fluctuating between 22‰ and 35‰. Salinity was at maximum (35‰) in November and at minimum (22‰) in October (Fig. 1). pH was in a narrow range of 7.4 to 8.5 units.

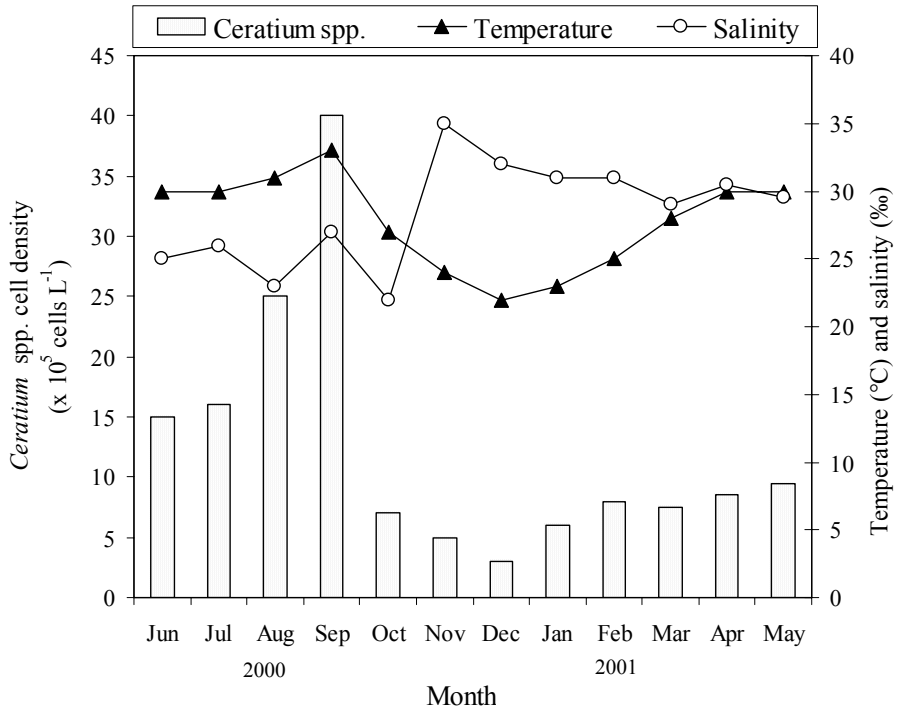

Fig. 1. Relationships among temperature, salinity and the seasonal abundance of *Ceratium* spp. at the mouth of the Maheshkhali channel, from June 2000 to May 2001.

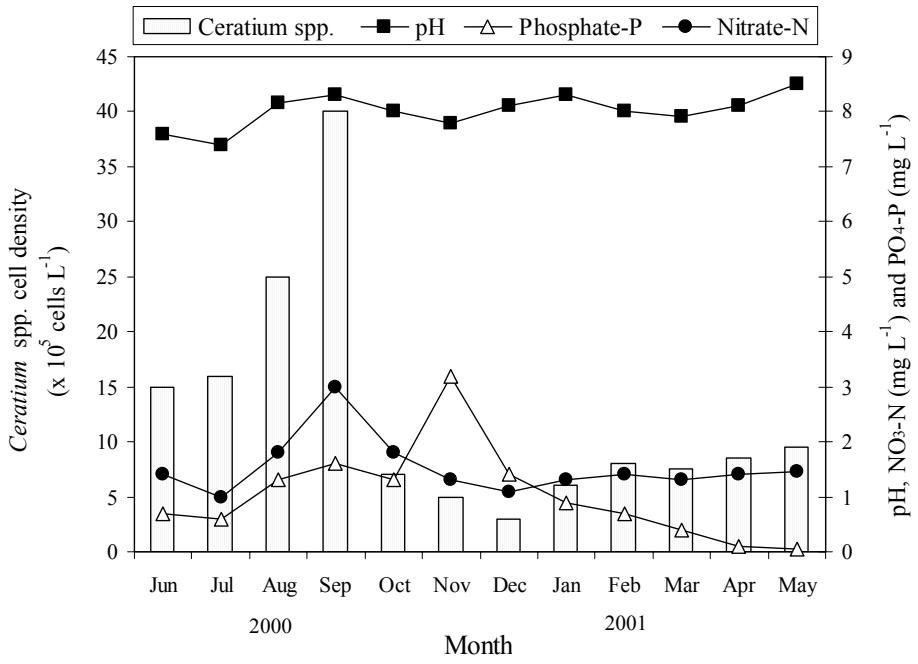

Fig. 2. Relationships among pH, NO_3-N, PO_4-P and the seasonal abundance of *Ceratium* spp. at the mouth of the Maheshkhali channel, from June 2000 to May 2001.

The concentration of PO_4-P fluctuated widely, ranging between 0.06 and 3.2 mg L^{-1}; the highest value was in November and the lowest in May (Fig. 2). During the study period, the NO_3-N concentration fluctuated between 0.8 and 3.0 mg L^{-1}, with the maximum in September when the cell density of *Ceratium* spp. was the highest (Fig. 2).

In September 2000, *Ceratium furca* formed a bloom in the Maheshkhali channel of the Bay of Bengal. During the bloom period, we found the highest temperature (33°C) and concentration of NO_3-N (3.0 mg L^{-1}) (Fig. 3). The maximum cell density of *C. furca* reached 36 × 10^5 cells L^{-1}, which contributed 90% of the *Ceratium* species and 70% of the total phytoplankton population.

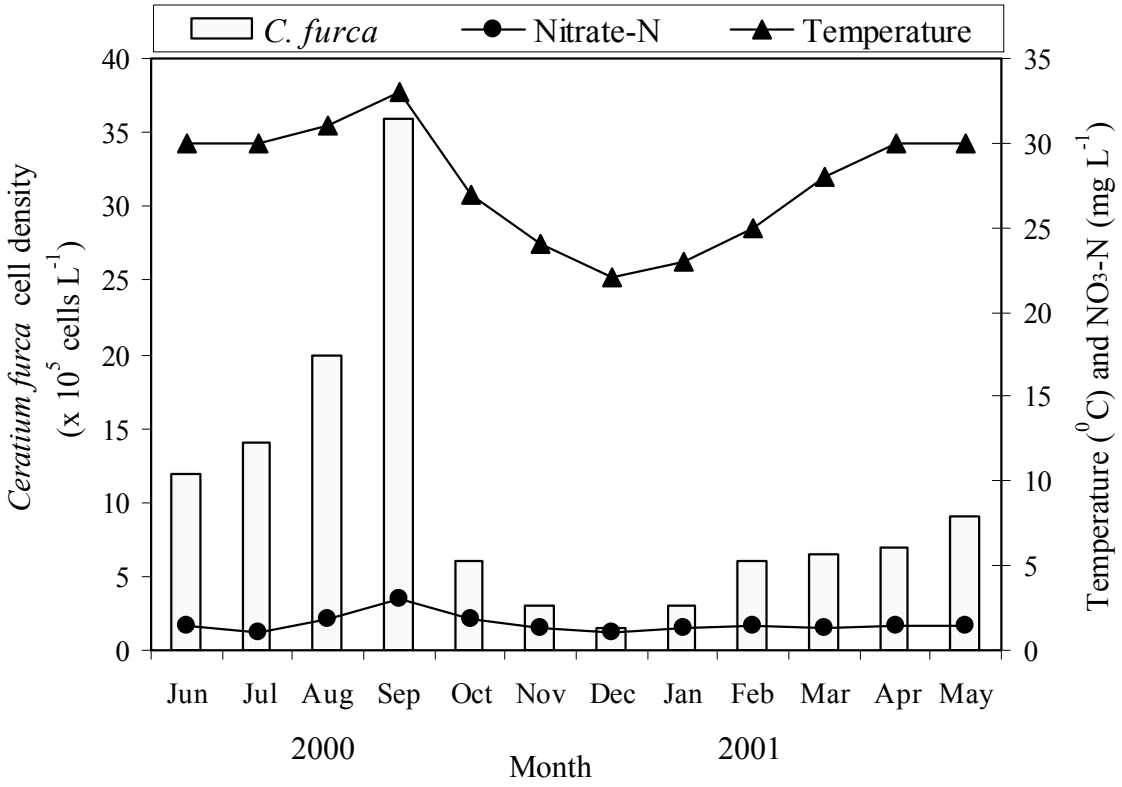

Fig. 3. Relationships among temperature, NO_3-N concentration and the seasonal abundance of *Ceratium furca* at the mouth of the Maheshkhali channel, from June 2000 to May 2001.

During the study period, diatoms and macrozooplankton showed an inverse relationship with *Ceratium* species (Fig. 4). At the time of the *Ceratium* bloom, the density of diatoms and macrozooplankton was comparatively lower (7.7 × 10^5 cells L^{-1} and 100 organisms L^{-1}, respectively).

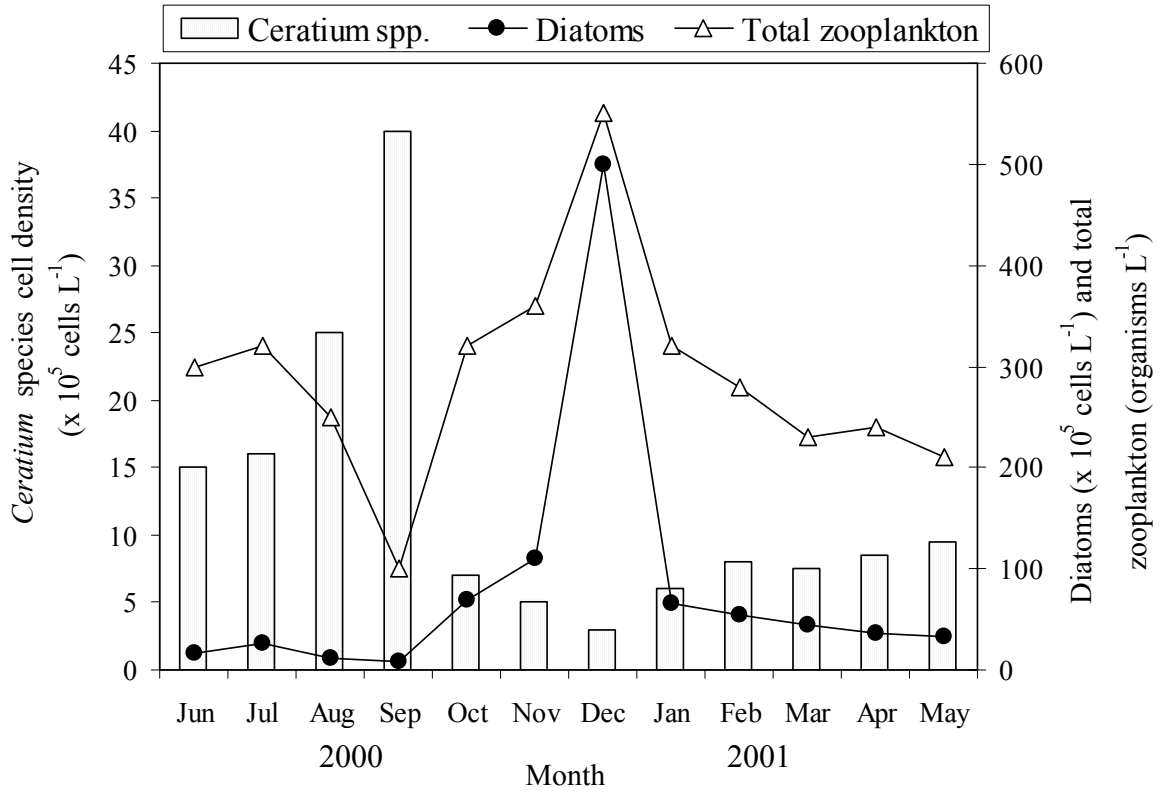

Fig. 4. Densities of *Ceratium* spp., diatoms and total zooplankton at the mouth of the Maheshkhali channel, from June 2000 to May 2001.

The three most common *Ceratium* species were *C. furca*, *C. tripos* and *C. fusus*, each of which varied seasonally. Among these three species, *Ceratium furca* was dominant during the monsoon and pre-monsoon seasons, whereas *C. fusus* was dominant during the post-monsoon season (Fig. 5). *Ceratium tripos* exhibited its highest abundance during the pre-monsoon months. The remaining four species, i.e. *C. trichoceros, C. azoricum, C. inflatum* and *C. hircus*, were less common at the mouth of the Maheshkhali channel (Table 1).

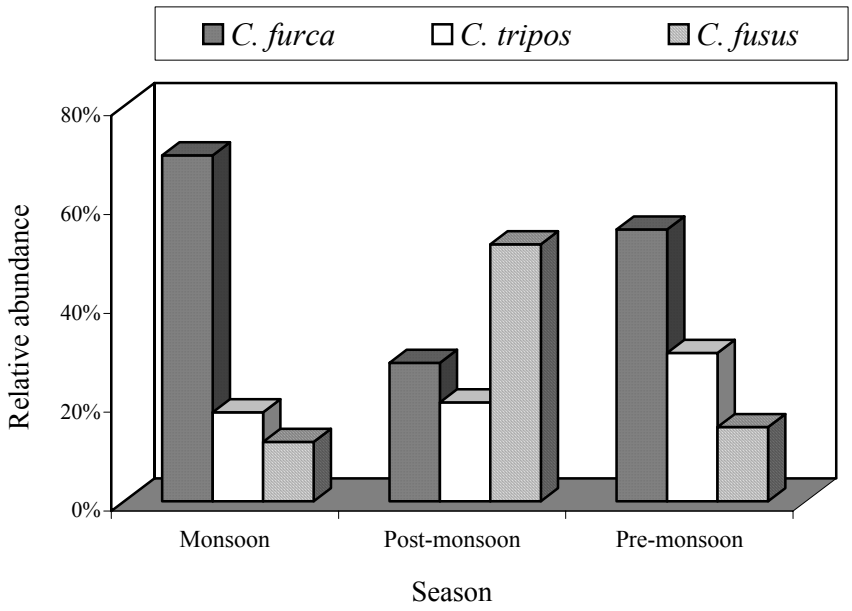

Fig. 5. Seasonal relative abundance values of the three most common *Ceratium* spp. at the mouth of the Maheshkhali channel, from June 2000 to May 2001.

DISCUSSION

Ceratium species were present throughout the sampling period and reached the highest density in September 2000. In the present study, temperature appeared to be an important factor affecting *Ceratium furca* blooms in the Maheshkhali channel. We recorded relatively high water temperatures during the bloom period. High temperatures in September might have created favourable conditions for the formation of a *C. furca* bloom in the Maheshkhali channel. A decline in the bloom coincided with a drop in water temperature, agreeing with the findings of Guerra-Martinez and Lara-Villa (1996), who reported that a bloom of *C. furca* was associated with high water temperature. Similarly, a *C. furca* bloom associated with high water temperature during December, 1980 was observed in Deukryang Bay, southern coast of Korea (Lee and Huh, 1983). In agreement with this, Gillbricht (1983) reported that a bloom of *Ceratium furca* in the southern North Sea developed from July to October 1981, when the temperature was high, and then declined, coincidental with a drop in water temperature.

The occurrence of a *Ceratium* bloom at the Maheshkhali channel of the Bay of Bengal assumes a greater significance, as it is the first report from Bangladesh. A mass mortality of marine animals associated with a bloom of *Ceratium tripos* in the New York Bight was observed by Mahoney and Steimle (1979). The low oxygen levels, and the probable subsequent production of hydrogen sulfide, caused extensive mortalities of marine animals, especially the surf clam *Spisula solidissima*. In Bangladesh, June to September is known as a monsoon season, during which time there is usually heavy rainfall, sometimes accompanied by floods. The *Ceratium* cell density was highest in summer months, which may be due to high temperatures and the availability of nutrients. During that monsoon season the increased nutrients, accumulated from

land runoff due to the monsoon rainfall, may have created a favourable condition for the maximum growth of *Ceratium* spp. Roden (1984) found a large autumn bloom of *C. tripos* in coastal waters of Ireland, reportedly due to heavy freshwater runoff. In the present study, the inorganic nutrients (NO_3-N and PO_4-P) might have greatly influenced the formation of the *C. furca* bloom at the Maheshkhali channel, Bay of Bengal. Concentrations of PO_4-P, and especially of NO_3-N, were high during the bloom period. A similar result was also found by Guerra-Martinez and Lara-Villa (1996), who reported that a bloom of *C. furca* was associated with an increase in the ratio of N:P. Likewise in Hong Kong, nitrogenous nutrients have been reported to be the major limiting factor in blooms of *Alexandrium catenella* (Whedon & Kofoid) Balech (Ho and Hodgkiss, 1993). Furthermore, it has been pointed out that the N:P:Si ratio in many coastal waters has changed during the last decade, favouring an increase in flagellate blooms (Schöllhorn and Granéli, 1993).

In this study, low densities of diatoms and macrozooplankton were observed in association with the *Ceratium* blooms. Santhanam and Srinivasan (1996) observed similar low densities of diatoms and zooplankton in association with blooms of *Dinophysis caudata* Saville-Kent in Tuticorin Bay, southern India. In the Maheshkhali channel, *Ceratium furca* was dominant among *Ceratium* species during the monsoon and pre-monsoon seasons, when the temperature was relatively high. High temperatures might therefore have created a favourable condition for the maximum cell abundances of *C. furca* during these seasons. *Ceratium fusus* was most abundant in post-monsoon months, when the temperature was relatively low and the salinity was high. These conditions may therefore have had a great effect on the cell abundances of *C. fusus* in post-monsoon months in the Maheshkhali channel.

The frequency of sampling during the study was limited, due to a lack of facilities. A greater sampling frequency (weekly, if possible) and more stations are needed to get better information about the occurrence and abundance of *Ceratium* species, as well as other noxious algae, and about their relationship with environmental factors. Additional comprehensive field studies will provide a greater understanding of the different noxious algal populations in coastal waters of Bangladesh.

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DISCUSSION GROUP SUMMARIES

REPORT OF DISCUSSION GROUP A

Monitoring Toxins in a Regulatory Setting

Moderator:
Michael Quilliam (Institute for Marine Biosciences, National Research Council, Halifax, N.S.)

Panel:
John Aasen (Norwegian School of Veterinary Science, Oslo, Norway)
Edward Black (Fisheries and Oceans Canada, Ottawa, Ont.)
Joanne Jellett (Jellett Rapid Testing Ltd., Chester, N.S.)
Rowena Linehan (Canadian Food Inspection Agency, Ottawa, Ont.)
Paulo Luciano (European Commission, Food and Veterinary Office, Grange, Ireland)
Russell Nicholson (Simon Fraser University, Burnaby, B.C.)
Nancy Peacock (Canadian Food Inspection Agency, Dartmouth, N.S.)

Rapporteur:
Philipp Hess (Marine Institute, Galway, Ireland)

INTRODUCTION

Harmful marine algae cause a multitude of problems due to their potential for producing marine biotoxins or harming marine organisms through different exposure mechanisms. The major public health problem is caused by the accumulation of toxins in shellfish that are harvested for human consumption. Further problems can be caused by the more complex exposure of farmed fish to harmful algae, leading to mass fish kills and large economic losses. The toxins produced by or in association with various algae are both of the hydrophilic and the lipophilic type. Test methods vary considerably for the different groups of toxins and depend on the approach taken, e.g. research, screening or confirmatory testing.

Canadian scientists have taken a leading role among international researchers on investigations into the mechanisms of toxicity, the ecology of harmful algae and methods of analysis for marine biotoxins. The diversity of toxic and harmful marine algae in Canadian waters and the multitude of the toxins in shellfish in Canada pose particularly challenging tasks to the government, agencies responsible for public health and surveillance, researchers and the aquaculture industry. The other factor specific to Canada is the extensive coastal region of the northern half of the North American continent, and thus the area that has to be covered for monitoring.

This discussion session addressed the following themes:

- Increasing diversity of toxins
- Cost of monitoring and legislative constraints
- Sampling and testing
- The European perspective
- Biological versus instrument-based/biochemical techniques for monitoring
- Standards and reference materials

These themes were all addressed within the context of regulatory monitoring, i.e. the surveillance program that is used to ensure public safety.

1. Increasing diversity of toxins (talk by M. Quilliam):

The diversity of toxins that have been identified in phytoplankton and shellfish from Canada is amazing. The hydrophilic toxins include domoic acid (DA) and its isomers (at least eight isomers have been confirmed), as well as the paralytic shellfish toxins (PSTs), of which 18 have been confirmed; more are likely to exist. The lipophilic toxins that have been confirmed in Canada include okadaic acid (OA), dinophysistoxin-1 (DTX1), pectenotoxins (PTXs) and spirolides. However, worldwide, there are other lipophilic toxins that have been found in shellfish. These are also of relevance to the Canadian monitoring system, either because some may already be contaminating the shellfish/fish or because the new species of algae that produce them may be introduced to Canadian waters. These toxin groups include azaspiracids (AZAs), yessotoxins (YTXs), ciguatoxins (CTXs) and brevetoxins (PbTXs). This diversity is further complicated by the presence in shellfish of metabolites of parent toxins, such as DTX-3, which are also relevant in terms of toxicity.

By analogy to the chemical diversity, there is also a wide variety of methods available to detect various groups or combinations of groups of toxins. Assays have been developed using live animals or cell cultures, and biochemical or immunological techniques. Live animal assays include the mouse and rat assay, whereas cell culture assays are typically based on cytotoxicity. The biochemical assays include receptor binding assays and enzyme inhibition assays (e.g. of PP2a), while the immunological assays comprise enzyme-linked immunosorbent assays (ELISAs), radioimmunoassays (RIAs) and lateral flow immunochromatography (LFICs). The chemical analytical techniques include liquid (LC), gas (GC) and thin layer chromatography (TLC), mass spectrometry (MS) and capillary electrophoresis (CE), as well as hyphenated techniques such as liquid chromatography coupled to mass spectrometry (LC-MS, GC-MS, CE-MS).

The diversity of compounds and techniques available for detection and quantitation leads to a number of possible strategies to conduct a National Monitoring Program. Regulatory agencies in most countries currently use prescriptive methods based on biological assays, in some cases accompanied by instrumental chemical methods for confirmation. One possible approach includes the use of rapid assays such as LFICs or PP2a for screening all samples, combined with chemical analyses for confirmation of positives. Another approach that has been suggested is the use of LC-MS for screening plus confirmatory work. It is clear that there is a need for an internationally accepted performance-based approach in which countries can choose their preferred techniques and validate their implementation with interlaboratory trials and/or certified reference materials.

2. Cost of monitoring and legislative constraints (talk by P. Hess)

The driving factors in any strategy to monitor shellfish toxins in a regulatory context are food safety, available methods, legislation and the resources needed for monitoring. The food safety aspect is obviously the overriding factor determining the methodology to be applied for testing marine biotoxins in most countries. However, the cost of a testing regime will also have an influence on the choice of method, distribution of sampling sites and frequency of sampling.

The example of the Irish National Monitoring Programme shows how annual numbers of samples can increase from just over 100 per annum in the late 1980s (2 staff) to over 3,000 per annum since 2000 (ca. 18 staff). The current cost of the National Monitoring Programme is in excess of € 1,500,000 per annum, while the total value of the industry (after export) was ca. € 50,000,000. This means that the monitoring program represents ca. 3% of the total value of the product, of which industry itself contributes one tenth of the program cost through its role in providing the samples. This is a high contribution by the State, and may not be sustainable in the long term. It will therefore require regular review in terms of the balance between State and industry. The tonnage produced by the shellfish industry in Ireland (ca. 35,000 t in 2001) is very comparable to the shellfish production in Atlantic Canada.

The legislation governing the surveillance of shellfish in Europe is currently being reviewed. Existing legislation is convoluted and has proven to lead to different systems in most countries, instead of a harmonized system across Europe. Although test methods are prescribed, the definitions are very loose. Thus, the only description for the technique to be applied for domoic acid is that this compound should be analyzed by HPLC. Similarly, the description of bioassays for DSP and PSP only states that "the customary bioassay" has to be used. A recent Commission Decision (225/2002-EC) has improved on the definition of test methods for DSP toxins in as much that tests other than the customary bioassay are permitted for official surveillance. For the first time, this decision has also set quantitative limits for a range of lipophilic toxins, including DSP toxins, YTXs, AZAs and PTXs. Most scientists consider this legislation a big step forwards toward a performance-based strategy to monitor toxins produced by harmful algal blooms. The only remaining difficulty in scientific terms is that the customary bioassay remains the reference method for any toxin in shellfish. With the exception of PSP toxins, this effectively means that a non-quantitative method, which has neither been internationally standardized nor validated, remains the standard against which the performance of other methods is compared, even if the other methods are fully quantitative and in the process of being validated both in-house and internationally.

Further review and research in Europe is likely to lead to a purely performance-based system. However, these changes are also likely to stretch over a number of years. Such resource and legislative restrictions are important to any country importing shellfish into the EU, and the question for the regulator is not only to find the most effective system to monitor marine biotoxins but also to find a system acceptable to the EU.

3. Sampling and testing

One of the central questions in many monitoring systems remains the area coverage and frequency of testing. If sampling stations are covering too large an area, they are not necessarily representative of this area and may lead to what can be considered "blanket closures". Equally, if the sampling frequency is insufficient, this may lead to extended closure periods, which are very damaging to the shellfish industry. Both factors are particularly important in Canada due to the large areas reaching over several provinces in Atlantic Canada alone. Other countries with long coastlines, such as New Zealand and Norway, have already switched to a testing regime largely based on LC-MS testing. However, both of these countries also have significant contributions by the industry for the monitoring system, which is not the case in Canada.

Scientists see a big advantage in using some of the alternative techniques, such as functional assays, to help in the screening of large numbers of samples. These tests are commercially available for ASP, PSP and to some extent also for DSP toxins. In addition, there is a version of tests, also referred to as kits, which may be used by lay people and thus increase the general public awareness of the problem and prevent incidences of shellfish poisoning in remote areas. However, the stated view of the regulatory agency responsible for national monitoring in Canada was that the use of these systems would be difficult to control and cannot be recommended by the agency for this reason. Discussions between the regulatory authorities, Health Canada and the scientific community are expected to continue in this area.

4. The European perspective

A representative from the Food and Veterinary Office (FVO) of the EU gave his personal insight into the European perspective from the angle of EU visits to countries exporting shellfish to the EU. In general, each country exporting shellfish to the EU is expected by the EU to produce shellfish to standards at least equivalent to EU standards. Specifically, this also means, in general, that all product destined for Europe must also have been demonstrated to be free of marine biotoxins occurring in the EU, e.g. ASP, PSP, DSP, AZP, YTXs and PTXs, and in particular according to Article 9 of Directive 91/492/EEC and according to Commission Decision 2002/225/EC. This is particularly important if the exporting country cannot produce a scientific demonstration that the toxic phytoplankton and these toxins have not (yet) been shown to occur in its waters/shellfish. This obviously means that the country hoping to export to the EU must be able to monitor those compounds in their shellfish. Due to the current lack of standards and reference materials, this also means that most countries will be obliged to continue using the mouse bioassay as a testing method for lipophilic toxins. However, the possibility of using alternative detection methods (e.g. HPLC, LC-MS, immunoassays and functional assays) was already given by the mentioned Decision 2002/225/EC of 15.03.2002 (Official Journal L75, of 16.03.2002), which some countries have implemented.

The expert from the EU-FVO also outlined that in his personal view there was a great lack of international coordination among competent authorities, research institutes and public health agencies that would be important to achieve, in order to advance the harmonization of sampling and analysis methods. In addition, there seems to be a dramatic lack of information on epidemiological data in this area and there is not even a harmonized procedure for the collation of information on diseases.

5. Mouse bioassay

The mouse bioassay was discussed as a technique for regulatory monitoring, and while no agreement was reached, the advantages and inconveniences can be outlined as follows.

In view of the multitude of toxin classes and the lack of standards, particularly among the lipophilic classes of toxins, the mouse bioassay seems ideal as a general toxicity screen since it assesses overall toxicity of a shellfish sample.

On the other hand, the mouse bioassay has been reported to give a number of both false positives (implications for industry) and false negatives (implications for public health). Furthermore, the use of animal testing has been banned by several countries for ethical reasons. Scientifically, the

test is also questionable because the possible interactions of different toxin groups have not been researched, neither in mice nor in humans. Finally, there is the possibility of systematic false positives for the mouse bioassay in the presence of low levels of YTXs, i.e. the test may show a positive at levels which have no health implication. An important question on the international scene is that the test has not been validated (nor standardized) and this may be difficult due to the number of toxin groups it can detect. In addition, the question of reference materials for mouse bioassays was brought up again and rejected due to a lack of funds in this area.

6. New techniques

The array of available alternative tests was discussed in Section 1, above. In principle, these techniques are ideal tools to increase the number of samples that can be analyzed in any given laboratory or even in the field. Thus, they have a good potential for helping to cover the long Canadian coastline and to increase the frequency of testing. These techniques can potentially be used by regulatory agencies, industry and the general public. Therefore, rapid assays and kits may in the long term play a major role in aiding the industry and in ensuring public health. Any technique used in a regulatory context must be validated prior to application for monitoring. Scientists therefore see the use of these techniques being phased in, in parallel with the mouse bioassay and confirmatory techniques such as LC-MS.

7. Standards and reference materials are essential to progress

The easiest way to achieve international harmonization in the area of marine biotoxin surveillance is the establishment of performance-based strategies for testing techniques. Ideally, these strategies are based on the toxicity of the toxin and the resulting permissible levels of these toxins in shellfish, rather than on a comparison to a particular test, e.g. the mouse bioassay.

As described above, there is a lack of toxicity data for a number of these toxins. This can only be addressed through further toxicological studies, which require purified toxin standards. The validation of methods – both in-house and internationally – equally requires the availability of standards and reference materials for all toxin groups in question. Therefore, Canadian scientists have appealed to regulatory agencies, industry and scientists worldwide to continue the cooperation in the production of standards and reference materials, which has led to great advancement in the area so far.

Discussion Group B:

Ballast Water and HABs

Editor's note: The following talk was given prior to the discussion on Ballast Water and HABs.

Implications of Ballast Water Discharge for the Introduction/Dispersion of Harmful Algal Species in Atlantic Canada

Claire E. Carver and André L. Mallet

Mallet Research Services, 4 Columbo Dr., Dartmouth, Nova Scotia, B2X 3H3 Canada

In a ballast water survey conducted from September 2001 to February 2002, samples were obtained from 98 ships arriving at 15 ports in the four Atlantic provinces. The ships (29 tankers, 21 bulk carriers, 17 container carriers and 31 general cargo carriers) were selected on the basis of their port of origin to comprise a representative cross-section of the foreign vessel traffic in Atlantic Canada. Examination of 235 ballast water samples revealed 423 phytoplankton taxa of which 20 (5%) were classified as harmful and 32 (8%) as potentially harmful. Among these 52 taxa were 13 species considered non-indigenous to Atlantic Canadian waters, including *Dinophysis fortii* and *Pseudo-nitzschia subpacifica*. Samples of ballast water originating from the Northeast Atlantic, Mediterranean and Pacific regions (container and general cargo carriers) had higher numbers of harmful taxa (2.9 taxa per sample) than those originating from the western Atlantic, including waters off the US east coast (2.1 taxa per sample) (primarily tankers and bulk carriers). Cell numbers, however, were typically lower in the samples from more distant regions, probably due in part to the greater mean age of the samples (20 d vs. 5 d). Given that container and general cargo carriers discharge small volumes of water (150 to 2400 m^3) relative to tankers and bulk carriers (750 to 46,407 m^3), the overall risk of introducing harmful taxa from these regions may also be lower.

Approximately 70% of the samples originated from ballast water which had been exchanged in coastal or oceanic waters en route to Atlantic Canada. This practice, although important for reducing the risk of transferring invasive coastal species, did not reduce the incidence of harmful phytoplankton taxa; estimates for exchanged water were slightly higher (2.3 taxa per sample) than for non-exchanged water (2.0 taxa per sample). A comparison of the harmful phytoplankton taxa in samples originating from three potential exchange zones (Southeast USA, Northeast USA and Atlantic Canada) suggested that exchanging ballast water in Canadian waters could, under certain conditions, promote the introduction/dispersion of undesirable taxa such as *Pseudo-nitzschia fraudulenta* into inshore regions.

REPORT OF DISCUSSION GROUP B

Ballast Water and HABs

Moderator:
Claire Carver (Mallet Research Associates, Dartmouth, N.S.)

Rapporteur:
Jennifer L. Martin (Fisheries and Oceans Canada, St. Andrews, N.B.)

The session was introduced by Claire Carver, who gave a 40 min presentation summarizing a ballast water study funded by Transport Canada (see her abstract on the previous page). Water was sampled for salinity and phytoplankton content from ballast tanks of 98 ships arriving at various ports in Atlantic Canada.

The discussion that followed included the importance of sampling ballast tank sediments, because cysts have been proven to reside within the sediments and to remain viable for extended periods of time. Sediments had not been sampled in the Transport Canada study, and difficulties of their sampling were acknowledged. When in dry dock, sediments may be flushed out of the ballast tanks; containment of dry dock effluents should therefore be mandated. The question was raised as to whether it would be best to leave the sediments undisturbed. Preliminary data from a sediment study undertaken by the European Union indicate that sediments are more of a threat than originally thought.

It was stressed that an historical database was required to determine whether a species was non-indigenous to an area. Knowledge of the system from which the ballast water originated was also important for determining the possible threat for introduction.

It was suggested that the following water parameters should be measured when sampling: salinity, chemistry, bacterial numbers, algal species composition, and proportion of live vs. dead algal cells. With progress in genetics and DNA sequencing, the group felt that determining strain variation was important, because a particular species might morphologically resemble a local strain, yet might differ from the molecular viewpoint. This could impact the toxicity potential, because not all strains have the same ability to produce toxin.

The point was raised that although ships have travelled for many years throughout the world, there are now increased concerns about the transport of ballast water. One suggested reason for concern is that traffic and trading patterns have changed in the past 1-2 decades. For example, today on Canada's east coast much of the traffic is north-south and from Asia and Africa. Another reason is increased ship traffic; since World War II, 85% of all goods are transported by ship. In addition, transit times are now shorter and larger volumes of ballast are being transported, so that there is an increased chance that more exotic phytoplankton species will be transported and more will survive the transit.

It was mentioned that *Pseudo-nitzschia fraudulenta* was observed in low proportions at four sites on the Northumberland Strait shore of Nova Scotia, in December 2001. This was the first time that *P. fraudulenta* was documented anywhere in the Gulf of St. Lawrence. In the month prior to this event, samples of ballast water originating from the northeast coast of the U.S.A., as well as off the coast of N.S., frequently contained *P. fraudulenta*. Given that ships carrying water from these regions are known to discharge this ballast in the Northumberland Strait region, it was postulated that the appearance of this potentially toxic algal species may have been linked to ballast water discharge.

Reference was made to a Ballast Water Background Paper (May 2003) prepared by the IOC of UNESCO International Panel on Harmful Algal Blooms (IPHAB) and edited by Gustaaf Hallegraeff, for input to the ICES-IOC-IMO Study Group on Ballast Water and Other Ship Vectors (http://www.ices.dk/reports/ACME/2003/SGBOSV03.pdf, pages 67-74). The report summarizes the known role of ballast water in the spread of HABs, focusing mainly on diatoms and dinoflagellates that can impact human health, fisheries and aquaculture. The Study Group recommended that ballast water exchange be continued until more cost-effective methods become available. Alternative methods being explored for treatment of ballast water include: filtration, heat treatment, ultraviolet irradiation, and chemical and biocidal inactivation. Although the report states that not enough information is available to estimate what constitutes a minimum algal cell inoculum, there was agreement that global ballast water standards proposing to allow 200 viable cells mL^{-1} will not be effective in reducing the risks for invasion.

A high percentage of the traffic to Atlantic Canada consists of vessels travelling in a north-south bound corridor. For ships traveling to the Great Lakes that are unable to exchange ballast water in the open ocean, exchange is presently only permitted in a specific region in the southern Gulf of St. Lawrence. Steps are being taken by Canadian and United States researchers and regulators to determine whether there are areas within the Economic Exclusion Zone (EEZ) where ballast water exchange might be environmentally and economically more feasible.

Information was presented about a Ballast Water Workshop to be held 27-28 October 2003, in Halifax, N.S. (http://massbay.mit.edu/ballastwkshp03/). This workshop will explore the feasibility of identifying areas along the northwestern Atlantic that may be suitable for ballast water exchange for vessels traveling along the coast. Speakers will address what is known about the physical and biological oceanography of that part of the ocean, summarize what the shipping community is doing to prevent introductions, examine the adequacy of data for risk assessment, and discuss how these issues are addressed elsewhere. Finally, they will focus on policy suggestions and recommendations for managing ballast water exchange in the northwestern Atlantic.

REPORT OF DISCUSSION GROUP C

The Aquaculture Industry Perspective

Moderator:
Maurice Mallet (Fisheries and Oceans Canada, Moncton, N.B.)

Panel:
Crystal McDonald (Prince Edward Island Aquaculture Alliance, Charlottetown, P.E.I.)
Florence Albert (Professional Shellfish Growers Association of New Brunswick / Association des Conchyliculteurs Professionnels du N.-B., Caraquet, N.B.)
Sirje Weldon (Aquaculture Association of Nova Scotia, Halifax, N.S.)

Editor's note: The Prince Edward Island Aquaculture Alliance, the Professional Shellfish Growers Association of New Brunswick, and the Aquaculture Association of Nova Scotia each gave individual presentations at the workshop. The three associations collaborated on writing the following summary of the information presented, and the issues raised, during the discussion.

Background of the Maritime Aquaculture Industry

The Maritime aquaculture industry is quite diverse in its approach to, and success with, aquaculture. Prince Edward Island's mussel industry has shown that with the right encouragement and support, aquaculture can be a very large part of the region's economic future. It captures the link that our rural, coastal communities still have with the sea and capitalizes on the entrepreneurial spirit and strong work ethic that is an integral part of the Maritime rural population.

If we take a quick glance at the aquaculture industry in each Maritime province, we will see that they each have their collective and individual strengths and challenges. A shared challenge is that posed by the environment. Invasive species and harmful algal blooms have the potential to present significant challenges for both the finfish and shellfish sectors. P.E.I.'s experience with the domoic acid crisis of 1987 still stands clear in the minds of the industry as a reminder of the seriousness of the issue being dealt with. Since 1987, Fisheries and Oceans Canada (DFO) and the Canadian Food Inspection Agency (CFIA) have worked diligently with the industry to ensure the safety of Canadian shellfish.

Today, each of the Maritime provinces has a significant investment in aquaculture production that has been of benefit to the local economies as well as to the wealth of the region as a whole. The following is a quick glimpse at the statistics for the region:

Prince Edward Island

- 1,114 lease sites
- 18,308 acres being cultured (predominantly mussel and oyster culture)
- $40 Million annual landed value
- 80% of North American mussel production

- 1,500 – 2,000 workers employed
- have had experience with ASP and PSP toxin closures

New Brunswick

- 550 existing leases (additional applications in the system)
- 700 acres being cultured
- $2.4 Million annual impact to the province in areas where much effort is being put into coastal development
- 2002 is the first experience with an ASP toxin closure

Nova Scotia

- 392 leases (finfish and shellfish)
- $30 Million value (88% finfish)
- 850 employed (skilled labour to high tech jobs)
- diverse industry (6 species of finfish, 5 species of shellfish)
- have had experience with ASP, DSP, PSP, and spirolide toxins, and some closures
- have also experienced fish kills due to toxic and harmful algal species

Impacts of closures to the Aquaculture Industry

In 1987, the outbreak of a new poisoning caused by eating blue mussels from Cardigan Bay, P.E.I., led to the discovery of amnesic shellfish poisoning (ASP). *Pseudo-nitzschia multiseries* was identified as the producer of the phycotoxin domoic acid. Mussels that had fed upon these toxic algae and had then been consumed by humans caused serious illness and three deaths.

The 1987 domoic acid "mussel crisis" on P.E.I. caused non-quantifiable costs in human life and suffering, and resulted in an immediate cessation of mussel harvesting for several months. Although these closures were completely supported by the industry, they caused significant loss of revenue for P.E.I. and the ensuing negative publicity impacted consumer confidence.

The 1987 experience with ASP on P.E.I. has made the entire Maritime shellfish industry aware that harmful marine algae are not to be taken lightly. Recognizing the potential health impacts of biotoxin-producing algae, the Maritime aquaculture industry is supportive of closures when harmful marine algae conditions require that products not be harvested.

Fortunately, the Maritime aquaculture industry was able to rebound after both the 1987 domoic acid crisis and the 2002 spring closure of much of the southern Gulf of St. Lawrence. However, these closures are not without short- and long-term costs to the industry, including:

- Research and monitoring costs
- Closure of harvest sites
- Disruption of supply to markets
- Layoff of employees in processing plants and grow-out operations
- Loss of revenue and the resulting financial strain on the growers, processors and local communities/economy
- Consumers' negative perception

Given these impacts to the industry, regulators need to ensure that closures are based on predetermined quantifiable data and not on a knee-jerk reaction to elevated levels of biotoxin within a specific shellfish species. The closure of an area to the harvest of all shellfish species, when only one species is showing elevated levels of biotoxins, compounds the impact that the closure has on these rural communities. Species-specific closures, while more costly to monitor and enforce, are a practical option for ensuring public health and industry sustainability.

What is required to manage the situation

Because the industry recognized that the majority of the participants at the workshop were researchers, comments were generally restricted to underlining the critical need for research support. The three industry associations were in agreement on most aspects of what was required to manage the situation, including policy changes that would need to be discussed with another audience on another day. The industry felt that the key requirements to manage appropriately harmful algae situations are:

Monitoring of phytoplankton

- Year-round monitoring to detect unexpected toxic algal blooms (broad spectrum)
- Monitoring of harmful phytoplankton that could seriously/negatively impact either finfish or shellfish culture
- Detection of exotic and endemic phytoplankton species
- Better understanding of variations in the toxicity of *Pseudo-nitzschia* species

Closures

- Manage by species of bivalve
- Site-specific closures and sampling
- "Precautionary closures" that ensure a margin of safety should be based on predetermined, quantifiable data, if possible, e.g. a combination of historical data (if any), hydrographic information, meteorological conditions, toxin levels in adjacent areas, and rate of increase in toxin levels

Uptake and depuration research

- Clearance and ingestion rates of phytoplankton cells by different bivalve species
- Uptake and depuration rates of various biotoxins by different bivalve species under various conditions (e.g. retention of domoic acid at different temperatures)
- Depuration plants as a solution to biotoxin closures
- Uptake/depuration rate variances with bottom, off-bottom and within processing plant holding systems, to determine the time required to clear the biotoxin

Communication

- Increased communication and coordination among regulators, researchers and industry
- A follow-up session with industry to review past experiences in dealing with harmful algae and how to improve the process
- Continuation of DFO's efforts to understand the production and distribution of domoic acid

Question and answer discussion highlights

The discussion was deferred until after all three industry presentations had been given. The following are the high points of that session:

- The New Brunswick industry indicated that while there was great collaboration during the 2002 spring closure due to domoic acid, the radical approach of closing such a wide area and preventing harvesting of all bivalve species was not acceptable due to the financial constraints it placed on the industry.
- Ireland has a lot of problems with PSP and other toxins and they have year-round monitoring in place because some of the toxins are seen during the winter months.
- Ireland has seen "oysters much, much less affected", so they are reducing the frequency of sampling for the oysters.
- If industry wants year-round and species-specific monitoring, then there might be a need to look at cost recovery methods used in other jurisdictions, because government cannot afford to pay for it (e.g. in New Zealand, industry pays for the biotoxin and phytoplankton monitoring programs, which are accredited by the government).
- In Newfoundland last June (2002), there was a continued closure of scallop harvest but government did not close mussel harvesting because mussels were found to be free of biotoxin when tested.
- DFO and CFIA have been discussing species-specific closures, but there needs to be some research done (i.e. there is very little information known about what oysters do regarding feeding/clearance of certain biotoxins at various temperatures).
- DFO and CFIA are working on putting a plan in place to study domoic acid uptake and depuration by mussels and oysters during the next toxic *Pseudo-nitzschia* bloom.
- The absence of senior CFIA staff (because of a conflicting meeting) during an important part of the discussion session was noted. At least one senior official with responsibility could have remained to hear the presentations of the industry perspective.
- Industry was questioned about its willingness to do monitoring tests and to pay for testing programs similar to what is done in New Zealand. It was explained that some initial discussions had taken place as part of a Canadian Aquaculture Industry Alliance shellfish project that had been completed the previous year. Industry also indicated that there would need to be a direct cost benefit (i.e. species-specific closures), but that the industry at all levels (seed, grow-out and processing) was already inundated with additional costs of production resulting from the problem of invasive species introduced from ballast water.
- Industry was urged to quantify the losses due to the spring 2002 domoic acid closure (e.g. product dumped because no longer salvageable, loss of clients or credibility, 6 to 8 week loss of income for small operators, etc.). It is important to make it clear what the losses are for the industry and region so that regulators will support a $3 to $4 Million monitoring program and additional studies.
- DFO, CFIA and provinces are to meet with industry representatives in the near future to discuss policy issues.

Photo Album from the 8th Canadian Workshop on Harmful Marine Algae

Photos by Catherine Vardy, Science Liaison Officer (Fisheries and Oceans Canada, Moncton, N.B.)


NORTH COAST WATER QUALITY & BIOTOXIN PROGRAM

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