TOXINS AND TOXICITY SESSIONS

Azaspiracid Poisoning: Aetiology, Toxin Dynamics and Bioconversion in Shellfish

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Abstract

The new human toxic syndrome azaspiracid poisoning (AZP) was declared following illness from the consumption of contaminated mussels (*Mytilus edulis*). To discover the aetiology of AZP, sensitive analytical protocols involving liquid chromatography–mass spectrometry (LC-MS) were used to screen marine phytoplankton for azaspiracids. The dinoflagellates in phytoplankton samples that tested positive for azaspiracids were manually separated to produce monocultures. *Protoperidinium crassipes,* was identified as the progenitor of azaspiracids in three samples collected 1999–2001. Three toxins, azaspiracid (AZA1-AZA3), were identified in extracts of 200 cells by LC-MS^3 using an ion-trap mass spectrometer. This discovery has significant implications as the *Protoperidinium* genus is ubiquitous and was previously considered to be toxicologically benign. A three-year study of the variation in the AZP toxin profiles in the various tissue compartments of mussels (*M. edulis*) and scallops (*Pecten maximus*) revealed significant differences between these species. Five new azaspiracids were identified in mussels but since they were not detected in phytoplankton, they are probably bioconversion products.

Introduction

It has been demonstrated that several polyether toxin groups are produced by dinoflagellates and these include okadaic acid and analogs (Yasumoto *et al.,* 1980; Yasumoto 2000), pectenotoxins and yessotoxins (Satake *et al.,* 1997). The new human toxic syndrome azaspiracid poisoning (AZP) was caused by the consumption of contaminated mussels (*Mytilus edulis*) from Ireland (Ofuji *et al.,* 1999). The isolated toxins, named azaspiracids, represent a new class of polyether compounds containing structurally unique features (Satake *et al.,* 1998).

In addition to the predominant toxins, AZA1-AZA3, two minor azaspiracids, AZA4 and AZA5, were identified as the hydroxylated analogs of AZA3 (Ofuji *et al.,* 2001). The potential widespread distribution of azaspiracids in Northern Europe has been confirmed with the identification of these toxins in shellfish cultivated in England, Norway, France and Spain (James *et al.,* 2002; Braña Magdalena *et al.,* 2003). Toxicological studies of AZA1 in mice showed acute morphopathological changes (Ito *et al.,* 2000) and serious chronic effects were observed including interstitial pneumonia and lung tumors (Ito *et al.,* 2002). An important research objective is the discovery of the aetiology of AZP in order to implement phytoplankton surveillance programs.

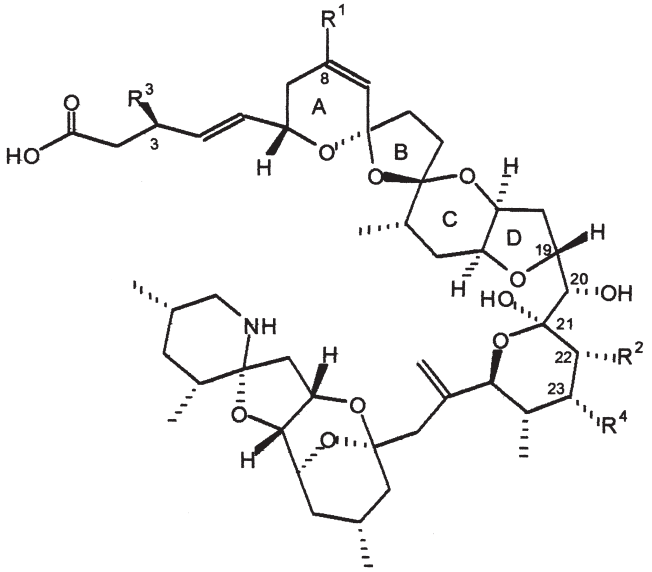

Figure 1 Structures of azaspiracids. AZA1 (R^1 = H, R^2 = CH_3); AZA2 (R^1 = R^2 = CH_3); AZA3 (R^1 = R^2 = H).

Materials and Methods

Phytoplankton sampling was carried out 1–10 km offshore of Baltimore and Glandore, County Cork, Ireland, during September, 1999–2001. The plankton net had mesh sizes of 50 µm (outer net) and 108 µm (inner net). The outer net, length 590 cm, had a diameter of 140 cm and the inner net, length 460 cm, had a diameter of 80 cm (James *et al.,* 1999). Chilled algae/seawater sample (50 mL) was homogenized with acetone. After evaporation, the supernatant was extracted with ethyl acetate (2 × 50 mL), evaporated and the residue was reconstituted with chloroform (1 mL). A diol solid phase extraction cartridge (Supelclean LC-Diol, Supelco, Dorset, U.K.) was conditioned with methanol (5 mL) followed by chloroform (5 mL). The extract was transferred to the cartridge, washed with chloroform (5 mL), and the toxins were eluted with chloroform/methanol (50:50 v/v, 6 mL). The solution was evaporated to dryness using nitrogen and reconstituted in methanol (1 mL). An aliquot (5 µL) was analyzed using recently developed LC-MS^3 methods for the determination of azaspiracids in shellfish, using ion-trap multiple tandem MS (Lehane *et al.,* 2002). Azaspiracids were determined using LC-MS^3 with the target parent and fragment ion combinations as follows: $[M+H]^+ \rightarrow [M+H-H_2O]^+ \rightarrow [M+H-2H_2O]^+$; AZA1 (*m/z* = 842.5 → 824.5 → 806.5); AZA2 (*m/z* = 856.5 → 838.5 → 820.5); AZA3 (*m/z* = 828.5 → 810.5 → 792.5).

Results and Discussion

The discovery of the aetiology of shellfish toxic syndromes

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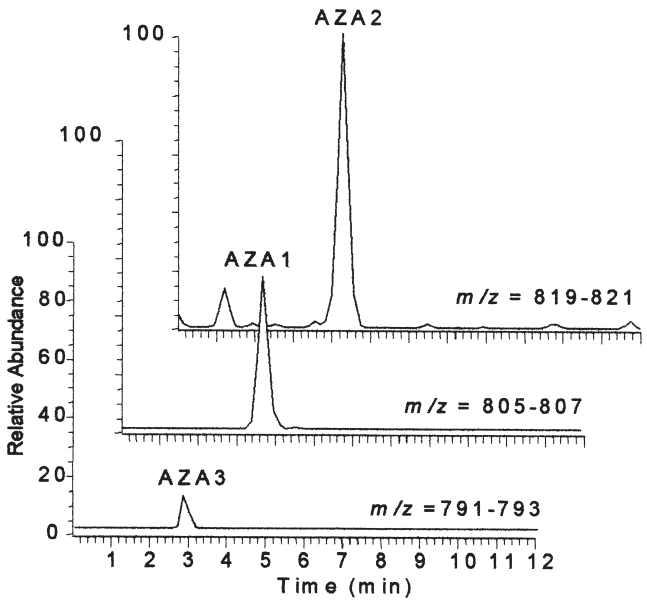

Figure 2 Chromatograms from the LC-MS3 analysis of an extract of 200 cells of *P. crassipes.*

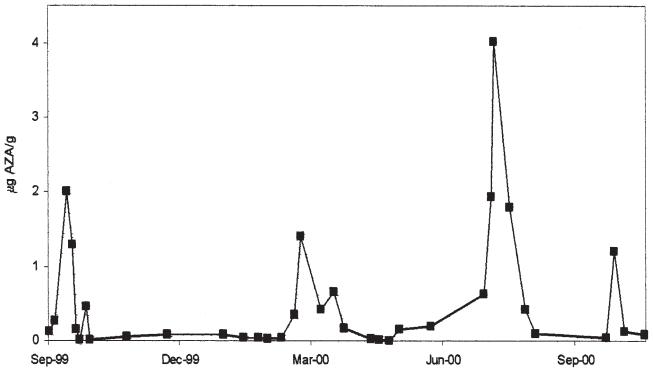

Figure 3 Temporal variation of azaspiracids in mussels (*M. edulis*)

is important to allow the implementation of phytoplankton surveillance programs to alert impending shellfish intoxications (Hallegraeff *et al.,* 1995). The dinoflagellates in phytoplankton samples that tested positive for azaspiracids were manually separated to produce monocultures. The dinoflagellate composition from these plankton trawls was mainly *Ceratium* spp. (30–97%), *Dinophysis* spp. (2–70%) and *Protoperidinium* spp. (0.5–3%). The only known toxin-producing organism among these was *D. acuta* (James *et al.,* 1999) and expectedly, the DSP toxins, okadaic acid and dinophysistoxin-2 were present. LC-MS3 methods proved highly sensitive and were readily applied to study azaspiracids in phytoplankton. This phytoplankton sample also contained three azaspiracids, AZA1, AZA2 and AZA3. The dinoflagellates in phytoplankton samples that tested positive for azaspiracids were manually separated to produce monocultures. Analysis of extracts from 200 picked cells led to the identification of *Protoperidinium crassipes* as the progenitor of azaspiracids. This was confirmed in samples collected each year during September, 1999–2001. AZA1 was usually the predominant azaspiracid in both phytoplankton and shellfish but in the example shown, Fig. 2, this sample of *P. crassipes* contained 60% AZA2. There was no evidence of the presence of AZA4 and AZA5 in any of the phytoplankton samples that were positive for azaspiracids and these compounds are probably produced by bioconversion in shellfish. The average content was 1.8–2.2 femtomole total AZA toxins per cell. This discovery has significant implications for both human health and the aquaculture industry since this phytoplankton genus was previously considered to be toxicologically benign.

The variation of azaspiracids in mussels at a single cultivation site in Bantry Bay, County Cork, is shown in Fig. 3. The periods when toxin were present at levels significantly greater than the regulatory limit, 0.16 µg/g, were late September, July and March. Several new azaspiracids were identified in mussels and these are 3- and 24-hydroxy analogs that are probably the products of bioconversion (see Diaz Sierra *et al.*).

Acknowledgements.

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Florida's Red Tide Dinoflagellate *Karenia brevis* May Modulate Its Potency by Producing a Non-Toxic Competitive Antagonist

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Abstract

Florida red tides are the result of blooms of the dinoflagellate *Karenia brevis* that produces a family of toxic compounds known as brevetoxins (PbTx). The observed variability in *K. brevis* blooms may depend on many factors such as strain, temperature and salinity. Recently, a naturally produced competitive antagonist for the brevetoxin binding site was isolated from *K. brevis* cells grown in culture, which may also explain the variable toxicity of red tide blooms. The antagonist AJB6.0P, also referred to as brevenal (Fig. 1), was found to comprise 2–10% of the total toxin biomass in cells grown in culture. AJB6.0P alone had low toxicity in a fish bioassay, while pre-exposure to the AJB6.0P increased the time to death for fish exposed to PbTx-2. A variety of spectroscopic methods were employed to determine the structure of the AJB6.0P including MS, NMR, UV and FT-IR. High resolution FAB mass spectrometry gave an MH^+ peak at 657.4043. The spectroscopic experiments determined that the AJB6.0P ($C_{39}H_{60}O_8$) is similar in structure to the hemibrevetoxin B, ($C_{28}H_{42}O_7$) (Prasad and Shimizu, 1989). This is the first report of a competitive non-toxic ligand produced by *K. brevis*, and may explain the variable potency of red tides in natural environments.

Introduction

Blooms of *K. brevis* cause massive fish kills and adverse health effects in humans and marine mammals exposed to the brevetoxins either by ingestion or through respiration of aerosolized toxins. Brevetoxins bind with high affinity and activate site 5 of voltage sensitive sodium channels on neuronal membranes (Baden, 1989). The density of the blooms and the intensity of the toxic effects have been observed to vary from bloom to bloom. At times, very small blooms or blooms with low cell counts have been associated with massive fish kills and severe respiratory irritation in beachgoers. Conversely, there have been very large blooms with high cell counts that have very few effects on fish or beachgoers (Steidinger, personal communication, 2002). Theories to explain the variation in the toxic effects of the blooms implicate growth phase of the cells in the bloom; variation in overall quantity of toxin produced; toxin profile differences; and environmental factors such as wind, salinity or temperature (Steidinger and Baden, 1984; Baden and Tomas, 1988). In this paper, we propose an additional factor that may affect the overall toxicity of the *K. brevis* blooms, namely a naturally occurring antagonist to the brevetoxins produced by the dinoflagellate itself, AJB6.0P (brevenal).

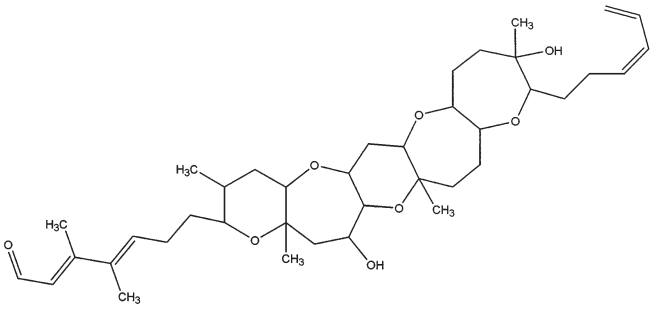

Figure 1 Structure of the new brevetoxin antagonist AJB6.0P (brevenal).

Materials and Methods

Extraction of PbT-3, PbTx-2 and AJB6.0P AJB6.0P and brevetoxins were extracted from eight 10 L carboys of *K. brevis* cultures (Wilson's 58 clone) using chloroform. The purification procedure for both the brevetoxins and AJB6.0P followed the procedures of Baden and Mende 1982, with the following differences for the purification of AJB6.0P. For final purification of AJB6.0P, a Phenomenex reversed-phase phenyl-hexyl column (0.8 × 25 cm, 99% MeOH, 3.4 mL/min, $\lambda = 215$) was used.

Isolation of the Antagonist from the Environmental Samples Samples (100 mL) were collected during a red tide bloom off the west coast of Florida USA. The seawater was extracted with ethyl acetate. The organic layer was filtered (0.2 µm filter) and dried, the samples were dissolved in 300 µL methanol and 100 µL aliquots were used for quantification by HPLC-UV. For separation of AJB6.0P, a Phenomenex reversed-phase l phenyl-hexyl column (0.4 × 25 cm, 90% MeOH, 1.4 mL/min, $\lambda = 215$) was used. Separation of the toxins was performed using a traditional C_{18} column (0.4 × 25 cm, 85% MeOH, 1.4 mL/min, $\lambda = 215$).

Fish Bioassay Male mosquito fish (n = 55) were used for this experiment. Fish were placed individually in 50 mL beakers containing 20 mL water. The test compounds (PbTx-2 and AJB6.0P) were dissolved in ethanol at a concentration 0.1 mg/mL and added to the fish in a total of 200 µL ethanol. The control fish received 200 mL ethanol. Fish

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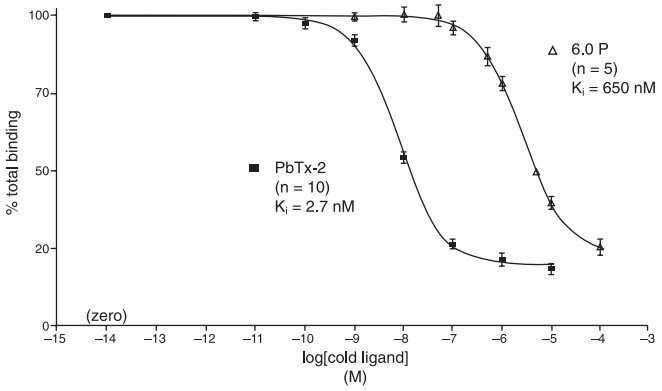

Figure 2 Synaptosome binding assay for AJB6.0P vs. ^{3}[H] PbTx-3.

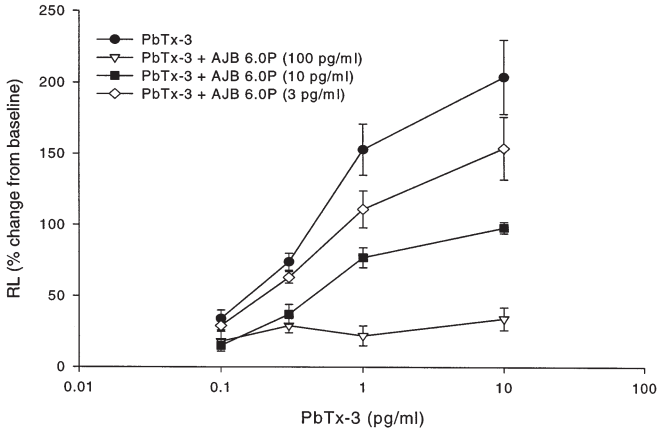

Figure 3 Effects of AJB6.0P on PbTx-3 induced lung bronchoconstriction in sheep.

were exposed to toxin alone (1 µg/mL water), AJB6.0P alone (1 or 2 µg/mL water), or both AJB6.0P (1.0 µg /mL water) and toxin (1.0 µg/mL water) with the AJB6.0P being added 3 minutes before the toxin. After addition of the different compounds the fish were monitored for 24 hours or until the time of death. Significant differences were determined using a two-way Student's *t*-test.

Synaptosome Binding Assay Competitive rat brain synaptosome assays were performed as previously described by Poli *et al.,* 1986. Nonlinear regression curves were generated by Graph Pad Prism® from receptor binding data.

Structural Identification of the Antagonist NMR spectra were obtained using a Bruker 400 MHz NMR. Five to 10 mg of AJB6.0P were dissolved in deuterated D6-benzene and the following NMR experiments were run: 1H-proton, 13C -carbon, 13C -DEPT, 1H -1H-COSY, 1H -1H-TOCSY, 1H-13C-HMQC, 1H-13C-HMBC. Additional NMR experiments were run in the following solvents: D6-Acetone, CDCl3 and D4-Methanol. A 2-D-Noesy was also run in D6-acetone to determine the 3-D structure of the side chains. HR-MS was obtained using FAB+ with a DCM/NBA/NaCl matrix and performed by the Mass Spectroscopy lab at UC Riverside, Riverside CA. UV absorbance maxima of samples (in MeOH) were determined using an HP1100 HPLC equipped with a diode array detector (MP was 90:10 MeOH:H2O, 1.4 mL/min, Agilent C18 analytical column). FT-IR absorbance spectra for AJB6.0P (0.5 mg) was prepared in a KBr pellet and obtained using a Matteson Cygnus 100 FTIR with WIN98 software.

Companion Studies Several companion studies were performed in concert with the studies reported above to determine if the antagonists would inhibit various effects of brevetoxins. The effect of AJB6.0P on inhaled brevetoxins in rats was reported by J. Benson *et al.,* 2003. The effects of AJB6.0P on brevetoxin-induced bronchoconstriction (Fig. 3) were reported by W. Abraham *et al.,* 2003.

Results

Two to ten percent of total toxin produced by *K. brevis* cells grown in culture was AJB6.0P. During two naturally occurring Florida red tide blooms, AJB6.0P concentrations ranged from 0.26 pg/cell to 5.80 pg/cell. In the same samples, the concentration of PbTx-2 + 3 ranged from 18 pg/cell to 74.6 pg/cell. AJB6.0P:toxin ratios ranged from 0.031 to 0.322 and may provide clues to the variation in toxicity seen in *K. brevis* blooms.

Synaptosome binding assays showed a displacement of the tritiated brevetoxin by AJB6.0P with 80% displacement occurring at approximately 1000 times the concentration of AJB6.0P to toxin (Fig. 2).

AJB6.0P effectively protected fish from an equal concentration of PbTx-2, prolonging life by 2.5 fold, possibly allowing them additional time to escape a from a red tide bloom (Table 1).

Inhalation experiments using a sheep model for asthma showed that AJB6.0P alone had no effect on airway resistance or airway constriction. Brevetoxin (0.1 pg/mL–10pg/mL) alone resulted in an increase in airway resistance ranging from 40% to 200% of baseline in asthmatic sheep. If AJB6.0P administration preceded the administration of the brevetoxin in doses ranging from 3pg/mL to 100 pg/mL the increase in airway resistance was attenuated at all doses of AJB6.0P and completely abolished at 100 pg/mL. At equal concentrations of brevetoxin and AJB6.0P there was a 75% drop in airway resistance as compared to brevetoxin alone (Fig. 3)

HR-MS of AJB6.0P showed a positive ion at (MH^+) 657.4043. The HR MS and NMR experiments indicated the molecular formula to be C39H60O8. UV absorption spectra (227 nm and 295 nm) and IR spectra (OH 3400 cm^{-1}, C-H on C=CH2 2970 cm^{-1}, C-H on CH2 2941 cm^{-1}, C-H on CH3 2873 cm^{-1}, R(C=O)H 1667 cm^{-1}, C=C 1618 and 1593 cm^{-1}, C-O-C 1085 cm^{-1}) were consistent with the structure of AJB6.0P. The ^{1}H, ^{13}C, DEPT, and HSQC NMR spectra showed that AJB6.0P contains 1 doublet CH^3, 5 singlet CH^3s, 11 aliphatic methylenes, 1 aliphatic methine, 3

Table 1 Fish bioassay.

Treatment	N	Time to Death (min ± s.e.m.)
Control	20	No deaths after 24 hrs
AJB 6.0P 1 mg/mL	10	No deaths after 24 hrs
AJB 6.0P 2 mg/mL	5	No deaths after 24 hrs
PbTx-2 1 mg/mL	10	7.5 ± 1.06
PbTx-2 1 mg/mL + AJB 6.0P 1 mg/mL	10	17.0 ± 2.84 $P < 0.01$

quaternary oxycarbons, 1 terminal olefinic methylene, 1 aldehyde, 5 olefinic methines, 9 aliphatic oxymethines and, 2 quaternary olefinic carbons. Thus, the doublet methyl resided on the aliphatic methine and the other 5 methyls resided on quaternary carbons. Based on carbon and proton shifts two of the methyls were attached to olefinic carbon and the other three were on aliphatic quaternary carbons. The structure of the two side chains was determined using COSY, TOCSY and HMBC spectra. Based on the NOSEY spectrum, the side chain with the aldehyde was determined to be in trans-configuration and the conjugated diene side chain was found to be in cis-configuration. The number or rings and ring structure was determined using COSY, TOCSY and HMBC spectra.

Discussion

In this paper, we described a non-toxic compound, AJB6.0P (Brevenal), isolated from cultures and natural *K. brevis* blooms that acts as a competitive antagonist to PbTx-3 in receptor binding studies. In addition, it acts as a functional antagonist in living organisms (sheep, rats and fish) and therefore has the potential for use as a therapy for brevetoxin poisoning. Its presence in natural samples provides another explanation for the variability in toxicity of *K. brevis* blooms found in nature and in culture and provides a potential mechanism for the regulation of composite toxicity of Florida red tides. Structural elucidation by spectroscopic means suggests the structure of AJB6.0P is a 6,7,6,7,7 poly ether ladder structure with two side chains and two secondary alcohols, and that it bears some structural resemblance to hemibrevetoxin (Prasad and Shimizu 1989).

Acknowledgements

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Detection and Identification of Paralytic Shellfish Poisoning Toxins in Florida Pufferfish Responsible for Incidents of Neurologic Illness

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Abstract

In March 2002, consumption of pufferfish caught in Titusville, Florida, led to several incidents of neurologic illness. The toxic agents were identified by liquid chromatography-mass spectrometry as the paralytic shellfish poisoning toxins saxitoxin, decarbamoylsaxitoxin, and gonyautoxin-5. Extremely toxic levels of up to 45,000 micrograms STX-equivalents per kg were found in the muscle tissue.

Introduction

On March 18, 2002, a 69-year-old New Jersey man and his 65-year-old wife consumed a meal of pufferfish shipped to them by a relative in Florida. Within minutes of eating the fish, both noticed "tingling" around their lips. In addition the woman complained of tingling of her tongue and the man complained of tingling of his fingertips. During the next 2 hours the symptoms increased in intensity and the woman developed vomiting. On arrival at the emergency department the couple continued to complain of the same symptoms. Their vital signs were completely normal. Despite the clear description of the fish, it was felt that since pufferfish on the east coast are commonly believed to not be contaminated with a neurotoxin, a liter of a 20% solution of mannitol was administered to each of them for the possibility that the toxin was ciguatera. The man continued to do well, while the woman, who had a history of chronic obstructive pulmonary disease, developed increased discomfort and developed a tachycardia of 109 and a blood pressure of 160/70. She was treated with nitropaste for the chest discomfort. Over the next 4–6 hours, the woman developed muscular weakness first in the lower extremities, progressing in an ascending fashion until her reflexes disappeared. Pulmonary function showed a rapid decrease in forced capacity (measured vital capacity dropped to 500 mL) and CO_2 retention and she was electively intubated and placed on a ventilator. Over the next 24 hours the man became asymptomatic and the woman regained her reflexes and voluntary movement. She was extubated at approximately 72 hours and discharged thereafter.

The pufferfish were caught by the man's brother, who had gone fishing off of a pier in Titusville, Florida. He used small pieces of shrimp as bait. He froze the fish and kept them frozen. When he drove to New Jersey he kept the fish on ice until he gave them, still frozen, to the couple in New Jersey and various family members in other states. The couple denied eating any other fish, shellfish or any contact of the fish with any shellfish or water used in cooking shellfish. They denied any exposure to known neurotoxin. Both the fisherman and the male victim stated that the fish were just like all the other pufferfish they had eaten all of their lives. Subsequent to these two cases, 15 additional suspected cases were uncovered in three states (Florida, New Jersey and Virginia), between January and the end of April, and all were similarly associated with pufferfish from Titusville (CDC 2002a, 2002b).

In Pacific regions, pufferfish toxicity is usually related to the presence of tetrodotoxin (TTX) and the symptoms do match those in the New Jersey cases. Some previous intoxications by pufferfish in Florida were attributed to TTX (Lalone *et al.,* 1963). Seven cases of pufferfish poisoning were reported in Florida during 1951–1974, including three fatalities (Benson, 1956; Burklew and Morton, 1971). However, pufferfish on the east coast of the USA were generally considered as safe to eat and in the year 2000, approximately 41 tons of pufferfish were sold in the USA with no reported toxic effects. Therefore, an investigation was launched to determine if TTX was the toxic agent in this 2002 event or if the toxicity was due to some other toxin present.

Materials and Methods

Samples and Standards Filets of pufferfish muscle tissue (no skin or other organs) were received from the NJ Township of Sparta Health Department. Tetrodotoxin standard was purchased from CalBioChem (La Jolla, CA) and PSP toxin standards were obtained from the NRC Certified Reference Materials Program (Halifax, NS, Canada).

Extraction Method 1: A literature method for TTX (Chen *et al.,* 2002) was used with slight alterations: 10 g of tissue was extracted with 10 mL methanol (with 1% acetic acid) by homogenizing with a Polytron followed by centrifugation. The supernatant was collected and the residue was further extracted with another 10 mL of solvent. Each extract was evaporated to near dryness using a Savant vacuum centrifuge and then re-dissolved in 2 mL of 1% aqueous acetic acid. After partitioning with four 3-mL portions of chloroform, the aqueous extract was filtered through an OASIS-HLB cartridge. Method 2: 10 g of tissue was extracted with 20 mL 0.05 M acetic acid by homogenizing with a Polytron. After centrifugation, the supernatant was filtered through an OASIS-HLB cartridge. Method 3: 10 g of tissue was extracted with 10 mL 0.1 M HCl by boiling for 5 min. The pH and weight of the extract was adjusted to 3 and 20 g, respectively. The extract was then filtered through Whatman filter paper.

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Liquid Chromatography-Mass Spectrometry (LC-MS) LC-MS experiments were performed using PE-SCIEX API165 and API4000 single and triple quadrupole mass spectrometers (Thornhill, ON) coupled to an Agilent 1100 HPLC (Palo Alto, CA). The LC column (2 × 250 mm) was packed with 5 µm TSK gel Amide-80 (TosoHaas, PA). Isocratic elution was performed with 65% B, where eluent A was H_2O and eluent B was 95% CH_3CN/H_2O, both containing 3.5 mM formic acid and 2 mM ammonium formate. The flow rate was 0.2 mL/min and a sample injection volume of 3 µL was used. Electrospray ionization and selected ion monitoring (SIM) detection were carried out using $[M+H]^+$ ions for PSP and TTX toxins (Quilliam *et al.,* 2001).

Liquid Chromatography-Fluorescence Detection (LC-FLD) The LC-FLD experiments were performed using an HP1046A fluorescence detector coupled to HP1090 HPLC (Agilent, Palo Alto, CA). The LC column (4 × 250 mm) was packed with 5 µm LiChrospher100 RP18. A step gradient was used for elution: 0% B for 20 min and at 20 min 0 to 100% B, where eluent A was 4 mM heptane sulphonate, 10 mM ammonium phosphate, pH 7.1 and eluent B was the same with 7% CH_3CN. A flow rate of 1 mL/min was used. Post column oxidation and fluorescence detection was performed according to the method of Oshima (1995).

Cell Assay The tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels was performed as described by Manger *et al.* (1993).

Mouse Bioassay Standard AOAC mouse bioassays were performed by Nancy Peacock, Canadian Food Inspection Agency (Dartmouth, NS). A twofold dilution of the extract prepared by method 2 above was submitted and then serially diluted to achieve a death time close to 15 minutes.

Results and Discussion

Figure 1a shows an LC-MS analysis of uneaten pufferfish received from the stricken New Jersey couple via the Health Department. The mass chromatogram for the $[M+H]^+$ ion of TTX at *m/z* 320 showed no significant peak at the expected retention time, thus indicating TTX was not the toxin responsible for this event. In this same analysis, all of the $[M+H]^+$ ions for PSP toxins were also monitored. Significant signals were observed in the mass chromatograms for saxitoxin (STX, *m/z* 300), decarbamoylsaxitoxin (dcSTX, *m/z* 257), and gonyautoxin-5 (GTX5, *m/z* 380), all at retention times matching those of standards. The identities

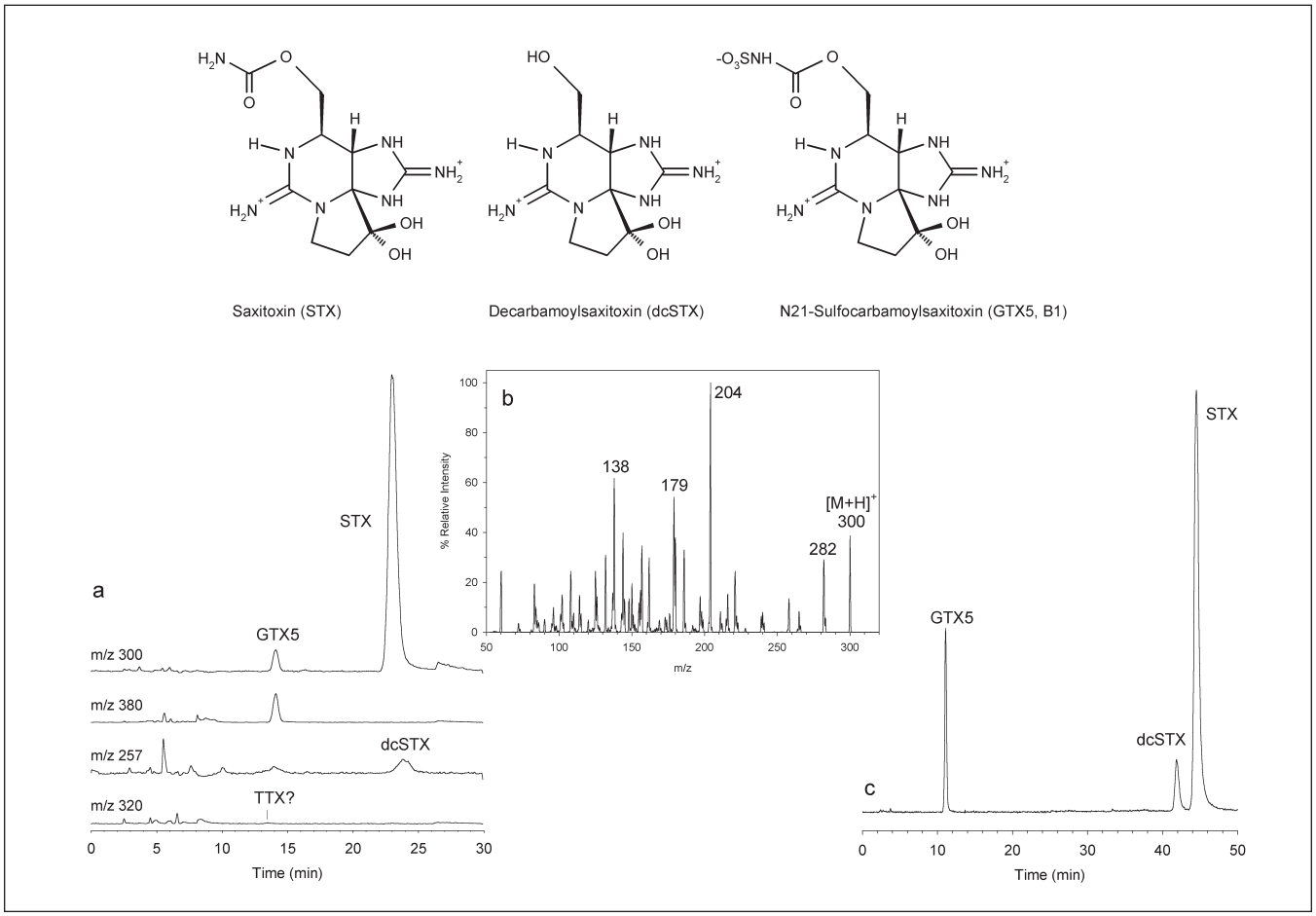

Figure 1 Results of the analyses of pufferfish extract and the structures of the three toxins observed: **a**, LC-MS mass chromatograms; **b**, MS/MS spectrum of the STX peak; **c**, LC-FLD chromatogram.

Table 1 Quantitation of saxitoxin equivalents in pufferfish responsible for poisoning of the NJ couple.

Analysis Method	Extraction Method	µg STXequiv/kg
LC-MS #1	#1: Methanol (TTX method)	20,000
LC-MS #2	#2: Acetic acid	36,000
Cell assay	#3: AOAC (boiling HCl)	26,000
Mouse assay	#3: AOAC (boiling HCl)	45,000

of these three toxins were each confirmed by tandem mass spectrometry, by matching fragment ion spectra of the $[M+H]^+$ ions with those of standards. Figure 1b shows the MS/MS spectrum of the STX in the pufferfish extract. The extract was also analyzed by liquid chromatography using gradient elution, post-column oxidation reaction and fluorescence detection. The LC-FLD spectrum (Fig. 1c) showed the same three toxins present with an exact match of standard retention times.

Two assay methods were also used to test for toxic agents: the neuroblastoma cell assay (Manger *et al.,* 1993) and the AOAC mouse bioassay. Both showed responses consistent with neurotoxic agent(s). The measured toxicity values from these assays matched quite well with the levels determined by LC-MS, considering that different sub-samples of tissue and extraction methods were used. Some quantitative results are summarized in Table 1.

Epidemiological studies have shown that severe symptoms can occur with ingestion of 2 mg STX-equivalents. For a meal of 100 g tissue and a concentration of 40,000 µg/kg tissue, the dose ingested by the New Jersey couple would have been 4 mg. Thus, the levels of PSP toxins observed in the pufferfish do explain the severity of the symptoms. The presence of STX in puffers has been reported previously in Pacific regions (Nakamura *et al.,* 1984; Sato *et al.,* 2000). The origin of these toxins has not yet been established in those studies. An investigation of the source of the toxins in the Florida pufferfish will be reported separately.

Acknowledgements

The authors gratefully acknowledge Bill Hardstaff, IMB, and Stephen Chung, visiting IMB from Hong Kong Government Laboratory, for technical assistance; Nancy Peacock, Canadian Food Inspection Agency, for mouse bioassay measurements; and Jan Landsberg and Karen Steidinger, Florida Marine Research Institute, for discussions.

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Lipophylic Toxins of Different Strains of Ostreopsidaceae and Gonyaulaceae

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Abstract

Production of lipophylic toxins of the yessotoxins group has been reported on strains of the dinoflagellates *Protoceratium reticulatum* and *Coolia monotis* and is suspected in *Lingulodinium polyedrum.* While *P. reticulatum* and *L. polyedrum* belong to the family Gonyaulaceae, *C. monotis* belongs to family Ostreopsidaceae, where species of the genus *Ostreopsis* produce lipophylic toxins of another group, the palytoxin group. Both yessotoxins and palytoxins are fused polyethers of very high molecular weight but without any known toxicological relation. In this work, the production of these toxins in strains obtained from different geographical regions is explored. Detection of yessotoxins was done by means of an HPLC analysis with derivatization and fluorimetric detection and by HPLC-MS. For the detection of palytoxins, HPLC and hemolytic assays were done. The strains of *P. reticulatum* analysed were from Europe and North America, and the strains of *L. polyedrum* were from the Atlantic coast of Spain. All were toxic, with *L. polyedrum* being the least toxic. This is the first report of dinoflagellates producing yessotoxins in Spain. Several European strains of *C. monotis* were analysed, but no cooliatoxin was detected. Strains of two species of *Ostreopsis* from the Mediterranean Sea and from Brazil have strong, delayed hemolythic activity that could be attributed to a palytoxin-like toxin (ostreocin).

Introduction

Palytoxin (PTX) is the most potent marine toxin known, and it belongs to a group of closely related, very poisonous aliphatic molecules with high molecular weights around 2600 Da (Habermann *et al.,* 1982). It acts through the Na+, K+ -ATPase of cell membranes, inducing channel or pore formation by the enzyme proteins. It has been primarily isolated from the marine zoanthids *Palythoa*. Recently PTX was found in the benthic dinoflagellates *Ostreopsis siamensis* (Usami *et al.,* 1995; Onuma *et al.,* 1998), *Ostreopsis ovata* (Granéli *et al.,* 2002) and *Ostreopsis mascarenensis* (Turquet *et al.,* 2002). Structurally, YTX is a disulfated polyether, nominally included in the diarrhetic shellfish poisoning (DSP) category, but YTX should be excluded from this group due to different properties. The causative organism of YTX was demonstrated in *P. reticulatum* (Satake *et al.,* 1997; Satake *et al.,* 1999), but although YTX was detected in field samples dominated by *L. polyedrum* (Ciminiello *et al.,* 1997), the production of this toxin in pure cultures was not proved. The present study was performed to determine palytoxin–like toxins in *Ostreopsis* spp. from different geographical locations (Mediterranean Sea and Brazil) and to test for the presence of toxins of the YTX group in other dinoflagellates in addition to *Protoceratium reticulatum,* like *L. polyedrum, C. monotis* and *Ostreopsis* spp.

Materials and Methods

Cultures of the strains shown in Table 1 were grown in L1 medium (Guillard and Hargraves, 1993) without silicates with a salinity of 34 psu, an irradiance of ~90 µmol quanta m^{-2} s^{-1} provided by daylight fluorescent bulbs and a 12:12 D:L regime. For palytoxins analysis in *Ostreopsis,* 10 L culture were harvested on the 20th day after inoculation. The whole volume was filtered through Whatman GF/C filters, resuspended in ethanol/water (8:2) and sonicated for homogenation and centrifugated, collecting supernatants at the end. Ethanol/water 80% extract was partitioned with hexane. The aqueous ethanol fraction was evaporated and the residue was again partitioned between water and butanol. The aqueous fraction was evaporated and the residue was resuspended in Acetonitrile/Acetic acid 1% (3:7) and was chromatographed on Vydac 201TP54 C18 column at 30°C, using Acetonitrile : 1% Acetic acid (3:7) as a mobile phase at a flow rate of 0.75 mL · min^{-1}. Elution of palytoxin from the column was monitored by UV-PDA at 230 nm and 263 nm following a method based on Usami et al (1995) and Tan and Lau (2000). Hemolysis assay was based on Bignami's method (1993) as modified by Onuma *et al.* (1999). Human blood in EDTA was diluted in PBS (1:100) and washed twice with PBS and the evaporate extract was diluted in pH 7.4 PBS supplemented with 0.1% BSA, 1 mM $CaCl_2$ and 1

Table 1

Strain	Species	Location
OS06BR	*Ostreopsis* cf. *ovata*	SW Atlantic
OS2V	*Ostreopsis* cf. *siamensi*s	Mediterranean
GG1AM	*Protoceratium reticulatum*	NE Atlantic
CCMP1720, CCMP1721	*Protoceratium reticulatum*	NW Atlantic
CCMP1889, CCMP404	*Protoceratium reticulatum*	NW Pacific
LP3AA, LP4V, LP5V, LP6V, LP7V, LP8V, LP9V, LP10V	*Lingulodinium polyedrum*	NE Atlantic
CM1V, CM2V, CM3V, CM4V, CM5V, CM6V	*Coolia monotis*	NE Atlantic
CCMP305, CCMP1345, CCMP1744	*Coolia monotis*	NW Atlantic

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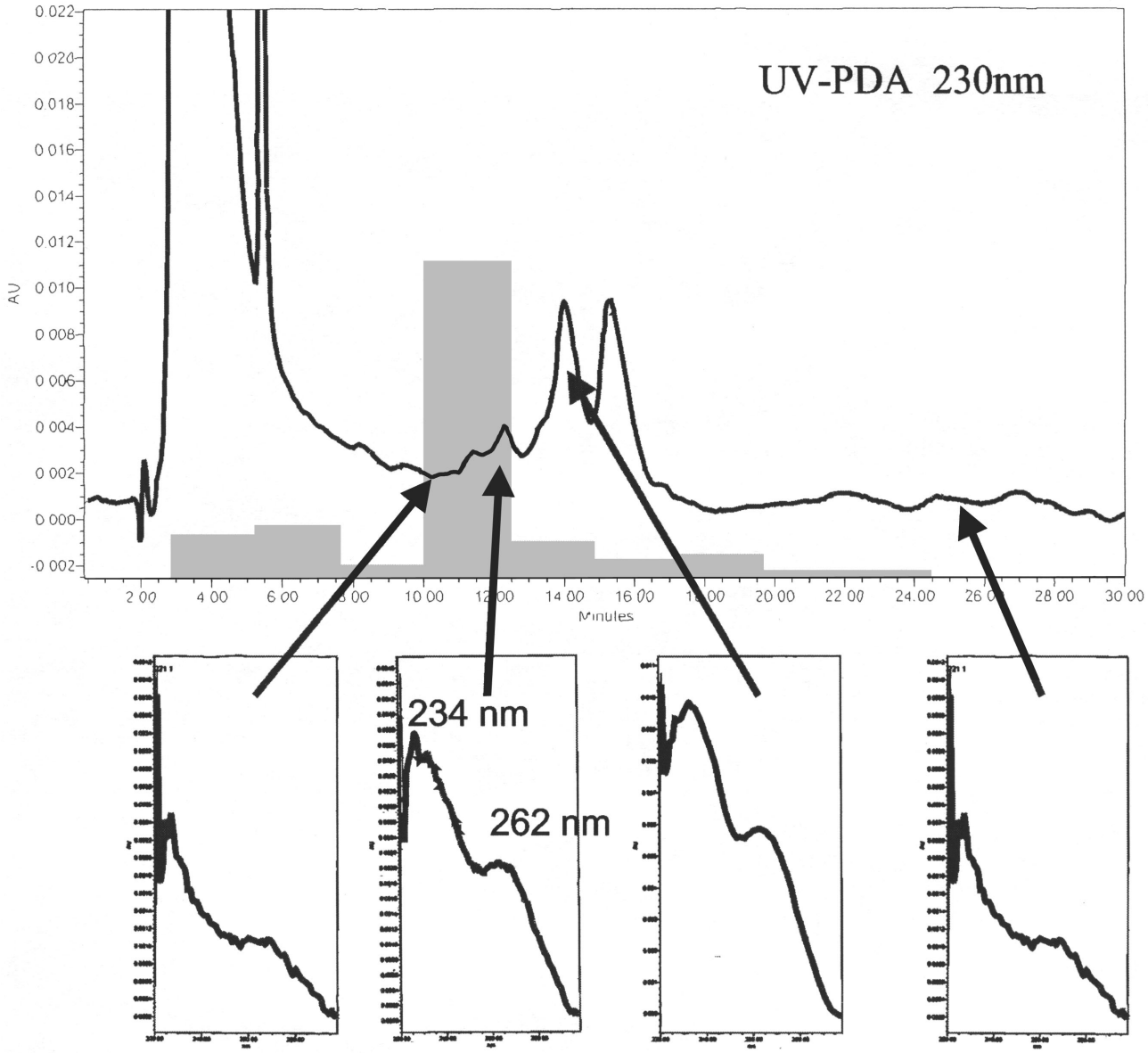

Figure 1 *Ostreopsis* cf. *ovata* (OS06BR). Chromatography was carried out on a Vydac 201TP54 C18 column. Elution of palytoxin-like substance from the column was monitored by UV-PDA absorption at 230 nm and was monitored by hemolysis assays of each fraction collected in a 2.5 min. portions (bar chart). Ouabain inhibited the hemolytic activity of the elution fraction from 10 to 12.5 min.

mM H_3BO_3. For hemolysis neutralization, assay blood cell suspension was mixed with sample solution (1:1), blank was prepared mixing diluted blood and saline PBS (1:1) and 100% hemolysis adding distilled water. After incubating at 37°C for 4 hours in a water bath, supernatant absorption was measured at 405 nm with microplate reader. For yessotoxins analysis, samples were extracted following a modification of Yasumoto and Takizawa (1997) method. Forty-five mL of each culture was filtered through Whatman GF/C filters and resuspended in 2 mL MeOH, and the cells were sonicated. The derivatization reaction was performed with 50 µL of 0.1% solution of DMEQ-TAD in CH_2Cl_2. YTX analysis were performed by LC-FLD using a Hypersil ODS 5 mm (4.6 × 150 mm) column using a mobile phase of 100 mM ammonium acetate (pH 5.8) and MeOH (3:7). The flow rate was 0.75 mL · min^{-1}, the column temperature was 35°C and the excitation and emission wavelengths were 370 nm and 440 nm, respectively. YTX confirmation was made by mass spectrometry.

Results and Discussion

The extracts of *Ostreopsis* showed strong delayed hemolytic activity that could be neutralized by ouabain, two criteria considered to indicate the presence of PTX. After each partition, the hemolytic assay was performed and the toxic fractions selected. The analysis of *Ostreopsis* spp. extracts by UV-PDA showed peaks possesing the characteristic UV spectra (absorbance maxima at 230 and 263 nm) of PTX. Elution from the column was also monitored by hemolytic assay (Fig. 1). This is the first record of toxicity of *Ostre-*

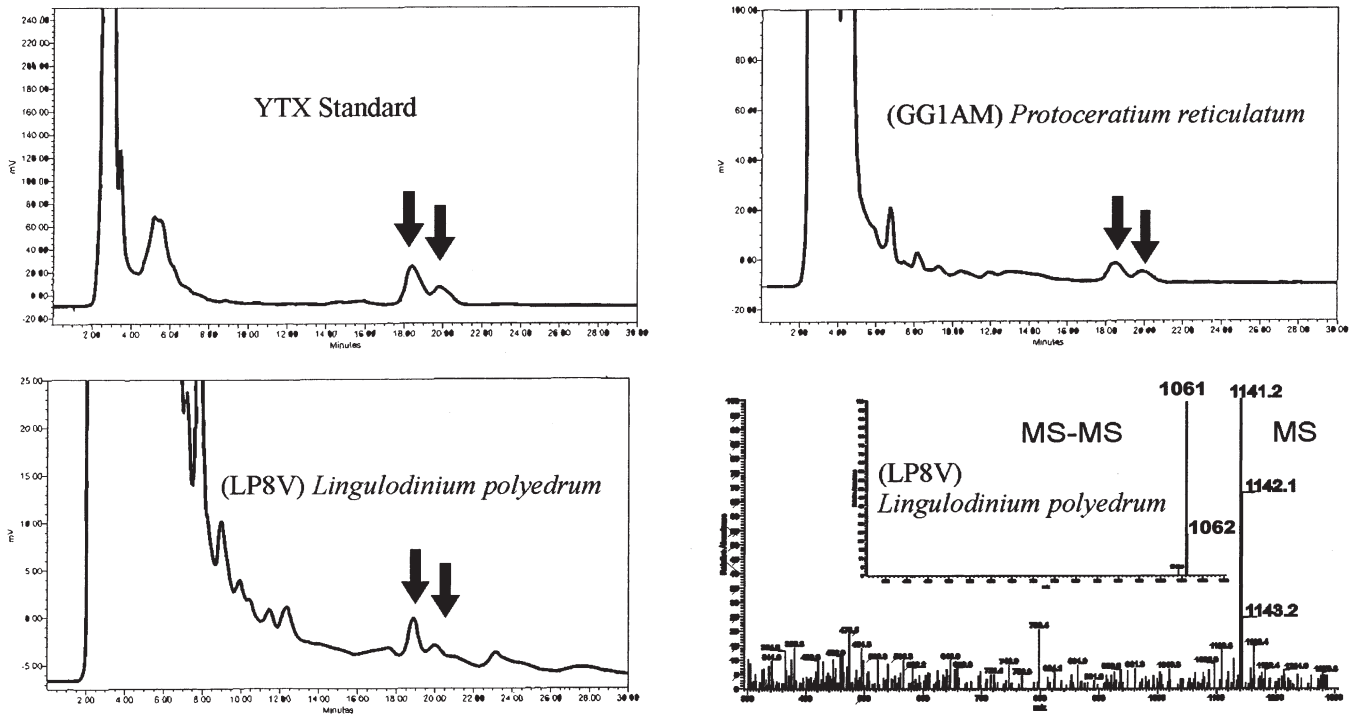

Figure 2 Chromatographs of YTX standard, YTX from (GG1AM) *Protoceratium reticulatum* and YTX from (LP8V) *Lingulodinium polyedrum* performed by LC-FLD and LC-MS of (LP8V) *Lingulodinium polyedrum.*

opsis spp. in the Mediterranean Sea, and this genus should be taken into account in monitoring programmes as it may introduce palytoxins in the food web and eventually cause human poisonings. The hemolyitic assay was also performed in extracts of *C. monotis, L. polyedrum* and *P. reticulatum,* and no activity was observed.

YTXs were detected in *P. reticulatum* and in lower concentrations in *L. polyedrum* by LC-FLD and proved by MS. Selected ion monitoring (SIM) for YTX was performed on ion *m/z* 1141, in MS-MS with the 1061 ion daughter (Fig. 2) but not in *C. monotis* or *Ostreopsis* spp. Although the production of YTXs by *P. reticulatum* had already been reported (Satake *et al.,* 1997), this work confirms Yasumoto's hypothesis that this species was also a producer of YTXs. High levels of YTXs were also detected in the culture media by LC-FLD and proved by MS. The production of YTXs by dinoflagellates looks very variable, as the same strains of *L. polyedrum* may produce the toxins or not due to unknown reasons, and we did not detect YTX in *Coolia*, although it was detected in an Australian strain (Holmes, 1995). Palytoxins look to be restricted to the family Ostreopsisdaceae and YTX to Gonyaulacaceae.

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Preparation of In-House Certified Materials (RM) to Be Used as a Tool in Quality Assurance of the Analytical Results in Paralytic Shellfish Poisoning Toxin Assays

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Abstract

In-house reference material (RM) was developed with Mediterranean cockles, *Acanthocardia tuberculatum,* naturally contaminated with paralytic shellfish poisoning (PSP) toxins. A total of 35 sachets, each one containing 50 g of homogenized meat, were prepared. 14.3% of the sachets were used for the homogeneity studies, allowing the assignation of a reference value for its PSP toxicity content by both Mouse Bioassay and HPLC. A stability study was performed at three time intervals: 0, 9 and 12 months, after storage at –20ºC. Five sachets were analysed at each time period by both analytical methods. The results (Mouse Bioassay and HPLC-FD) concerning homogeneity, stability, assigned values and uncertainty obtained in the development of this RM for its PSP toxicity content are shown.

Introduction

Because of the threat that marine biotoxins represent to human health, the reliability of the test results in seafood is an important goal to achieve. International Standard ISO/IEC 17025:1999 establishes the requirements to be accomplished by the analytical laboratories willing to demonstrate their technical competence and capability to produce validated acceptable results. Compliance with these requirements by analytical laboratories and their accreditation by institutions that have signed agreements for the mutual acknowledgement with their equivalent ones from other countries, should facilitate the acceptance of their respective analytical results. To achieve this goal, analytical laboratories must develop quality control procedures for proving the acceptance of the assays performed. International Standard ISO/IEC 17025:1999 recommends, among its general strategies, the routine use of Certified Reference Materials (CRM) and/or secondary RM. Some drawbacks related to CRM (availability, adequacy, traceability, expiration date, price, etc.) mean that the preparation of in-house certified Reference Materials will be an alternative option for the quality assurance of the analytical results.

The aim of this work was to develop an in-house reference material for their paralytic shellfish poisoning (PSP) toxicity content, to be used as a part of the internal quality control system for analytical methods used in the toxicity determination of molluscs samples. Mediterranean cockles, *A. tuberculatum,* naturally contaminated with paralytic shellfish poisoning (PSP) toxins were used for this purpose.

Materials and Methods

The PSP standards used in this study were as follows: STX from the Food and Drug Administration (US); GTX1, GTX2, GTX3, GTX4, GTX5, NeoSTX from the National Research Council (Canada); and dcSTX provided by the Community Bureau of Reference of the EU for an intercomparison study.

Preparation of the In-House Reference Material Batches of "Mediterranean cockle" (*A. tuberculatum*) naturally contaminated with PSP toxins, were collected from harvesting areas in the southwest coast of Spain and kept frozen (–20°C). The molluscs were thawed and drained; then the edible meat was removed from the shells, cut into small pieces and blended. The homogenized meat was split up in 50-g aliquot portions, packed in polyethylene bags and sealed. Every sachet was identified with a code number and frozen at –20°C.

Homogeneity Study and Assignation of PSP Toxicity Reference Value and Its Uncertainty Since the whole content of each sachet is intended to be used for each quality control, only the between-unit homogeneity of the batch was checked. Therefore, the content of each one of five (14.3%) sachets or RM were extracted and analyzed by Mouse Bioassay (MB) (AOAC, 2000) and also by HPLC-FD (Lawrence, 1995) using the same extracts. To test the homogeneity, the standard deviation of the results obtained with both methods was compared with that obtained in the validation of the methods. Toxin concentrations determined by HPLC were converted into toxicity values according to Oshima (1995).

We considered as assigned PSP toxicity value for the RM the mean analytical values obtained in the respective homogeneity studies.

According to the point of view of the Nordic Committee for Food Analysis (NMKL, 1997), we estimated the uncertainty of the mean values on the basis of the internal reproducibility (standard deviation) established in the validation of the methods: $U = K \times RSD \times c$ (U = expanded uncertainty; K = 2; RSD = relative standard deviation; c = concentration).

Stability Study Once the homogeneity of the material was verified, the stability at –20ºC was checked at three time intervals (zero, nine and twelve months) by determining the

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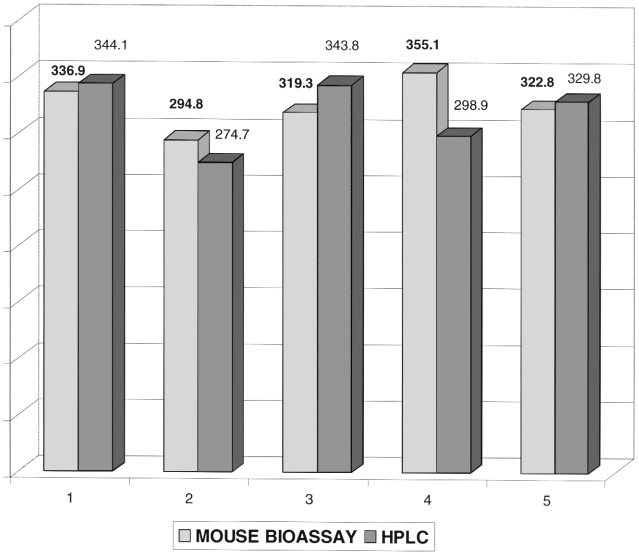

Assigned PSP toxicity value and its uncertainty
MB: 325,8 ± 57,0 (µg STX equiv. /100 g)
HPLC: 318,3 ± 28,6 (µg STX equiv. /100 g)

Figure 1 Individual results in µg STX equiv /100 g obtained in the homogeneity study by MB and HPLC-FD.

total PSP toxicity content. Time zero corresponded to the mean value in the homogeneity study. Five sachets were analyzed each time by MB and HPLC, which represented 42.9% of the whole RM batch. Trend analyses ($t_{0,95}$ test) were applied to detect any significant trend in the stability.

Results and Discussion

Figure 1 shows the individual results (MB and HPLC-FD) obtained in the homogeneity study. The standard deviation of the results with the MB method, was below or comparable to that obtained in the validation of the method. For HPLC, this was also true as far as the contribution of STX and dcSTX to the sample toxicity was considered. The toxin profile revealed GTX5 as the predominant toxin, followed by dcTSX and STX. Other toxins detected were GTX2,3, GTX1, dcGTX2,3, C1,2. Taking into account the internal reproducibility for the evaluation of the expanded uncertainty (see above), then the assigned values for the PSP toxicity content were 325,8 ± 57,0 and 318,3 ± 28,6 µg STX equiv. / 100 g, by MB and HPLC-FD, respectively.

Results of the stability study are summarized in Fig. 2. The toxicity values where plotted versus storage time and the regression line were calculated. The slope of the regression lines did not differ significantly from zero ($t_{0,95}$ test) by MB and HPLC; therefore, no evidence of instability was detected after twelve months' storage period at –20°C, when using the constancy of the sample toxicity as stability criterion.

We conclude that the preparation of in-house RM is a practical and feasible tool to be used as a part of the quality control system applied to analytical methods used for the toxicity determination of mollusc samples contaminated with PSP toxins.

The use of these RM must be accompanied with the corresponding checking by means of controls.

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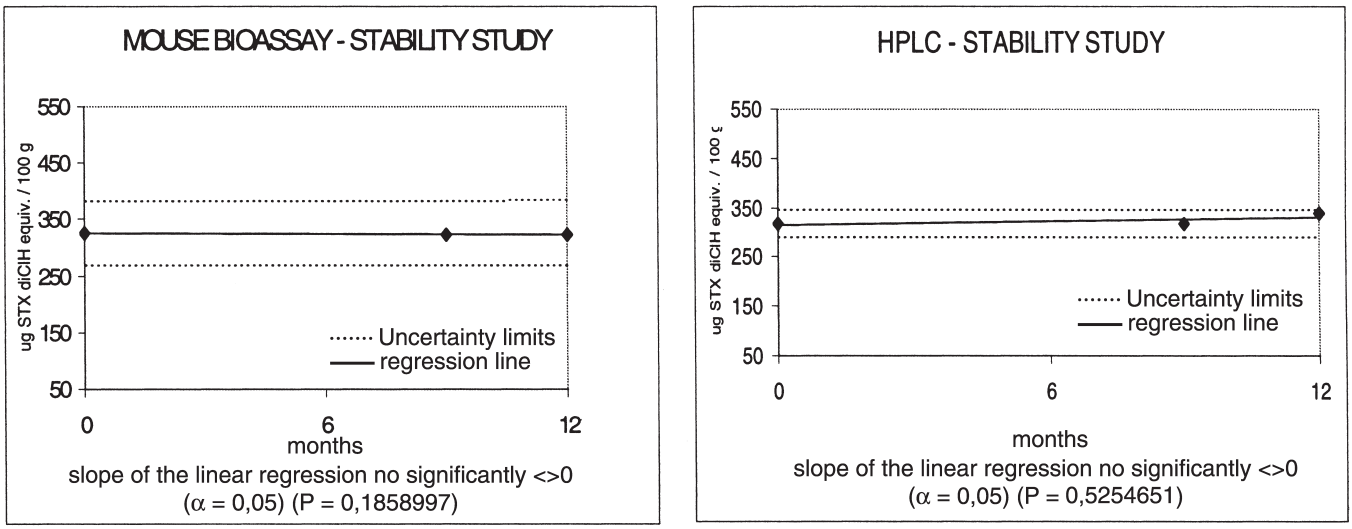

Figure 2 Stability study: PSP toxicity values obtained by MB and HPLC-FD, plotted versus storage time.

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Mass Culture of New Zealand Isolates of *Pseudo-nitzschia australis* for Production of a New Isomer of Domoic Acid

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Abstract

An unusual isomer of DA (iso-DA) was detected in shellfish along with domoic acid (DA) collected from the Marlborough Sounds and Bay of Plenty, New Zealand (August 2001), during routine monitoring for marine biotoxins by LC-MS analysis. The causative organism was identified as *Pseudo-nitzschia australis,* using DNA probes targeted at species specific rRNA combined with LC-MS analysis of Si-depleted isolate cultures. In order to obtain sufficient material for chemical characterisation of iso-DA, mass cultures were grown in a step-up system in 10 L barrels by adding Si-deplete f/2 medium to *P. australis* inoculum over a 3 week period. Approximately one third of the iso-DA was present in the supernatant following gentle filtration (Whatman's GFC glass fibre filter). Cell concentrate or filtrate were acidified to pH 2.5, applied to preconditioned 500 mg C18 SPE columns (Phenomenex, USA), and the columns washed with acidified water and eluted with 20% acetonitrile in water. However, the large amounts of supernatant being put through the column necessitated an improved method of iso-DA production, particularly as production by *P. australis* decreased with time. Addition of copper to the culture medium was assessed in an effort to boost yield.

Introduction

The diatom genus *Pseudo-nitzschia* is known to be responsible for domoic acid (DA) contamination of shellfish on occasion in New Zealand. Sea water samples are routinely collected from 70 sites throughout New Zealand for phytoplankton monitoring for marine biotoxin risk assessments. Shellfish flesh samples are collected at the same time from corresponding sites for biotoxin testing for regulatory clearance of shellfish (Rhodes *et al.,* 1998, 2002b).

Routine analyses of shellfish were carried out by HPLC-UV between 1993 and 2001, and during that time no novel isomers of DA were reported. Since June 2001 DA analyses have been carried out by LC-MS and, in August 2001, a novel DA isomer, referred to as iso-DA, was detected in Greenshell™ mussels (*Perna canaliculus*), scallops (*Pecten novaezealandiae*), and Pacific oysters (*Crassostrea gigas*) harvested from the Marlborough Sounds and the Bay of Plenty (Holland *et al.,* 2002).

Pseudo-nitzschia australis has been the major species of concern in New Zealand. Several other species do produce DA, but most at lower concentrations per cell, and some species are non-toxic (Rhodes *et al.,* 2002b). DNA probe assays, using fluorescent *in situ* hybridization, are routinely requested when *Pseudo-nitzschia* blooms occur, for species identification and thus a better risk assessment of potential DA contamination. Cells of *Pseudo-nitzschia* were observed in sea water samples collected at the time of the iso-DA contamination of shellfish, and many of these isolates were successfully cultured. The resulting clonal cultures were identified by DNA probe assay and analysed by LC-MS to determine whether they produced DA and/or iso-DA.

Chemical characterization of the isomer and determination of its toxicology requires bulk material and so mass cultures were undertaken. However, over time in culture, DA and DA isomer production per cell decreased. Increases in DA production have been reported by Rue and Bruland (2001) following copper addition to toxic *Pseudo-nitzschia* cultures, and copper addition to cultures was therefore assessed to determine whether toxin production could be similarly increased with *P. australis.*

Materials and Methods

Micro-Algae Sea water samples were collected from sites around New Zealand with hose or bottle samplers. Live cells were isolated by micro-pipette into f/2 medium (Guillard 1975) and incubated at 100 μmol m^{-2} s^{-1} (14:10 h light:dark), 19°C. Cultures were maintained in the Cawthron Microalgae Culture Collection.

Whole cell (*in situ*) hybridisation with species-specific large-subunit ribosomal RNA (LSU rRNA)-targeted oligonucleotide probes tagged with FITC (auD1, puD1, muD1, muD2, heD2-2, frD1, deD1, UniC and UniR), were used to identify cultured *Pseudo-nitzschia* isolates (refer Scholin *et al.,* 1996; Scholin *et al.,* 1997; Miller and Scholin 1998).

Clonal isolates were stressed for production of DA and iso-DA by culturing for five days under silicate limitation (f/2 medium minus $Na_2SiO_3.5H_2O$), then sub-culturing (40% inoculum) into f/2 –Si for a further 9 days. Cultures were analysed for iso-DA by LC-MS. Mass cultures were grown in 10 L barrels with an initial inoculum of 100 mL (grown in f/2 medium). Step-wise additions of f/2 –Si medium were made, doubling the culture every few days until day 20 of growth. Cultures were harvested by filtration (Whatman's GFC glass fibre filter). Analysis was by LC-MS.

Copper additions were made to clonal isolates of *Pseudo-nitzschia australis* (CAWB51, 52), *P. fraudulenta* (CAWB56, 57) and *P. pungens* (cultures since died) in f/2 -Si to enhance isomer and DA production. Final concentrations of $CuSO4.5H_2O$ were 40 μM, 80 μM and 120 μM.

Biotoxin Analysis Cultures of *Pseudo-nitzschia,* grown under silicate limitation, were frozen directly or harvested gently by filtration as above. Cultures, or cell concentrate and cell filtrate, were frozen, then thawed and sonicated for 1 min. (×2) prior to extraction. Aliquots of cell concentrate

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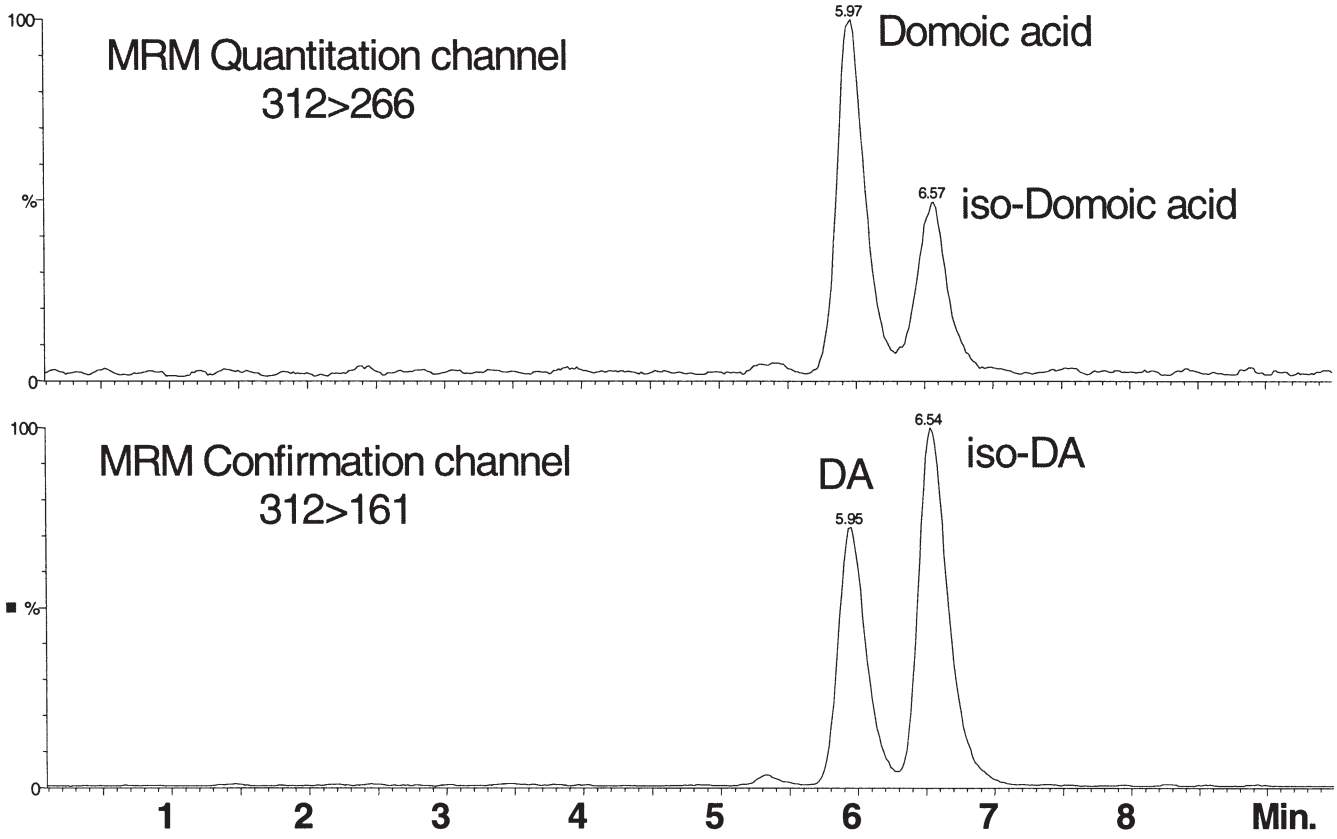

Figure 1 Tandem LC-MS chromatogram for a C18-SPE concentrate fraction from a *Pseudo-nitzschia australis* culture (CAWB 51) with copper addition producing domoic and iso-domoic acid.

or filtrate (50 mL) were acidified (pH2.5) with dilute HCl and applied to preconditioned 500 mg C18 SPE columns (Phenomenex, USA). Following a wash with acidified water (5 mL) the columns were eluted with 20% acetonitrile in water (2.0 mL). The extracts were then analysed for DA and iso-DA by LC-MS/MS with electrospray ionisation (Holland *et al.,* 2002).

Results and Discussion

Pseudo-nitzschia isolates obtained in August 2001 from sites where iso-DA was detected in shellfish flesh were successfully established in culture. The results of rRNA-targeted species-specific DNA probe assays determined the presence of *P. australis, P. fraudulenta* and *P. pungens* in the Marlborough Sounds (northern South Island) and those same three species plus *P. multistriata* in the Bay of Plenty (eastern North Island). Scanning electron microscopy of frustules has confirmed the species as *P. australis* (clonal isolates CAWB 51, 52).

All isolates of *P. australis* tested from the Marlborough Sounds and the Bay of Plenty produced both iso-DA (max. conc. 1.5 pg cell^{-1}) and DA (max. conc. 1.1 pg cell^{-1}) with iso-DA concentrations varying from one-third to treble the concentration of DA (Rhodes *et al.,* 2002). However, not all *P. australis* isolates from other regions produced the isomer; for example, only one of two DA-producing isolates from the North Island site of Tairua produced iso-DA, and then at low concentrations (0.1 pg cell^{-1}; Rhodes *et al.,* 2002a).

The greatest proportion of iso-DA and DA was detected in the cell concentrate (ranging from 68–87% of total toxin concentration; Fig. 1). The balance, detected in the cell culture medium, was difficult to extract due to the high salt concentrations in the medium. The addition of extra copper to the culture medium to increase isomer production, on the basis that DA was enhanced by the addition of this trace metal (Rue and Bruland 2001), was successful for *P. australis.* The increases in both DA and iso-DA were proportionally similar, with toxin production showing greatest increases between days 8 and 21 of growth (following sub-culturing after 5 days of initial growth on f/2 –Si).

The maximum concentration of iso-DA per cell was with the addition of 80 µM $CuSO_4$.5H2O and occurred on day 21 of growth, although there was considerable variation between replicate cultures (4.17–9.05 pg cell^{-1}; Fig. 2). Significant differences were detected between 80 µM and 200 µM copper additions on days 6 and 11 of growth, but although at other growth stages differences were not significant, the highest concentrations were always recorded for cultures with 80 µM copper additions. Concentrations of iso-DA per cell decreased after day 21 of growth and cells began to show signs of deterioration (*e.g.,* chloroplast disintegration). However, cultures with additions of copper

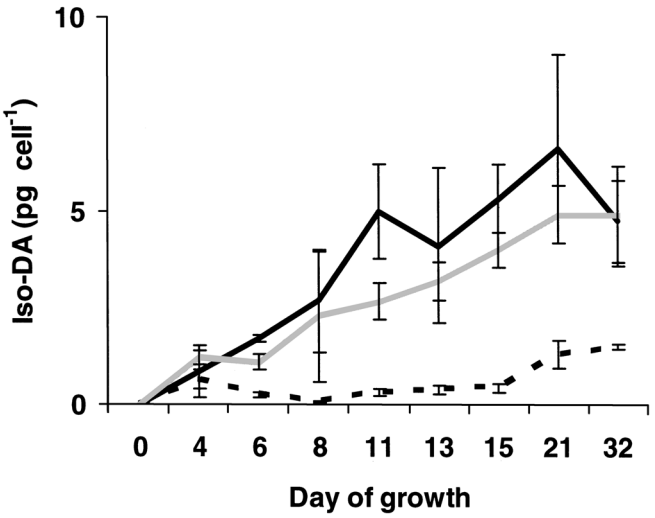

Figure 2 Effect of copper on production of iso-DA, an isomer of domoic acid, by *Pseudo-nitzschia australis* (CAWB 52). Culture medium: f/2 (Guillard 1975) minus Si with final conc. $CuSO_4.5H_2O$ of 40 μM (- - -), 80 μM (—) or 200 μM (—)

above the standard 40 μM were still producing significantly more iso-DA than cells cultured in standard f/2 –Si medium (Fig. 2).

Pseudo-nitzschia fraudulenta, P. pungens and *P. multistriata* isolates tested were all negative for DA and iso-DA as determined by LC-MS (level of detection < 0.05 pg cell^{-1}). DA was detected by polyclonal DA ELISA at the level of detection of the assay (1.0×10^{-4} pg cell^{-1}) in both *P. pungens* and *P. fraudulenta,* and at 0.6 to 2.2 pg cell^{-1} in *P. australis* isolates, during an earlier study (Rhodes *et al.,* 2002a). However, no iso-DA or DA was detected in the *P. pungens* and *P. fraudulenta* cultures following copper addition.

Spectroscopic information obtained thus far has established that iso-DA is a geometrical isomer of DA with unconjugated double bonds (Holland *et al.,* 2002). Once sufficient material is available, full chemical characterisation of iso-DA and a comparison with the DA isomers described from the red alga *Chondria armata* (Zaman *et al.,* 1997) will be carried out as well as comparative toxicity testing of the iso-DA against DA. Until the toxicity is known, iso-DA will be treated additively with DA for regulatory purposes in New Zealand.

Acknowledgements

Thanks to Krystyna Ponikla, curator of the Cawthron Micro-algae Culture Collection, the Cawthron phytoplankton and biotoxin monitoring teams for result updates, and Cath Ross, AgResearch, Hamilton, NZ, for ASP ELISAs. The use of Ministry of Health and Marlborough Shellfish Quality Programme testing results is acknowledged. This research was funded by the Foundation for Research Science and Technology (Contract No. CAWX0005).

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New Advancements in Detection and Structural Elucidation of Marine Biotoxins from Adriatic Mussels

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Abstract

A number of polyether toxins have been isolated and characterized in the course of our studies on mussels from Northern Adriatic Sea. Some of them represent new additions to the DSP-class of biotoxins and seem to be peculiar to the Adriatic sea. Their structures were elucidated by 1D and 2D NMR techniques in combination with MS experiments. A liquid chromatography-mass spectrometry (LC-MS) method for direct detection of yessotoxins was also proposed.

Introduction

DSP outbreaks, associated with blooms of harmful microalgae, have occurred in the Adriatic Sea with alarming frequency since 1989 (Boni *et al.,* 1990). These cause serious threat to human health and severe economic losses for shellfish industries. In order to prevent or minimize such damage, continuous monitoring of toxicity in shellfish and structure elucidation of the causative toxins are prerequisites. Instrumental analysis is indispensable for analyzing toxic shellfish, because toxin profiles may change in both their chemical structure and toxicological effects year after year. A research program based on instrumental analysis has been initiated in Italy since 1990 to carefully investigate DSP contamination in mussels from the Adriatic Sea. Since then, a number of polyether toxins have been isolated and characterized, some of which represent new additions to the DSP-class of biotoxins and seem to be peculiar to the Adriatic region (Fig. 1).

Materials and Methods

Toxic mussel samples were collected along the Cesenatico coasts (Emilia Romagna, Italy) when toxicity was detected. After each collection, the hepatopancreases were removed, homogenized with a Waring™ blender, and extracted with acetone at room temperature. The extracts obtained after removal of the solvent were dissolved in 80% MeOH and partitioned against *n*-hexane. The hydromethanolic layers were finally partitioned between 40% MeOH and CH_2Cl_2. Each dichloromethane soluble material was fractionated by repeated bioassay-guided column chromatography on ODS and Toyopearl HW-40 SF column. Final HPLC purification was done on a RP 18 column (Phenomenex-Luna 5u C18; 250 mm × 4.60 mm) using as eluent CH_3CN/MeOH/H_2O 1:1:2. UV lamp set at 230 nm was used to detect yessotoxins.

Results and Discussion

The toxin profile in Adriatic mussels has completely changed in the last years. In the early 1990s okadaic acid and its analogues were found to be responsible for human intoxication (Fattorusso *et al.,* 1992). Yessotoxins (YTXs) have become the predominant Adriatic toxins since 1995. In addition to yesssotoxin (YTX) (Ciminiello *et al.,* 1997), homoYTX, 45-hydroxyhomoYTX (Satake *et al.,* 1997), and 45-hydroxyYTX (Ciminiello *et al.,* 1999), we isolated and structurally determined several new analogues of YTX

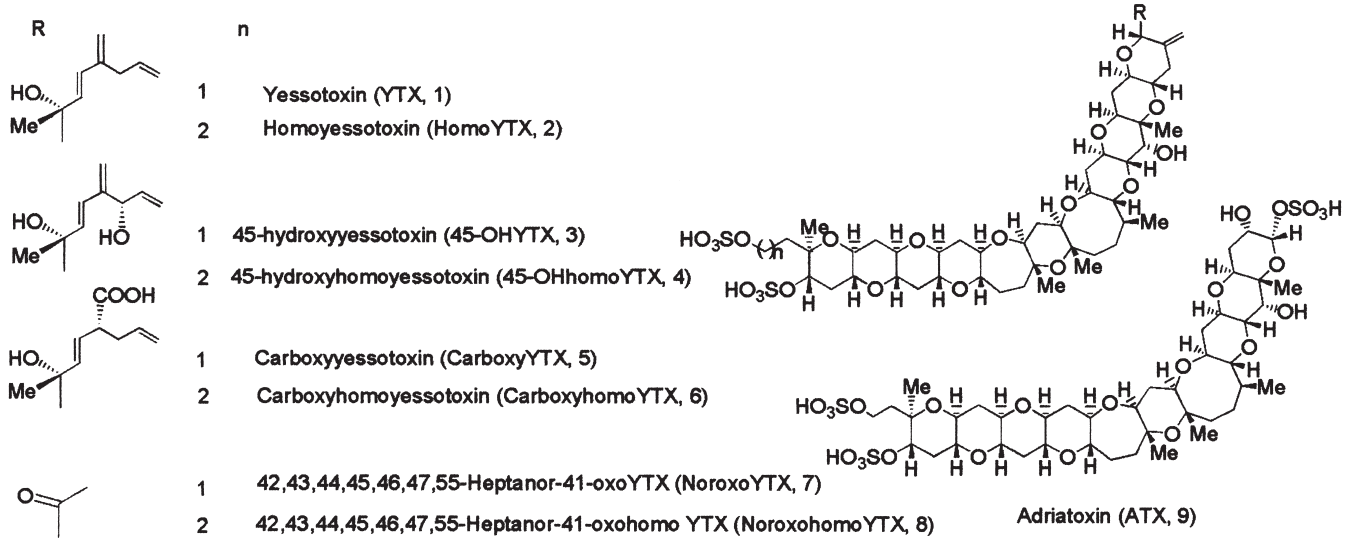

Figure 1 Structures of yessotoxins occurring in Adriatic mussels.

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from the hepatopancreases of mussels of the Adriatic sea, such as adriatoxin (Ciminiello *et al.,* 1998), carboxyyessotoxin (Ciminiello *et al.,* 2000a), carboxyhomoyessotoxin (Ciminiello *et al.,* 2000b) and 42,43,44,45,46,47,55-heptanor-41-oxohomoyessotoxin (Ciminiello *et al.,* 2001). All the new analogues have been isolated in pure form and their chemical stereostructures determined on the basis of spectral evidence, particularly 1- and 2-dimensional ^{1}H NMR, as well as MS and MS/MS experiments. These analogues represent new additions to the class of YTXs, and seem to be peculiar to the Adriatic Sea, since they have not been reported in any other country. Their toxicology remains to be investigated.

To decrease the time required for the analysis of toxic mussels, the combination of liquid chromatography and mass spectrometry (LC/MS) was considered. This approach is advantageous in that it detects intact, underivatized toxins and related compounds in relatively crude extracts of both shellfish and plankton samples. This technique was extremely appropriate for Adriatic toxins, since the most common analytical methods for detection of YTXs require derivatization of each toxin with a fluorescence label followed by HPLC analysis (Yasumoto *et al.,* 1997). Unfortunately, the method is not reliable for those derivatives which lack a conjugated diene functionality in the molecule. Therefore, we tested the suitability of the LC/MS method developed by Quilliam (2001) for detection of lipophilic toxins to separate and detect okadaic acid and all yessotoxins isolated so far. The technique used a single chromatographic run of 25 min and was both selective and sensitive, with a detection limit of 70 pg for YTX (Ciminiello *et al.,* 2002a). The method has a part-per-billion detection level and can detect the possible presence of the new analogues.

LC/MS data on the toxic mixture obtained from *Mytilus galloprovincialis* collected in 1998 from one sampling site located along the Emilia Romagna coast of Italy revealed that a novel YTX analogue was present in the mixture. An inspection of the MS/MS spectrum of the unknown peak showed the typical fragmentation pattern of the backbone skeleton of yessotoxin, but no sulfate loss was observed for this peak, thus suggesting the presence of only one sulfate ester group in the molecule. These data suggested that the peak under investigation was due to a desulfo-YTX, the only uncertainty being in the desulfated position. NMR experiments are required to unambiguously assign 1-desulfo- or the alternative 4-desulfo-YTX to the above peak. However, to the best of our knowledge, this is the first report of a desulfoYTX derivative from Italian mussels. Similarly the LC-MS analysis of DSP-infested mussels (*Mytilus galloprovincialis*) collected in June 2001 yielded the same materials. The total ion current (TIC) chromatogram showed a significant chromatographic peak of a potentially new analogue. Careful analysis of the LC-MS/MS spectra of this compound suggested it to be 42,43,44,45,46,47,55-Heptanor-41-oxoyessotoxin, the homologue in the YTX series of the noroxohomoYTX, that we have previously isolated and fully characterized. This hypothesis was supported by a comparison of the chromatographic and mass spectral properties of the involved compounds, which eluted in the same experimental conditions at almost the same retention time and whose MS/MS spectra appeared to be almost superimposable, as long as they were shifted of 14 mass units (Ciminiello *et al.,* 2002b). So, the proposed LC-MS method allowed us not only to hasten the analysis of toxic samples but also to advance effective structural hypothesis even when full structure elucidation of new toxins by NMR spectroscopy is hampered by the limited amount of available material.

In conclusion, our studies have revealed a very interesting, uncommon and changeable scenario of shellfish toxicity in Italy. The toxin profile in mussels from the Adriatic Sea differs from that of mussels from other countries where the DSP phenomenon has been deeply studied and where the new analogues of YTX have not been reported until now.

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Liquid Chromatography–Mass Spectrometry for the Analysis of Anatoxin-A, Homoanatoxin-A and Their Degradation Products

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Abstract

Anatoxin-a and homoanatoxin-a readily degrade in sunlight and high pH to non-toxic degradation products, dihydroanatoxin-a, dihydrohomoanatoxin-a, epoxyanatoxin-a and epoxyhomoanatoxin-a. Fluorescent derivatisation of the six anatoxins can occur with 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) which has led to the development of sensitive analytical methods with good chromatographic separation of six anatoxins under isocratic conditions. Liquid chromatography coupled to mass spectrometry (LC-MS) allows the rapid and sensitive determination of the anatoxins with minimal sample clean-up. This was achieved using an ion-trap spectrometer with MS^n capability. MS^{2-3} selectively detects the anatoxins and enhances the signal-to-noise in field samples. Analytical methods have been developed for both the free and derivatized toxins. LC-MS^n was applied to the rapid determination of anatoxins in cyanobacteria and freshwater samples collected throughout Ireland. Nano ESI quadrupole-time-of-flight (QqTOF) MS was used for confirmation of toxin identity and to aid assignments for the major fragment ions.

Introduction

Cyanobacterial neurotoxins have been implicated in animal deaths from drinking contaminated water (Skulberg *et al.,* 1984), (Carmichael, 1989), (Codd *et al.,* 1989). Anatoxin-a (AN) is produced predominantly by the genera *Anabaena, Planktothrix (Oscillatoria)* and *Aphanizomenon,* while homoanatoxin-a has been reported only in *Planktothrix formosa* (Skulberg *et al.,* 1992). Most studies of freshwaters contaminated by anatoxins from cyanobacterial blooms have been carried out using liquid chromatography with ultra-violet detection (LC-UV), but the toxin degradation products cannot be detected by this method (Zotou *et al.,* 1993). Anatoxin-a, 2-acetyl-9-azabicyclo[4.2.1]non-2-ene, is an alkaloid and was the first toxin from cyanobacteria to be structurally elucidated (Devlin *et al.,* 1977). It has a high toxicity and it is a potent nicotinic agonist (Carmichael *et al.,* 1979). Typical symptoms in animals include gasping and convulsion, and rapid death can occur due to respiratory arrest. HMAN differs from AN by having an additional methylene unit on the side-chain (Fig. 1A inset). HMAN

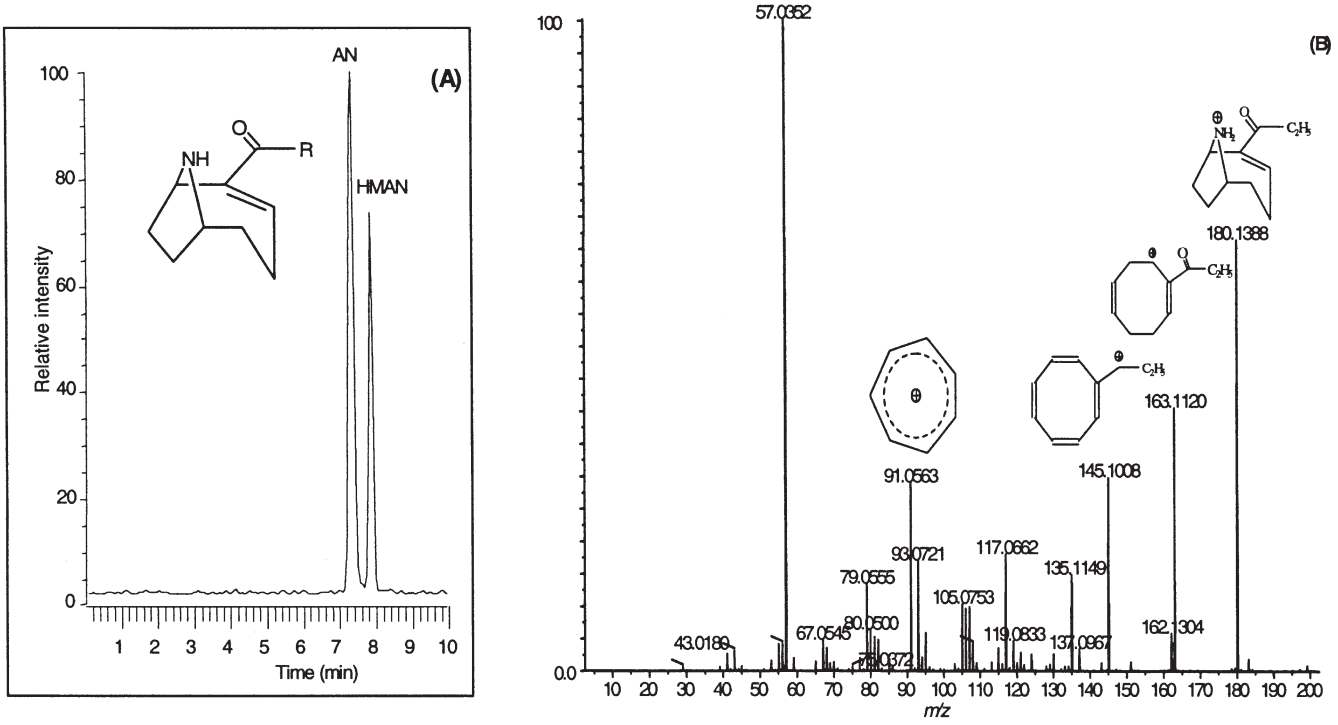

Figure 1 **A** Chromatogram showing the separation of anatoxin-a (AN, R = CH_3) and homoanatoxin-a (HMAN, R = C_2H_5) using LC-MS^2. **B** Spectrum and main peak assignments for HMAN using high mass accuracy nano ESI quadrupole-TOF MS.

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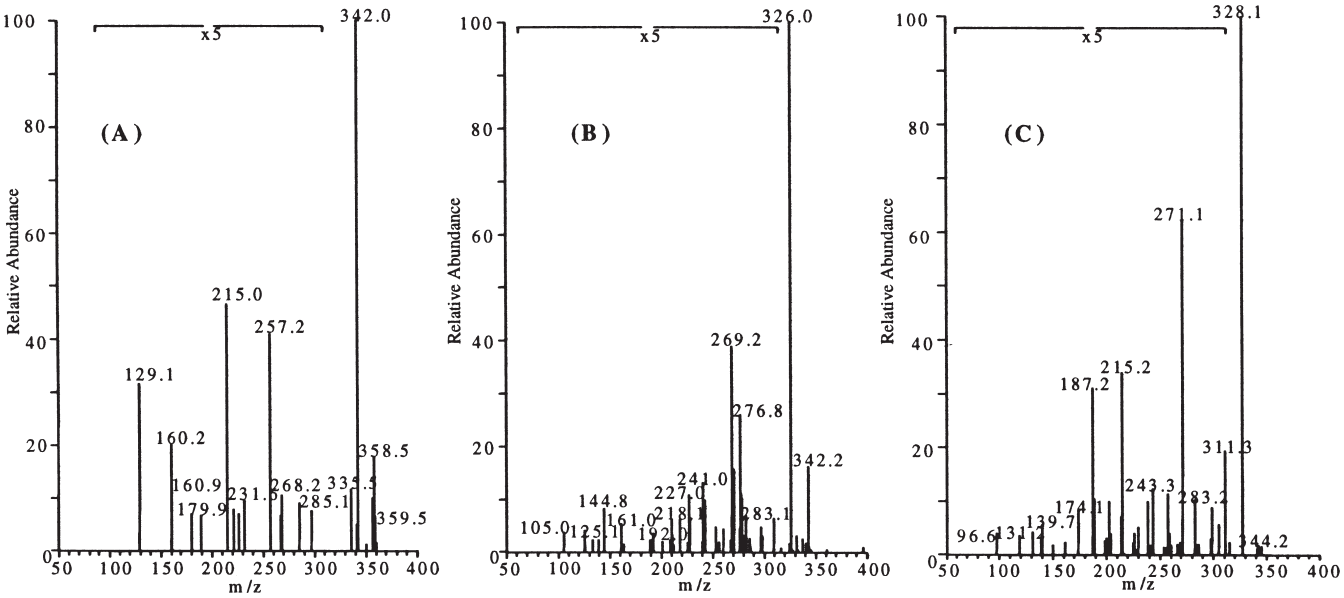

Figure 2 MS^2 spectra of derivatised products with NBD-F; **A** NBD-epoxyhomoanatoxin ($[M+H]^+ = 358$); **B** NBD-homoanatoxin-a ($[M+H]^+ = 342$); **C** NBD-dihydrohomoanatoxin ($[M+H]^+ = 344$).

has a similar toxicity to AN and both toxins have recently been found in Irish lakes (James *et al.,* 1997), (Furey *et al.,* 2003). AN degrades readily, especially in sunlight and at high pH, to dihydroanatoxin-a, epoxyanatoxin-a (Smith and Lewis, 1987). These non-toxic degradation products are undetectable using LC-UV, the most commonly used analytical method for anatoxins, and this may explain why there are few reports of these products. The most sensitive method for the simultaneous determination of AN, HMAN and their dihydro- and epoxy- analogs is fluorimetric LC which involves the facile derivatization using 4-fluoro-7-nitro-2,1,3-benzoxadiazole (NBD-F) (James *et al.,* 1998). A number of liquid chromatography-mass spectrometry (LC-MS) methods have been used for the determination of anatoxins in cyanobacteria (Hormazábal *et al.,* 2000), (Draisci *et al.,* 2001). This report discusses the application of electrospray multiple tandem MS (LC-MS^n) for the determination of anatoxins in cyanobacteria and water.

Materials and Methods

Standard anatoxin-a (AN) was purchased (Calbiochem-Novabiochem, Nottingham, UK) and HMAN was isolated from a culture of *Planktothrix formosa* (NIVA-CYA-92), as previously described (James *et al.,* 1998). LC-grade acetonitrile and water were purchased from Labscan (Dublin, Ireland). Anatoxins were extracted using a procedure similar to that published previously (Harada *et al.,* 1989). Liquid chromatography-multiple tandem mass spectrometry (LC-MS^n) was carried out using an Alliance 2690 LC (Waters Corporation, Milford, MA, USA), which was linked to an LCQ ion-trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). Isocratic chromatography was performed using acetonitrile-water (15:85) containing 0.05% TFA, at a flow rate of 400 µL/min using a C18 column (Luna-2, 250 × 4.6 mm, 5 µm, Phenomenex, Macclesfield, UK) at 35°C. MS analysis was performed using an electrospray ionization (ESI) source and data were acquired in positive mode. The MS was tuned using a standard solution (AN, 10 µg/mL), which was infused at 3 µL/min with monitoring of the $[M+H]^+$ ion at *m/z* 166. Multiple tandem MS produced collision-induced dissociation (CID) spectra, which were obtained by trapping the $[M+H]^+$ ion for each toxin and then used in subsequent fragmentation experiments to produce characteristic spectra for each toxin. The optimized relative collision energies (% RCE) were 30% for MS^2 and MS^3. Both AN and HMAN were determined using the following target parent and product ion combinations in the MS: AN: *m/z* 166, 149, 131, 107; HMAN: *m/z* 180, 163, 145, 135, 107 (Nano electrospray (ESI) quadrupole hybrid time-of-flight).

Results and Discussion

Positive ESI mass fragmentation of AN and HMAN targeted the molecular-related ion species, $[M+H]^+$, at *m/z* 166 (AN) and *m/z* 180 (HMAN). An advantage of the ion-trap MS is the ability to switch between a full-scan MS and MS^n scan of fragments without a significant loss in sensitivity. In this study, multiple tandem MS (MS^n) was used for the repeated trapping and fragmentation of ions. Interpretation of the spectra allowed the selection of candidate ions for subsequent MS experiments. The structure assignments for the major ions were confirmed using nano ESI quadrupole-time-of-flight (QqTOF) MS. These assignments and the corresponding ions for HMAN, determined at high mass accuracy, are shown in Fig. 1B. In MS^2 mode, AN produced a major fragment ion due to ammonia loss at *m/z* 149 $[166\text{-}NH_3+H]^+$; MS^3 yielded the fragment ions for AN at *m/z* 131 $[166\text{-}NH_3\text{-}H_2O+H]^+$, *m/z* 121, 107, 105, 91, 81 and 79.

Similarly, for HMAN the MS^2 gave major fragment ions at *m/z* 163 $[180\text{-}NH_3+H]^+$, *m/z* 145 $[180\text{-}NH_3\text{-}H_2O+H]^+$, *m/z* 135, 120, 107, 91. MS^3 spectra were obtained by trapping and fragmenting the ion at *m/z* 163. The ions at *m/z* 107 $[M\text{-}NH_3\text{-}COCHR]^+$ and *m/z* 91 $[C_7H_7]^+$ were observed for both toxins in the MS^3 spectra. Linear correlations were obtained for AN in each MS mode and sensitivity increased when using LC-MS^2 and LC-MS^3 compared to LC-MS. This improvement is attributed to the collapse in background signal in multiple MS modes. Five scan events were performed simultaneously: a) full scan MS, b) MS^2 (AN), c) MS^2 (HMAN), d) MS^3 (AN), e) MS^3 (HMAN). The selected ions were *m/z* 149 and *m/z* 163 in MS^2 with *m/z* 131 and *m/z* 145 selected in the MS^3 mode for AN and HMAN, respectively. These ions were generated by trapping and fragmenting the product ions from the MS stage, *m/z* 166 (AN) and *m/z* 180 (HMAN). Good reproducibility data were obtained for AN, using both LC-MS^2 and LC-MS^3 modes. The relative standard deviation (% RSD, n = 5) values were ≤2 at 5.0 µg/mL and ≤7.0 at 0.10 µg/mL, respectively. The detection limit (S/N = 3) was 0.6 µg/L. The chromatographic separation of AN, HMAN and their dihydro- and epoxy- analogs is problematic but the NBD derivatives of these compounds were completely resolved using reversed phase LC. The MS^2 spectra for HMAN and analogs are shown in Fig. 2. The characteristic spectra that were obtained for each of these compounds should be useful for the confirmation of the presence in homoanatoxin-a degradation products which have not yet been found in nature.

Acknowledgements

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Genotoxicity Investigation of Chlorinated Degradation Products of a Cyanobacterial Toxin, Cylindrospermopsin

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Abstract

Cylindrospermopsin (CYN), a potent cyanobacterial hepatotoxin produced by *Cylindrospermopsis raciborskii* and other cyanobacteria, is regularly found in water supplies in many parts of the world and has been associated with the intoxication of humans and livestock. Water treatment via chlorination can degrade the toxin effectively but result in the production of several byproducts. In this study, male and female Balb/c mice were injected via the intraperitoneal (IP) route with a single dose of 10 mg/kg 5-chlorouracil and 10 mg/kg 5-chloro-6-hydroxymethyluracil; these two compounds are the predicted chlorinated degradation products of CYN. DNA was isolated from the mouse livers and examined for strand breakage by alkaline gel electrophoresis (pH 12). The median molecular length (MML) of the DNA distributed in the gel was determined by estimating the midpoint of the DNA size distribution by densitometry. The toxicity of 5-chlorouracil (as measured by DNA strand breakage) was significantly influenced by time from dosing. There was no significant difference in MML between mice dosed with 5-chloro-6-hydroxymethyluracil and the controls. In another experiment, mice were dosed with 0, 0.1, 1, 10 and 100 mg/kg body weight 5-chlorouracil and 0, 0.1, 1, 10 and 20 mg/kg 5-chloro-6-hydroxymethyluracil via IP injection. The heart, liver, kidney, lung and spleen were removed, fixed and examined under electron microscopy. Liver was the main target organ. The EM results revealed marked distortion on the nuclear membrane of liver cells in mice dosed with 1.0 mg/kg 5-chlorouracil or 10 mg/kg 5-chloro-6-hydroxymethyluracil, or higher.

Introduction

Cyanobacteria may produce toxins that may present a hazard for drinking water safety. Cylindrospermopsin (CYN) is a potent cyanobacterial hepatotoxin produced by *Cylindrospermopsis raciborskii* and other cyanobacteria such as *Umezakia natans* and *Aphanizomenon ovalisporum* (Duy *et al.,* 2000; Shaw *et al.,* 2000). CYN is a tetracyclic alkaloid, possessing "a tricyclic guanidine moiety combined with hydroxymethyluracil" (Ohtani *et al.,* 1992). It has a molecular weight of 415 Daltons and is zwitterionic. It is therefore highly water-soluble, indicating that difficulties exist for removal of this toxin using conventional treatment techniques such as flocculation and filtration. Of the water treatment procedures, chlorination, possibly micro/ultrafiltration, and especially ozonation are the most effective in breaking down this cyanobacterial toxin (Hitzfeld *et al.,* 2000). Under experimental conditions using samples with a solution pH of 6–9, a residual chlorine concentration of 0.56 mg/L was sufficient to degrade >99% of CYN (Senogles *et al.,* 2000). This type of water treatment, however, may result in the formation of a number of chlorinated byproducts: one is 5-chlorocylindrospermopsin; the other predicted byproducts are 5-chlorouracil (5ClUra) and 5-chloro-6-hydroxymethyluracil (5Cl6HMUra) (Wickramasinghe *et al.,* 2001). In this study, the genotoxic potential of two predicted degradation byproducts of CYN, namely 5ClUra and 5Cl6HMUra, were examined.

Materials and Methods

Balb/c mice (2 months old) of 25–30g body weight were used for the dosing experiments. All mice were acclimatized for at least one week before experimentation. 5ClUra was purchased from Sigma Chemical Co. (St. Louis, MO, USA). 5Cl6HMUra was synthesized by Dr. W Wickramasinghe of NRCET. 5ClUra was dissolved in corn oil to produce 6 mg/mL, 600 µg/mL, 60 µg/mL and 6 µg/mL solutions. Via IP injection, mice were administered the above solutions to achieve doses of 0.1 mg/kg, 1 mg/kg, 10 mg/kg, 100 mg/kg, respectively. Mice dosed with pure corn oil were treated as the vehicle control for 5ClUra. 5Cl6HMUra was dissolved in saline and administered to mice via IP injection to achieve doses of 0 mg/kg, 0.1 mg/kg, 1 mg/kg, 10 mg/kg and 20 mg/kg. Mice dosed with saline were treated as the solvent control for 5Cl6HMUra. Mice were autopsied after 96 hr exposure and the liver, heart, spleen, kidneys and lungs of each mouse were put into 10 mL fixative solution (2.5% glutaradehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate trihydate, pH 7.8) and processed for electron microscopy examination. Mortality of the experimental animals was also recorded over the 96-hour period.

For the genotoxicity assays, male and female Balb/c mice were injected via the IP route with a single dose of 10 mg/kg 5ClUra or 10 mg/kg 5Cl6HMUra. These doses were chosen based on data from acute toxicity studies (Banker *et al.,* 2001) Animals were selected randomly and sacrificed at 6 hr, 12 hr, 24 hr, 48 hr and 72 hr. Livers were removed from the abdominal cavities of individual animals, frozen in liquid nitrogen and stored at –80°C until further analysis. DNA was extracted and purified with Proteinase K, phenol and chloroform treatment. Each DNA sample was subjected to electrophoresis under alkaline conditions (pH 12) following the procedures described in Shen *et al.* (2002). The median molecular length (MML) of the DNA distributed in the gel was determined by estimating the

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midpoint of the DNA size distribution by densitometry (Black *et al.,* 1996).

Eight DNA samples were examined for each treatment. A two-way analysis of variance (ANOVA) was used to test the null hypothesis that time from dosing and treatment of the degradation products does not cause significant change in DNA integrity (as measured by MML). In the event that ANOVA indicated a significant effect, the dataset was further analyzed using a pair-wise Tukey test. Statistical significance was accepted at $P < 0.05$.

Results and Discussion

No death occurred within the 96-hr exposure period with respect to either chlorinated degradation product. No apparent change in the appearance and behavior was observed in the exposed as compared to the control animals, with the exception that one mouse dosed with 20 mg/kg 5ClUra trembled and moved slowly inside the cage. On this basis, the second lowest dose (*i.e.,* 10 mg/kg) was used in the subsequent genotoxicity test for both degradation products.

Mouse bioassay indicates that the acute IP LD_{50} is 2 mg/kg after 24 hrs (Ohtani *et al.,* 1992) and 0.2 mg/kg after 5 days for CYN (Banker *et al.,* 2001; Ohtani *et al.,* 1992). In this study, the LD_{50} of 5ClUra and 5Cl6HMUra were at least 500 and 100 times higher than the LD_{50} of CYN, respectively. Based on these results, it is postulated that the intact pyrimidine ring is an essential molecular component for the toxicity of CYN and its degradation products. The hydroxyl group at position 7 in cylindrospermopsin is also essential for its acute toxicity as it has been shown that deoxycylindospermopsin (Norris *et al.,* 1999) is much less toxic than cylindrospermopsin. We therefore concluded that after water treatment with chlorine under appropriate condition, the acute toxicity of the water contaminated with CYN was reduced considerately to levels that should not produce unacceptable risks to humans in most situations.

No toxicological effects on heart, lung, kidney and spleen were observable based on electron microscopy. Histological alterations were, however, observed in the liver. Therefore, similar to CYN, the liver appeared to be the target organ for this compound. The most marked alteration was in the cell nuclei. Based on this observation, liver tissues from animals exposed to 0.1, 1 and 10 mg/kg 5ClUra were processed for further examination. In the control mice, the hepatocyte contained a round or oval nucleus with variable amounts of dispersed and peripheral heterochromatin and a single prominent nucleolus. The nuclear membranes were smooth and had numerous nuclear pores. There were no abnormal morphological changes in hepatocytes of the control mice. When mice were administered 0.1 mg/kg of the compound, appearance of all the nuclei still remained normal. When the mice were administered 1 mg/kg of the 5ClUra, fewer normal nuclei could be found. Nucleolus and condensed chromatins were attached to the nuclear membrane. Almost all nucleus membrane changed into irregular shapes. Based on the above morphological standpoint, it could be concluded that the threshold dose was 1 mg/kg. When the mice were administered 10 and 100 mg/kg, all the nuclei were damaged.

There were no abnormal changes in the group treated with 0.1 mg/kg 5Cl6HMUra compared with the control. In the 1 mg/kg group, polynuclei and dispersed heterochromatins could be observed though the shape of the nucleus remained normal. In the 10 mg/kg group, almost all the nuclei showed an irregular shape. In the groups dosed with 10 and 20 mg/kg 5Cl6HMUra, accumulation or aggregation of cell organelles could be found. From the standpoint of histological alterations, it could be concluded that the threshold dose was 1 mg/kg.

CYN toxicity in mice is often characterized by an appearance of foamy lipid vacuolation in the liver, and periacinar coagulative necrosis was also consistently observed. CYN also causes extrahepatic lesions involving the kidney, heart and thymus (Falconer *et al.,* 1999; Terao *et al.,* 1994). The main target of CYN was the liver, while thymus, kidneys and heart were also affected (Terao *et al.,* 1994). It has been suggested that the toxicity of CYN is associated with four consecutive phases of pathological changes in the liver. The initial phase was that of inhibition of protein synthesis; the second phase of membrane proliferation was followed by the third phase of fat droplet accumulation and finally, phase of cell death. In addition, ribosomes on the membrane of the rough surfaced endoplasmic reticulum in the hepatocytes were detached from the membrane and accumulated in the cytoplasm. Nucleoli in the nuclei of the hepatocytes became dense, rounded and reduced in size. The symptoms in the hepatocytes of mice dosed with 5ClUra and 5Cl6HMUra appeared to be different from those of CYN-intoxicated animals, suggesting that different toxicity mechanisms are involved for the degradation byproducts as compared to their parent toxin molecule (CYN). One of the established mechanisms of toxicity of CYN is inhibition of protein synthesis. No evidence has been produced that this mechanism is operating with the chlorouracils.

There was no consistent change in MML value over time for mice exposed to 5ClUra. A significant decrease in MML value was observed 6 hours post dosing, suggesting an impact on DNA integrity due to exposure to 5ClUra (Fig. 1). There is, therefore, some evidence that 5ClUra may be genotoxic, as would be expected due to incorporation of this uracil into DNA (Pal *et al.,* 1981). Furthermore, Pal *et al.* (1981) reported that mice exposed to 5ClUra through drinking water showed heavy incorporation of the base in the liver and testes DNA (1 in 250 nucleotides), although no obvious adverse effects were observed. Notwithstanding, possible long-term effects associated with the incorporation of 5ClUra into DNA molecules do deserve further investigation. Based on our results, there was no apparent effect on DNA integrity due to single exposure to 5Cl6HMUra (Fig. 2).

In conclusion, we have shown that potential chlorination byproducts of CYN have considerably reduced acute tox-

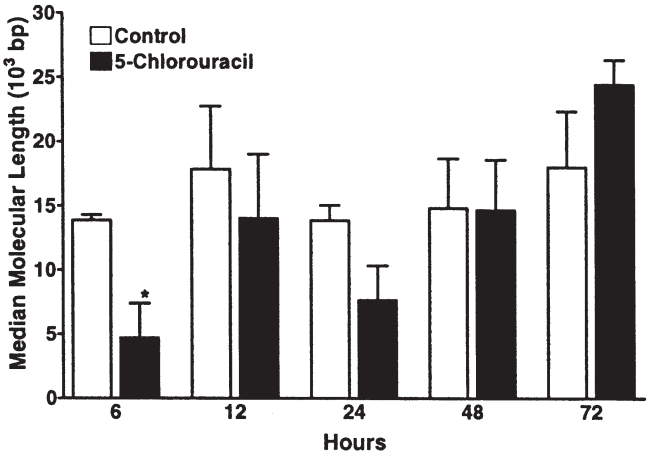

Figure 1 Median molecular lengths of DNA from exposed mice (treated with 10mg/kg 5-chlorouracil) and control mice (treated with 10 mg/kg corn oil) in different time course (n = 8). Significance level: * = $P < 0.05$.

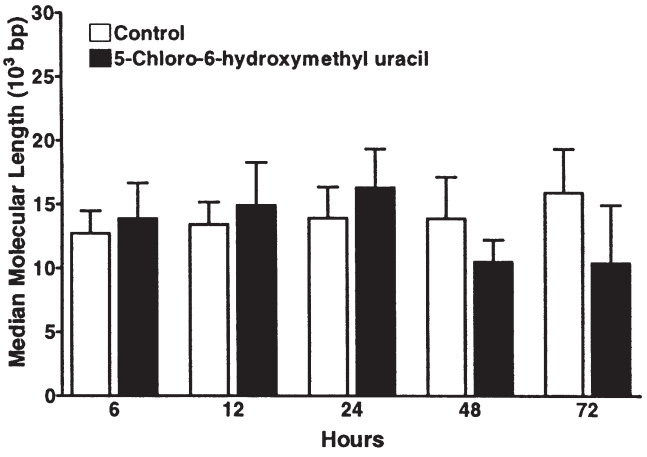

Figure 2 Median molecular lengths of DNA from exposed mice (treated with 10 mg/kg 5-chloro-6-hydroxymethyluracil) and control mice (treated with 10 mg/kg saline) in different time course (n = 8).

icity compared with CYN, but that incorporation into DNA is likely to result in the observed nuclear changes and possible effects on DNA integrity. Overall, our findings cannot eliminate the possibility that some degradation products may have genotoxic potential, and suggest that this aspect should be thoroughly investigated to provide for assessing the risks associated with cyanobacterial toxins and their derivatives following treatment by chlorination.

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Laboratory Analyses of Nutrient Stress and Toxin Accumulation in *Pseudo-nitzschia* Species from Monterey Bay, California

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Abstract

Here we present results from laboratory experiments using *Pseudo-nitzschia* spp. isolated from Monterey Bay, California, grown under constant temperature and irradiance. We demonstrate substantial clonal variability and variable toxicity depending on harvest time and nutrients. We also show that Si-limitation can directly affect photosynthetic performance and looks functionally like iron and nitrogen limitation as assessed by variable fluorescence (decreasing variable fluorescence with increasing stress). This response negatively correlates with domoic acid accumulation in batch and chemostat experiments.

Introduction

There have been at least two recent occurrences (1998 and 2000) of toxigenic strains of *Pseudo-nitzschia* spp. in Monterey Bay, California (Scholin *et al.,* 2000). Studies conducted on these bloom events have shown evidence of trophic transfer of the phycotoxin domoic acid (DA) to marine seabirds and mammals (Gulland *et al.,* 1999; Scholin *et al.,* 2000. Despite the frequent occurrence of blooms and the large number of studies (both field and laboratory), there is still no conclusive link to a specific "trigger" of toxin production. This may be in part because of the enormous variability in the ambient oceanographic conditions or because of the range of physiological responses evident from natural isolates of *Pseudo-nitzschia* spp. Here we demonstrate that the natural range of responses to nutrient stressors and effects of culturing methodology may account for much of the observed variability in laboratory and field analyses.

Materials and Methods

Data presented represent growth of *Pseudo-nitzschia* spp. in f/2 medium enriched with silicic acid (batch cultures), Si-limited medium (continuous cultures) and synthetic seawater medium (semicontinuous batch cultures). For batch culture experiments, seventeen toxigenic strains of *Pseudo-nitzschia* (*P. multiseries* and *P. australis*) isolated from Monterey Bay, CA in 2001–2002 were made available courtesy of M. Hughes (UCSC). Clones were grown in one liter of medium with f/2 enrichment, and were maintained under 12 h light (ca. 100 µmol photons m^{-2} s^{-1}) at 15°C. Cultures were monitored daily for cell density using *in-vivo* chl *a* fluorescence. Successive transfers were made in exponential growth. For testing DA accumulation in two strains of Mu-411P, eight transfers in log phase were made, and at the final transfer half the cells were harvested for DA content. The remainder was kept in the same conditions for 48 hours. The experiment was then terminated, and DA content was measured on these cells in early stationary phase. Cell numbers were calculated from a regression of *in-vivo* chl *a* fluorescence measurements and cell counts conducted by microscopy.

Chemostat runs were carried out using a uni-algal non-axenic strain of *P. australis* (Au-221A) and *P. multiseries* (Mu-6 and Mu-411P). 750 mL plexiglas water-jacketed

Table 1 Growth parameters from isolates maintained under identical conditions.

Isolate	Clone ID	Isolation Date	Max Biomass (cells/mL)	Mean growth rate (d^{-1})	S.D.
P. australis	Au-211M	Feb-02	15727	0.76	0.26
P. australis	Au-211J	Feb-02	16167	0.66	0.05
P. australis	Au-211K	Feb-02	16991	0.33	0.18
P. australis	Au-211D	Feb-02	15287	0.61	0.16
P. australis	Au-211B	Feb-02	18146	0.54	0.07
P. australis	Au-211L	Feb-02	17321	0.40	0.24
P. australis	Au-1004V	Oct-01	19630	0.60	0.18
P. australis	Au-211A	Feb-02	13198	0.26	0.25
P. australis	Au-211C	Feb-02	17101	0.47	0.10
P. australis	Au-1004W	Oct-01	17266	0.58	0.14
P. australis	Au-1004A	Oct-01	17156	0.40	0.25
P. australis	Au-1004F	Oct-01	18036	0.46	0.16
P. multiseries	Mu-411A	Apr-01	27436	0.60	0.23
P. multiseries	Mu-411I	Apr-01	25567	0.35	0.13
P. multiseries	Mu-411O	Apr-01	20949	0.48	0.03
P. multiseries	Mu-411P	Apr-01	21994	0.59	0.12
P. multiseries	Mu-420B	Apr-01	26776	0.48	0.24

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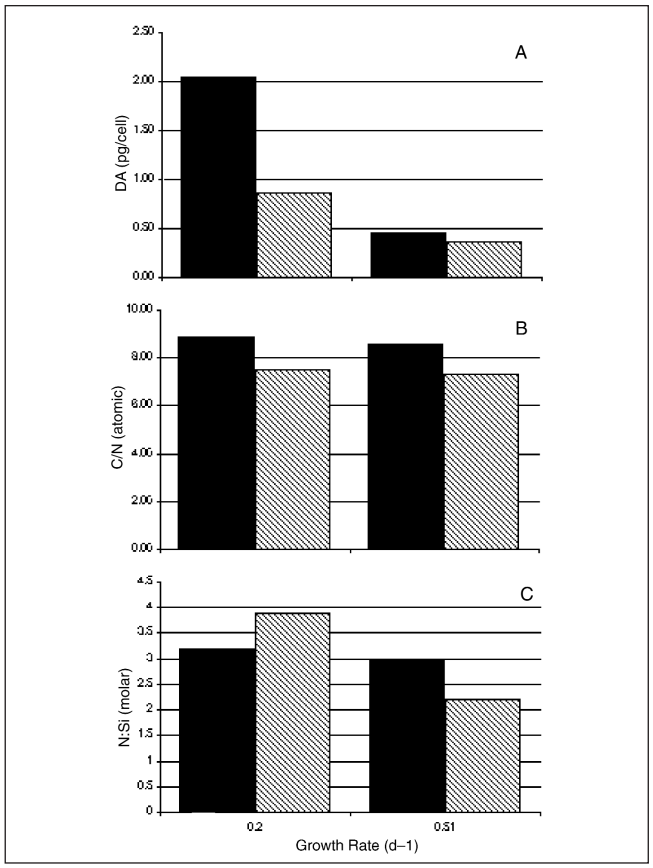

Figure 1 Biochemical composition of chemostats (Mu-411P; solid; Au-221A, striped) harvested early (3 days; solid) and late (10 days; striped) during steady-state. **A**: DA per cell. **B**: C:N composition. **C**: Si:N composition.

cylindrical chemostat vessels were maintained under 24 h light (ca. 100 µmol photons $^{-2}$ s^{-1}) at 15°C, and monitored daily for cell density using fluorescence and cell counts. Dilution rates were calculated by measuring the effluent volume collected from the vessels daily. The growth media consisted of 0.2 µm filtered seawater augmented with L1 nutrient additions or f/2 nutrient additions limited by silicic acid. The chemostats were run at variable dilution rates (ca. 20%-80% of µmax). For analysis of harvest date on toxin accumulation (Fig. 1), one set of chemostats was harvested at 3 days steady-state (constant cell density), and the second 7 days later, using Mu-411P and Au-221A. Data in Fig. 2 are for Mu-6 and Mu-411P; *P. australis* show similar trends (data not shown).

For chemostat/batch experiment presented in Fig. 3, replicate semicontinuous cultures of *Pseudo-nitzschia multiseries* (Mu-411P) were maintained in exponential growth phase over a 29 day period in Guillard's f/2 enrichment medium with 50 µM silicic acid (same as for the chemostats), but with no nutrient limitation (growth was controlled by washout). After reaching steady state, an aliquot was harvested for DA, variable fluorescence, and other parameters (below), and the pumps were shut off, allowing the cultures to enter batch (Si-limited) conditions. A subset of the sample was harvested daily for variable fluorescence, DA, and

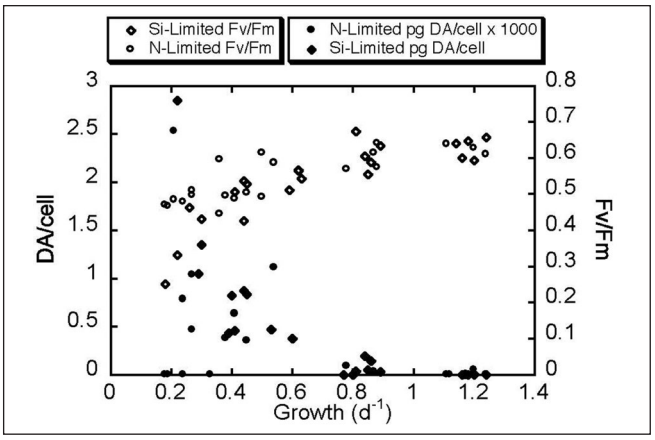

Figure 2 Particulate DA and Fv/Fm plotted versus growth rate for N-limited and Si-limited chemostats, Mu-411P and Mu-6. N-limited DA values are fg/cell, Si-limited values are pg/cell.

cell density. Cell numbers were determined using optical density (800 nm) with a subset of cell counts to validate the optical density measurements. Successive additions (4, 8, and 10 days from entering batch mode) of silicic acid (days 4 and 8) or f/2 trace-metal stock (day 10) were added to assess nutrient stress.

Samples collected for most experiments also include pigments, macronutrients, biogenic silica, and particulate CHN. Because of detection issues, dissolved DA was not available for every sample. When measured, dissolved concentrations were typically <1% of the total DA in chemostats; in batch cultures, dissolved DA was typically <10% of the total but was more variable. For this contribution, particulate DA values are reported. Particulate DA concentrations were determined using the FMOC-HPLC method described by Pocklington *et al.* (1990). Variable fluorescence (Fv/Fm) may be considered an indicator of cell health, or impairment of the photosystem II system, and is a direct estimate of photosynthetic competency (Schreiber, 1986; Kolber *et al.,* 1998). Estimates of variable fluorescence were conducted using a Walz PAM 101/102/103 instrument and validated with DCMU-excitation fluorescence. Samples were dark-adapted >30 min. at 15°C for Fo estimates (Schreiber, 1986; Lippemeier *et al.,* 1998).

Results and Discussion

Clonal Variability In this study, 17 clonal isolates from Monterey Bay were maintained under identical growth conditions. There is a high degree of variability in growth of different clones of *Pseudo-nitzschia* isolated from the same location on the same day and reared under identical temperature and irradiance (Table 1). Mean growth rates were similar for *P. multiseries* and *P. australis* (Table 1), although *P. australis* maximal cell densities were typically lower.

Domoic Acid Accumulation Cellular DA content in semicontinuous cultures of *P. multiseries* after eight consecutive transfers show that Si-limitation can trigger up to a 30-fold increase in particulate DA concentrations from late exponential to early stationary phase (0.1–0.3 pg/cell vs. 2.5–3.2

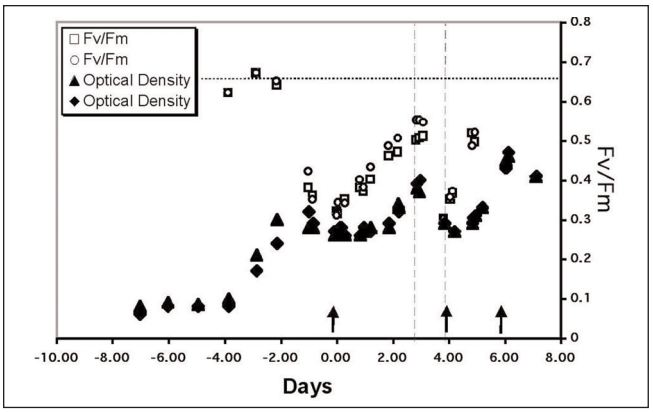

Figure 3 Replicate Si-limited chemostat-batch (Mu-411P). The horizontal dashed line indicates maximum Fv/Fm value in healthy cultures. Arrows indicate silicic acid (day 0, 4) and trace-metal (day 6) additions. Vertical dashed lines indicate dilution with unenriched medium.

pg/cell). Strain and species variability is also pronounced, with typical early stationary particulate DA values ranging from <0.1 to >15 pg/cell for isolates grown under the same conditions. To determine whether the observed increase in DA content per cell in the semicontinuous batch transfers was a function of growth rate or acclimation, we conducted a series of chemostat experiments at low (0.2 d^{-1}) and high (0.51 d^{-1}) growth rates. By harvesting "early" (3 days in steady-state) vs. "late" (10 days), particulate DA again changed as a function of growth rate, with up to a threefold decrease in toxin accumulation (Fig. 1). The chemostats exhibited essentially constant cell density, pigment per cell (not shown), and elemental composition (Fig. 1). The dramatic changes in DA suggest that physiological adaptation (*i.e.,* toxin production) takes much longer to stabilize than commonly used estimators of steady-state, such as density and biochemical composition (Fig. 1).

Variable Fluorescence Si-limited and N-limited chemostats (*P. multiseries* clone Mu-6) were maintained under a range of nutrient-limited growth conditions (Fig. 2). Variable fluorescence was measured after harvesting, again using the criteria of 3 days with cell densities within one standard deviation, and showed a good relationship (negative correlation) for both growth rate and toxin accumulation under both N and Si limitation. Although N-limited cultures were ca. 1000× lower in DA accumulation, the general pattern (increasing accumulation with decreasing growth rates) was similar to Si-limitation. These results suggest that variable fluorescence could be a useful indicator for toxin accumulation. It also strongly suggests that Si-limitation of diatoms impacts photosynthetic performance, possibly due to regulatory feedback mechanisms (*e.g.,* Lippemeier *et al.,* 1998) or through inhibition of a carbon-concentrating mechanism (*e.g.,* Milligan and Morel, 2002).

To determine whether Si-limitation was really affecting photosynthetic performance, duplicate chemostats of *P. multiseries* clone Mu-6 were maintained until steady-state was achieved and then grown as batch cultures (Fig. 3). The cells were allowed to become Si-limited and were then pulsed (2×) with silicic acid additions (ca. 10 μM), demonstrating that Si-limited Fv/Fm variability is inducible and reversible. Trace-metal additions (Fig. 3) showed no response in any measured parameter, indicating that although the Fv/Fm signal was similar to the response expected from iron limitation (Behrenfeld *et al.,* 1996), there was no apparent metal stress.

Conclusions

In this study we demonstrated that there is variability in growth among different clones and species of *Pseudo-nitzschia,* isolated from the same area and grown under identical conditions. Si limitation correlates with increased DA accumulation in batch, semicontinuous, and continuous cultures, as expected. There was a surprising amount of variability in DA accumulation, however, depending on exactly when the cells were harvested, which was not well correlated with cell density or biochemical composition. Variable fluorescence is a good indicator of nutrient stress and presumably DA production, with Fv/Fm declining as DA accumulation increases. We suggest that cellular toxicity may largely be dependent on the culturing methods and may take much longer to stabilize relative to other physiological parameters. This highlights the importance of consistent methodology and careful intercomparisons when analyzing different strains and experiments.

Acknowledgements

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Pseudo-nitzschia australis, Mytilus edulis, Engraulis anchoita, and Domoic Acid in the Argentine Sea

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Abstract

In July 2000, a bloom of *Pseudo-nitzschia australis,* identified by Transmission Electron Microscopy (TEM), reached an abundance of 2.8×10^4 cells L^{-1} at a fixed monitoring station near Mar del Plata adjacent to the Argentine Sea. Domoic acid (DA) was confirmed by HPLC in natural samples of phytoplankton, in mussels (*Mytilus edulis*), and in muscle and the gastrointestinal contents of anchovies (*Engraulis anchoita*). The maximum load of DA detected in the mussels (7.7 μg g^{-1}) was below the regulatory level for human consumption (20 μg g^{-1}). The concentration of this neurotoxin was high in the gastrointestinal content of anchovies (76.6 μg g^{-1}), which also contained *P. australis* frustules, while muscle concentrations were lower (4.9 μg g^{-1}). Bloom development coincided with a decrease in temperature (from 17 to 10°C), homogeneization of the water column during winter, and when salinity values were at a maximum (33.9–34.1‰). Nutrient concentrations were high throughout the water column. These results confirm that natural populations of *P. australis* produce significant amounts of DA and that this neurotoxin is transferred to benthic and pelagic filter-feeding organisms. These results are discussed in comparison with similar events in other areas of the world.

Introduction

Pseudo-nitzschia australis was first described by Frenguelli (1939) in the San Matías Gulf, Argentina (42°15′S, 62°13′W) and identified by Hasle (1965) in samples from Quequén (38°30′S, 59°W). Sar *et al.* (1998) later confirmed the species designation by studying the Frenguelli samples. In the Argentine Sea, most of the earlier records of *Pseudo-nitzschia* were identified as *Nitzschia seriata* and *N. delicatissima,* but these have been considered erroneous by Ferrario *et al.* (1999) because electron microscopy was not used for identification. Studies on the distribution of *Pseudo-nitzschia* species in the Argentine Sea were intensified (Negri and Inza, 1998; Ferrario *et al.,* 1999) when Amnesic Shellfish Poisoning (ASP) became increasingly evident globally. Since 1998, whenever *Pseudo-nitzschia* species are detected in the plankton samples, the content of domoic acid (DA) in mussels is determined at INIDEP. DA was first confirmed in 2000 (Montoya *et al.,* 2000).

Among the species producing DA, *Pseudo-nitzschia australis* is known for its harmful effects on biota by transferring toxins through pelagic fish, *e.g.,* the massive death of sea lions in Monterey Bay (Bates, 2000; Scholin *et al.,* 2000). The most important pelagic fish in the Argentine Sea is the anchovy *Engraulis anchoita,* which occurs from southern Brazil (24°S) to Patagonia (48°S), and is a key species providing food for other fish, marine birds, and mammals (Hansen, 2000). Although anchovies are almost exclusively zooplanktivorous, anchovy stomach contents containing only diatoms, including *Pseudo-nitzschia australis* (formerly identified as *Nitzschia pseudoseriata* in Angelescu [1982]) have been documented (Fig. 1–Anchovies 7 October 1978).

The objective of this study was to assess the associated environmental conditions when a *P. australis* bloom occurred in an area near its type locality. The transfer of toxins by the species to higher trophic levels, both benthic (mussels) and pelagic (anchovies), was also investigated. These results were compared to similar events elsewhere.

Materials and Methods

Oceanographic cruises were conducted at a fixed monitoring station (EPEA station, 38°28′S, 57°41′W) throughout the year 2000 (Fig. 1). Temperature and salinity profiles were measured with a CTD (Seabird SBE 19). Qualitative samples of phytoplankton were collected by a 25 μm mesh net.

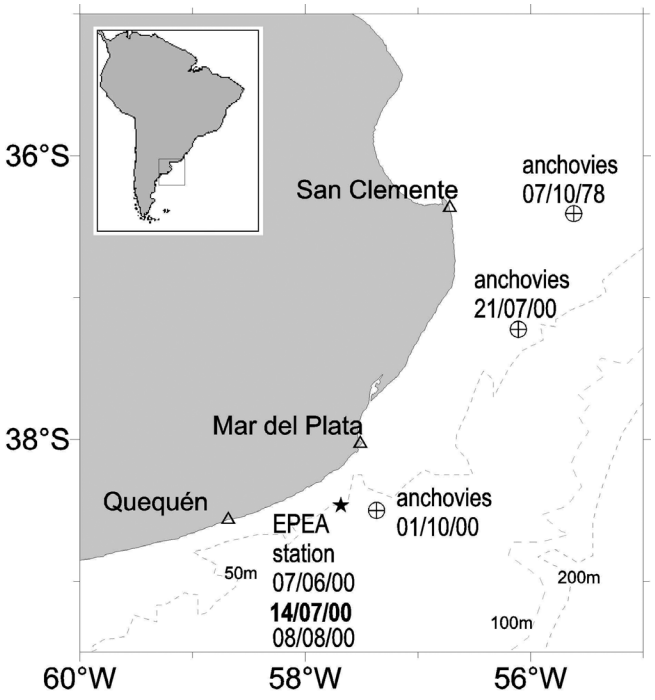

Figure 1 Position of the Estación Permanente de Estudios Ambientales (EPEA station) and the anchovy samples.

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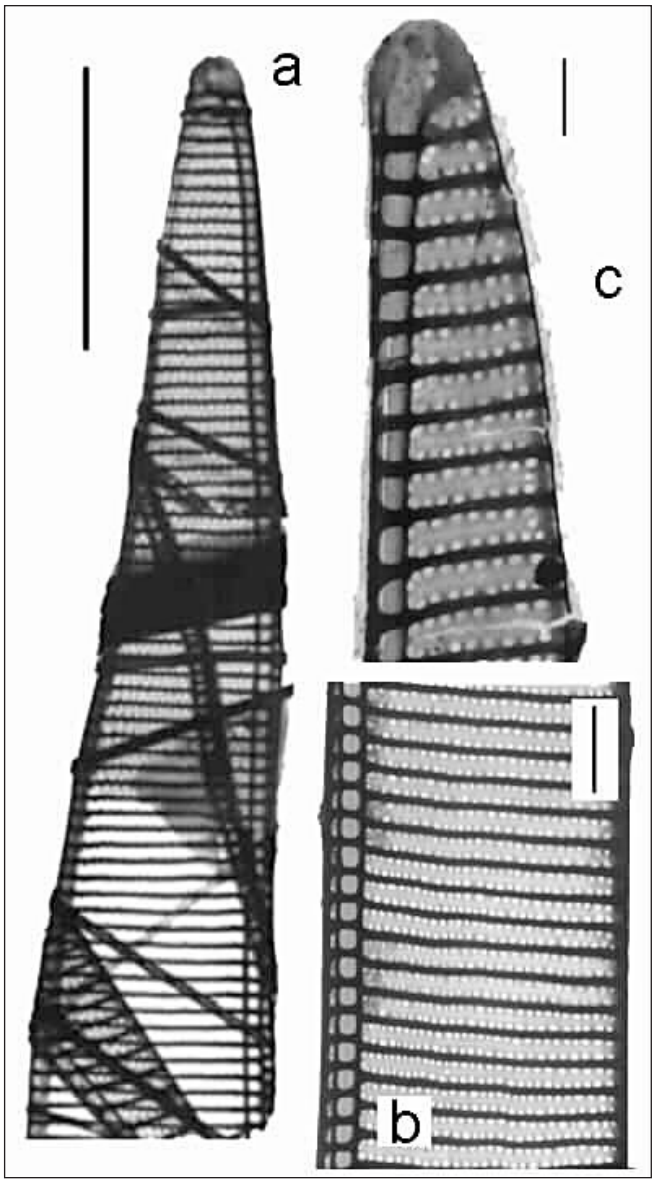

Figure 2 *Pseudo-nitzschia australis,* TEM. **a**: Valve view; **b**: Central part of the valve showing poroids in two rows; **c**: View of the valve pole. Scale bars: **a**, 10 µm; **b**, 5 µm; **c**, 1 µm.

Water samples were collected at different depths with Niskin bottles for the determination of chlorophyll *a* and for a quantitative analysis of phytoplankton. Species at each depth were enumerated with an inverted microscope (Olympus IX70) after sedimentation. Samples for transmission electron microscopy (TEM-Hitachi HU 11C1) were cleaned and prepared according to Ferrario *et al.* (1999) prior to observation. Chlorophyll *a* was measured using the fluorometric method (Holm-Hansen *et al.,* 1965). Mussels (*Mytilus edulis*) were sampled at the EPEA station by dragging while anchovy samples were collected during stock assessment cruises in the area. DA concentrations were determined by High Performance Liquid Chromatography (HPLC) with diode array detection (Wright and Quilliam, 1995).

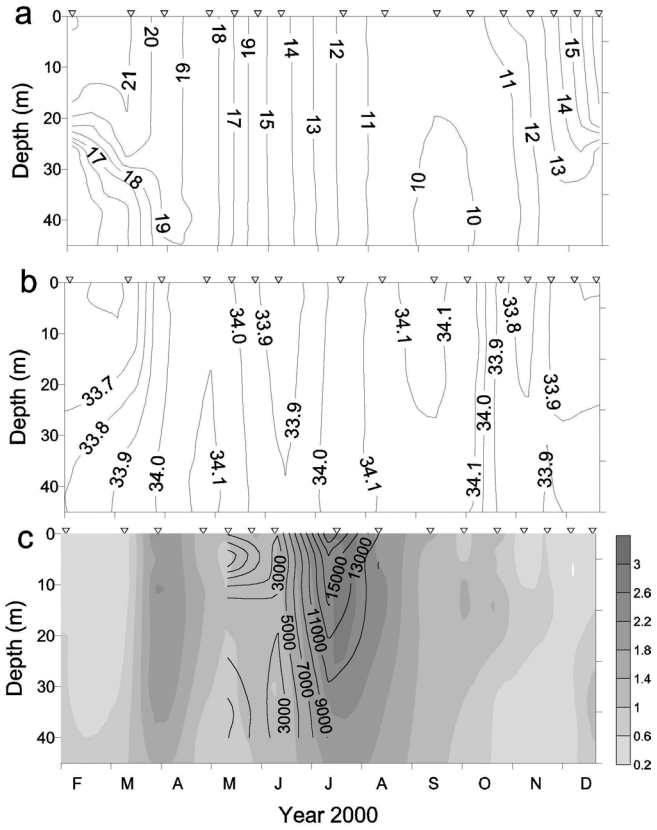

Figure 3 Temporal profiles of temperature (°C), salinity (‰), and chlorophyll *a* (µg L^{-1}) and *Pseudo-nitzschia australis* concentration (cells L^{-1}) at the EPEA station.

Results and Discussion

Increasing cell concentrations of one species of *Pseudo-nitzschia,* later identified by TEM as *P. australis* (Fig. 2), were recorded from May to July throughout the surveyed period. The occurrence of *P. australis* coincided with a temperature decrease from 17° to 10°C (Fig. 3a) and with a largely homogeneous water column ($\Delta\sigma_t$: 0.037 to 0.003 between bottom and surface). The maximum abundance (2.8×10^4 cells L^{-1}) was registered at temperatures from 11° to 12°C and salinity values that were the highest reached during the year (33.9 to 34.1 ‰) (Fig. 3b). The period of maximum cell concentration was also characterized by a high nutrient availability (Carreto *et al.,* this Proceedings).

Previous quantitative data of *P. australis* in the Argentine Sea indicated cell counts of $<10^4$ cells L^{-1} from June to November (Negri and Inza, 1998). Ferrario *et al.* (1999) recorded the species in coastal waters between Mar del Plata and San Clemente during winter (June–July) and also by the end of summer (February–March). The sample from which Frenguelli performed the original description of the species was dated 29 September 1932, and those from Quequén analyzed by Hasle (1965) were collected by Professor Enrique Balech in June 1929 and August 1961. These results, in agreement with those of the present study, indicated that the development of the species at this area

takes place principally during the winter season. Bates *et al.* (1998) cited the occurrence of *P. australis* during the warm season (end of summer and fall) and during spring-summer related to upwelling events. Scholin *et al.* (2000) found the highest concentrations at the beginning of spring (May) in Monterey Bay. Findings by Trainer *et al.* (2001) have shown a succession of harmful *Pseudo-nitzschia* species and toxicity events along the west coast of USA, taking place off California during spring (May) and moving northwards up to Washington State during summer (August). This succession is apparently related to several upwelling systems located along the coast that may favor the blooming of these species.

Phytoplankton biomass at the EPEA station showed two peaks; the first in autumn, immediately after the thermocline breakdown, with the most abundant taxa, diatoms *Hemiaulus* and *Chaetoceros,* and the second in July–August, reaching a maximum value of chlorophyll *a* of the period (3.3 μg L^{-1}, Fig. 3c). Then, the dominant species were *P. australis* and *Coscinodiscus waillesii* (4 July), *P. australis* and *Chaetoceros socialis* (8 August) and *Leptocylindrus danicus* (8 September). On all these three sampling dates, diatoms represented 80–90% of the phytoplankton biomass (μgC L^{-1}, estimated from cellular volume).

Domoic acid was found in net phytoplankton samples at the EPEA station concomitant with the highest abundance of *P. australis* recorded throughout the period (14 July). At the same time, DA (7.7 μg g^{-1}) was also detected in *Mytilus edulis.* A few days later (21 July), the gastrointestinal contents of *Engraulis anchoita* fished within the area (Fig. 1) contained the same species, *P. australis* and *C. waillesii,* found in water samples collected at the EPEA station. The muscle and the gastrointestinal contents of anchovies analyzed by HPLC yielded DA concentrations of 4.9 μg g^{-1} and 76.6 μg g^{-1}, respectively. Anchovies migrate to the Mar del Plata fishing area during winter, coinciding with the occurrence of *P. australis* in the plankton (Hansen, 2000; Negri and Inza, 1998). DA was not detected in a subsequent anchovy sample analyzed (Fig. 1–Anchovies 1 October), when *P. australis* was absent from the water column. Anchovies constitute an important prey item for penguins, other marine birds, and mammals in the Argentine Sea. Nevertheless, until now there has been no evidence of toxin transfer to these higher trophic levels.

Results of *P. australis* concentrations in the water and of toxin levels in anchovy stomachs found in this study are comparable with those reported by Scholin *et al.* (2000). When DA concentration in anchovy stomach contents was 76.6 μg g^{-1}, the abundance of *P. australis* in plankton samples varied between 1.3 and 2.8×10^4 cells L^{-1}. This DA concentration is comparable to the approximate 4.0×10^4 cells L^{-1} in the plankton estimated by Scholin *et al.* (their fig. 1a). These authors were able to conduct an intensive sampling throughout the whole bloom period (ca. 1 month), identifying and quantifying different *Pseudo-nitzschia* species. Although plankton samples in our study correspond only to a fixed location, the presence of *P. australis* and *C. waillesii* in the stomach contents of anchovies in the nearby area suggests an expansion of the phenomenon.

Results by Scholin *et al.* (2000) and Trainer *et al.* (2001) have shown the harmful effect of *Pseudo-nitzschia* species on the biota. The wide distribution of *P. australis* found on the Atlantic coast of South America (Odebrecht *et al.,* 2001; Negri and Inza, 1998; Ferrario *et al.,* 1999), together with this finding of DA toxicity in *M. edulis* and *E. anchoita* on the Buenos Aires shelf area, shows a potential risk of toxin transfer to higher trophic levels in the region. Toxicity events in the biota may have been missed because of the large extent of the coastal area, the width of the Argentine continental shelf, and the scarce human population along the coast.

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Study on Domoic Acid in Portuguese King Scallops (*Pecten maximus*)

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Abstract

The king scallop, *Pecten maximus,* is a bivalve mollusc from the North Atlantic that retains a residual toxicity of domoic acid (DA) for long periods of time. Scallops have a high commercial value in several countries. However, in Portugal they are not much exploited, and they could represent an important alternative resource. Since August 2001, evaluation of DA toxin accumulation in *P. maximus* harvested from the Portuguese west coast, Setúbal area, has been carried out by HPLC-UV with a diode array detector. The anatomical distribution of toxicity was studied. The highest DA concentration was found in the hepatopancreas followed by the male gonads, which presented levels about ten times lower (hepatopancreas >> male gonads > gills > mantle > female gonads > adductor muscle). The hepatopancreas had 77% of the total amount of toxins. Seasonal variability in toxicity was also detected. A decrease in the toxin content during the winter months was found in all organs, with the toxin concentration in the hepatopancreas remaining the highest and always above the regulatory limit of 20 ppm. These data show that if the hepatopancreas is removed, the total content of DA becomes lower than the regulatory limit and scallops can be commercialised.

Introduction

Domoic acid (DA), the compound responsible for amnesic shellfish poisoning (ASP), was first studied in Portuguese shellfish in mid-1995. In accordance with that study, DA monitoring was introduced in Portugal in 1996 (Vale and Sampayo, 1996). Monitoring of phytoplankton, including blooms of *Pseudo-nitzschia* spp., has been carried out since 1986 (Sampayo *et al.,* 1997). In 2001, a king scallop, *Pecten maximus,* bed was found in the Setúbal area. Only certain organs from this species, such as the adductor muscle and the gonads, are generally consumed, which makes it difficult to regulate harvesting and processing. The regulatory limit for DA is 20 µg DA/g tissue; however, species like *Pecten maximus* and *P. jacobeus* may be harvested with a concentration of DA in the whole body exceeding 20 µg/g but lower than 250 µg/g, under restrictive conditions (Commission Decision 2002/226/EC). In order to evaluate DA toxin accumulation in Portuguese king scallops between August 2001 and May 2002, HPLC-UV analyses were carried out on the whole body and on separate tissues.

Materials and Methods

Sample Collection As part of the monitoring program since August 2001, scallop samples were collected in the Setúbal area (1 mile offshore, 23 m deep) in August, September, and November of 2001 and in February, April, and May of 2002 (designated as samples A to F, respectively).

Toxin Extraction and HPLC Analysis In order to determine ASP toxicity in each sample, HPLC-UV analyses were carried out on homogenates of 10 scallops each of the whole soft body, whole body with the hepatopancreas (HP) removed, and the edible parts (muscle and gonads). Domoic acid distribution in different tissues (hepatopancreas, adductor muscle, female and male gonads, gills, and mantle) was also evaluated, analyzing each tissue of each scallop individually. Special care was taken with the adductor muscle and gonads to prevent contamination from other tissues. No black material was left either on the adductor muscle or gonads. Extractions were carried out according to the method of Quilliam *et al.* (1995) with some modifications (Vale and Sampayo, 2001).

Results and Discussion

The variability of DA content in the scallop samples collected during the study period is presented in Fig. 1. In August (sample A) and September 2001 (sample B), a maximum concentration of DA was found. A decrease in DA

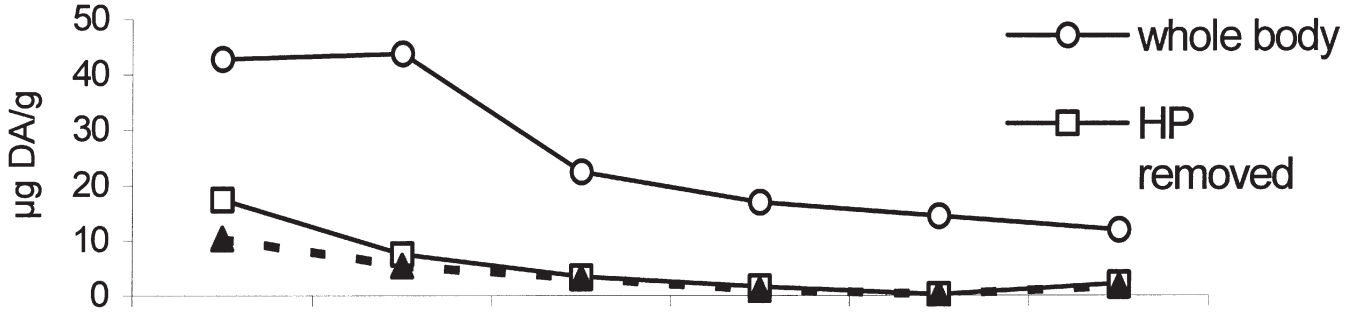

Figure 1 Seasonal variability of DA concentration in the scallops (whole body, whole body with HP removed, and the edible parts). Sample A = August 2001, Sample B = September 2001, Sample C = November 2001, Sample D = February 2002, Sample E = April 2002, Sample F = May 2002.

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Table 1 DA concentrations (mean + SD, mg/g tissue) detected in different scallop tissues of the six monthly samples.

Sample	Date	HP	M	FG	MG	G	MT
A (n = 26)	2001/08/02	286.1 ± 103.6	7.7 ± 4.4	16.2 ± 13.8	33.9 ± 20.5	24.6 ± 15.9	35.3 ± 14.2
B (n = 20)	2001/09/12	371.4 ± 134.0	4.3 ± 3.2	6.7 ± 4.7	15.9 ± 9.0	20.1 ± 14.1	9.5 ± 7.1
C (n = 10)	2001/11/28	158.0 ± 59.3	2.7 ± 2.2	4.0 ± 2.5	9.8 ± 7.3	7.6 ± 3.5	2.2 ± 0.9
D (n = 7)	2002/02/12	139.2 ± 49.4	1.0 ± 1.3	0.6 ± 0.3	3.8 ± 2.1	3.7 ± 4.1	1.6 ± 1.3
E (n = 4)	2002/04/13	123.5 ± 42.6	nd	nd	1.1 ± 1.0	0.3 ± 0.3	nd
F (n =8)	2002/05/14	46.2 ± 23.4	1.3 ± 0.7	2.6 ± 0.8	4.3 ± 2.1	4.9 ± 2.1	4.0 ± 2.5

HP: hepatopancreas; M: adductor muscle; FG: female gonads; MG: male gonads; G: gills; and MT: mantle. nd: not detected.

content was observed after this time, corresponding to the winter and spring months. From February 2002 (sample D), DA levels were below the regulatory limit in the whole body.

HPLC-UV analyses were also carried out on the different tissues of each scallop. High DA concentrations were found in the hepatopancreas (HP), with the highest concentration of 625 µg DA/g tissue being detected in a scallop from sample B. The other organs had a toxin content ten times lower (Table 1). The male gonads (MG) had the second highest DA level, followed by the gills (G) and the mantle (MT). The lowest DA concentrations were detected in the female gonads (FG) and in the adductor muscle (M). Between individuals of the same sample, the DA concentration found in the different organs varied widely from one scallop to another, especially in the HP.

In the main edible part, the adductor muscle, DA was never detected above the regulatory limit. However, when the adductor muscle was combined with the gonads, an increase in DA content was observed. The highest DA concentration was found in the HP of sample B scallops, but it was in scallops from sample A that the adductor muscle and gonad combination revealed higher DA concentrations. These results show the difficulty in predicting the toxicity of the edible parts (adductor muscle and gonads) (Fig. 2).

Because the main toxin accumulation was determined to be in the HP, a sharp toxin decrease was found after this organ was removed from the whole body samples (Fig. 1). In all monthly samples, DA concentration was lower after HP removal. Nevertheless, sample A showed DA concentrations close to the regulatory limit, reaching 17.4 µg DA/g. The highest difference in DA content was found in sample B. When only the edible parts were analysed, results were lower than the regulatory limit in all samples.

From August 2001–May 2002, 77% of the total amount of toxin was accumulated in the HP. In the most toxic samples it was observed that DA was shared with the other organs. In sample A, 60% of the toxin was found in the HP, about 20% in the MT and 10% in the M (Fig. 3). Although high DA concentrations have been found in the MG, this organ, due to its small size, was only responsible for 2% of the total toxin accumulation. The percentage of DA content in the HP was much higher during periods of low toxin concentration (Fig. 1), in some cases accounting for almost 100% of the toxin measured (Fig. 3).

The DA values found in the present study are not as high as those obtained by Arévalo et al (1998) in Spanish scallops. The highest concentration we have detected in the whole soft body was 43.8 µg DA/g, well below the limit of 250 µg DA/g that determines some harvesting restrictions (Commission Decision 2002/226/EC). Even in the period from February to May 2002, DA levels in the whole body were found to be under the regulatory limit, which suggests that this location may be an interesting one to develop for scallop aquaculture.

Some Portuguese shellfish resources are over-exploited (Gaspar *et al.,* 2002) so scallops can represent an alterna-

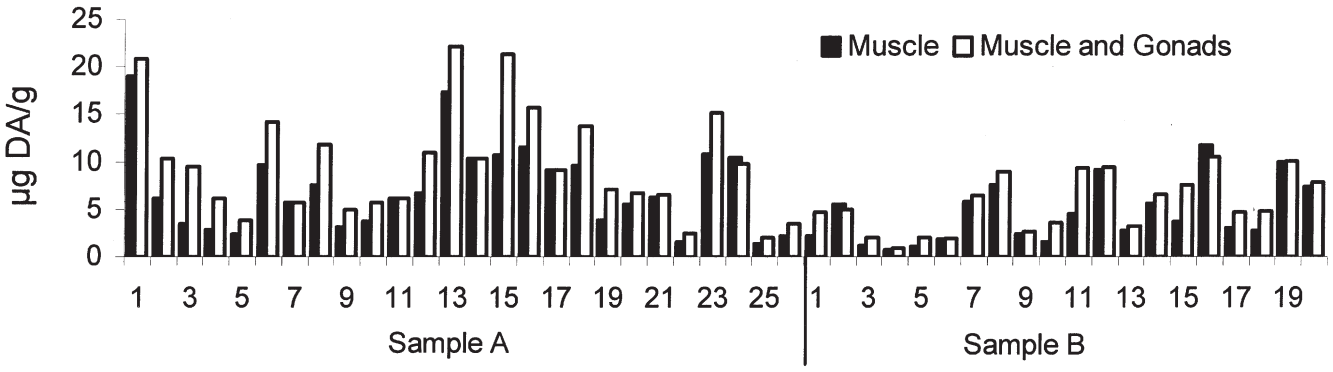

Figure 2 DA concentration in the adductor muscle and in the combination of adductor muscle with gonads, from scallops of sample A and B.

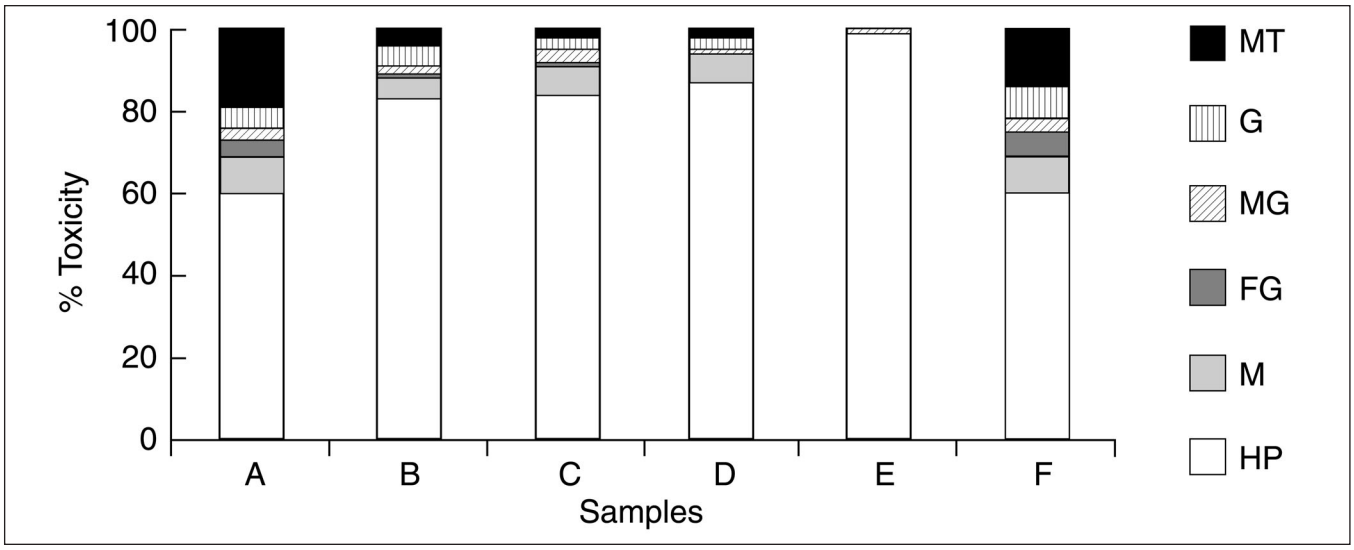

Figure 3 Toxicity percentage among the different scallop tissues in the six samples studied between August 2001 and May 2002.

tive resource with a high commercial value. As shown in our results, marketing of whole scallops may be a risk to public health due to the high DA concentrations that were detected. Because the HP is responsible for the majority of the total amount of toxin present in scallops, its removal may lead to safe levels. Safe marketing of the adductor muscle and gonads is feasible under a close regulatory regime to monitor their DA content to ensure public health safety.

Acknowledgements

The Portuguese Foundation for Science and Technology (FCT) supported this study through a doctoral grant to the first author. Programs "Sanitary Health of Bivalve Molluscs" and "Safety, Surveillance and Quality of Bivalve Molluscs" (QCAIII/med.4/PROPESCAS Program) also supported this work.

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Geographic Strain Variation in Toxin Production in *Karlodinium micrum* (Dinophyceae) from Southeastern Estuaries of the United States

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Abstract

A total of 17 cultured isolates and natural bloom samples containing the ichthyotoxic dinoflagellate *Karlodinium micrum* were screened for the presence of recently discovered toxic compounds to test the hypothesis that the main toxin from Chesapeake Bay, Maryland, USA, isolates and water samples differed from isolates and water samples collected from other Southeastern estuaries of the United States. For the Chesapeake Bay, it was found that in four cultured isolates and four bloom samples, two with fish kills and two without, KmTx 1 was the main toxin in terms of amount and potency. For samples collected from North Carolina, South Carolina, and Florida estuaries, in seven isolates and two water samples (both collected during fish kills), KmTx 2 was the main toxin. KmTx 2 was not detected in any KmTx 1-producing strains, and KmTx 1 was not detected in any KmTx 2-producing strains. Based on this data, there does appear to be a geographic strain variation in toxin production among *K. micrum* populations from Southeastern estuaries of the United States.

Introduction

For decades, *Karlodinium micrum* (Leadbeater *et* Dodge) Larsen has been associated with fish mortalities in temperate latitudes worldwide (Braarud 1957; Nielsen 1993; Landsberg, 2002). Recently in the United States, high densities (>30,000 cells mL^{-1}) of this organism have been observed to co-occur with fish mortalities, both aquaculture and non-aquaculture related, typically in shallow, poorly flushed, estuarine tributaries (Deeds *et al.,* 2002; Kempton *et al.,* 2002; Goshorn *et al.,* this Proceedings).

In an attempt to determine the cause of repeated fish kills in an estuarine aquaculture facility in Maryland, USA, we have recently shown that *K. micrum* produces a unique suite of compounds (putatively called karlotoxins) which possess hemolytic, cytotoxic, and ichthyotoxic properties (Deeds 2003; Deeds *et al.,* 2002). In collaboration with Kempton *et al.* (2002), we found that the main toxin (KmTx 2) isolated from South Carolina, USA, cultured isolates and fish kill samples was similar but not identical to the main toxin isolated previously from several Maryland, USA, isolates (KmTx 1). This study was undertaken to test the hypothesis that there is a geographic strain variation in toxin production (KmTx 1 vs. KmTx 2) among *K. micrum* isolates from estuaries of the southeastern United States. To accomplish this, eleven *K. micrum* isolates were acquired from estuarine waters ranging from Maryland to Florida. In addition, we were fortunate to acquire and test six *K. micrum* natural bloom samples, four of which were collected during fish kills.

Materials and Methods

The following *K. micrum* isolates were cloned by single cell isolation: GE (syn. CCMP 1974) and GE 2-1 (syn. CCMP 1975), both acquired from D. Stoecker, Horn Point Environmental Laboratory, Cambridge MD; MD5CR0599 and MD6CR0599, both acquired from M. Johnson, Horn Point Environmental Laboratory, Cambridge MD; and 010410-B1 (syn. CCMP 2282), 010410-C6 (syn. CCMP 2283), and JW020205-B4, acquired from J. Wolny, South Carolina Department of Natural Resources. All were grown at 20°C, under 100 $\mu E\ m^{-2}s^{-1}$ illumination, in f/2-Si-enriched artificial sea water (Instant Ocean Brand), either 12 or 32 PSU depending on original isolation, plus 1% soil extract, prepared according to Deeds *et al.* (2002). All were harvested for toxin extraction / identification, at cell concentrations ca. 1×10^5 cells mL^{-1}, according to procedures described in Deeds *et al.* (2002). Additional *K. micrum* isolates Cell J, HR1NovC4, PIM05JulC4, and F205AprD2 were all grown at 23°C, under 50 $\mu E\ m^{-2}s^{-1}$ illumination, in Gulf Stream, USA, water diluted to 15 psu. Each of these four isolates were grown in parallel either autotrophically, by adding f/2-Si nutrient mixture, or mixotrophically, by periodically adding *Rhodomonas* (CCMP 767) as the sole food source. All were harvested at concentrations of $3–5 \times 10^4$ cells mL^{-1} by gentle filtration using Whatman GF/F filters. Toxin extraction / identification was performed on frozen and thawed 500 mL filtrate samples, according to procedures described in Deeds *et al.* (2002). Four water samples, collected by the Maryland Department of Natural Resources and Department of the Environment, were also included in this study. Two were collected during fish kills associated with high densities (>30,000 cells mL^{-1}) of *K. micrum,* and the other two were collected during routine sampling. Additional information on these samples is contained in Table 1. Another water sample associated with a fish kill was sent to our laboratory (frozen) for analysis by researchers at the North Carolina State University Pamlico Aquaculture Field Laboratory. The sample had been collected five days after a large unexplained kill of hybrid striped bass in a pond receiving water from South Creek, a tributary of the Pamlico River, North Carolina, USA. On the day of the kill, *K. micrum* densities were reported to be ca. 3.5×10^4 cells mL^{-1}. Fish kills due to *K. micrum* had not previously been reported from this facility. The final two samples were collected during a mixed fish kill in a South Carolina brackish retention pond and contained 7×10^4 cells mL^{-1} *K. micrum.* Isolate

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Table 1 *K.micrum* samples screened for presence of KmTx 1 and KmTx 2.

Map Code	Isolate / Sample	Collection Location	Isolation Date	Salinity (PSU)	Main Toxin	Map Code	Isolate / Sample	Collection Location	Isolation Date	Salinity (PSU)	Main Toxin
1	CCMP 1974	Chesapeake Bay, MD	5/95	12	KmTx1	9	Cell J[e,f]	Neuse River, NC	7/99	15	KmTx2
2	CCMP 1975	HyRock Farm, MD	7/96	12	KmTx1	10	Water Sample[g]	NCSU Field Lab	6/02	6	KmTx2
3	MD5CR-0599	Choptank River, MD	5/99 2282	12	KmTx1	11	CCMP	Hilton Head, SC	3/01	32	KmTx2
4	MD6CR-0599	Choptank River, MD	5/99 2283	12	KmTx1	12	CCMP	Hilton Head, SC	3/01	32	KmTx2
5	Water Sample[a]	Fishing Creek, MD	6/02	9	KmTx1	13	Water Sample[h]	Charleston, SC	2/02	11	KmTx2
6	Water Sample[b]	Sharp's Island, MD	4/02	15	KmTx1	14	JW020205-B4[i]	Charleston, SC	2/02	15	KmTx2
7	Water Sample[c]	Oak Creek, MD	6/03	6	KmTx1	15	F205Apr D2e	St. Johns River, FL	4/01	15	KmTx2
8	Water Sample[d]	Goose Creek, MD	7/03	8	KmTx1	16	PIM05-JulC4e	St. Johns River, FL	7/00	15	KmTx2
						17	HR1Nov-C4e	St. Lucie River, FL	11/00	15	KmTx2

[a]Mixed fish kill, 4×10^4 cells mL^{-1} *K. micrum*. [b]Routine sampling, 7×10^3 cells mL^{-1} *K. micrum.* [c]Suspect area, no fish kill, 1.6×10^5 cells mL^{-1} *K. mirum.* [d]Fish kill (3600 silversides), 5.7×10^4 cells mL^{-1} *K. micrum.* [e]Two samples each, one autotrophic, one mixotrophic. [f]Genbank numbers AF352365, AF352367 (Litaker *et al.,* 2003). [g]Sampled five days after kill, 3.5×10^4 cells mL^{-1} *K. micrum* during kill. [h]Mixed fish kill, 7×10^4 cells mL^{-1} *K. micrum.* [i]Isolated from fish kill (h), kill described in (Kempton *et al.,* 2002).

JW020205-B4 was cultured by single-cell isolation from this water sample by J. Wolney (SC DNR). Prior to processing in our lab, all isolates and water samples were positively identified as containing *K. micrum* using a modified Taqman assay with PCR specific probes as described in Tengs *et al.* (2001).

Results

For all of the isolates and water samples collected from the Chesapeake Bay, Maryland, USA, KmTx 1 was found to be the main hemolytic toxin, in terms of amount and potency. For all other isolates and water samples collected from North Carolina, South Carolina, and Florida, KmTx 2 was found to be the main toxin (Table 1). KmTx 1 was not detected in any KmTx 2-producing strains and KmTx 2 was not detected in any KmTx 1-producing strains.

The estimated amount of toxin per cell for all of the isolates tested varied greatly (ca. 0.1–1 pg $cell^{-1}$). No trends in amount of toxin per cell were found between isolates containing KmTx 1 vs. KmTx 2 as the main toxin, nor were any trends observed for the amount of toxin per cell compared to length of time in culture. Compared to cultured isolates, the estimated amount of toxin per cell for water samples tested in this study, with the exception of the North Carolina fish kill sample, was substantially greater (5–12 pg $cell^{-1}$). The sample sent from the NCSU Aquaculture Field Laboratory, collected five days after the fish kill, contained trace amounts of KmTx 2 only. The isolates, Cell J, F205AprD2, PIM05JulC4, and HR1NovC4, were each found to contain KmTx 2 as the primary toxin regardless of trophic state. Furthermore, although the amount of toxin per cell varied among individual isolates, within the range previously reported for cultures, no major differences were observed for the amount of toxin per cell based on their mode of nutrition.

Discussion

In this study, *K. micrum* clonal isolates and bloom samples, from sites both with and without fish kills, were screened from the Chesapeake Bay, Maryland; The Neuse and Pamlico river estuaries, North Carolina; Charleston and Hilton Head, South Carolina; and the St. Johns and St. Lucie river systems, Florida (Fig. 1). After testing a total of eleven isolates and six bloom samples, we found that KmTx 1 was the main toxin in cells from the Chesapeake Bay and KmTx 2 was prevalent in all other southeastern USA samples.

KmTx 1 (formerly Tox A), along with another hemolytic fraction (Tox B), which has not yet been fully purified, was first discovered as part of an investigation into the cause of repeated fish kills in Maryland, USA associated with blooms of *K. micrum* (Deeds *et al.,* 2002). KmTx 2 was first discovered as part of an investigation into the cause of a mixed fish kill along a tributary of Charleston Harbor, South Carolina, USA (Kempton *et al.,* 2002). Using model vertebrate systems, the biological activities of KmTx 1 and KmTx 2 appear to be similar (Fig. 2, unpublished data), yet these two compounds have distinct HPLC retention times and differing spectral characteristics (UV max: 224 nm for KmTx 1 and UV max: 235 nm for KmTx 2) (Kempton *et al.,* 2002). It now appears that KmTx 1 and 2 also have distinct geographic distributions.

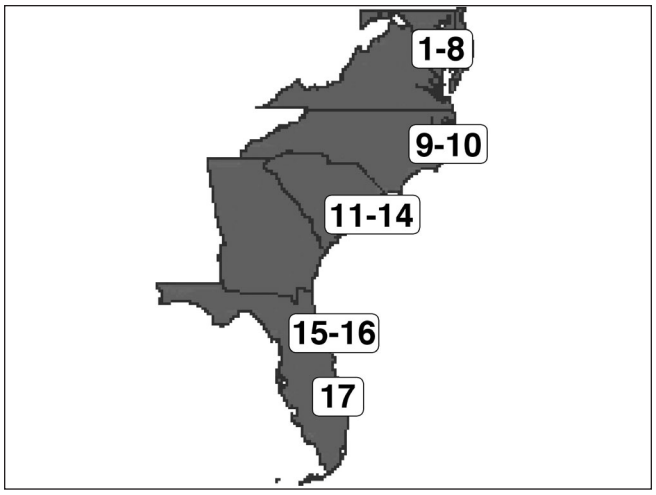

Figure 1 Southeastern coast of the United States. Numbers represent sampling locations and correspond to Table 1.

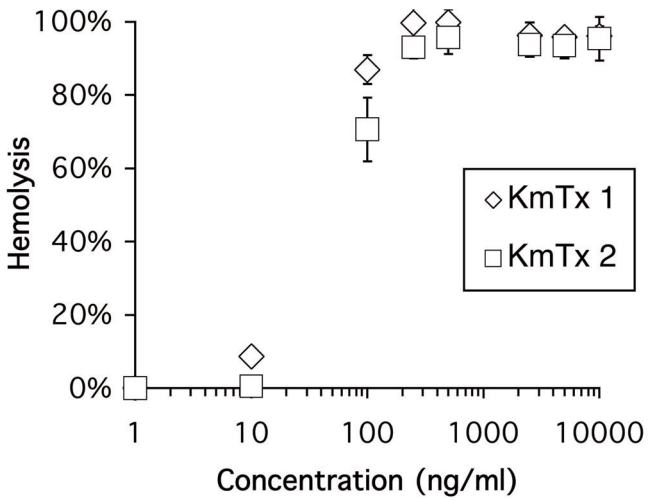

Figure 2 Dose response curves for KmTx1 and KmTx2 based on lysis of rainbow trout erythrocytes. (n = 3) bars = S.D.

An interesting observation from this research has been that water samples containing high concentrations of *K. micrum* collected directly from waters in which a fish kill had recently occurred contained 5–100 times the amount of toxin, on a per cell equivalent, than all of the cultured isolates we have tested thus far. Due to the fact that both KmTx 1 and KmTx 2 are easily released from cells, it has been difficult to assess the exact amount of toxin contained within a cell as opposed to being present in the surrounding water. By simply measuring the amount of hemolytic activity present in undisturbed cultures it has been estimated that >90% of the toxins are typically stored within the cells (Deeds 2003; Deeds *et al.,* 2002). However, due to these technical difficulties it cannot be discriminated whether the high amounts of toxin present during fish kills are due to higher toxin-producing populations of *K. micrum* or prolonged toxin build up in these waters due to cell disruption. Once released from cells and into the culture media containing cellular debris and all associated bacteria, hemolytic activity is lost (at room temperature) over a period of 24–48 hours (Deeds 2003; Deeds *et al.,* 2002). Therefore, it is unlikely that strains with low toxin production could generate sufficient toxin to reach levels observed to be associated with fish kills, particularly at the warm temperatures (>20°C) at which this organism typically blooms (Li *et al.,* 2000; Goshorn *et al.,* this Proceedings).

Continued research into the factors regulating *K. micrum* blooms and toxin production may yield insights into why these observed strain variabilities occur.

Acknowledgements

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The Production of Brevetoxin and Brevetoxin-Like Compounds During the Growth Phases of *Karenia brevis*

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Abstract

Blooms of the marine dinoflagellate *Karenia brevis* are associated with red tides in the Gulf of Mexico. Interestingly, the toxicity of blooms, as determined by the number of fish killed, does not always correlate with the cell counts of *K. brevis.* In this study, cultures of *K. brevis* were allowed to grow for a period of six and a half weeks and cell counts were taken twice a week. Quantitative HPLC analysis of cell culture extracts was performed for PbTx-2, PbTx-3 and AJB6.0P, a non-icthyotoxic compound that is a competitive inhibitor of brevetoxin binding to rat brain synaptosomes. The production of PbTx-3 did not significantly change throughout the observed time period. The production of PbTx-2 did show significant difference between lag phase and day thirty-five in stationary phase. The production of AJB 6.0P did significantly change from lag to stationary phase or log to stationary phase.

Introduction

Brevetoxins, the group of neurotoxins thought to be responsible for fish kills during Florida red tides, are broadly classified into two groups based on structural differences in the polyether backbone: A-type brevetoxins (*e.g.,* PbTx-1) and B-type brevetoxins (*e.g.,* PbTx-2). It has been observed that there is variability in the production of different toxins among different clones of the same species of *K. brevis* (Baden *et al.,* 1988). It has also been shown that there is a significant change in the ratio of PbTx-2 to PbTx-3

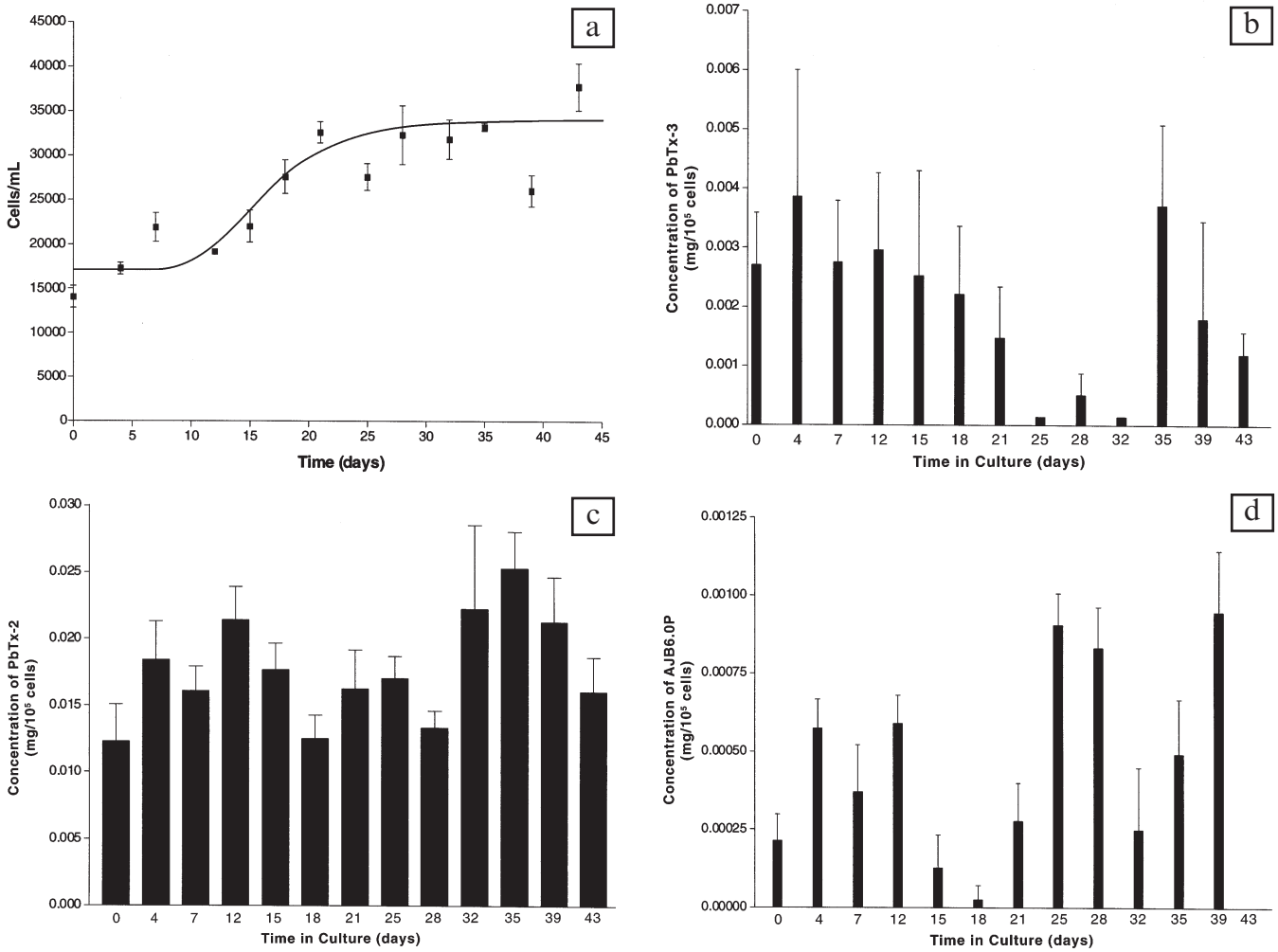

Figure 1 Effects of time in culture on cell number and production of PbTx-2, PbTx-3, and AJB6.0P in *K. brevis* cultures. Each measured value represents the mean ± s.e.m. (n = 5 cultures). **a** Cell growth over period of study. **b** Age of culture vs. PbTx-3 concentration (mg/10^5 cells). **c** Age of culture vs. PbTx-2 concentration (mg/10^5 cells). **d** Age of culture vs. AJB6.0P concentration (mg/10^5 cells).

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throughout the growth phases of the same clone (Roszell *et al.,* 1989). However, these ratios were not determined on a per-cell basis. The purpose of this study was to determine the concentration profiles of PbTx-2, PbTx-3, and AJB6.0P (a brevetoxin antagonist) per cell during different growth phases in culture in an attempt to conduct a more controlled study of toxin production in Wilson's diploid clone of *K. brevis.*

Materials and Methods

Twenty-four mL aliquots of *K. brevis* culture were taken twice a week for six and a half weeks from each of five different 10 L cultures of Wilson's clone of *K. brevis.* Four mL were used to determine cell counts using the Coutler Multisizer II counter. From the remaining 20 mL, an aliquot containing 10^5 cells was extracted for each determination. After sonication, samples were extracted 1:1 with ethyl acetate, filtered (0.2 mL) and dried under vacuum at room temperature. The residue was dissolved in 100 mL MeOH and injected onto a HP11OO HPLC coupled to an HP UV diode array detector. The mobile phase was a linear MeOH:H_20 gradient from 75% MeOH to 100% MeOH over 13 minutes and sustained at 100% MeOH for 2 minutes. Quantification was achieved by comparison of peak area to standard curves of PbTx-2, PbTx-3, and AJB6.0P measured at wavelengths of 215 and 295. B-type brevetoxins, AJB6.0P, and cell counts were observed graphically to determine trends. Data were analyzed for statistical significance by one-way ANOVA and post tests using GraphPad Prism.

Results and Discussion

The cultures exhibited lag phase (from day 0 to day 12), log phase (from day 12 to day 28), and stationary phase (from day 28 to 43) growth (Fig. 1a). Although an apparent decrease in PbTx 3 was visible at the end of log phase and throughout stationary phase (Fig. 1b), this difference did not achieve statistical significance compared to day 0 (ANOVA, Dunnett's $P > 0.05$). Significant differences in PbTx-2 were observed on day 35 (stationary phase) relative to day 0 (Fig. 1c). Significant differences in AJB6.0P production (Fig. 1d), relative to day 0, were observed at the end of log phase (day 25) and throughout stationary phase (days 28 and 39) (Dunnett's, $P < 0.05$). Significant differences in AJB6.0P production relative to days 15 and 18 (log phase) were observed at the end of log phase (day 25) and in stationary phase (days 28 and 39). Statistical differences in production of type-B brevetoxins in comparison to day 0 (with the exception of day 35 in PbTx-2) were not observed. Several repetitions of the study would help to clarify the trends that are apparent but not significant. The significant increase in the non-icthyotoxic compound that is a competitive inhibitor to brevetoxin, AJB6.0P (Bourdelais *et al.,* this Proceedings), during stationary phase may help to further enlighten the discrepancies between fish kills and cell counts throughout the duration of a *K. brevis* bloom off the coast of Florida.

Acknowledgements

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Transformation and Photodegradation of Domoic Acid in Seawater

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Abstract

Domoic acid (DA, Amnesic Shellfish Poisoning Toxin, ASP Toxin) is a widespread, naturally occurring marine toxin produced by several species of diatoms belonging to the genus *Pseudo-nitzschia*. The toxin is a glutamate agonist and acts by binding to various glutamate receptors. Consistent with this, a part of the DA structure resembles the endogenous neurotransmitter glutamate. In addition to this moiety, DA also contains an unsaturated side chain and it has been shown that the geometry of this conjugated diene system is crucial to biological activity. In a comparative study, it was shown that the natural geometrical isomer (Z, E : domoic acid) is the most potent of these isomers. These geometrical isomers can be produced in the laboratory by brief irradiation of DA solution with UV light (254 nm). However, more recently, we have shown that exposure of DA in seawater to natural sunlight also results in rapid formation of these geometrical isomers. In addition, our preliminary photodegradation studies revealed that a series of decarboxylated derivatives are also produced. These lipophilic derivatives, presumably lacking the side chain carboxyl group, are of particular significance since it is not known if they may be more toxic that the parent algal metabolite.

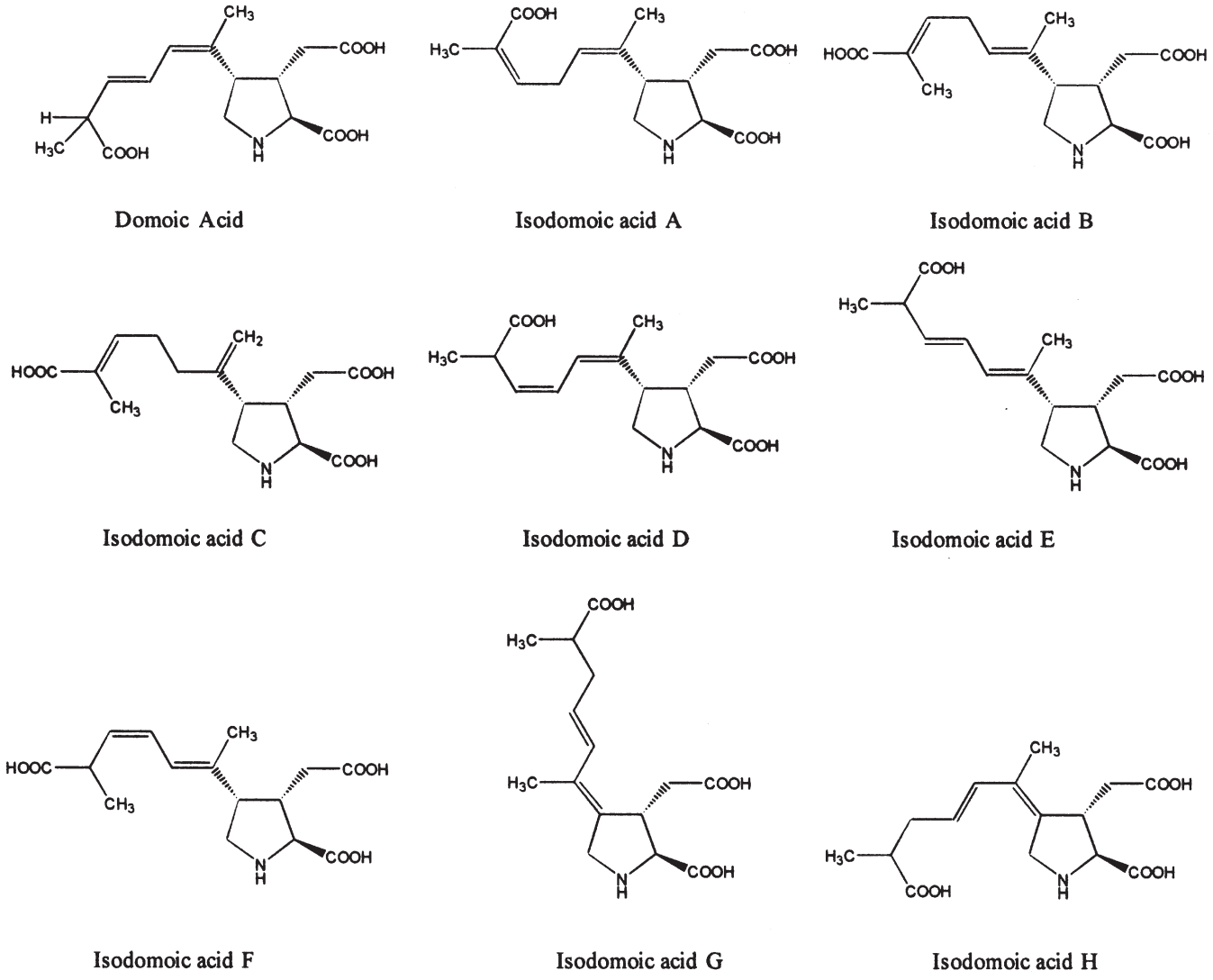

Figure 1 Structure of domoic acid and its various isomers.

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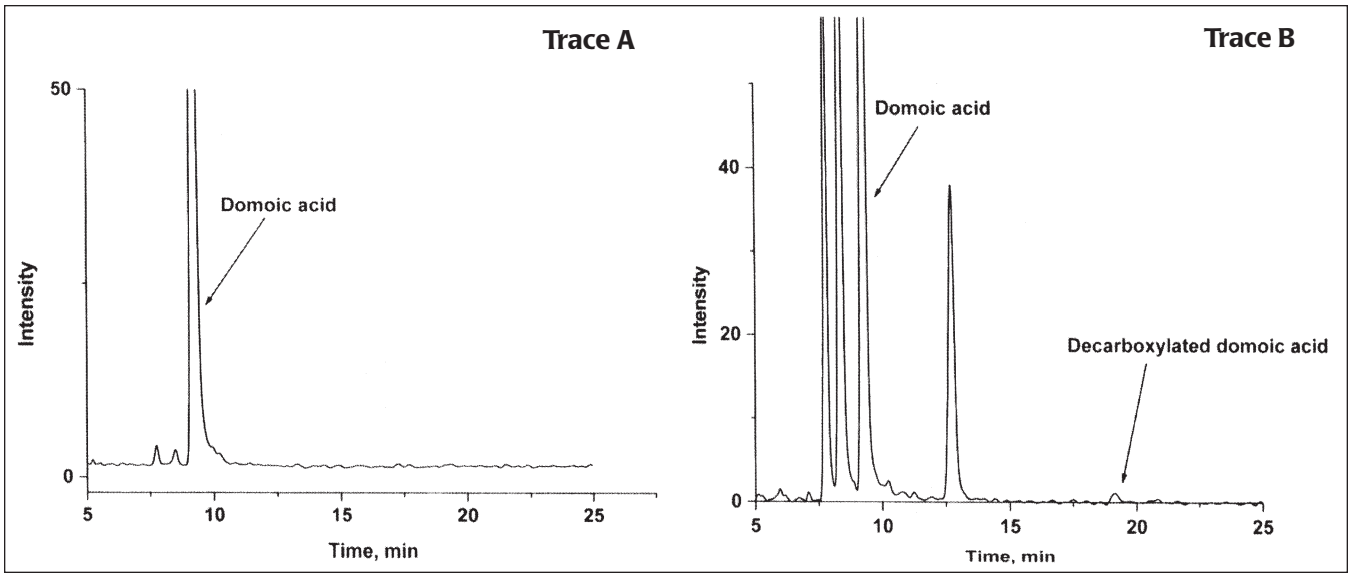

Figure 2 HPLC of domoic acid and its geometrical isomers before and after irradiation with artificial sunlight. **A** DA before irradiation and **B** DA and isomers isodomoic acid D, E, and F, following irradiation with artificial sunlight for 24 hours in seawater. Conditions of HPLC runs: Vydac C18 analytical column (250 × 10 mm); DAD detection wavelength: 242 nm; t = 40°C; isocratic elution 10% ACN/0.1% TFA in water.

Introduction

Domoic acid is the major toxin produced by the diatom *Pseudo-nitzschia* sp. and can occur in other marine species following ingestion of the toxic diatom. Other isomers such as isodomoic acids A, B and C have been discovered, as well as domoilactones A and B, although all these compounds were isolated from the red alga *Chondria armata* (Zaman, 1997). Also, three geometrical isomers (isodomoic acids D, E and F) and the C5' diastereomer have been isolated in small amounts from shellfish (Wright, 1995). Later studies on the constituents of *C. armata* yielded two additional isomers, isodomoic acid G and isodomoic acid H (Zaman, 1997). The structures of these isomers are presented in Fig. 1.

Studies on the potency of selected DA isomers indicated that they are less potent than DA itself (Hampson, 1992). In general, for maximum potency, the double bond closest to the ring should have the Z configuration. It is known that DA is converted to three geometrical isomers upon irradiation with UV light at 254 nm under laboratory conditions (Wright, 1995). The goal of our experiments was to determine if these DA geometrical isomers could be formed in the natural environment. This was investigated by irradiating DA in seawater using artificial sunlight, and observing the HPLC profile of the resulting mixture of products. Using LC/MS detection methods, the presence of DA derivatives other than its geometrical isomers was also examined. The irradiation experiments were conducted for 1, 3, 6, 12 and 24 hours with the control samples kept in the dark for the same amount of time.

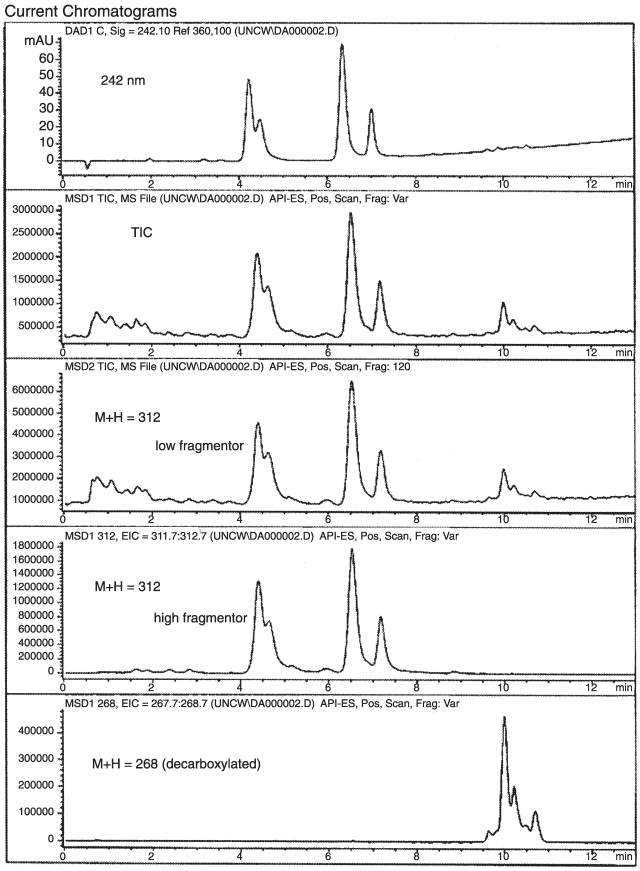

Figure 3 HPLC-MS of domoic acid and its geometrical isomers after irradiation with sunlight. Conditions of HPLC runs: Zorbax C18 analytical microcolumn; DAD detection wavelength: 242 nm; t = 40°C; isocratic elution 60% ACN/0.1% TFA in water.

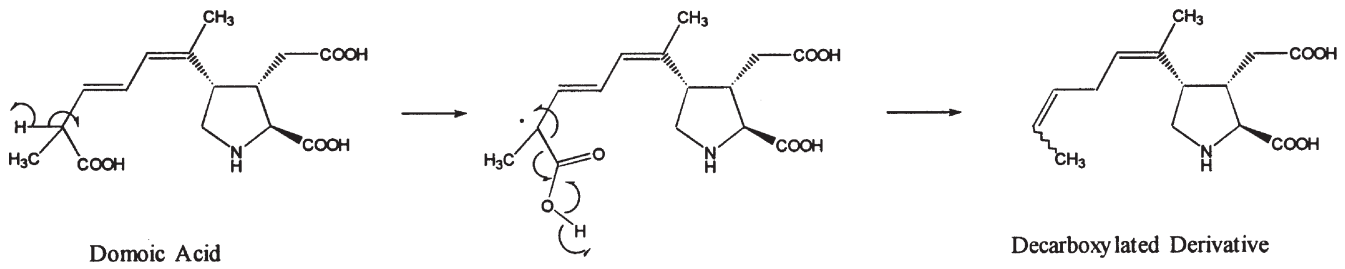

Figure 4 Proposed mechanism for the decarboxylation of domoic acid following irradiation with sunlight.

Results and Discussion

It was shown that DA is converted to its geometrical isomers under irradiation with artificial sunlight in seawater with a significant increase of isomer peak areas (by HPLC) after just one hour of irradiation. The peak areas of DA isomers increased steadily during 24 hours of irradiation with a corresponding decrease in the area of the DA peak (Fig. 2). The obtained results have significant environmental implications since the toxicity of the isomers is known to be lower than that of DA, which may be important for detoxification taking place during the natural degradation of DA. Furthermore, although DA is known to chelate Fe and Cu, which may be of environmental importance (Rue, 2001), nothing is known of the ability of the photoisomers to chelate metal ions. Furthermore, it is also possible that certain metal ions may modulate the photoisomerization process, and this will be the subject of future studies at our laboratory.

Another important result of this study is the observation of the formation of a group of less polar products with a RT of ~19 mins (see Fig. 2). The increased lipophilicity of these compounds, coupled with their MW (*m*/*z* 267), strongly suggests that these products are decarboxylated isomers of DA. The loss of the side chain carboxyl group is most likely, perhaps resulting in rearrangement of the conjugated diene system, as suggested by the reduced UV molar absorptivity for these products (see Fig. 3). Toxicity studies on these new decarboxylated isoforms will be performed in the future. A schematic with a proposed mechanism for the generation of the decarboxylated derivatives is shown in Fig. 4.

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Brevetoxin Degradation and By-Product Formation via Natural Sunlight

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Abstract

We investigated the effects of solar radiation on brevetoxin (PbTx2). Our findings suggest that natural sunlight mediates brevetoxin (PbTx2) degradation and results in brevetoxin by-product formation via photochemical processes.

Introduction

Harmful algal blooms (HAB) of the dinoflagellate *Karenia brevis* are an annual concern in the US, affecting all areas of the Gulf of Mexico (GOM). *Karenia brevis* produces brevetoxin (PbTx), responsible for mass morbidity and mortality among marine wildlife such as fish, birds and marine mammals and the causative agent of Neurotoxic Shellfish Poisoning (NSP) in humans (Forrester *et al.,* 1977; Baden, 1983; O'Shea, 1991; Bossart *et al.,* 1998; Wilson *et al.,* 1999). Brevetoxins are among nature's most potent naturally occurring voltage gated sodium channel (VGSC) agonists, pharmacologically active in nanomolar concentrations. There are at least 10 distinct brevetoxins which vary in metabolic stability, pharmacological activity and potency (Gawley *et al.,* 1995; Rein *et al.,* 1994), characteristics which may be modified via metabolic and environmental processes as well as (as this paper will suggest) photochemical processes. While the impacts of brevetoxin(s) upon marine wildlife and humans are well known, a complete etiology of brevetoxicosis remains poorly defined and the transport and fate of brevetoxin in the environment has not been well characterized. In this study the effect of solar radiation on brevetoxin degradation was explored.

Materials and Methods

Samples (100 mL) of seawater, sea-surface microlayer and deionized water spiked with 150 µg to 450 µg of brevetoxin (PbTx2) were subjected to solar radiation (both natural and simulated sunlight) and dark conditions for varying periods of time (2 to 96 hours). Following exposures the samples were extracted with ethyl acetate (1:1) and the organic layer dried before resuspending in methanol. The suspended samples were filtered (0.2 µ nylon membrane filter) and analyzed via HPLC. Photochemical byproducts were further assayed via HPLC-MS, ^{1}H-NMR and enzyme linked immunosorbent assay (ELISA). HPLC grade methanol, acetone, ethyl acetate and deionized and HPLC grade water were used in the extraction (described above) and HPLC analyses. A Hewlett-Packard 1100 HPLC (using Chemstation software) was used with a 5 µM C18 reverse phase column (Agilent Technologies) and a diode array detection wavelength of 215 nm. Either isocratic or gradient mobile phases of 85:15 MeOH;H_2O (flow of 1.4 mL/min.) were employed in sample analysis and byproduct isolation. Artificial sunlight exposures were made using a Spectral Energy LPS 256 SM and LH 1153 solar simulator generating a solar spectrum from 300 nM to 700 nM. Measurements of the natural sunlight at the exposure location (Wilmington, N.C., noonday sun on June 21st) were PAR-1670 µE/m^2/sec, UVA-3060 µW/cm^2 and UVB-290 µW/cm^2. The measured solar simulator spectra was PAR-1830 µE/m^2/sec, UVA-4590 µW/cm^2 and UVB-820 µW/cm^2.

Results

The HPLC profiles of PbTx2 spiked aqueous solutions subjected to solar radiation differed markedly from the PbTx2 spiked aqueous solutions kept under dark conditions (Fig. 1). At least 18 chemical entities were detected in the solar exposed samples which were not detected, or present in markedly lower concentrations, in samples stored under dark conditions. These photochemical by-products were consistently observed in each assay (n = 17). Figure 1 plots the average profile change of the solar exposed versus dark conditions samples over a 24 hr period (not all by-products are shown for clarity). The plots are based on peak area determined by HPLC chromatograms. Semi-quantitative analysis indicates brevetoxin (PbTx2) concentrations were reduced ~35% after 24 hrs of exposure to natural sunlight. The photochemical by-products (denoted in Fig. 1 by ByPrd 3.2, etc.) were found to represent from 2% to 13% of the total material in the sample after 24 hrs of exposure. In contrast the average PbTx2 degradation was ~3% under dark conditions. Subsequent experiments (n = 2) of 96 hr natural sunlight exposures yielded profiles where by-products represented ~75% of the total material in the sample and PbTx2 represented ~25% of the total material in the sample. Thus, brevetoxin (PbTx2 spike) concentrations were found to decrease while photochemical by-products were found to increase when samples were subjected to solar radiation. Four of the photochemical by-products were isolated in adequate quantities and purity to permit preliminary characterization. Each of the four products demonstrated a positive enzyme linked immunosorbent assay (ELISA) for brevetoxin. ^{1}H-NMR spectra for each of the four by-products suggested similarities to known brevetoxin standards (PbTx2 and PbTx9). HPLC-MS analyses of the photoproducts suggests the majority of these brevetoxin derivatives may be novel; most (19 of 21) of the by-product masses detected did not correlate to any known brevetoxins or brevetoxin metabolites in published literature. The major ions detected were mz (m + h) = 577,579, 621, 848, 877, 885, 895, 899, 904, 912, 913, 914, 927, 930,

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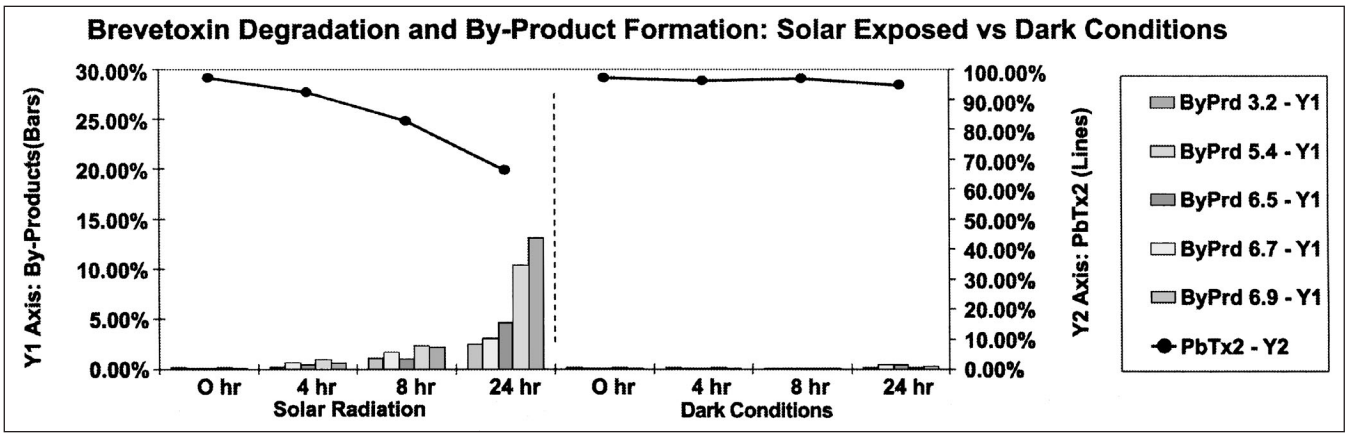

Figure 1 Average PbTx2 profile changes over 24 hr exposure to solar radiation and dark conditions (*note dual axis). Photochemical by-products (not all shown for clarity) are denoted by "ByPrd" (bars). The Y2 axis is for the PbTx2 spike concentration (~35% reduction), the Y1 axis is for the concentration of the photoproducts (2% to 13% increase in solar exposed). PbTx2 and by-products shown as a percentage of total material in the sample. In the solar exposed samples, photoproduct concentrations were found to increase while PbTx2 concentrations declined with time. The samples subjected to dark conditions show markedly less activity. There was no difference in PbTx2 degradation or by-product formation under natural and simulated sunlight.

944, 949, 958, 962, 972, 973, 981. In summary, the photochemical by-products/derivatives are of brevetoxin (PbTx2) origin and those analyzed demonstrate structural similarities to the PbTx2 parent compound.

Discussion

Results from this work suggest natural sunlight plays an important role in brevetoxin (PbTx2) degradation in the natural environment. Our findings show solar radiation mediates PbTx2 degradation and photochemical by-product formation via first order photochemical processes. Approximately 18 PbTx2 degradation products, the majority of which appear to be novel, were found. These photoproducts were not observed to degrade over the time frames studied and appear to be more stable than the parent compound, PbTx2; however, no similar data exist for the stability of these photoproducts in the natural environment. The biological activity of these photochemical by-products is not yet known; work on their biological activity is currently being conducted at Duke University.

During the course of this study Welker and Steinberg (1999) reported the indirect photolysis of microcystins, potent cyanobacterial hepatotoxins. Their findings indicated that, unlike the results here, photodegradation was via indirect photolysis and required the presence of humic substances. More recently it was shown that domoic acid, a potent neurotoxin produced by the diatom *Pseudo-nitzschia multiseries,* also undergoes photodegradation, forming various geometrical isomers as well as decarboxylated products (Campbell *et al.,* 2002). Hence, it is apparent that solar radiation can play a role in the degradation of a variety of aquatic toxins. These findings will hopefully augment our understanding of bloom dynamics and marine biotoxin speciation in natural waters.

Acknowledgements

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Effects of Temperature on Production of Brevetoxins and Brevetoxin-like Compounds

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Abstract

Cultures of *Karenia brevis* were grown and maintained at 15°, 22°, 24° and 26°C. Samples were taken once a week to determine the effect of temperature on production of brevetoxins and AJB6.0P (brevenal, a recently discovered brevetoxin-like compound defined as a competitive non-toxic ligand produced by *K. brevis*). After 4 weeks, cultures maintained at 15°C exhibited a steady decline in the number of cells per mL. Also, only the culture maintained at 15°C exhibited any significant difference from control (24°C) in toxin concentration.

Introduction

The marine dinoflagellate *Karenia brevis* has been implicated in fish kills in the Gulf of Mexico for nearly half a century. Brevetoxins, the toxins associated with *K. brevis,* are the agents thought to be responsible for the toxic effects in fish and mammals. It has been established that changes in environmental conditions affect total toxin production in marine dinoflagellates (Baden and Tomas, 1988; Ogata *et al.,* 1989). Studies have examined the effects of temperature and salinity on bloom formation and growth (Vargo *et al.,* 2001). It is reasonable to expect that the conditions affecting growth also affect toxin production. Specifically, little is known about the effects of water temperature on the production of specific brevetoxins. In this study, the results of culture temperature on toxin profile will be examined.

Materials and Methods

The concentrations of PbTx-2, PbTx-3, and AJB6.0P were measured in cultures grown at 15°, 22°, and 26 °C, and were compared to toxin concentrations of control cultures maintained at 24°C. Three 1-L containers of culture were maintained at each temperature under a constant supply of cool white fluorescent light. The cultures grown below 24°C were slowly acclimated to the final temperature by lowering the temperature of the water bath by two degrees per day. The cultures grown at 26°C were equilibrated in a water bath after one day of growth at 24°C. After one week of growth at the final temperature, a 10-mL sample was taken from each culture ("week 1"). Additional samples were taken at one-week intervals (weeks 2–5 and 9–11). Four mL were used for a cell count using a Beckman-Coulter Multisizer IIe and 6 mL were extracted with 6 mL of ethyl acetate. The organic layer was dried under vacuum. The residue was dissolved in 100 µL MeOH and analyzed by HPLC. Toxin concentrations were determined using an HP 1100 HPLC coupled to an HP uv diode array detector and set for wavelengths of 215, 245, 254 and 295. The HPLC mobile phase was a MeOH:H_20 gradient from 75% MeOH to 100% MeOH over 13 minutes and sustained at 100% MeOH for 2 minutes. Concentrations of toxin and AJB6.0P (brevenal, see Bourdelais *et al.,* this Proceedings) were determined by measuring peak areas and comparison to standard curves for each analyte. Data were analyzed by one way ANOVA using GraphPad Prism.

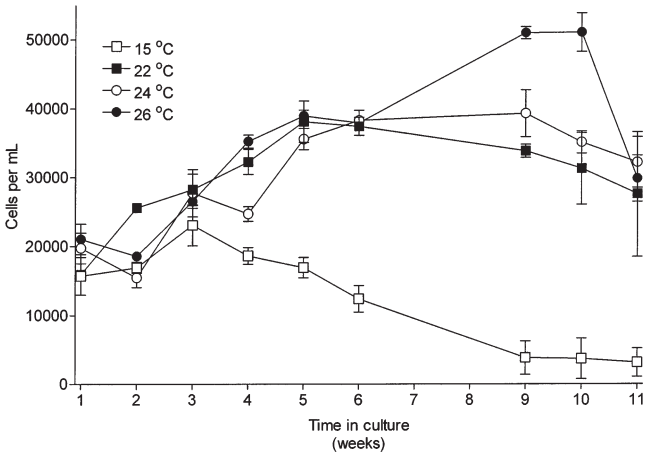

Figure 1 Effect of ambient temperature on cell growth. Each point represents the mean ± s.e.m. of data from three cultures.

Results and Discussion

The 24°C control culture exhibited log phase growth through week 6, was in stationary phase through week 9, and then began to decline (Fig. 1). The 22°C culture paralleled the control culture very closely. The 26°C culture exhibited significantly greater growth versus control at weeks 9 and 10. The 15°C culture exhibited a steady, significant decline in cell number from week 4 (Fig. 1). Statistical analysis revealed significant differences in cell numbers over time compared to the 24°C control culture (ANOVA, Tukey's or Dunnett's $P < 0.05$).

Only the 15°C culture exhibited any significant change (ANOVA, Tukey's $P < 0.05$) in toxin concentration over time (Fig. 2). Compared to the control culture at 24°C (Fig. 2a), the only significant differences (Dunnett's $P < 0.05$) in PbTx-2 concentrations were at weeks 3, 5 and 6 in the 15°C culture (Fig. 2b). Significant differences were observed in PbTx-3 concentrations at weeks 5 and 11 in the 15°C culture. Differences from control in AJB6.0P concentration were observed only in the 15°C culture at weeks 2, 3, 5 and 6. Cultures grown at 22°C and 26°C exhibited no differences from control in cell growth or toxin concentration. We believe that low temperature stress may

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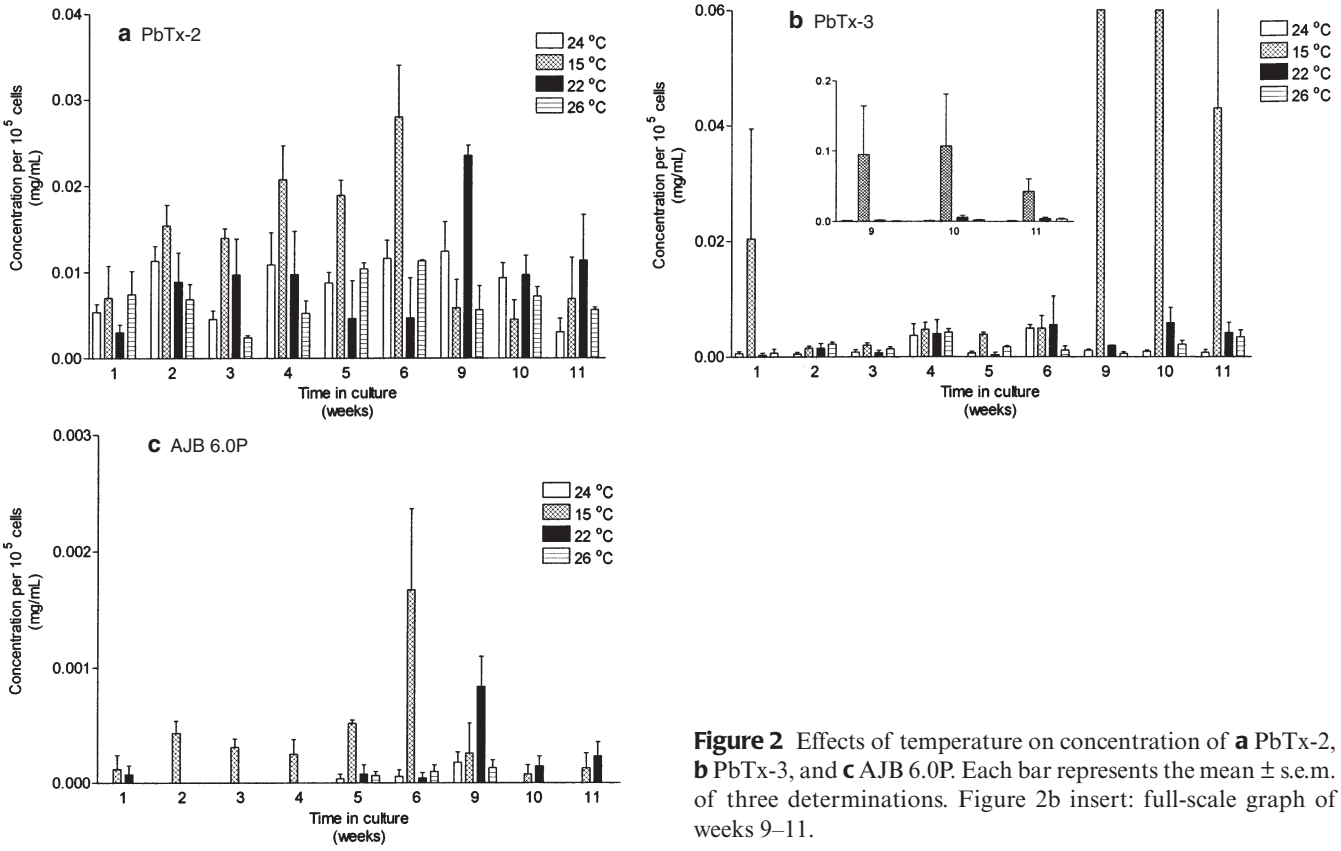

Figure 2 Effects of temperature on concentration of **a** PbTx-2, **b** PbTx-3, and **c** AJB 6.0P. Each bar represents the mean ± s.e.m. of three determinations. Figure 2b insert: full-scale graph of weeks 9–11.

induce toxin and AJB6.0P production. Previous studies have shown that *Karenia brevis* does not thrive at water temperatures less than 19°C. Cultures grown at 15°C exhibited a decrease in cell number but an increase in toxin concentration per cell relative to control cultures at 24°C. Although cell numbers were decreasing at 15°C, toxin concentration per cell was significantly higher relative to control. As the number of viable cells decreased, toxin levels did not change appreciably.

Acknowledgements

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Synthesis, Binding Assays and Toxicity of New Derivatives of Brevetoxin B

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Abstract

Nine new derivatives of brevetoxin B were synthesized. Six of them were examined for their affinity for Site 5 of the synaptosomal voltage-sensitive sodium channels (VSSC) and their toxicity to mosquito fish, *Gambusia affinis.* Three derivatives studied seemed to be less effective at displacing tritiated PbTx-3 from rat brain synaptosomes and three others were more effective. One derivative, having an opened A-ring, was 100-fold less effective.

Introduction

Brevetoxins (Lin Y. Y., 1981; Shimizu Y., 1986) (Fig.1) are produced by the marine dinoflagellate *Karenia brevis,* an organism linked to the "red tide" outbreaks in the Gulf of Mexico and elsewhere. Blooms of this species are often associated with catastrophic consequences for marine and terrestrial life, including humans. A human disease known as neurotoxic shellfish poisoning has been ascribed to the consumption of shellfish contaminated with these toxins.

The brevetoxin B backbone has a trans-fused polycyclic ether ring system which contains six-, seven- and eight-membered ether rings, 23 stereocenters and three carbon-carbon double bonds. They manifest their neurotoxicity by altering the gating and sodium ion permeability of voltage-sensitive Na^+ channels (VSSC) in excitable membranes. Numerous studies investigating the effects of toxin binding to VSSC indicate that these toxins bind specifically to Site 5 associated with domain IV of the channel (Poli *et al.,* 1986; Rein *et al.,* 1994a; Trainer *et al.,* 1991). Binding of brevetoxins to VSSC results in (i) a shift of the activation voltage for channel opening to more negative values (*i.e.,* channels are opened at normal resting potential), (ii) inhibition of the inactivation of opened channels, resulting in persistent activation or prolonged open times, and (iii) induction of subconductance states. As part of the continuing effort to more completely elucidate the effects of changes in brevetoxin structure on binding to and function of VSSC, new derivatives of a toxin readily available from cultures of *K. brevis* (PbTx-3) have been synthetized.

Results and Discussion

Chemical Modification Examination of the structure of the brevetoxin B backbone (Fig. 1) reveals three readily accessible sites for chemical modification: the A-ring α,β-unsaturated lactone, the H-ring double bond and the K-ring side chain. Several brevetoxin derivatives have been investigated (Rein *et al.,* 1994b) leading to the hypothesis that reduction of the H-ring double bond induces a significant change in the shape of the molecule resulting in loss of toxicity and binding affinity. For that purpose, no reactions involving the H-ring double bond were performed.

The first two new derivatives were obtained by basic protection of the terminal alcohol on the side chain. Treatment of PbTx-3 with tetrahydropyran in the presence of a catalytic amount of *p*-toluensulfonic acid furnished, after extraction and purification, compound 1 and the unexpected derivative 2 (Scheme 1). The C-37 hydroxyl was thought to be unreactive based on prior experience. Nevertheless, under mild conditions, compound 2 was isolated with good yield.

Binding of brevetoxin to VSSC is believed to occur with the K-ring (sidechain) end of the molecule pointed outwards (Matile *et al.,* 1996) and therefore the A-ring positioned down with the C-1 carbonyl pointed only in one direction because of the sp^2 character of its double bond. Therefore, introduction of more rotational freedom on the A-ring carbone 1 was thought to allow the carbonyl to point in other directions and influence binding to VSSC. To test this hypothesis, we attempted to synthesize a derivative such as 4 (Scheme 2). The lactone on the A-ring of PbTx-3 was reduced into the lactol 3 with good yield (Scheme 2), and (carbethoxymethylene)triphenylphosphorane was then added to a solution of 3 in THF/DBU and heated under reflux (Edmunds A. J. F., 2000). After extraction and purification by HPLC, one major compound was isolated and identified by NMR and mass spectrometry as 5. To date,

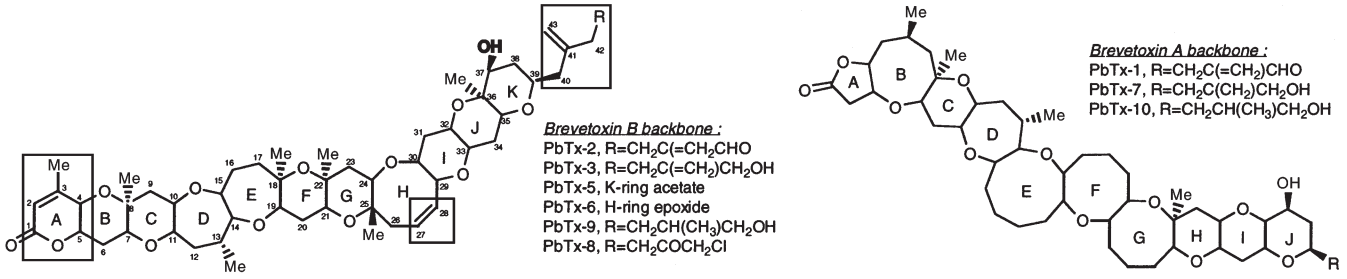

Figure 1 Brevetoxin structure

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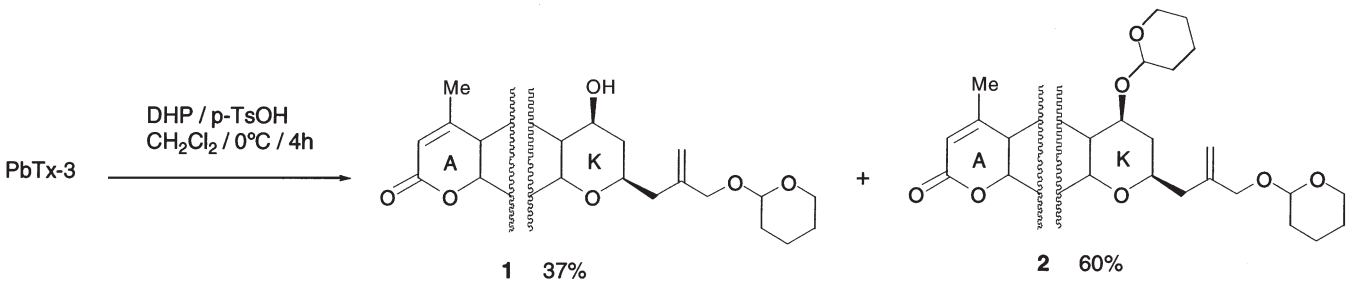
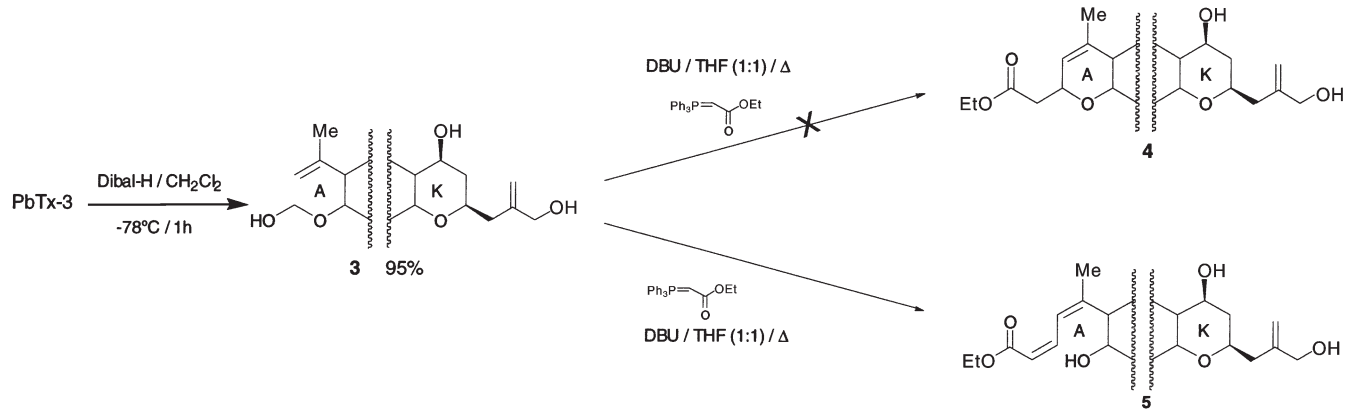
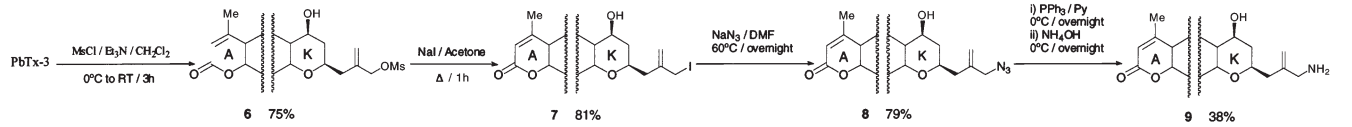


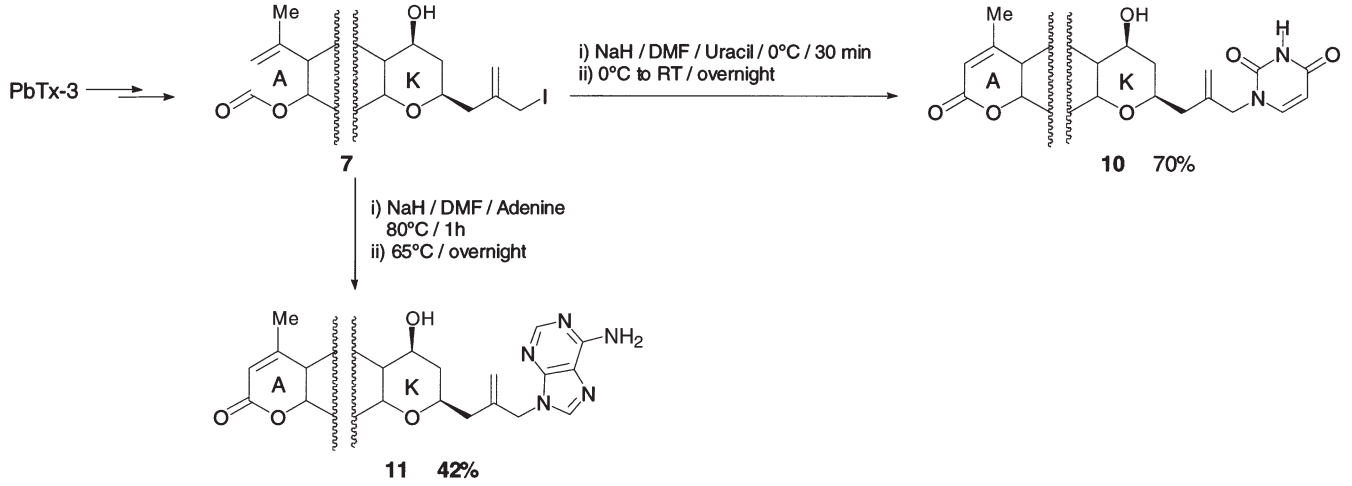
Scheme 1 Chemical modification on the side chain.



Scheme 2 Chemical modification on the A-ring.



Scheme 3 Synthesis of the azide and amine.



Scheme 4 Synthesis of Uracil-PbTx and Adenine-PbTx.

no traces of 4 were found. The closure of the derivative to re-form the A-ring and furnish 4 is under investigation.

Photolabeling studies were also initiated. The synthesis of the photoaffinity probe p-azidobenzoyl brevetoxin has been reported in a previous work (Trainer *et al.,* 1994) and labeled the binding site of brevetoxin on VSSC. Although the labeling was specific, the p-azido group on brevetoxin introduced certain limitations. The photoreactive moiety possessed significant rotational freedom and was quite far from the toxin backbone. In this study, the attempt was made to repeat the above work using a photoaffinity probe closer to the K-ring. Compound 8, which contains the azide di-

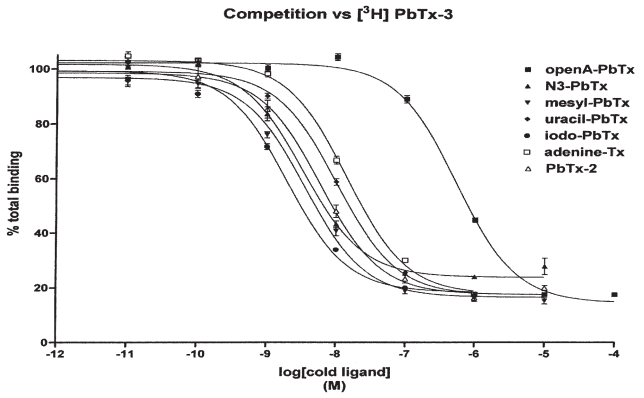


Figure 2 Inhibition of Binding of [^{3}H]-PbTx-3 to site 5 of the voltage-gated sodium channel by the derivative toxins.

Table 1 Binding and icthyotoxicity data for derivative toxins.

Compound	EC_{50} (nM)	LC_{50} (nM)
PbTx-2	5.9	not done
5	540.0	>2 μM
6	3.6	39.7
7	2.0	not done
8	3.6	19.8
10	10.0	>0.1 μM
11	14.0	>0.1 μM

rectly linked to the toxin backbone, was synthetized as described in Scheme 3. The primary alcohol on the K-ring sidechain of PbTx-3 was first activated by formation of the mesylate 6. The mesylate was then replaced by an iodide to provide 7 (81% yield) and finally by an azide to give 8 with 79% yield after purification. The structures of the azide and all the intermediates were confirmed by NMR and mass spectroscopy. The disposable azide was then easily reduced into the amine 9 in the presence of triphenylphosphine and ammonia according to the Staudinger reaction.

Finally, two nucleotide derivatives, 10 and 11 (Scheme 4), have been synthetized. Uracil (Zimmerman M. N., 2000) or adenine (Hakimelahi G. H., 2001) were treated with NaH in DMF at 0°C and 80°C, respectively, then the iodide 7 was added dropwise and the mixture was stirred overnight. Extraction and purification by HPLC afforded 10 and 11 with 70% and 42% yield, respectively. The structure of 10 and 11 were confirmed by NMR and mass spectroscopy.

Binding Assays and Toxicity Inhibition curves showing the displacement of triated PbTx-3 by competitor ligands (5–11) are shown in Fig. 2. From the latter, the EC_{50} values summarized in Table 1 were calculated. When sufficient quantities were available, LC_{50} for G. affinity were calculated according to the method described by Weil (Weil C. S., 1952).

Mesyl (6), iodo (7) and azide (8) are somewhat more effective at displacing tritiated PbTx-3 from rat brain synaptosomes and seem to be slightly more toxic than the natural toxin PbTx-2. The uracil (10) and adenine (11) derivatives are somewhat less effective displacers whereas the opened A-ring compound (5) is 100-fold less effective.

Conclusion

We have reported the synthesis of new derivatives of the brevetoxin B backbone. Our attempts to modify the A-ring opened it, resulting in a new product. We have synthetized a new photoaffinity probe in three steps with 48% overall yield. This photoaffinity probe will be used to obtain structural information about the VSSC. The reduction of the azide has provided the amine, a more polar compound than PbTx-3. The synthesis of two nucleotide derivatives has also been described. An iodide intermediate derivative has been made that was then converted to the azide and the nucleotide derivatives. The presence of the iodide-leaving group allows addition of nucleophiles and will be the starting point for the synthesis of many new derivatives.

Acknowledgements

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Production of Gymnodimine by *Karenia selliformis* (Haywood *et al.*)

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Abstract

The production of gymnodimine in particulate and dissolved fractions of batch cultures of the planktonic dinoflagellate *Karenia selliformis* was investigated. Gymnodimine was produced at a relatively constant rate, throughout most of the growth cycle. During the early growth phase, up to 50% of gymnodimine was dissolved in the culture medium, although as cell numbers and gymnodimine concentrations increased, this proportion rapidly declined and through the exponential and stationary phases, >70% of gymnodimine was associated with the cells. Gymnodimine appears to be a rather stable constituent of the cells; however, because significant amounts are also dissolved in the medium, a possible ectocrine function for this compound is suggested.

Introduction

Gymnodimine (GYM) is a spiroimine compound (Fig. 1; Seki *et al.,* 1995), produced by the planktonic dinoflagellate *Karenia selliformis* (Haywood *et al.,* in press). Since the original description of gymnodimine, two additional analogues (gymnodimine B and C) have been described (Miles *et al.,* 2000) and it has been found to be a common, low-level contaminant in a variety of shellfish species throughout New Zealand (MacKenzie *et al.,* 1996; Stirling, 2001). Gymnodimine has low oral toxicity but a high (LD^{50} 96 µg/kg) fast-acting potency when administered by intraperitoneal injection (Miles *et al.,* 1999) to mice in the standard bioassay for diarrhetic shellfish poisoning (DSP) toxins. Mainly for this reason, (*i.e.,* it confounds the interpretation of the results of DSP-screen mouse biossays), it is a concern to shellfish quality regulators although it is considered to present little, if any, hazard to human consumers of shellfish. The biosynthesis and physiological role of this compound, and any ecological advantage it may confer on the dinoflagellate, is as yet unknown. Gymnodimine has structural features in common with other marine bioactive compounds, namely the pinnatoxins (Uemura *et al.,* 1995), prorocentrolides (Lu *et al.,* 2001) and spirolides (Hu *et al.,* 1995). There are few data on the dynamics of production of the lipid soluble polyketide derived spiroimine toxins, either because the causative organisms are unknown, difficult to culture, or they have only recently been discovered and this work has yet to be accomplished. Because gymnodimine is easily and abundantly produced by *K. selliformis* cultures, studies on its production may provide a model for the production of, and reveal a commonality of purpose for, these other compounds produced by microalgae.

Materials and Methods

Five replicate batch cultures of *K. selliformis* (Cawthron Culture Collection isolate CAWD79) were grown in GP+Se medium (Loeblich and Smith, 1968) in 2 litre Erlymer flasks at 18°C. The cultures were illuminated under fluorescent lights (Cool White TLD 58W/33; approximately 100 µE m^{-2} s^{-1}; 12:12 hr photo-period). The cultures were sampled at 3–5 day intervals for cell counts (sub-samples were preserved with Lugol's iodine) and measurement of the total and dissolved gymnodimine content. Counts were carried out under an inverted microscope, after the settling of appropriately diluted volumes in Utermohl chambers. For the measurement of total gymnodimine, 10 mL samples of the culture medium were sonicated prior to analysis. For the measurement of dissolved gymnodimine 2 mL was gently filtered by gravity through 13 mm 3 µm polycarbonate filters (Osmonics Inc.) prior to analysis of the filtrate. The quantity of gymnodimine in the particulate fraction was obtained by subtraction of the dissolved from the total estimates. To examine the rate of degradation of gymnodimine in solution under the culture conditions an exponential phase culture was held in a water bath at 55°C for 2 minutes and vigorously sonicated to kill and disrupt the cells. Two flasks containing this solution were incubated alongside the cultures, and samples were withdrawn at two day intervals for 6 days for total gymnodimine quantification. Gymnodimine was quantified using a Waters 2790 LC system coupled to a Micromass Quattro Ultima triple quadrupole mass spectrometer. All samples were diluted 1:10 with 10% acetonitrile and filtered prior to injection into the instrument. Chromatography was carried out using a 5 µm, Phenomonex Luna C18 column (150×2 mm^2) and an isocratic mobile phase of 25% acetonitrile and 4 mM ammonium hydroxide and 50 mM formic acid. The electrospray ionization interface (ESI) was operated in positive mode and the diagnostic masses of parent and daughter ions (parent > daughter: 508.4 > 392.3 and 508.4 > 490.3) were measured by the mass spectrometer in multiple reaction monitoring (MRM) mode. Quantification of peaks was by comparison with a quantitative gymnodimine reference material kindly provided by Dr. Chris Miles of Ag-Research Hamilton, NZ.

Results and Discussion

Under the culture conditions used in this experiment, a maximum growth rate of 0.18 divisions/day was observed during the exponential phase, and a maximum cell density of 5.3×10^4 cells/mL was achieved (Fig. 2). The optimum growth conditions for this culture have yet to be determined. Although it is expected that a higher light intensity than that

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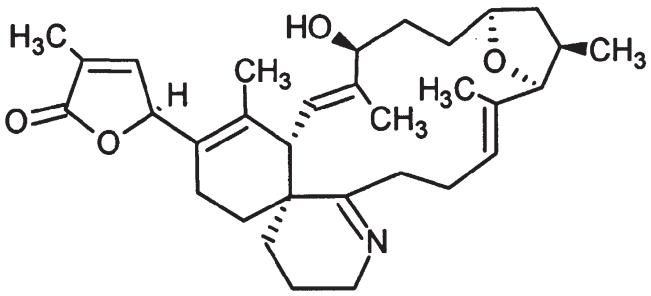

Figure 1 Molecular structure of gymnodimine.

used in this study may result in a higher growth rate, it is unlikely that a higher final biomass would be achieved.

In stationary phase, a maximum average yield of 0.77 ± 0.18 µg GYM/mL was observed (Fig. 3) in these cultures, under these conditions. During the early growth phase, up to 50% of the gymnodimine was free in the medium, though this proportion rapidly decreased and throughout the remainder of the growth cycle most of the gymnodimine (>70%) was in the particulate fraction. This proportion increased up to 85% as the cultures entered the stationary phase (Fig. 3).

Immediately after inoculation when the cell numbers and concentration of total gymnodimine in the cultures were low, estimates as high as 101 pg total GYM/cell and 55 pg particulate GYM/cell were made, though these declined rapidly (Fig. 4) to 13.8 and 7.5 pg of total and particulate GYM/cell respectively during the mid log phase. Excluding the early growth phase, an average of 11 pg particulate GYM/cell during log and stationary phases was estimated though there was a slight increase in the cell content (up to

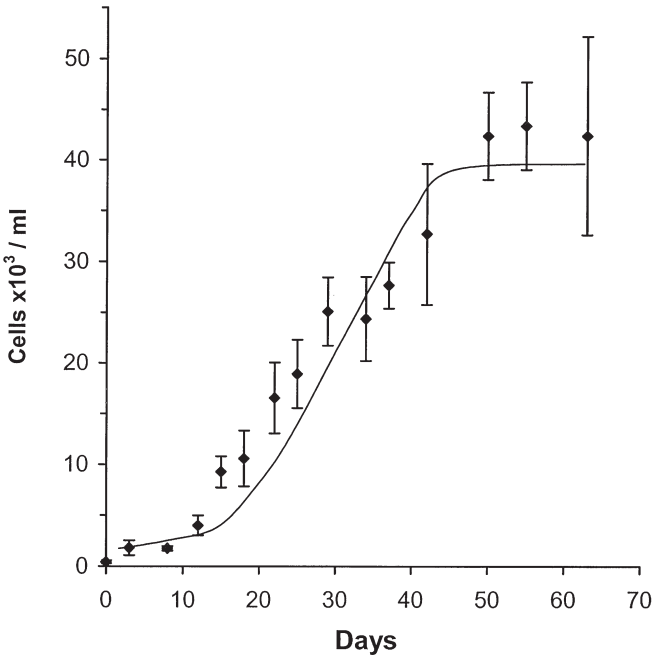

Figure 2 Growth curve of *Karenia selliformis.*

17 pg particulate GYM/cell) of gymnodimine as the cultures entered the stationary phase (Fig. 4). Gymnodimine is known to be unstable at neutral to high pH (Miles *et al.,* 1999) and since the pH of the medium was in the range of 8.1–8.7 it was likely that significant decomposition was taking place in the dissolved fraction during the course of the experiment. This was confirmed by the decomposition experiment, during which the gymnodimine concentration was observed to decline at a rate of about 5% per day. The

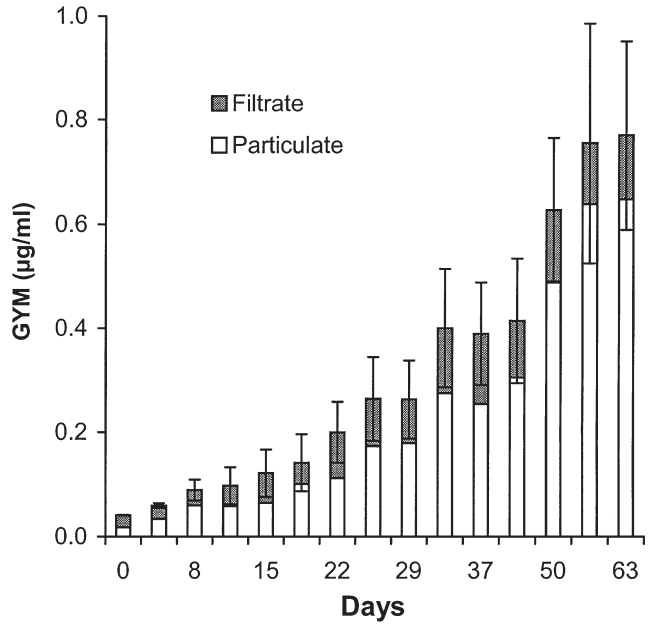

Figure 3 Production of gymnodimine throughout the growth cycle.

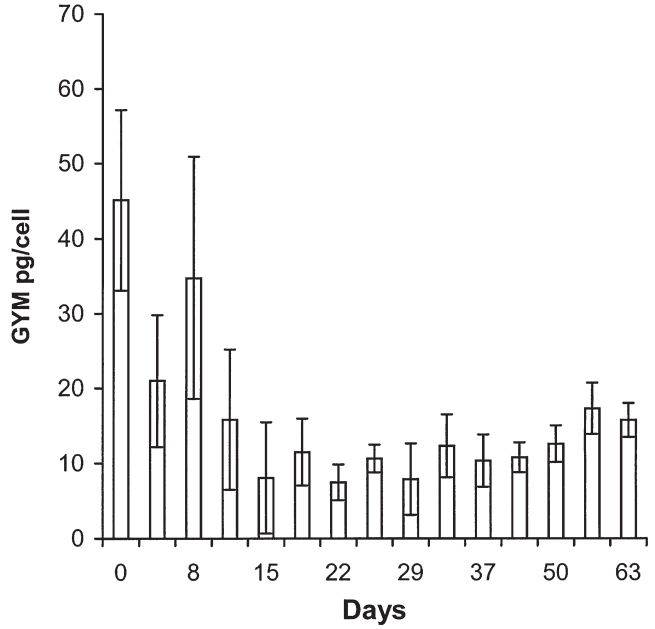

Figure 4 Cell content of gymnodimine (in the particulate fraction).

concentration of gymnodimine in the dissolved fraction therefore represents the net result of continuing loss from the cells and decomposition in the medium. Whether the dissolved gymnodimine is a result of an active excretion process, passive leakage from the cells or release due to cell lysis, is unknown. Despite its instability the absolute concentration of gymnodimine in the dissolved fraction did increase throughout the growth cycle and it appears to be sufficiently stable, and its rate of entry into the dissolved faction sufficiently rapid, for net accumulation in the medium to take place. However, except during the early phases of the growth cycle, the majority of the gymnodimine was within the particulate fraction and it appears from this data that the gymnodimine is a relatively constant constituent of the cells themselves, though its actual anatomical location and function remain unknown. To put the gymnodimine content into perspective two independent estimates of the biomass of *K. selliformis* cells were made. *K. selliformis* cells are on average 15 μm long × 12.5 μm wide × 6.5 μm thick and have an estimated cell volume of about 650 μm^3. Calculation of the cell carbon content from the relationship for athecate dinoflagellates of Menden-Deuer and Lessard (2000) gives an estimate of about 136 pg C/cell. An estimate of cell carbon biomass based on C, H, N analysis of cell concentrates during the exponential phase gave a carbon content of 271 pg C/cell. The average gymnodimine content of 11 pg GYM/cell equates to 8.3 pg C/cell or, using the above biomass estimates about 3–6% of the total carbon content of the cell. Gymnodimine is a potent bioactive compound, which elicits symptoms in laboratory animals which suggest the site of action is at neuro-muscular junctions (R. Munday, pers. comm.), and blooms of *K. selliformis* have on at least two occasions in New Zealand been associated with mass mortalities of marine fauna including fin-fish, abalone, surf clams and mussels (MacKenzie, 1994). Cultured cells of *K. selliformis* are lethal to oyster larvae and are distasteful, and after prolonged exposure may be lethal, to mature Greenshell mussels (MacKenzie, unpublished data). Its effect on other microalgae and grazers of *K. selliformis* has yet to be tested. Although it has not been definitively established that gymnodimine itself is the cause of these effects the relatively high proportion of the compound in the cells and its presence in significant quantities in solution suggests it may very well have an ectocrine function that confers some ecological advantage on *K. selliformis.*

Acknowledgements

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Preliminary Investigations into Oxidation of Paralytic Shellfish Poisons (Saxitoxins and Derivatives) in Drinking Water by Chlorine

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Abstract

The fresh water cyanobacterium *Anabaena circinalis* produces saxitoxin (STX) and several other toxins with similar basic structural skeleton. Collectively, these toxins are known as Paralytic Shellfish Poisons or PSPs. These toxins are water soluble and can escape into the water body after cell lysis. The presence of these toxins in drinking water is a serious threat to human health. The present work has shown that Paralytic Shellfish Poisons (PSPs) in drinking water can be removed by chlorination at high pH (>9.0), provided a residual of 0.5 mg/L of free chlorine is present after 30 minutes of contact time.

Introduction

Saxitoxin is a very potent neurotoxin. This toxin binds to the voltage gated sodium channels in nerve cells with high affinity and prevents neurotransmission at both the neuronal synapses and neuromuscular junctions, ultimately causing respiratory paralysis and death (Kao 1993).

Saxitoxin is a tricyclic alkaloid containing two guanidinium moieties as depicted in Fig. 1. The toxin interacts with a carboxylate group located in the mouth of the Na^+-channel through one of the guanidinium moieties present in the toxin, thereby inhibiting the function (Stryer, 1988). The interaction between the toxin and Na^+-channel is considerably more complex, involving a network of hydrogen bonding and other favourable steric interactions.

The dangers of saxitoxin (STX and analogues) to public health is twofold. Saxitoxin is produced by some marine dinoflagellates including *Alexandrium tamarense, Gymnodinium catenatum* and *Pyrodinium bahamense* (Shimizu, 1977; Harada *et al.,* 1982; Oshima *et al.,* 1987). Filter-feeding bivalves such as mussels and clams may ingest these dinoflagellates, thereby accumulating the toxins. The toxins may then enter the human body through the consumption of contaminated seafood.

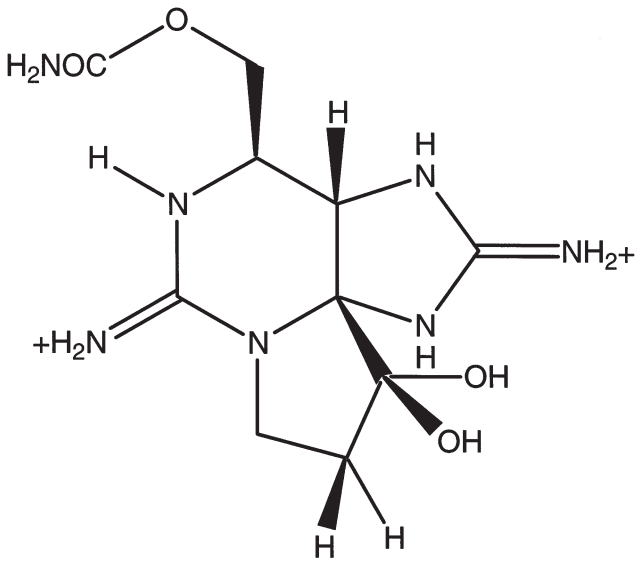

Figure 1 The structure of saxitoxin.

The second mechanism of toxicity to humans can occur via drinking water. *Anabaena circinalis,* a cyanobacterium found in Australia, produces saxitoxin (Humpage *et al.,* 1994; Onodera *et al.,* 1996). In addition to saxitoxin, it also produces related analogues which are toxic to a lesser degree. These include C1, C2, GTX2, GTX3, GTX5 (B1) and decarbamoyl STX, all of which have the same basic tricyclic skeleton similar to saxitoxin but with different substituents (Negri *et al.,* 1997; Velzeboer *et al.,* 1998; 2000). During a bloom, it is possible for these toxins to find their way into water reservoirs, thereby creating a public health hazard.

The standard chlorination techniques practiced in most water treatment plants will not remove the toxin completely. During systematic investigations into algal toxins in our laboratory, we have investigated the removal of saxitoxin and its analogues from drinking water by chlorination under different pH conditions.

Materials and Methods

A freeze-dried sample of *Anabaena circinalis* collected from a previous bloom was used in the study. (Coolmunda Dam Queensland, Australia, 1997 bloom). A raw water sample collected from a local reservoir was used in the study (Lake Samsonvale, North Brisbane; GPS co-ordinates of the site are 27°17.260 S, 152°54.627 E). This water had a pH of 7.6 and a chlorine demand of 2.0 mg/L and contained no detectable levels of PSPs.

To the freeze-dried toxin material (250 mg), we added milliQ water (30 mL) and the suspension was sonicated for 30 minutes. This was then centrifuged and the supernatant liquid separated. The pellet was re-extracted with 50 mM acetic acid (2 × 30 mL). With each extraction the sample was sonicated for 15 more minutes. All extractions were then combined and ultracentrifuged for 1 hour at 35 000 rpm at 4 C. The supernatant liquid was carefully separated and

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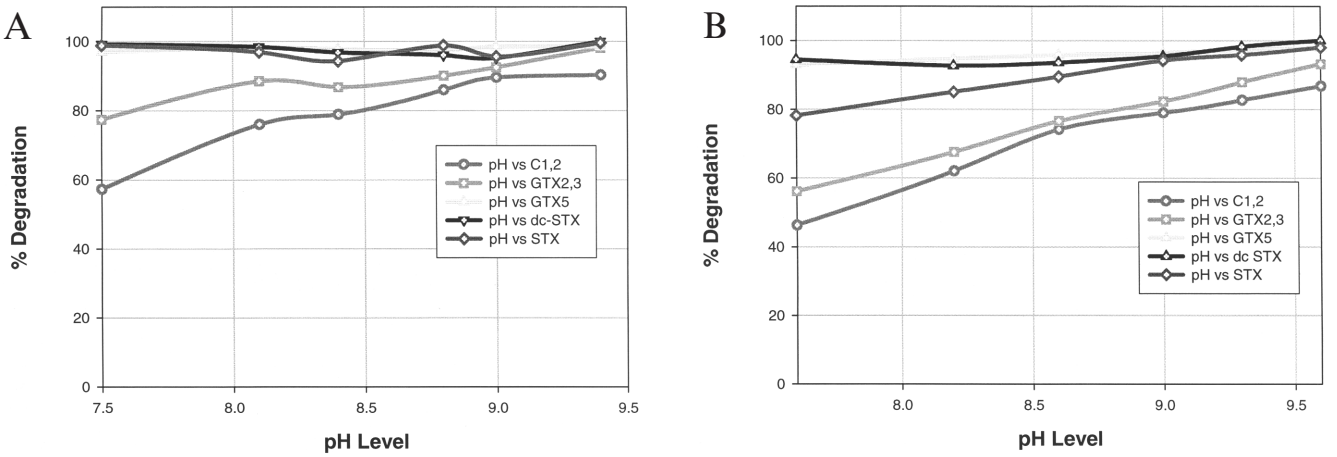

Figure 2 Degradation of PSPs at different pH levels. **A**, with cellular material; **B**, cell free extract.

lyophilised to reduce volume (final volume adjusted to 10.0 mL with milliQ water). (Preliminary work indicated that two extractions of 50 mM acetic acid will release all the toxin.) This material, which contained the toxins C1,2, GTX 2,3, GTX5 (B1), decarbamoyl STX and STX, was used in subsequent chlorination experiments. Chlorinations were done in duplicate using NaOCl as the chlorine source. The aim was to have a chlorine residual of about 0.5 mg/L after 30 minutes of contact time. A preliminary experiment established the initial chlorine concentration to be about 7.5 mg/L.

Raw water (49.5 mL) contained in a Falcon tube was added to the above toxin extract (500 µL). A subsample was taken at this point, and the toxin concentration was measured to be as follows: C1,C2 = 226, dcSTX = 0.55, GTX2,3 = 26.0, B1(GTX5) = 17.2 and STX = 5.5 µg/L. NaOCl solution was added to give an initial Cl_2 concentration of 7.2 mg/L. The solution pH was immediately adjusted with 100 mM KOH to the desired level. The samples were stirred in the dark (with caps closed) for 30 minutes. At this stage, a portion of the sample (15 mL) was neutralised with a solution of $Na_2S_2O_3$ (25 µL of 1.0 M solution) to remove excess chlorine. This process was repeated for all pH values. The residual chlorine was measured using Cl_2 powder pillows. These values varied from 0.3 to 0.85 mg/L. A portion of the neutralised solution (10.0 mL) was then carefully lyophilised to reduce volume. The final volume was adjusted to 1.0 mL. All toxin concentrations were determined by pre-column oxidation using HPLC (Lawrence, 1991) and fluorescence detection. The standards used were obtained from NRC Canada. A toxin mixture kept at pH 8.0 but without chlorine did not show significant toxin degradation.

The above experiment was repeated without removing the cellular material (*i.e.,* omitting the ultracentrifugation step). An initial experiment established the chlorine demand for this to be about 20 mg/L. Initial toxin concentration was measured to be as follows: C1,C2 = 265, dcSTX = 0.63, GTX2,3 = 29.0, B1(GTX5) = 20.0 and STX = 5.7 µg/L.

Results and Discussion

The percentage of degradation of toxins with and without cellular material at various pH levels is depicted in Figs. 2A and 2B, respectively. Data for Fig. 2B are shown in Table 1; data for Fig. 2A are not shown.

Table 1 Degradation data for PSPs with cell free extracts at different pH levels.

Sample	C1, C2 µg/L (% deg)	dcSTX µg/L (% deg)	GTX2, 3 µg/L (% deg)	GTX5 µg/L (% deg)	STX mg/L (% deg)	Initial Cl2 Dose µg/L	Residual Cl2 Level after 30 min µg/L
Initial Toxin Concentration	226	0.55	26	17.2	5.5		
pH 7.6	121 (46)	0.03 (95)	11.4 (58)	1.2 (93)	1.2 (78)	7.2	0.28
pH 8.2	85 (62)	0.04 (93)	8.4 (69)	0.9 (95)	0.8 (85)	7.2	0.29
pH 8.6	58 (74)	0.04 (93)	6.1 (77)	0.7 (96)	0.6 (89)	7.2	0.31
pH 9.0	47 (79)	0.03 (93)	4.6 (85)	0.6 (96)	0.3 (95)	7.2	0.35
pH 9.3	39 (83)	0.01 (98)	3.1 (88)	0.4 (98)	0.2 (96)	7.2	0.85
pH 9.6	29 (87)	0.00 (100)	1.7 (92)	0.4 (98)	0.1 (98)	7.2	0.58

As shown by Figs. 2A and 2B, the removal of PSPs as a function of pH was not linear, with the degree of removal increasing rapidly at around pH 8.5. The more effective removal at higher pH was not expected as chlorine is known to be a weaker oxidant at high pH. However, this may be attributed to the toxin molecule being present in an unprotonated form at higher pH, and this form is more susceptible to oxidation.

It is possible to conclude from these data that for high level removal of saxitoxin and its analogues produced by *Anabaena circinalis,* a pH of 9 (or higher) is required, with a residual chlorine level of 0.5 mg/L after 30 minutes of contact time. Water filtration plants using chlorine as the disinfectant agent will therefore need to operate under this relatively severe pH regime to ensure removal of saxitoxin and its analogues during blooms of *Anabaena circinalis.*

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Persistence of Paralytic Shellfish Toxins in Freshwater Environments

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Abstract

This paper presents data on the persistence and degradation of paralytic shellfish toxins (PST) produced by *Aphanizomenon* sp. LMECYA31 (STX, neoSTX, dcSTX and GTX5) in aqueous solutions. Toxin degradation was determined both in sterile/deionized water under different pH (3, 7, and 9) and temperature (20°C and 30°C) conditions and in the cyanobacterial cell-free culture media under different conditions of bacterial contamination and protein content. Results indicate that PST may persist in water for time periods greater than 2 months. However, PST degradation rates are influenced by both temperature and pH and may be reduced in the presence of bacteria and other organic compounds.

Introduction

The production of PST in freshwater environments has been associated with several cyanobacteria bloom-forming species found in many countries (Sivonen and Jones, 1999). Field and laboratory studies have revealed that those species can produce high levels of PST (Kass and Henriksen, 2000; Dias *et al.,* 2002). The health risks associated with bloom occurrences may be particularly high after bloom collapse or following water treatment with algicides, since both promote toxin release to the water. However, little is known about PST persistence and degradation in freshwater environments. Jones and Negri (1997) evaluated the stability and conversion of some cyanobacterial PST (C1+2, dcGTX2+3, GTX2+3) in water, under temperature conditions similar to those found in natural environments. The aim of the present study was to determine the persistence of PST (STX, dcSTX, neoSTX and GTX5) in water under different conditions of temperature, pH, bacterial contamination, and protein content.

Materials and Methods

Production of Toxins Toxins were obtained from large-scale non-axenic cultures of *Aphanizomenon* sp. LMECYA31 strain (Pereira *et al.,* 2000). After reaching the stationary growth phase, cyanobacterium cells were separated from the culture medium by decantation followed by filtration through glass fiber membranes (Millipore). Cyanobacterial-free culture medium contained the dissolved PST released after cell lysis during late stages of growth. PST from the biomass were extracted as described in Dias *et al.* (2002). The extract was further clarified through a solid phase C18 column (Sep-Pak, Waters) to remove pigments and hydrophobic contaminants and then filtered through a Centricon YM3 membrane (Millipore) to remove the protein fraction.

Toxins Incubation The semi-purified PST extract (pH = 3) was diluted in distilled-deionized (MilliQ) water. This toxin solution was sterile filtered through 0.2 µm membrane filters and divided into four fractions (10 mL each). These fractions, carried out in duplicate, were incubated under a 16/8h L/D cycle (light intensity 30 µE $m^{-2}s^{-1}$) and under the following conditions: (1) pH 3, 20°C; (2) pH 7, 20°C; (3) pH 9, 20°C; (4) pH 7, 30°C. The pH was adjusted with NaOH 1M.

Cyanobacterial-free culture medium was divided into 3 fractions (500 mL each), carried out in duplicate, and incubated at 20°C, under the light/dark cycle referred to above. Fraction (1) was non-axenic and non-deproteinized; fraction (2) was previously sterilized through 0.22 µm membrane filters but not deproteinized; fraction (3) was previously sterilized and deproteinized: proteins were precipitated by the addition of trichloroacetic acid 5% v/v and the medium was re-adjusted to pH 7 (pH of conditions 1 and 2) with 10 mM phosphate buffer. Axenicity and bacterial contamination of all fractions were confirmed by plating on nutrient agar (TSA) for one week at 22°C.

Toxin Quantification Aliquots were taken periodically from all fractions throughout the incubation period (62 days), including time 0. Samples were acidified to pH 3 with 0.5 M acetic acid and preserved at –20°C until toxin quantification. Toxin concentration and degradation profile was quantified by HPLC-FLD analysis carried out according to the method described by Oshima (1995). Toxin concentrations were determined by comparing peak areas for each toxin with those of the toxin standards. PST standard mixtures (Oshima, 1995) were a generous gift from Prof. Oshima, Tohoku University, Japan.

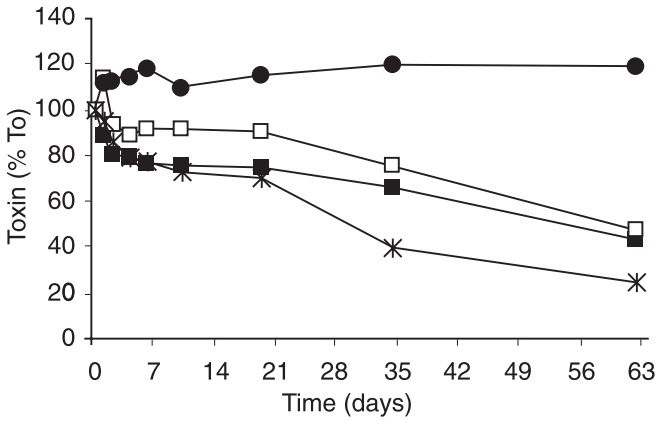

Figure 1 Variation of total PST levels (% of initial) in sterile/deionized water at (●) 20°C, pH 3; (■) 20°C, pH 7; (□) 20°C, pH 9 and (✶) 30°C, pH 7.

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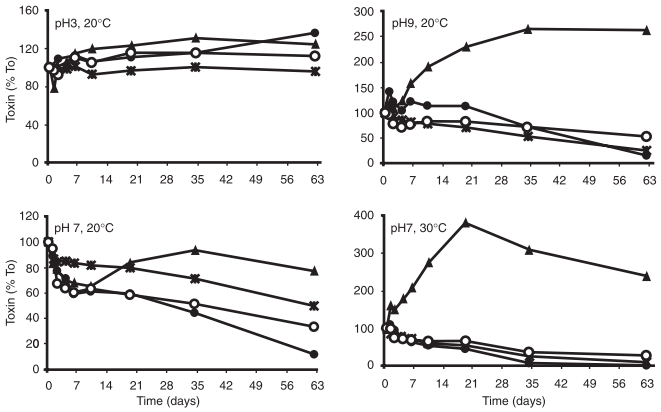

Figure 2 Variation of each PST component (% of initial) in sterile/deionized water under different pH and temperature conditions. Legend: (○) STX; (●) neoSTX; (▲) dcSTX; (✶) GTX5.

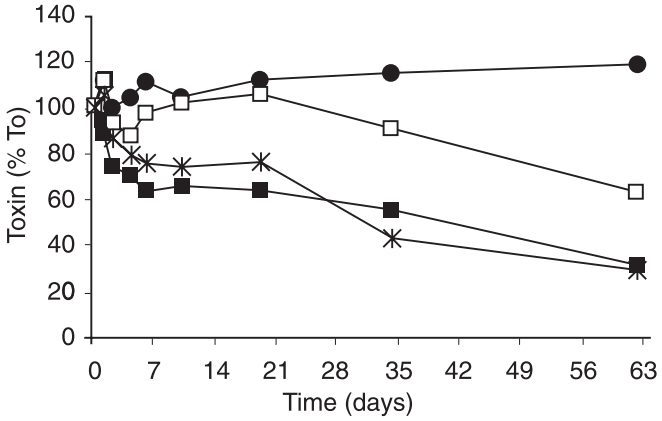

Figure 3 Variation of total toxicity (μM STX equivalents as % of initial) in sterile/deionized water at (●) 20ºC, pH 3; (■) 20ºC, pH 7; (□) 20ºC, pH 9 and (✶) 30ºC, pH 7.

Results

Influence of pH and Temperature on Toxin Degradation Figure 1 shows the variation (% of initial) of total PST concentration in water throughout the incubation period under different pH and temperature conditions. At pH 3, PST were fairly stable. Under both neutral (pH 7) and alkaline (pH 9) conditions, total PST concentration decreased exponentially with time according to first order degradation kinetics (ANOVA, $P < 0.05$). At 30°C (pH 7), PST decay also followed first order kinetics (ANOVA, $P < 0.05$). However, the effect of increasing the incubation temperature by 10°C was a twofold increase in the PST degradation rate. Under pH 7 conditions, the half-lives of total PST at 20°C doubled the half-lives reached at 30°C (Table 1).

Concentration of GTX5, neoSTX, and STX decreased exponentially with time under both neutral and alkaline conditions (Fig. 2). However, this decay was coupled with an increase of dcSTX that reached levels beyond the initial concentration in both alkaline (pH9) and high temperature (30ºC) incubation settings. The combined effect of these differences in the overall toxicity is shown in Fig. 3.

Influence of Bacteria and Organic Matter on Toxin Degradation All PST dissolved in the cyanobacterial-free culture media degraded according to first order kinetics (ANOVA, $P < 0.05$), exhibiting half-lives much shorter and degradation rates much higher than in sterile MilliQ water (Table 1).

Figure 4 shows the variation of total PST levels (% initial) in the cyanobacteria-free culture media under the different incubation settings. Results suggest that the elimination of bacteria from the culture medium enhanced the degradation of PST. This enhancement was most significant in the presence of the protein fraction. Nevertheless, residual levels of PST were detected in all culture media after one month of incubation.

Discussion

PST toxins were highly stable in acidic solution, as previously noted by others (Alfonso *et al.,* 1994). PST- producing cyanobacterial blooms are not typically known to occur in acidic natural environments. However, cyanobacteria can be exposed to reduced pH conditions in water treatment processes. The collapse of cyanobacterial blooms often contributes to a slight alkalinization of the surrounding

Table 1 Degradation rate (k) and half-life time ($t_{1/2}$) of PST under different incubation conditions.

	k (d^{-1}) [$t_{1/2}$ (d)]				
Incubation condition	Total PST	GTX5	NeoSTX	dcSTX	STX
Sterile / deionized water					
20ºC pH7	0.0107 [65]	0.0092 [75]	0.0301 [23]	–	0.0139 [50]
20ºC pH9	0.0113 [61]	0.0213 [33]	0.0302 [23]	–	0.0077 [90]
30ºC pH7	0.0218 [32]	0.0322 [22]	0.073 [9]	–	0.0208 [33]
Culture medium					
Non-axenic and non-deproteinized	0.089 [8]	0.086 [8]	0.115 [6]	0.137 [5]	0.094 [7]
Axenic and non-deproteinized	0.240 [3]	0.229 [3]	0.222 [3]	0.384 [2]	0.312 [2]
Axenic and proteinized	0.126 [5]	0.130 [5]	0.787 [1]	0.112 [6]	0.092 [8]

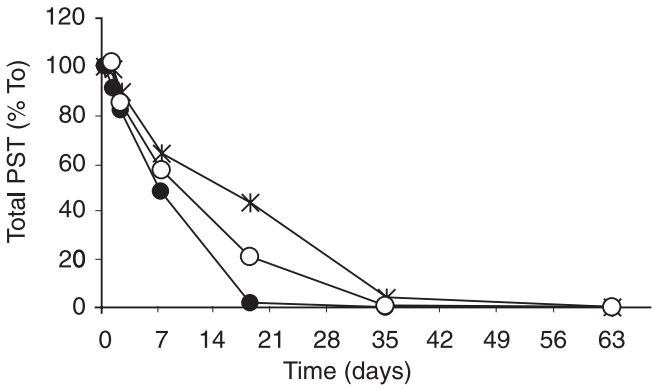

Figure 4 Variation (% of initial) of total PST levels in (✶) non-axenic and non-deproteinized culture medium; (●) axenic and non-deproteinized culture medium; (○) axenic and deproteinized culture medium.

medium. In sterile MilliQ water, PST persisted for an extended time period under both neutral and alkaline conditions, decaying to 50% of the initial concentration within one to two months of incubation. These results are consistent with those obtained by Jones and Negri (1997), who reported a persistence of other PST (C2+3, GTX2+3, dcGTX2+3) in aqueous solutions for 90 days at pH 7. However, our results are not in agreement with the high instability of PST in basic solutions (pH not specified) reported by Falconer *et al.* (1989). This may be explained by the alkaline conditions in our experiment not being strong enough to cause the rapid breakdown of toxins. Interestingly, an increase of dcSTX throughout incubation time was observed under both neutral and alkaline condition, especially at pH 9. This might be due to a desulfocarbamoyl reaction that converts less toxic GTX5 into the more toxic dcSTX (Jones and Negri, 1997). For this reason, toxicity decay in alkaline solutions was slower: at pH 9, toxicity levels decreased 40% after 2 months of incubation, while at pH 7 the decrease during that period was 70%. Increasing the temperature from 20°C to 30°C (at pH 7) caused a twofold to threefold increase in the degradation rates of GTX5, neoSTX and STX. However, the conversion of GTX5 into dcSTX was also considerably higher at 30°C, and therefore toxicity decay was similar at both temperatures. In many freshwater reservoirs, high water temperatures are often achieved during summer months when dense cyanobacterial blooms are also frequent (Mur *et al.,* 1999). The effects of water temperature and pH on the toxicity decay, after the collapse of a toxic bloom, should therefore be considered in order to correctly evaluate the risks of human exposure to PST through drinking water.

A broader range of variables, including not only the physical and chemical properties of the water, but also biologic and organic factors, may affect the degradation of cyanobacterial toxins in natural fresh waters. We simulated natural conditions, incubating PST in the culture medium where the toxic Aphanizomenon sp. LMECYA31 previously grew. Under these experimental conditions, PST degradation was considerably higher than in sterile MilliQ water. The differences may be attributed to the higher potential of toxin oxidation due to the presence of organic compounds dissolved in culture media (Jones and Negri, 1997). In natural freshwater environments, the levels of dissolved organic compounds increase significantly after bloom collapse. Under these conditions, it is expected that the reduction of PST to 50% of its initial concentration may occur within three to eight days, although residual levels may persist for more than one month.

Although the potential role of bacteria in the biodegradation and bioconversion of PST has been previously described (Kotaki *et al.,* 1985), our results do not indicate that PST degradation in the culture medium was mediated by bacterial activity. If PST degradation was linked to bacterial activity, toxin loss would not be modelled by first order kinetics. Conversely, none of the PST components increased throughout the incubation time, indicating that no interconversions of toxins had occurred. It is possible that the presence of cyanobacterial compounds released into the culture media might have inhibited potential bacterial activities. According to Jones *et al.* (1994), the synthesis of bacterial enzymes responsible for the degradation of microcystins from a *Microcystis aeruginosa* extract is apparently repressed by the catabolites present in that extract. However this hypothesis was not tested for PST. The observed increase in PST degradation when bacteria were removed from the culture medium might also suggest that toxin breakdown is catalyzed by enzymes that could be inhibited by the presence of proteolytic bacteria. The decrease of toxin degradation observed after deproteinization of culture medium supports this hypothesis, but further studies are needed.

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The Effects of Iron Limitation on Growth and PSP Toxin Production in *Alexandrium fundyense*

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Abstract

The effect of iron limitation was examined in the red tide dinoflagellate *Alexandrium fundyense.* Cells were grown in chelexed artificial seawater with f/2 micronutrients and varying concentrations of added iron. Cultures with higher levels of iron (11 μM) showed signs of iron toxicity, whereas cultures grown at concentrations of added iron less than 100 nM showed iron limitation. Upon iron-limitation, cultures showed a marked decrease in their toxin content and a change in their toxin composition. The decrease in toxin per cell was similar to that reported for N-limitation, suggesting that iron-limited cultures may be deficient in the nitrogen assimilation pathway needed to support toxin formation.

Introduction

PSP toxin formation in *Alexandrium* species is affected by a number of environmental variables (Cembella, 1998). Phosphate deprivation and low temperature increase the amount of toxin per cell (Boyer *et al.,* 1987; Anderson *et al.,* 1990; Siu *et al.,* 1997), presumably by decreasing the cellular division rate while leaving the toxin production rate constant. In contrast, nitrogen deprivation decreases the toxin content per cell (Boyer *et al.,* 1987; Taroncher-Oldenberg *et al.,* 1997). Saxitoxin and the related PSP toxins have a high nitrogen content (32% by weight), and this decrease under nitrogen is likely due to the inability of the cell to supply sufficient nitrogen to support toxin biosynthesis. Iron can also be a limiting nutrient for harmful algal blooms (Boyer and Brand, 1997). It is a key element in the respiratory pathway needed for energy production and the enzymes used for nitrogen assimilation. Biologically available iron can be low in coastal systems (Wells, 1989) and may be important in red tide formation. For these reasons, we were interested in examining the effects of iron on growth and PSP toxin formation in the dinoflagellate *Alexandrium fundyense.*

Materials and Methods

Culture Conditions *Alexandrium fundyense* (GTCA28) was grown in acid-washed polycarbonate flasks using chelexed artificial seawater (ASW) with f/2 trace nutrients and containing varying concentrations of added iron chelated with twice the iron concentration of EDTA or NTA. The initial experiments used 35 mL polycarbonate tubes containing 0, 1.17, 11.7, 117, 1170 or 11,700 nM added iron that could be inserted directly in a Turner Designs 10-AU fluorometer without opening the tubes to limit iron contamination. To deplete residual iron and induce iron limitation, batch cultures were grown until late exponential phase, then 10% of the culture volume was serially transferred through at least 3 culture cycles to dilute media carry-over and internal pools of iron. Later experiments utilized acid-washed 1-L polycarbonate flasks to obtain sufficient biomass for toxin analysis. These 1-L cultures were initially grown in chelexed media containing 50 nM iron, which was then used to inoculate the cultures containing 0, 1, 10, 100, or 1000 nM added iron, again sequentially transferred through at least 3 culture cycles. All of the cultures were grown at 17°C with a 14:10 hr photoperiod at 55 μmol quanta m^{-2} s^{-1} light intensity. Cell growth was measured by *in vivo* fluorescence and direct cell counts under 400× magnification using a light microscope. Cultures were harvested in late exponential phase and the PSP toxins determined by HPLC using both post column chemical oxidation and electro-chemical oxidation using the isocratic solvent systems of Oshima (Boyer and Goddard, 1999). The C-toxins were measured using both the Oshima C system, and by difference after the addition of 0.1 N HCl to convert the N-21 sulfo-toxins to their corresponding de-sulfo derivatives.

Results and Discussion

Representative results for the 35 mL growth experiments are shown in Figure 1. These experiments were repeated several times using both EDTA and NTA as chelators. In general, the cultures grew better in 117 or 1170 nM added iron when compared to 11 μM added iron normally pres-

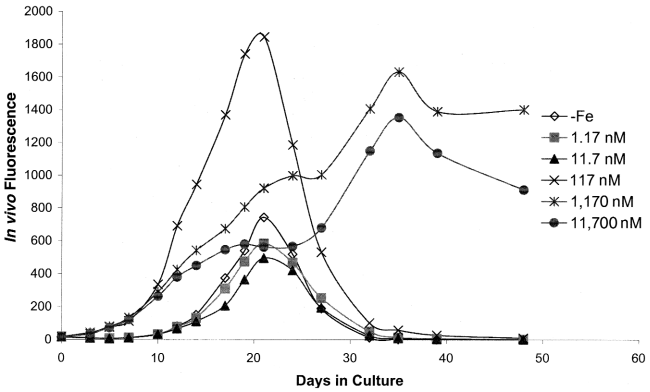

Figure 1 The growth of *Alexandrium fundyense* in 35 mL polycarbonate tubes containing various levels of iron chelexed with EDTA. Each point represents the average of three replicates.

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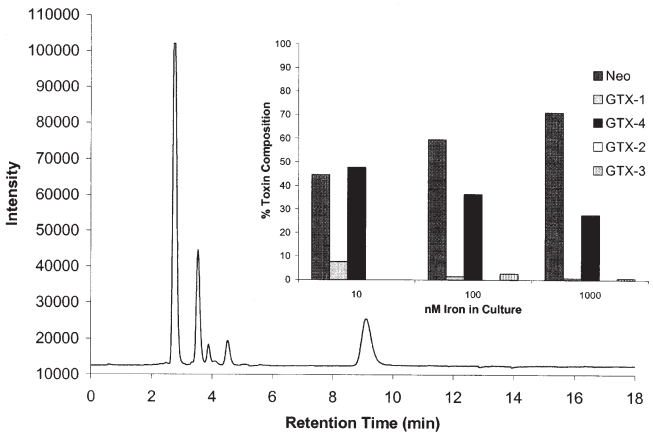

Figure 2 The HPLC-PCRS trace using the Oshima A solvent system for the 1000 nM culture. STX was not detected, even using the more sensitive HPLC-ECOS method. Gonyautoxins eluting between 3-5 min were identified using the Oshima B solvent. Toxin composition for the three treatments is shown in the insert. The peak at 2.5 min did not co-migrate with any "C" toxin using Oshima C, nor did it disappear upon addition of acid, indicating it is not a saxitoxin derivative.

ent in f/2. These results suggest that higher levels of iron may inhibit growth of *Alexandrium* species. Levels of added iron less than 117 nM could not support continual growth of *Alexandrium fundyense* in batch cultures.

Representative growth rates and the ratio of variable to maximal fluorescence for the 1-L batch cultures grown for toxin isolation are shown in Table 1.

No significant differences in growth rate and variable fluorescence were observed between cultures grown with 100 nM and 1000 nM added iron. In contrast, cultures serially transferred through 10 nM Fe showed negligible growth and an Fv/Fm less than 0.2, indicative of iron limitation. Addition of iron restored growth of these cultures to maximal levels. To determine the effect of iron on toxin production, toxin concentrations and compositions were determined at day 22 for three different iron treatments. These results are shown in Table 2 and Figure 2.

Alexandrium fundyense, grown under rigorous trace-metal clean conditions in batch culture, has a narrow range of optimal iron concentrations. Iron concentrations below

Table 1 Growth rate and variable fluorescence for 1-L batch cultures grown under three different conditions of added iron. Growth rates (div d^{-1}) were calculated by a linear regression of semi-log plots between days 5 and 20. DCMU enhanced fluorescence (Fv/Fm) was calculated for day 15.

Added iron	Growth Rate (div d^{-1})	Fv/Fm (s.d., n = 3)
10 nM Fe	~0	0.15 (0.03)
100 nM Fe	0.070	0.39 (0.02)
1000 nM Fe	0.063	0.44 (0.04)

Table 2 The change in toxin per cell with added iron as determined by HPLC analysis (n = 3). Saxitoxin, B1, B2, GTX-2 and C1-4 were not observed in any of the samples.

	Toxin Content in fmol toxin $cell^{-1}$ (% of total)		
Toxin	10 nM Fe	100 nM Fe	1000 nM Fe
NEO	3.96 (45%)	7.85 (60%)	23.6 (71%)
GTX-1	0.67 (8%)	0.19 (1%)	0.24 (1%)
GTX-4	4.24 (48%)	4.80 (36%)	9.19 (28%)
GTX-3	0	0.34 (3%)	0.23 (1%)
Total	8.87 (100%)	13.18 (100%)	33.26 (100%)

100 nM did not supply sufficient iron to maintain maximal grow rates in sequential transfers through new media. *A. fundyense* also showed decreased growth in full strength f/2 (11 μM added iron) when compared to media containing lower concentrations of iron. It is unknown if this inhibition was due to the high iron concentration (iron toxicity) or due to the higher chelator concentrations present in these cultures. Lower concentrations of iron in some of the newer medium formulations such as "Pro99" (CCMP, unpublished) may be better suited for growth of *Alexandrium* species.

Toxin production in *A. fundyense* was sensitive to the levels of available iron. Toxin per cell decreased as the cells were increasingly iron-limited, suggesting that iron was necessary to provide either the energy or nitrogen needed for toxin biosynthesis. Similar results have been observed for another nitrogen containing toxin, domoic acid, where iron limitation also decreased toxin production in batch cultures (Bates *et al.,* 2001). This particular *Alexandrium* isolate showed a very unusual toxin composition, lacking both saxitoxin and the N-sulfo toxins. This composition is different from that originally reported for isolate GTCA28 (Anderson *et al.,* 1994) and may represent a change in composition after nearly 15 years in culture. Toxin composition also changed with iron limitation and was not a stable trait for this culture.

Acknowledgements

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Characterization of Sensitivity to PSP Toxins in North American Populations of the Softshell Clam *Mya arenaria*

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Abstract

Our prior research demonstrated significant differences in sensitivity to paralytic shellfish poisoning (PSP) toxins and thus capacity for toxin uptake between two test populations of softshell clams, *Mya arenaria,* which correlated with their long-term history of toxin exposure in the field. Resistant clams were prevalent in the population recurrently affected by PSP, and rare in that with no PSP history. The present study uses a rapid, non-destructive burrowing index to characterize the toxin sensitivity of Pacific and Atlantic North American populations of *M. arenaria* in relation to their PSP history, and thus test the validity of our initial findings over a wide geographic scale. Generally, good agreement was found between sensitivity determinations based on the burrowing index and *in vitro* nerve response to saxitoxin. Mapping of the latitudinal distribution of toxin resistance allowed identification of sensitive clam populations in SE Nova Scotia, Canada and central Long Island Sound, USA, regions with no history of PSP, and in Puget Sound, an area only recently affected by toxic blooms. In contrast, resistant populations (high capacity for toxin accumulation) were found throughout the Bay of Fundy and Gulf of Maine, regions historically affected by PSP. We hypothesize that the occurrence of resistant populations as far south as NE Cape Cod, which experience less frequent and intense toxic events, may be related to larval transport and thus gene flow via a southwestward coastal current in the Gulf of Maine. Parallel studies are aimed at determining the molecular basis for toxin resistance in this species.

Introduction

Significant differences in sensitivity to paralytic shellfish poisoning (PSP) toxins and in toxin accumulation between two populations of softshell clams, *Mya arenaria,* from Atlantic Canada, were found to correlate with their history of toxin exposure (Bricelj *et al.,* 2000; MacQuarrie and Bricelj, 2000). Resistant clams, which accumulated up to eight times higher toxicities over two weeks of laboratory toxification, were dominant in the population with a long-term history of PSP (Lepreau Basin, LB, Bay of Fundy) and rare in the population with no PSP history (Lawrencetown estuary, LE, Nova Scotia) (MacQuarrie, 2002). Sensitive clams suffered significant toxin-induced mortalities, and reduction in feeding and metabolic rates. We hypothesize that genetic adaptation to toxins, via selection of more resistant clams, occurs in PSP-affected areas.

The objectives of the present study were to a) compare two indices of individual toxin sensitivity: the *in vivo* burrowing index, and *in vitro* nerve test, and b) map the distribution of toxin sensitivity of North American populations of *M. arenaria,* measured by the burrowing index, in relation to their PSP history, in order to test and extend the validity of our findings from early studies over a wide geographical scale.

Materials and Methods

The burrowing index measures, under standardized conditions (16°C, 30 ppt salinity, coarse sand substrate), the ability of juvenile clams (averaging ~35 mm in shell length) to re-burrow when exposed at the sediment surface for 24 hours to *Alexandrium tamarense* [strain PR18b, 60–100 pg saxitoxin equivalents (STX eq) cell^{-1}, 100 cells mL^{-1}]. Juvenile *M. arenaria* were used because burrowing capacity declines in adults of this species. The burrowing index is rapid, non-destructive and allows characterization of resistance of large sample sizes. The percentage (%) of clams that can burrow (resistant phenotype) by the end of two hours is compared to that of controls fed nontoxic algae (n = 60 to 100 clams divided equally between two tanks per treatment). Clams are acclimated to laboratory conditions for at least three weeks prior to toxification. The nerve test was adapted from that described by Twarog *et al.* (1972), using STX standard provided by IMB's Certified Reference Materials Program (CRMP).

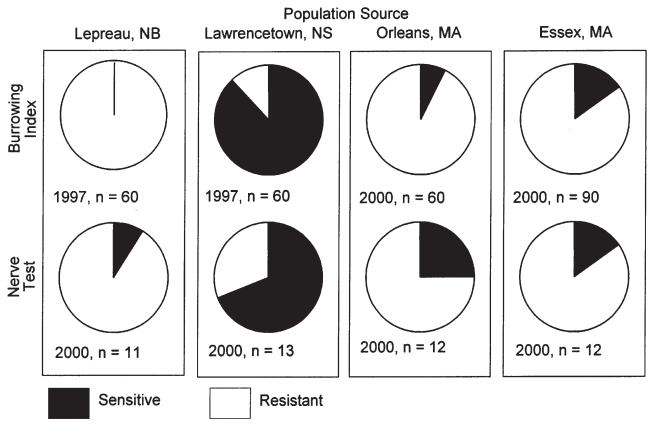

Figure 1 Comparison of results (% resistant vs. sensitive clams) obtained using the burrowing index and nerve test. NB = New Brunswick; NS = Nova Scotia; MA = Massachusetts; numbers = year of collection of the *Mya* cohort (the same year class was tested with both methods for the two MA populations); n = number of clams tested per treatment.

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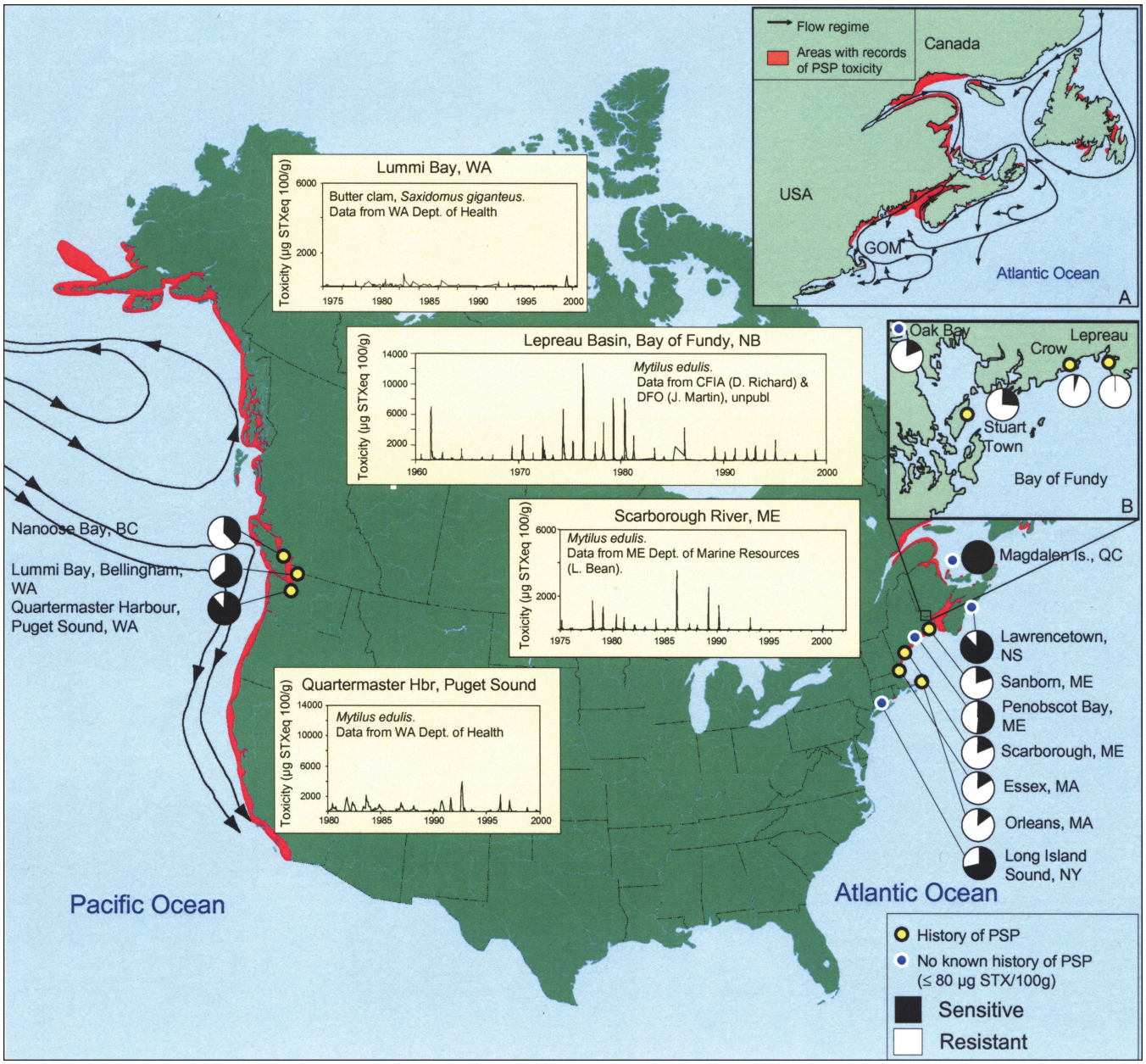

Figure 2 Percent of toxin-resistant and sensitive clams (pie charts) in *M. arenaria* populations. **A** General pattern of non-tidal surface circulation on the NW Atlantic (from Anderson *et al.,* 1994). **B** Detail of sampling sites in the Bay of Fundy, Canada. Insets show historical records of PSP toxicity in bivalves for selected sampling locations: *Mytilus edulis* data shown where available (note that this sentinel species generally attains toxicities two to four times more than co-occurring *Mya*). Note differences in the toxicity scale for Lummi Bay and Scarborough River monitoring sites. Areas of the coastline with records of PSP are marked in red (modified from Bates, 1997 and Anderson *et al.,* 1994). Circulation in the NE Pacific is redrawn from Horner *et al.* (1997).

Results and discussion

The nerve test measured action potentials of isolated, cerebro-visceral connectives exposed to increasing concentrations of STX to determine the concentration required to block conduction of the nerve action potential. Nerve action potentials of sensitive clams were fully blocked by 10^{-5} g STX mL^{-1}. Nerves of resistant clams were fully blocked at concentrations of 10^{-4} g STX mL^{-1} or greater. The nerve response is innate (constitutive) rather than induced by toxin exposure. Good agreement was found between the percent of resistant vs. sensitive clams determined with the two indices (Fig. 1).

On the Atlantic coast, sensitive *Mya* populations (71–98% sensitive clams) occur in Mt. Sinai Harbor, central Long Island Sound, New York (Bricelj *et al.,* 1996), and southeast Nova Scotia, offshore in the Magdalen Islands, Québec; regions with no known history of PSP (Fig. 2).

Resistant populations (high toxin uptake capacity) pre-

vail in the Bay of Fundy (inset B) and Gulf of Maine (GOM), regions historically affected by PSP. The Penobscot Bay toxin-free "sandwich area" (Shumway *et al.,* 1988) shows intermediate resistance compared to other Maine populations. This and the occurrence of resistant populations as far south as Cape Cod, where PSP outbreaks are more recent, less frequent, and less intense, may be related to larval transport via a southwestward coastal current in the Gulf of Maine (inset A). Similarly, the predominantly resistant population occurring in Oak Bay, in the upper reaches of Passamaquoddy Bay, where there are no records of PSP outbreaks (inset B), is attributed to larval transport via the residual counterclockwise circulation gyre and intense tidal mixing characteristic of the Bay of Fundy.

The relationship between percent resistance and history of PSP is less clear on the Pacific coast where *M. arenaria* was introduced in the late 1800s (Palacios *et al.,* 2000) and toxicity records are more limited: a predominantly sensitive population occurred in central Puget Sound, WA, an area only recently affected by PSP (since the 1970s), and in Lummi Bay, WA, where historical toxicities are extremely low. Sampling of west coast *M. arenaria* populations was also more limited, as this species is generally not dominant in local habitats, recruitment is sporadic, and populations are often composed of larger/older clams.

We suggest that toxin resistance in *Mya* has evolved as a complex function of PSP history (intensity, duration and frequency of occurrence) as well as physical circulation patterns controlling gene flow from pelagic larval dispersal. Investigation of the mechanism for toxin resistance at the molecular level, via sequencing of the sodium channel gene, is in progress (Connell *et al.,* 2002).

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Depuration and Transformation of PSP Toxins in the South African Abalone *Haliotis midae*

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Abstract

Abalone were grown 1) on diets of artificial feed, 2) with their *in situ* food source, kelp, which contained paralytic shellfish poisoning (PSP) toxins, and 3) in filtered seawater to investigate toxin depuration and transformation under feeding and starving conditions. The abalone were toxic at the start of each treatment with saxitoxin (STX), neosaxitoxin (NEO), gonyautoxins (GTX) 2+3 and 1+4, and B1 present. When fed artificial feed the abalone depurated at a rate of 6.3 mg STX eq 100 g^{-1} tissue d^{-1}; however, no depuration was observed in organisms fed kelp or starved. Toxin transformations occurred in abalone for each treatment. Initially, abalone toxin content was dominated by GTX1+4 and B1. In abalone fed artificial feed, STX and B1 congeners were negligible. Toxin content was not significantly different between abalone in the kelp-fed or starvation treatments, with the congeners NEO and B1 dominant. Knowledge of the toxin source and an improved understanding of abalone toxin depuration and transformation are still required, yet the need to include non-traditional vectors such as abalone in routine monitoring programs is clearly evident.

Introduction

Dinoflagellates of the genus *Alexandrium* are the most common PSP toxin sources, and suspension-feeding bivalves are the usual vectors. However, there are increasing reports of toxins in organisms other than shellfish (Shumway, 1995; Landsberg, 2002). PSP toxins were first detected in abalone off the NW coast of Spain in 1991 (Martinez *et al.,* 1993) and off the coast of South Africa in 1999 (Pitcher *et al.,* 2001). The toxin profile in the South African abalone *Haliotis midae* was very different from that of the local *Alexandrium* population, a potential toxin source. Toxins have been detected, however, in samples of the kelp *Eklonia maxima,* the primary *in situ* food source for the abalone (Fig. 1; Etheridge, 2002); thus, kelp is also considered a putative source of the abalone toxicity. Though the exact source of toxins to the abalone remains unknown, the goals of this study were 1) to determine the toxin depuration rate in abalone under feeding and starving conditions and 2) to quantify toxin transformations in the abalone.

Materials and Methods

Abalone were obtained from a south coast abalone farm where they were fed a diet of *E. maxima.* The animals used in experiments were approximately 2 cm in length, and the average wet weight of tissue was 0.6 ± 0.3 g. The abalone (n = 7 or 8, depending on the treatment) were incubated in aerated 500 mL flasks containing 300 mL of 0.7 mm filtered seawater, and they were exposed to one of the following conditions in the laboratory for two weeks: 1) commercial artificial feed obtained from the abalone farm, 2) the kelp *E. maxima,* and 3) filtered seawater. These represented non-toxic feeding, toxic feeding, and starving conditions, respectively. Samples were extracted and analyzed for toxins using high performance liquid chromatography (HPLC) (Etheridge, 2002). Toxins are reported as mmol toxin 100 g^{-1} wet weight or tissue, and toxicity in mg STX eq 100 g^{-1} tissue is also estimated from HPLC-based concentrations using conversion factors of Oshima (1995). Given the facile epimerization of GTX1 and GTX4, GTX2 and GTX3, C1 and C2, and C3 and C4 (Hall *et al.,* 1990), the toxin content is reported with epimer pairs combined. Departures from sample means are represented with standard error values, and differences between treatments were assessed using the Student's *t*-test.

Results and Discussion

The toxins STX, NEO, and GTX1+4 were present in the kelp sample (Fig. 1). The abalone were initially toxic (160 ± 38 µg STX eq 100 g^{-1} tissue), exceeding the legal limit for commercial markets (Fig. 2). The animal-to-animal variation was very high (cv = 0.52). When abalone were fed artificial feed only, they became less toxic (72 ± 13 mg STX eq 100 g^{-1} tissue); the average depuration rate over the 2-week period was 6.3 µg STX eq 100 g^{-1} tissue d^{-1} (Fig. 2). There was no significant difference in toxicity between the abalone at initial conditions and those in the kelp-fed and starved

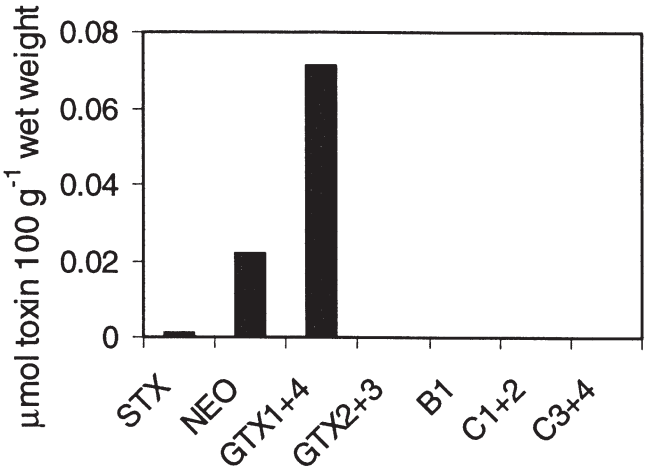

Figure 1 Toxin content of the kelp *E. maxima,* in µmol toxin 100 g^{-1} wet weight.

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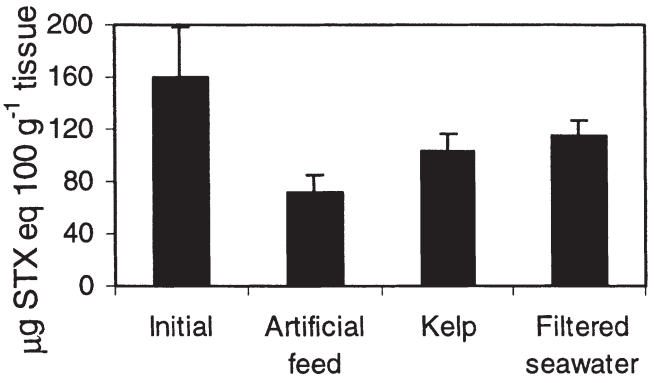

Figure 2 Mean abalone toxicity and standard error in µg STX eq 100 g^{-1} tissue for initial conditions and all treatments. The artificial feed treatment was significantly different from the initial conditions and other treatments ($P < 0.1$).

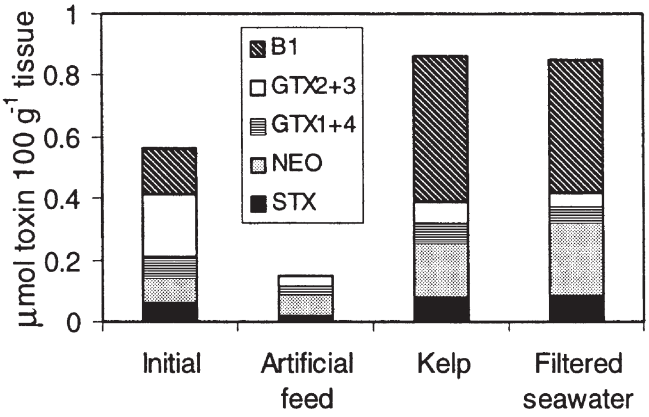

Figure 3 Toxin content expressed as µmol toxin 100 g^{-1} tissue for abalone at initial conditions and exposed to artificial feed, kelp, and filtered seawater treatments.

treatments (Fig. 2). The average coefficient of variation for all treatments (cv = 0.42) was less than the initial conditions.

The dominant congeners in abalone at initial conditions were GTX2+3 and B1 (Fig. 3). Toxins STX and B1 were negligible in abalone fed artificial feed (Fig. 3). Toxin content in abalone fed kelp and those starved was not significantly different, but they were dominated by NEO and B1 (Fig. 3).

The initial toxicity of the abalone was much higher than expected based on earlier reports for abalone from the south coast of South Africa. Results also demonstrated that there is considerable variability in toxicity among individual organisms, consistent with other abalone results (Bravo *et al.,* 1999; Pitcher *et al.,* 2001) and for other shellfish in general (*e.g.,* Cembella *et al.,* 1993; White *et al.,* 1993). The observed variability has significant implications for monitoring procedures and highlights the importance of designing protocols that allow for reliable sampling strategies for monitoring shellfish toxicity.

The only treatment where depuration was observed was for abalone fed non-toxic artificial feed. That the starved animals did not depurate suggests that toxin may be retained in the digestive tract or that depuration is dependent upon healthy metabolism. After two weeks, the abalone fed artificial feed depurated to just below the regulatory limit for harvesting. Therefore, we hypothesize that incubating abalone with artificial feed for a slightly longer period before making them commercially available may allow sufficient depuration, safely below the regulatory limit. It is notable, however, that abalone farmers have reported toxins in abalone raised on artificial feed. Thus, the use of artificial feed as a management strategy requires further investigation.

The toxins STX, NEO, and GTX1+4 were present in the kelp sample. It is possible that the toxins were produced by the kelp or organisms living in/on the kelp (*e.g.,* bacteria or epiphytes). Further investigations of the role kelp may play in toxin production are necessary. Though the toxin profile in kelp did not exactly match that found in abalone, it does not mean that kelp is not the source of the toxin. It has been demonstrated that toxin transformation can occur in shellfish (*e.g.,* Bricelj *et al.,* 1991; Cembella *et al.,* 1993); therefore, it is likely that abalone may also transform the toxins, producing a toxin profile that differs from the source.

Unlike previous studies in which only STX and dc-STX were detected, we observed a suite of toxins present in the abalone, including STX, NEO, GTX1+4, GTX2+3 and B1. Biotransformations may be the cause of these discrepancies in toxin profiles. During the 2 weeks, biotransformations were observed with the most obvious changes associated with the congeners GTX2+3 and B1. For abalone in the kelp only and starvation treatments, the relative amount of GTX2+3 decreased, whereas B1 represented a higher percentage of total toxins. Abalone exposed to artificial feed showed a decrease in STX, GTX1+4, GTX2+3, and B1, suggesting that depuration of these toxins occurred.

The detection of PSP toxins in abalone provides a new and additional risk to consumers. Abalone toxicity will add further to the existing damage to the economy caused by HABs (Anderson *et al.,* 2000). It is necessary to determine the toxin source and understand the possibility of toxin depuration and transformation in these organisms in order to protect public health and ensure the safety of commercial seafood products. Results to date support the need for including non-traditional vectors such as these gastropods in routine monitoring programs for PSP.

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The Variability of Paralytic Shellfish Poisoning Toxin Distribution in Cockles (*Acanthocardia tuberculatum*): Implications for the Evaluation of the Toxicity

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Abstract

In January 1989, paralytic shellfish poisoning (PSP) toxins were detected in shellfish along the Mediterranean shore of southern Spain and northern Morocco. The causative organism of this outbreak was identified as *Gymnodinium catenatum* Graham. The cockle *Acanthocardia tuberculatum* ("Mediterranean cockle," "corruco") is a bivalve mollusc largely exploited in the affected areas. Routine monitoring has shown that PSP levels in this mollusc frequently exceeded the regulatory level of 80 µg STX equiv./100 g meat. Previously published data suggest a possible individual variation in the PSP toxicity of molluscs from the same area. This variability could lead to a misinterpretation of actual toxicity of molluscs batches. A study on *A. tuberculatum* was carried out to document this individual variability. It also explored the assessment of PSP content using samples collected at different time intervals. Furthermore, the distribution of the PSP toxins in the different tissues and portions of the foot, which has been shown to accumulate the highest amount of the toxins, was studied.

Introduction

In January 1989, paralytic shellfish poisoning (PSP) toxins associated with *Gymnodinium catenatum* Graham were detected in cockles ("Mediterranean cockle," "corruco") *Acanthocardia tuberculatum* along the Mediterranean shore of southern Spain and northern Morocco. Routine monitoring has shown that PSP levels in this mollusc frequently exceeded the regulatory level of 80µg STX equiv./100 g meat. Possible individual variation in the PSP toxicity of molluscs from the same area could lead to misinterpretation of actual toxicity of mollusc batches. A study on *A. tuberculatum* was carried out to document this individual variability in toxicity, to assess PSP content at different time intervals, and to determine the distribution of the PSP toxins in the different tissues and portions of the foot, which has been shown to accumulate the highest amount of toxins.

Methods and Materials

Samples All studies were carried out on *A. tuberculatum* samples collected at a depth of 10–12 metres using a shellfish dredge. Cockles were collected at two different locations in the southern Spain littoral zone between Cádiz and Málaga. A total of 89 animals were selected at random and frozen until individual analysis was conducted. Cockles were classified according to their shell height (in mm) in different ranges (see Figs. 1, 2). Six individuals were also studied to determine the toxin distribution in the following tissues: foot, adductor muscle, mantle, siphon, gill and digestive gland. In order to compare variation in the toxicity levels in the distal, middle and proximal portions of the locomotor organ, the foot from each of 49 individuals was divided into these three portions (Fig. 3), and each portion was separately analysed. In each case, the level of toxicity obtained from the distal portion of the foot was given a value

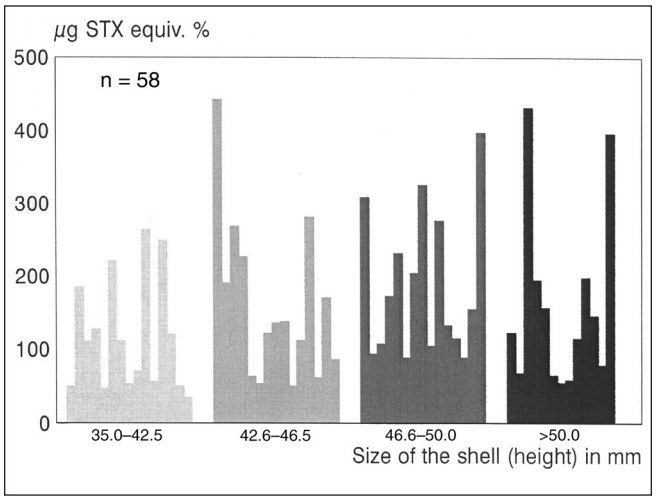

Figure 1 Individual variability. Distribution by size. Area of capture: La Línea (Cádiz).

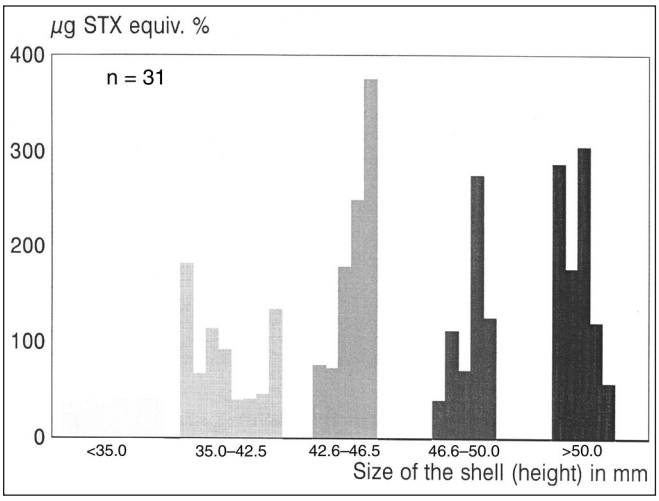

Figure 2 Individual variability. Distribution by size. Area of capture: Estepona (Málaga).

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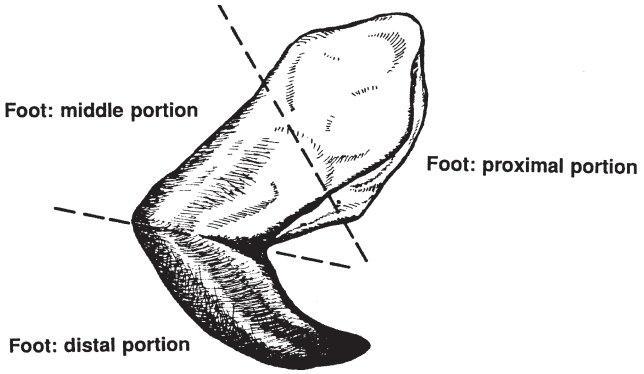

Figure 3 Foot of *A. turberculatum.*

of 1. The mean value of toxicity of the middle and proximal portions was normalised and expressed as a ratio of 1 (Table 1).

Analytical Methods Analyses were performed by the mouse bioassay (AOAC, 2000). This method was adapted to the amount of the edible portion of each individual. The results are given as amount (µg) of saxitoxin equivalent extrapolated to 100 g of edible meat. This was done to facilitate the relative comparison of toxicity amongst mollusc species or mollusc tissues. Mouse bioassay extracts were also used for the fluorometric assay (Burdaspal, 1991) and HPLC with pre-column peroxide oxidation and fluorescence detection (Lawrence and Menard, 1991; Lawrence *et al.,* 1995). Overall potency was calculated using individual toxin concentrations from HPLC analysis and the intrinsic potencies (Oshima, 1995) of each of the saxitoxin derivatives.

Results and Discussion

Substantial variability in the distribution of PSP toxin within the *A. tuberculatum* samples was demonstrated. This variability influences the analytical evaluation of mollusc toxicity. Our data suggest that different factors may contribute to this observed variability.

Individual Variability There are differences in the toxicity of the cockles captured within the same area (Figs. 1, 2). A wide variability in individual toxicity levels was found within each selected size range. The variability does not depend significantly upon the size (age) of the molluscs, except in the case of the smallest molluscs from Estepona (Málaga) (Fig. 2), where the toxicity detected was negligible or was close to the detection limit of the mouse bioassay.

Tissue Variability Using results from the mouse bioassay, the level of toxicity (mg STX equiv./100g) in the different tissues varied as follows: Foot > mantle and siphon > gill > digestive gland > adductor muscle (n = 6).

Our results are different from those obtained for *Spisula solidissima* (Cembella and Shumway, 1993).

A study on the distribution of toxins in the various tissues was also performed using HPLC (see Lawrence and Menard, 1991; Lawrence *et al.,* 1995). Decarbamoylsaxitoxin, saxitoxin and gonyautoxin-5 were detected in all tissues with the highest levels found in the distal portion of the foot. More than the 60% of these toxins were concentrated in the foot, followed by the mantle and the digestive gland. Except for some traces found in the digestive gland, gonyautoxins-2/3 were only detected in the distal portion of the foot. The digestive gland contained traces of C1/C2 which could not be detected in the other tissues. In all tissues except the gills, gonyautoxin-5 was the main toxin. In the gill, decarbamoylsaxitoxin was predominant and had relatively more concentration of saxitoxin and less N-sulfocarbamoyl toxins (gonyautoxin 5) when compared with other tissues. Tissues were increasingly toxic in the following order: Foot > mantle and siphon > digestive gland > adductor muscle > gill.

Locomotor Organ (Foot) Variability In all individuals studied (n = 49) the highest level of toxicity was found in the distal portion of the foot (intense red color), followed by the middle and proximal portions. Furthermore, the difference is larger with the greater the toxicity detected in the distal portion.

The data obtained in this study support the hypothesis that this bivalve mollusc, following ingestion, binds and accumulates the PSP toxin in organs other than the digestive gland. This difference clearly affects the rate at which toxin is eliminated. When molluscs from the same area show such variability in PSP toxin accumulation, there can be a degree of inaccuracy in the determination of toxicity during monitoring (White *et al.,* 1993). The possibility that toxicity of the analytical sample is not representative of the mollusc population constitutes a potential health hazard for

Table 1 Variability in the toxicity of the locomotive organ (foot).

Toxicity range		Distal Portion			Middle Portion			Proximal Portion		
µg STX equiv./100 g meat	n	M	S (n^{-1})	CV (%)	M	S (n^{-1})	CV (%)	M	S (n^{-1})	CV (%)
69–200	12	1	–	0	0.66	0.17	25.6	0.48	0.13	26.5
200–400	14	1	–	0	0.49	0.18	37.1	0.25	0.13	51.6
400–700	13	1	–	0	0.43	0.16	37.0	0.22	0.13	5.6
700–1324	10	1	–	0	0.36	0.09	24.0	0.19	0.06	33.7
69–1324	49	1	–	0	0.49	0.19	38.2	0.28	0.16	56.5

M: Average; S: Standard deviation; CV: Coefficient of variation

the consumer. Therefore, we recommend the establishment of certain conditions related to the sampling and preparation of the samples prior to the analysis, similar to those normally applicable to other natural toxins with an irregular distribution in food products (*e.g.,* mycotoxins). The sample chosen from the mollusc population must be as large as possible; it has to be composed of multiple subsamples randomly selected from different parts of the overall capture area; the sample should be thoroughly homogenised and blended until the finest size of particles is achieved; and from this homogenate, the analytical sample should be obtained.

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PSP Toxin Accumulation by the Edible Shore Crabs *Telmessus acutidens* and *Charybdis japonica* at Onahama, Japan

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Abstract

Several species of shellfish including edible shore crabs (*Telmessus acutidens* and *Charybdis japonica*) and bivalves (mussels *Mytilus galloprovincialis,* oysters *Crassostrea gigas* and Japanese scallops *Chlamys farreri nipponensis*) were collected from the Pacific coast at Onahama, central Honshu, Japan, to investigate the accumulation of PSP toxins during the bloom season of toxic dinoflagellates. The maximum visceral toxicity of *T. acutidens* collected in 1999 was 80.0 MU/g by mouse bioassay. The toxicities of mussels *M. galloprovincialis* and scallops *C. farreri nipponensis* collected at the same site in 1999 were 9.6 MU/g and 5.2–10.2 MU/g, respectively. The presence of PSP toxins in the crab viscera was identified by high performance liquid chromatography with fluorescent detection (HPLC–FLD) and electrospray ionization mass spectrometry (ESI–MS); this is the first observation of PSP toxins in *T. acutidens.* In 2000, the crabs *Charybdis japonica* and *T. acutidens* were again collected at the same site, and PSP toxins were investigated by HPLC–FLD. Low levels of PSP toxins were detected in the viscera of the two crab species as well as in mussels collected at the same time. *Telmessus acutidens* and *C. japonica* are known to prey on mollusks, and these crabs were observed to prey on mussels at the sampling site. Therefore one source of toxicity for the crabs was assumed to be from toxic prey organisms such as bivalves.

Introduction

Several species of crustaceans have been reported to accumulate PSP toxins (Shumway, 1995). PSP toxin was located mainly in the hepatopancreas of American lobsters (*Homarus americanus*) in eastern Canada (Watson-Wright *et al.,* 1991; Desbiens and Cembella, 1995), and the source of the toxin was suggested to be from toxic bivalve molluscs (Shumway, 1995). Kelp crab (*Pugettia producta*) and red rock crab (*Cancer productus*) were also reported to become toxic when dinoflagellate blooms occurred (Jonas-Davies and Liston, 1985). However, in Japan, little attention has been paid to the accumulation of PSP toxins by crustaceans during the bloom season of toxic dinoflagellates. Because many species of crustaceans are consumed as seafood in Japan, it is necessary to investigate their toxin accumulation. We therefore collected edible shore crabs, *Telmessus acutidens* and *Charybdis japonica,* together with bivalve mollusks that comprised their shellfish prey, from the Pacific coast at Onahama, Japan.

Materials and Methods

Crabs (*Telmessus acutidens* and *Charybdis japonica*) and bivalves (mussels *Mytilus galloprovincialis,* oysters *Crassostrea gigas,* and Japanese scallop *Chlamys farreri nipponensis*) were collected at Onahama, Japan, by SCUBA diving in 1999 and 2000. *Alexandrium* spp. was monitored from 23 March to 10 May 1999 and from 3 April to 22 May 2000 (Table 1). The test solution for the toxicity mouse bioassay (Kawabata, 1978) was extracted from the viscera of the crabs and from whole edible tissues of bivalve specimens, using 0.1N HCl. The extract used for the toxicity mouse bioassays was passed through a cartridge column (Sep-Pack C18, Waters) and an ultrafiltration membrane (Ultrafree C3GC, Millipore), and then the filtrate was applied for high performance liquid chromatography with fluorescent detection (HPLC-FLD) analysis by the method of Oshima (1995). Standards used were GTX1-4, dcGTX2, dcGTX3, C1, C2, neoSTX (provided by the Fisheries Agency of Japan), STX (provided by Dr. Noguchi of former Nagasaki University) and dcSTX (provided by Professor Oshima of Tohoku University). For electrospray ionization mass spectrometry (ESI-MS) analysis, the extract was partially purified by successive treatment with activated charcoal and a Bio-Gel P2 (Bio-Rad Laboratories) column (Kotaki *et al.,* 1981). ESI-MS was performed on a SSQ7000 mass spectrometer equipped with an atmospheric pressure ion source, and an electrospray ionization (ESI) interface (Finnigan MAT, CA, USA) was employed for detection. The

Table 1 Concentrations of *Alexandrium* spp. in 1999 and 2000 at Onahama.

Year	Date (month/day)	*Alexandrium* spp. (cells/liter) × 10^3
1999	3/23	N.D.*
	4/5	2.46
	4/19	1.07
	4/26	0.14
	5/10	0.03
	5/19	N.D.
2000	4/3	N.D.
	4/17	0.01
	5/8	0.01
	5/15	N.D.
	5/22	N.D.

*N.D.: Not detected

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Table 2 Toxicities of crabs and bivalves collected at Onahama in 1999.

No.	Species	Date (month/day)	Toxicity (MU/g)
Oh-1	*T. acutidens*	4/19	80.0
Oh-2	*T. acutidens*[a]	4/19	30.0
Oh-3	*M. galloprovincialis*[b]	4/21	9.6
Oh-4	*C. gigas*	4/21	<2.0[c]
Oh-5	*M. galloprovincialis*[b]	4/25	<2.0[c]
Oh-6	*C. farreri nipponensis*	4/25	5.2
Oh-7	*C. farreri nipponensis*	4/25	10.0
Oh-8	*C. farreri nipponensis*	4/25	10.2

[a]Two specimens, [b]three specimens combined for analysis. [c]over 15 min until mice died.

spray voltage was set at +4.5 kV, and the heated capillary temperature was maintained at 250°C. Full-scan spectra were acquired in the positive ion mode over the mass ranges of *m/z* 200–700 for GTXs, and in the negative ion peak mode for C toxins over the same range. Sample introduction was via a Rheodyne 7125 injector with a 25-µl loop through a reversed phase column (Mightysil RR-18, Kanto Chem.). The mobile phase consisted of distilled water and acetonitrile (95:5, v/v) with 0.1% acetic acid.

Results and Discussion

In 1999, the highest concentrations of *Alexandrium* spp. were 2.46×10^3 cells/L on 5 April, whereas in 2000, the concentrations detected were 0.01×10^3 cells/L on 17 April and 8 May (Table 1). In 1999, the toxicities of the crab *T. acutidens* were 30.0 MU/g and 80.0 MU/g in viscera, whereas the toxicity levels in whole edible tissues of mussels *M. galloprovincialis* and *C. farreri nipponensis* were 9.6 MU/g and 5.0–10.2 MU/g, respectively (Table 2).

One of the toxic sources for the crabs was assumed to be toxic prey organisms such as bivalves. This assumption was based on the fact that the crab is a predatory species of bivalves and abalone (Tohoku National Fisheries Research Institute, 1982, pers. comm.), and because we observed crabs preying on mussels at the sampling site. HPLC-FLD chromatograms of the crab viscera and the PSP toxin standards are shown in Fig.1. The peaks 1–7 corresponded with standard toxins GTX4, GTX1, GTX3, GTX2, C1, C2, and STX, respectively. The peaks corresponding with dcGTX2, dcGTX3, and dcSTX were not detected in the crab viscera (data not shown). The peaks corresponding to GTXs were partially purified and analyzed by ESI-MS in positive ion mode (Fig. 2).

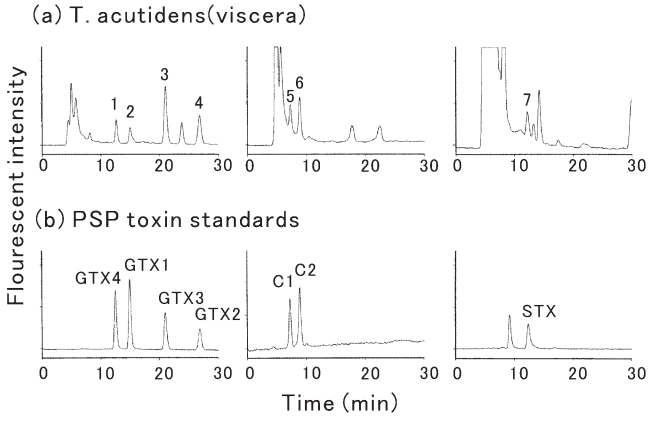

Figure 1 HPLC chromatograms of the extract from the crab viscera of *T. acutidens* (**a**) and PSP toxin standard (**b**).

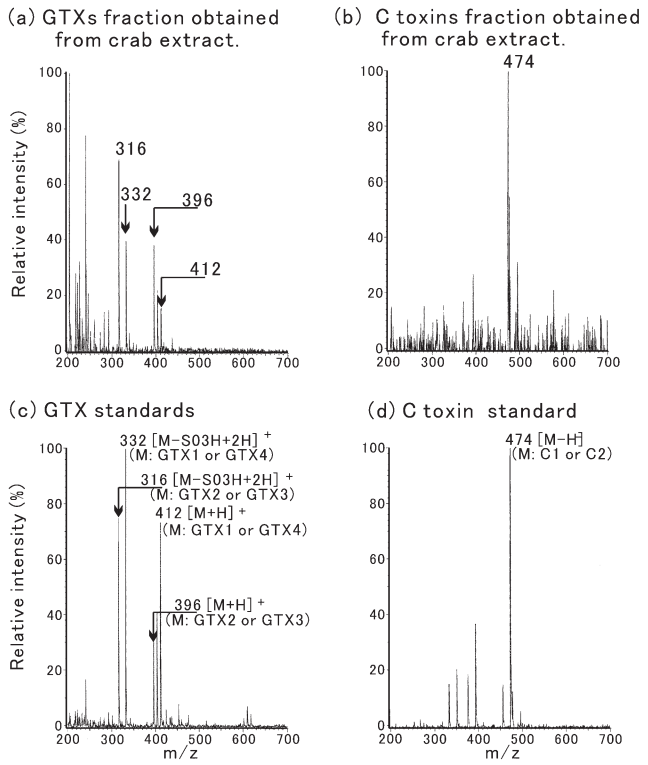

Figure 2 ESI-MS spectra of purified extract of the GTXs fraction (**a**) and C toxins fraction (**b**) obtained from the crab. GTXs standard (**c**) and C toxins standard (**d**) were also analyzed in same condition.

The ions at *m/z* 316, 332, 396, and 412 were detected both in the crab sample (Fig. 2a) and in the standards for GTXs (Fig. 2c), which were typical mass fragments of GTXs (Onodera *et al.,* 1996; Quilliam, 1996; Lagos *et al.,* 1999). The peaks corresponding to C1 and C2 were also purified and analyzed in negative ion mode (Fig. 2b), and the major ion peak was detected at *m/z* 474. The peak was considered to be the deprotonated molecule of C1 and C2 (Onodera *et al.,* 1996). A sufficient amount of the purified fraction of the peak corresponding to STX was not obtained from the extract; therefore, the extract was re-analyzed by HPLC-FLD without the oxidizing reagent. Both the peak and the STX standard disappeared (data not shown), as was found in a previous study (Onodera *et al.,* 1996; Lagos *et al.,* 1999). The results clearly indicated the presence of PSP toxins in the crab viscera, thus providing the first observation of PSP toxins in *T. acutidens.* In 2000, the crab *C. japon-*

Table 3 PSP toxin concentration in visceral tissues of two species of crabs in 2000.

Species	Date	Toxin (nmole/g)					
		GTX1	GTX2	GTX3	GTX4	C1	C2
T. acutidens	4/17	–[a]	–	–	–	–	–
T. acutidens	4/17	–	Tr.[b]	Tr.	–	–	–
T. acutidens	4/17	–	Tr.	Tr.	–	–	–
T. acutidens	4/17	–	–	–	–	–	–
T. acutidens	4/17	–	–	Tr.	–	–	–
T. acutidens	4/17	–	–	Tr.	–	–	–
T. acutidens	4/17	–	–	–	–	–	–
T. acutidens	5/11	0.16	Tr.	0.15	0.17	Tr.	0.68
C. japonica	5/11	–	–	Tr.	–	–	–
C. japonica	5/11	–	–	Tr.	–	–	–
C. japonica	5/11	–	–	Tr.	–	–	–
C. japonica	5/11	–	–	Tr.	–	–	–
C. japonica	5/11	–	–	–	–	–	–
C. japonica	5/11	–	Tr.	Tr.	–	–	–
C. japonica	5/11	–	–	–	–	–	–

–[a]: not detected, Tr.[b]: less than 0.1 nmol/g.

ica, which is also known to be a predatory species of molluscan shellfish (Kojima, 1981), was collected with *T. acutidens* at Onahama. PSP toxin concentrations in the two species were analyzed by HPLC-FLD, and trace amounts of PSP toxins were detected in both crab species (Table 3).

PSP toxins were also detected in mussels *M. galloprovincialis* collected at the same time (data not shown). However, we could not collect *C. japonica* when the shellfish prey were highly toxic. Sampling will be continued in the future to confirm the possible mode of toxin accumulation in crabs.

In this study, we found PSP toxin accumulation in the crabs *T. acutidens* and *C. japonica.* These two crab species are commercially fished and are also caught by recreational fishing in some districts in Japan. Further research needs to be conducted to evaluate the risk of toxin accumulation in these species. In particular, *T. acutidens* was considered a risk for human intoxication, since toxicity in the crab viscera was 20-fold higher than the regulatory limit (4.0 MU/g) for bivalves in Japan.

Acknowledgements

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Toxicity of the Aqueous Extract of *Alexandrium fraterculus* (Balech) Balech

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Abstract

Alexandrium fraterculus (Balech) Balech is a chain-forming armored dinoflagellate commonly found in the Brazilian Current. It has been suggested that this species may be involved in mussel contamination by PSP on the Uruguayan and Brazilian coasts. In order to investigate the toxicity of *A. fraterculus,* we isolated two strains from Santa Catarina Coast, southern Brazil (48°36´S, 26°47´W), where annually 12,000 metric tons of the Mytillidae *Perna perna* are produced. The strains were maintained in non-axenic *K* media plus soil extract at 20°C in a 12/12 light/dark cycle at 48 µE cm/m^2/s. Strain 1 (May 1999) was tested for PSP production by post column HPLC analysis and the mice bioassay method from AOAC. The results did not confirm the presence of paralytic shellfish toxins, at least for the experimental conditions, but toxicity was observed because mice died after 8 hours. Strain 2 (October 1999) was tested for the toxicity of a saline extract (7% NaCl) obtained from growing cells (log phase) by 5 different assays: hemolytic test, anti-mitotic and chronic tests in sea urchin larvae (*Lytechinus variegatus*), chronic test in the mussel larvae (*Perna perna*), and PSP based on the AOAC mice bioassay. The results showed that the extract was not hemolytic, anti-mitotic, nor toxic to mice (acute). On the other hand, the concentration, equivalent to 9.7 and 800.0 cel.mL^{-1} in the extract, delayed the larvae development of *P. perna* and *L. variegatus,* respectively. These results, and those obtained during a monitoring program, suggested that *A. fraterculus* does not produce paralytic shellfish toxins, but does produce other substances, yet to be identified, that are toxic to invertebrate larvae.

Introduction

Santa Catarina State is the largest mussel producer on the Brazilian coast, with an annual production in the year 2000 of about 12,000 metric tons of the Mytilidae *Perna perna.* Other cultured species include the oysters *Crassostrea gigas, C. rysophorae* and the Pectinidae *Lyropecten nodosus.* Studies on harmful algae started in 1995, following the rapid growth of shellfish aquaculture off the Santa Catarina coasts, in the beginning of the 1990s. As a result of a pilot HAB monitoring program concentrated at Armação do Itapocoroy bight, PSP and DSP have been found in cultured mussels, sometimes at levels higher than those established as safe for human consumption (*i.e.,* 400 MU · 100 g^{-1} and presence, for PSP and DSP respectively) (Proença *et al.,* 1998, Proença *et al.,* 1999).

Until now, toxins found either in mussels or in the water column included okadaic acid, saxitoxin, GTX2, GTX3, GTX1, GTX4, C1, C2 and domoic acid (Proença *et al.,* 1998, Proença *et al.,* 1999, Proença *et al.,* 2001, Proença unpublished). The toxins have been associated with known toxic algae species such as *Dinophysis acuminata, D. acuta, Gymnodinium catenatum* and *Pseudonitzschia* spp. Up to the moment, *G. catenatum* is the only confirmed PSP producer found in the region. The toxin profile of *G. catenatum* isolated from Armação do Itapocoroy bight did not match the one found in mussels in 1997, when PSP levels were higher than 400 MU · 100 g^{-1}, indicating another source of contamination in the region.

Dinoflagellates from the genus *Alexandrium* are among those which produce PSP and cause economic losses in several parts of the globe where aquaculture and natural stocks are exploited. *Alexandrium fraterculus* (Balech) Balech is a warm water chain-forming armored dinoflagellate found in Pacific coastal areas of Japan, Korea, Thailand, and Philippines and in the Atlantic Ocean between southeastern Brazil and northern Argentinean littoral (Balech, 1995). Chains of *A. fraterculus,* formed by several individuals have been frequently observed in the shallow waters of the Santa Catarina coast (Rörig *et al.,* 1998). The only data available about toxin production of *A.* (=*Protogonyaulax*) *fraterculus* concerns the toxin carried with an isolate from Senzaki Bay, in Japan (Noguchi *et al.,* 1985), which did not confirm toxin production from that strain. However, on the Uruguayan coast, the presence of this dinoflagellate coincided with outbreaks of toxic mollusks at a time when no other suspect organisms were observed in the plankton (Balech, 1995). The aim of this paper was to investigate the production of paralytic shellfish toxins and the toxicity of the aqueous extract of *A. fraterculus* occurring on Santa Catarina's coast.

Materials and Methods

Two strains of *A. fraterculus* were isolated from Armação do Itapocoroy bight (48°36′ Lat., 26°47′ Long) in different periods of the year. Cells were grown in K media plus soil extract and maintained at 48 µE · m^{-2} · s^{-1} in a 12:10 LD period (given by fluorescent lamps) at 20°C, at 34 ppt salinity.

Strain 1 (isolated in May 1999) was tested for PSP production by HPLC and mice bioassay based on the AOAC (1990). Cells growing at the exponential phase were concentrated on fiberglass filter and extracted in 0.1 N HCl by sonication, giving final concentration of 1.2×10^5 cells · mL^{-1}.Toxin analysis was carried out by the ion paring RF-HPLC with fluorescent detection (FLD) and post-column derivatization method based on Oshima (1995). Toxin identification was determined by comparing retention times

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with the 6 available standards pursued from CNRC/Canada.

A second strain (2) was isolated in October 1999. For this strain, cells growing at the exponential phase were retained on fiber glass filter and extracted by sonication in the physiological saline solution (0.9%), giving an initial concentration equivalent to 1.7×10^5 cell · mL^{-1}. The toxicity or toxin presence in this extract was tested by 5 different methods: a) HPLC post-column derivatization method (Oshima, 1995); b) mice bioassay based on the AOAC (1990); c) hemolytic activity (Rangel *et al.,* 1987); d) antimitotic and chronic test using the sea urchin larvae of *Lytechinus variegatus* (Freitas and Sawaya, 1986) and e) chronic test using D-shaped larvae of the mussel *Perna perna* (Reis Fo, 1999). Hemolytic activity was tested on erythrocytes suspension obtained from mice blood. Negative and positive controls were performed using saline solution at 0.9% and Triton 100 at 1%, respectively. For the invertebrate tests, a negative control was performed using a culture of the diatom *Chaetocerus gracilis* grown in the same conditions as *A. fraterculus.*

Results and Discussion

From the extract of strain 1 we did not observe the presence of PSP toxins either by HPLC or by the bioassay. None of the 6 PSP toxins tested were present in the chromatographic runs. In the bioassay, the immediate and conspicuous PSP symptoms produced after the IP inoculation were not observed, but the extract presented some toxicity, as tested mice died after 8 hours of assay.

The absence of PSP toxins was confirmed in the aqueous extract from strain 2. No peaks were identified within the elution times for the tested standards. Different from strain 1, we did not observe any acute toxicity on mice in a 24-hour bioassay. On the other hand, toxicity was observed on the assays of *L. variegatus* and *P. perna* larvae. Normal *P. perna* larvae development was interrupted with a minimal observed effect concentration (OEC) equivalent of 97 cells · mL^{-1}. Results showed a dose-dependent relationship. The aqueous extract also inhibited the normal development of *L. variegatus* larvae at an OEC of 800 cells · mL^{-1}. No hemolytic effect was observed.

The results obtained indicate that considering the experimental conditions, *A. fraterculus* does not produce PSP toxins. More concentrate extracts could be tested as well as different laboratory growing conditions. On the other hand, we have not found any evidence for PSP in mussels during the occurrence of *A. fraterculus* in the water column in Armação do Itapocoroy bight. The putative absence of paralytic shellfish toxins production by *A. fraterculus* from Santa Catarina's coast is supported by the finding of Noguchi et al (1985), who did not find PSP toxicity in the strain from Senzaki Bay, Japan. These findings suggest that this species can be excluded from the suspicious PSP-causing species in the region. At this moment, *Gymnodinum catenatum* is the only PSP producer found off Santa Catarina coast (Proença *et al.,* 2001). Previous toxin profiles from contaminated mussels cultured in the region indicate the presence of other unidentified toxic species different from *G. catenatum* (Proença *et al.,* 1999). The toxic effect on invertebrate larvae found indicate that *A. fraterculus* may not produce PSP, but we can consider it a potentially harmful species to the marine biota. Today, regional mussel culture is facing a problem of larvae recruitment of natural stocks. Normally, seeds for growth are collected at rocky shores, with a small part collected from culture structures. This practice, together with natural bank exploitation, is reducing the overall recruitment. We suggested, based on our results, that an eventual bloom of the studied species could indirectly negatively affect the activity.

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The Characterization of Two New Spirolides Isolated from Danish Strains of the Toxigenic Dinoflagellate *Alexandrium ostenfeldii*

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Abstract

Following their discovery, two new spirolides SP-1 and SP-2 from cultured isolates of *Alexandrium ostenfeldii* obtained from Limfjorden, Denmark, were isolated and structurally characterized by using LC/MS methodology.

Introduction

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 (Hu *et al.,* 1995). 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 (Uemura *et al.,* 1995; Takada *et al.,* 2001; C.-K. Lu *et al.,* 2000; Seki *et al.,* 1995). 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 and Tangen was identified as the cause of spirolide toxicity in Nova Scotia (Cembella *et al.,* 1999; Cembella *et al.,* 2000). 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 (Hu *et al.,* 1995; Hu *et al.,* 1996; Hu *et al.,* 2001). Using LC/MS methodology, spirolides have now been detected in cultures of *A. ostenfeldii* isolated from Limfjorden, Denmark. Examination of the LC/MS profiles of extracts from these Danish cultures has revealed the presence of desmethyl spirolide C, but also two previously unidentified spirolide components

Materials and Methods

Isolation of Spirolides from* A. ostenfeldii *Biomass Two isolates of *A. ostenfeldii* (LF 37 and LF 38) obtained from Limfjorden, Denmark were incubated in 2 L Fernbach flasks at 16°C under a photon flux density of 120 µmol m^{-2} s^{-1} on a 14:10 h light:dark photocycle. Cells were harvested in late exponential growth phase by gravity filtration on a 20 µm Nitex sieve. The cells were concentrated by centrifugation at 4,000 g for 20 min at 5°C in 15 mL Falcon centrifuge tubes.

The initial identification of spirolide compounds in the culture was performed on a PE-SCIEX API 165 single quadrupole mass spectrometer (Thornhill, Ont., Canada) equipped with a pneumatically-assisted electrospray ionisation source coupled to an HP 1100 liquid chromatograph (Agilent, CA, USA). A Hypersil C8 (50 × 2 mm) column was eluted isocratically with 70% A (50 mM formic acid, 2 mM ammonium formate, 0.02% trifluoroacetic acid) and 30% B (50 mM formic acid, 2 mM ammonium formate, 0.02% trifluoroacetic acid, 95% acetonitrile) at a flow rate of 200 µL/min.

The two new spirolide toxins were isolated from *A. ostenfeldii* biomass using a procedure similar to that described by Hu *et al.* (2001). Briefly, the wet cells of LF 38 (61.4 g) were extracted three times by adding methanol followed by sonication. The methanolic supernatants were pooled following centrifugation, evaporated to dryness, dissolved in water, and partitioned three times with dichloromethane to yield the toxin-containing dichloromethane fraction. This was fractionated using a Sephadex LH-20 column that was eluted with methanol. Fractions containing spirolides were pooled, and evaporated to dryness. Following dissolution in 30% methanol/ water the fraction was subjected to a C_{18} flash chromatography column which was conditioned and eluted with 40% acetonitrile/water (0.1% trifluoroacetic acid). Fractions containing spirolides were combined, concentrated and purified using a Vydac 201TP510 C_{18} HPLC column which was eluted isocratically with 30% acetonitrile/water (0.1% trifluoroacetic acid) and monitored at 210 nm. The yields of SP-1 and SP-2 spirolides from the LF 38 strain were determined by proton NMR quantitation, to be 5.6 and 6.3 mg respectively. NMR spectra were measured on a Bruker DRX-500 spectrometer in CD_3OH at 500.13 MHz (^{1}H) and 125.7 MHz (^{13}C). The workup of 72.5 g of wet biomass of the LF 37 strain yielded ~0.7 mg of SP-1 and no SP-2.

Results and Discussion

LC-MS analysis (Fig. 1) of Danish isolates LF37 and LF 38 of A. ostenfeldii detected 13-desmethyl spirolide C and at least two other spirolide-like compounds (SP-1 and SP-2) in the methanol extracts of wet cells of these cultures. A fragment ion occurring at *m/z* 164 in the MS/MS spectra of both peaks SP-1 and SP-2 indicated the presence of spirolide C analogues which contain vicinal dimethyl groups in the cyclic imine ring (Fig. 2). The isolation of SP-1 and SP-2 was undertaken to determine the structure of these novel spirolide analogues.

The molecular formulae of SP-1 and SP-2 were found

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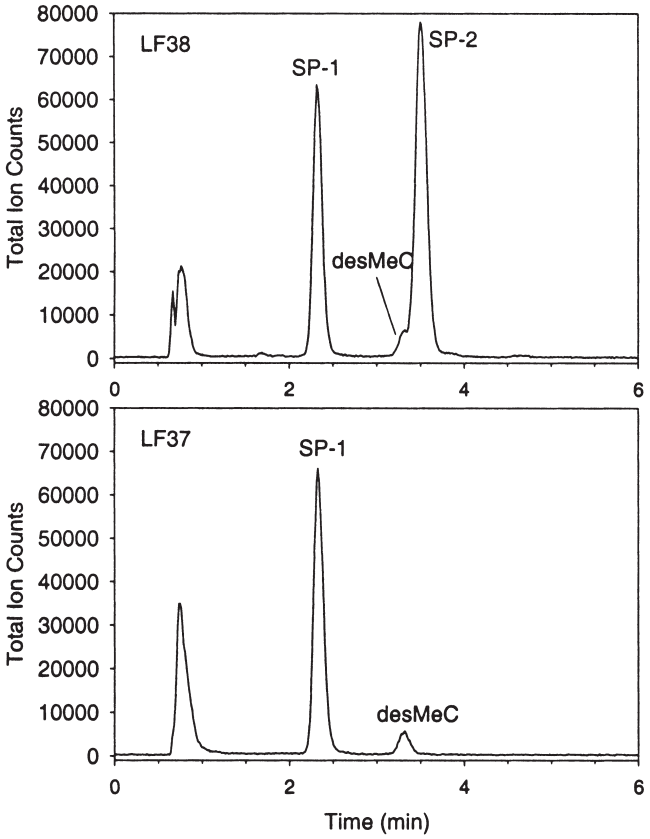

Figure 1 LC-MS analysis of methanol extract of *A. ostenfeldii* strains LF37 and LF38.

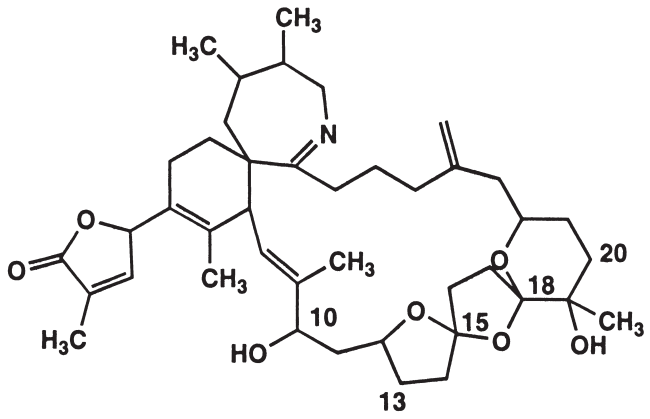

Figure 2 Structure of 13-desmethyl spirolide C.

by HRMS to be $C_{41}H_{59}NO_7$ ([M+H]$^+$ 678.4375, calc. 678.4370, Δ = 0.8 ppm) and $C_{42}H_{61}NO_7$ ([M+H]$^+$ 692.4564, calc. 692.4526, Δ = 5.4 ppm) respectively. FTIR results supported the presence of hydroxyl groups (3470 cm^{-1}), C = O and/or C = N group (1684 cm^{-1}) and a γ-lactone ring (1746 cm^{-1}). Using NMR data (^{1}H, ^{13}C DEPT, and HSQC) the carbons of SP-1 were determined to be distributed as five methyl, sixteen methylene, ten methine and ten quaternary carbons. Using the same analysis SP-2 was found to contain an additional methylene carbon. Through inspection of ^{1}H and ^{13}C chemical shifts, and COSY NMR data we were able to confirm the presence of the γ-lactone ring, an imine, and a vinyl double bond in both structures. The six remaining ^{13}C resonances between 122 and 149 ppm were assigned to three double bonds. Using COSY and TOCSY experiments we were able to elucidate six spin systems that were then connected using HMBC experiments. This allowed us to determine all of the structural features of SP-1 and SP-2 (Fig. 3) except for their trispiroketal ring systems. SP-1 and SP-2 were determined at this stage to be 13-desmethyl spirolide C derivatives that were both missing methyl groups at the carbon equivalent to carbon 19.

SP-1, also named 13,19-didesmethyl spirolide C, was assigned a 5,5,6-trispiroketal ring system based on the remaining unassigned ^{13}C shifts at 118 (C15) and 110 (C18) ppm which were characteristic of 5,5 and 5,6-spiroketal carbons, respectively. The two remaining quaternary ^{13}C shifts of SP-2 at 109 and 101 ppm, however, indicated the respective presence of 5,6 and 6,6 spiroketal carbons. The assignment of a 5,6,6 trispiroketal ring system to SP-2 was

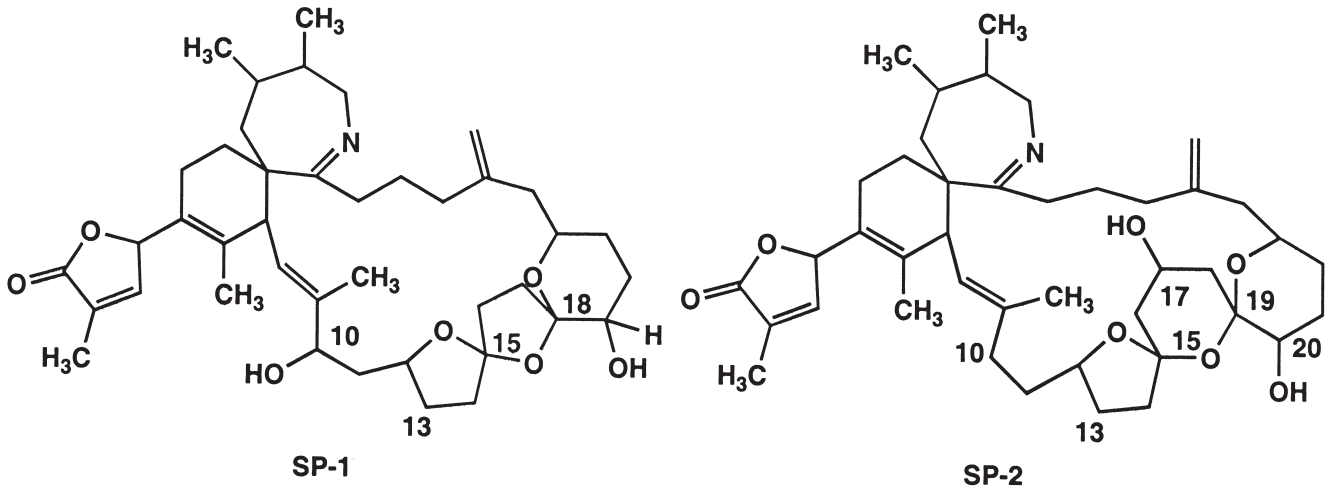

Figure 3 Structures of SP-1 and SP-2.

in agreement both with the spin systems that had been determined earlier indicating the presence of a hydroxyl group at C17 and with the observation that SP-2 contained one more methylene group than SP-1. The fragment ions observed in the mass spectra of each were in agreement with the proposed structures in Fig. 3.

To date, there is no strong evidence that links the presence of spirolides in the tissues of shellfish with human intoxication. At present we are investigating the mechanisms of action of these and other spirolides to determine their toxicological effects.

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Isolation and Structure Elucidation of New and Unusual Saxitoxin Analogues from Mussels

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Abstract

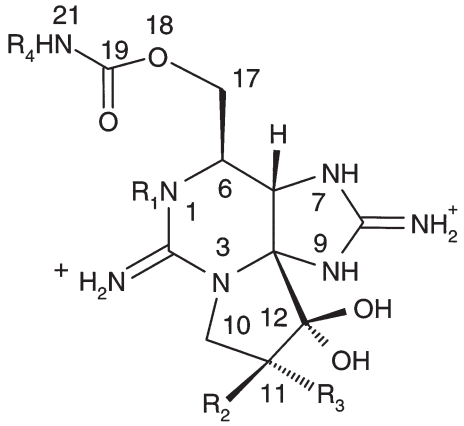
Three new paralytic shellfish poisoning (PSP) toxins were isolated from toxic mussels and, based on liquid chromatography-mass spectrometry and ^{1}H and ^{13}C NMR spectroscopy, were deduced to be 11β-hydroxy-N-sulfocarbamoyl saxitoxin (M1β); 11,11-dihydroxy-N-sulfocarbamoyl saxitoxin (M3); and 11,11 dihydroxy-saxitoxin (M4).

Introduction

In June 2000, a dense bloom of *A. tamarense,* a known producer of PSP toxins (Fig. 1), was found to be responsible for a massive kill of aquacultured salmon (Cembella *et al.,* 2002). During the investigation of this event, samples of wild mussels (*Mytilus edulis* and *M. trossulus*) collected from the vicinity of the salmon cages showed high toxicity (up to 67,000 µg saxitoxin equivalents per kg tissue) by mouse bioassay. Subsequent analyses by hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS), a recently developed method for the rapid, selective and sensitive detection of all PSP toxins (Quilliam *et al.,* 2001), showed that both plankton and mussels contained several known PSP toxins: STX, NEO, GTX1-4, GTX5, C1, and C2. The mussels also contained five saxitoxin-related compounds not present in the plankton. These five compounds were assigned codes M1 to M5. This report presents our work on the identification of four of these compounds.

Materials and Methods

Standard solutions of PSP toxins were provided by the Certified Reference Materials Program (NRC, Halifax). Mussels samples were collected in Shelburne harbor on June 15, 2000 (courtesy of N. Lewis and A. Bauder). Extraction (50 g of mussel tissue) with 0.1 M acetic acid and partitioning against dichloromethane was followed by a combination of Biogel P2 column chromatography (eluted with 0.1 M acetic acid) and preparative HILIC using MS detection. This led to the isolation in pure form of M1β (0.2 mg), M3 (0.1 mg), M4 (0.1 mg), and M5 (0.03 mg). All LC-MS and LC-MS/MS experiments were performed using PE-SCIEX API 165 and III+ single and triple quadrupole mass spectrometers (Thornhill, Ont., Canada) equipped with a pneumatically assisted electrospray (ionspray) ionization source coupled to an HP1090 liquid chromatograph (Agilent, CA, USA). The columns used (2 × 250 mm for analytical and 7.8 × 300 mm for preparative work) were packed with 5 µm TSK gel Amide-80 (TosoHaas, PA, USA). Isocratic elution was performed with 65% B, where eluent A was water and eluent B was a 95% acetonitrile/water, both containing 2 mM ammonium formate and 3.6 mM formic acid (pH 3.5 for A). The flow rate was 0.2 mL/min (0.8 mL/min for preparative) and a post column split was employed to deliver approximately 20 mL/min to the ion spray interface. A sample injection volume of 5 µL was used. Selected ion monitoring (SIM) and selected reaction monitoring (SRM) detection were carried out with a 140 ms dwell time. NMR spectra were measured on a Bruker DRX-500 spectrometer in D_2O/0.1M CD_3COOD, pH 2.0 solution and in 9/1 H_2O/D_2O/0.1M CD_3COOD, pH 3.9 solution. ^{1}H and ^{13}C NMR spectra were recorded



Code	Compound	R_1	R_2	R_3	R_4
STX	Saxitoxin	H	H	H	H
NEO	Neosaxitoxin	OH	H	H	H
GTX1	Gonyautoxin-1	OH	H	OSO_3^-	H
GTX2	Gonyautoxin-2	H	H	OSO_3^-	H
GTX3	Gonyautoxin-3	H	OSO_3^-	H	H
GTX4	Gonyautoxin-4	OH	OSO_3–	H	H
GTX5 (B1)	N21-sulfocarbamoylsaxitoxin	H	H	H	SO_3^-
GTX6 (B2)	N21-sulfocarbamoylneosaxitoxin	OH	H	H	SO_3^-
C1	N21-sulfocarbamoyl gonyautoxin-2	H	H	OSO_3^-	SO_3^-
C2	N21-sulfocarbamoyl gonyautoxin-3	H	OSO_3^-	H	SO_3^-
C3	N21-sulfocarbamoyl gonyautoxin-1	OH	H	OSO_3^-	SO_3^-
C4	N21-sulfocarbamoyl gonyautoxin-4	OH	OSO_3^-	H	SO_3^-
M1β	11β-hydroxy-N21-sulfocarbamoyl-saxitoxin	H	OH	H	SO_3^-
M2β	11β-hydroxy-saxitoxin	H	OH	H	H
M3	11,11-dihydroxy-N21-sulfocarbamoyl-saxitoxin	H	OH	OH	SO_3^-
M4	11,11-dihydroxy-saxitoxin	H	OH	OH	H

Figure 1 Chemical structures of known and new PSP toxins.

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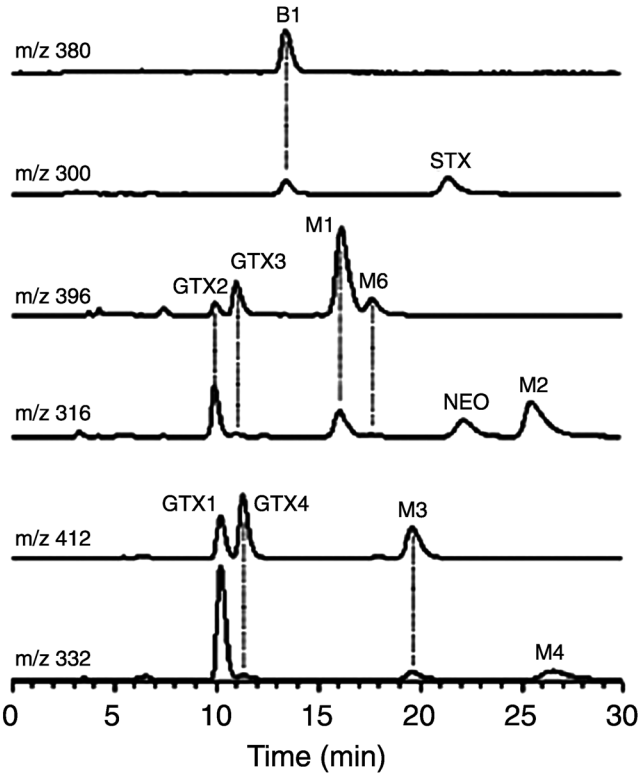

Figure 2 HILIC-MS analysis in SIM mode of mussel tissue extract after an initial Biogel P2 column cleanup.

at 500.13 MHz (^{1}H reference to CHD_2COOD at $\delta_H = 2.03$) and at 125.77 MHz (^{13}C reference to dioxane at $\delta_C = 67.6$), respectively. ^{1}H COSY, TOCSY and ^{1}H/^{13}C HSQC NMR experiments were carried out to unambiguously assign signals.

Results and Discussion

Figure 2 shows the HILIC-MS analysis of an extract that has been taken through an initial Biogel P2 column, which eliminated the C1-C2 toxins. Preparative work toward isolation in pure form of the presumably new compounds (M1-M5) was carried out.

The electrospray ionization (ESI) mass spectra of M2 showed an $[M+H]^+$ ion at *m/z* 316. The MS/MS product ion spectrum of *m/z* 316 had ions at *m/z* 298 (loss of H_2O), *m/z* 237 (loss of $NH_2COOH + H_2O$), *m/z* 220 (loss of $NH_2COOH + H_2O + NH_3$), and *m/z* 196 (loss of $NH_2COOH + NHCO + NH_3$). This spectrum and the compound's retention time matched exactly with those of 11β-hydroxy-STX. Therefore, this compound was identified as 11β-hydroxy-STX and thereafter coded M2β.

The ESI mass spectra of M1 showed an $[M+H]^+$ ion at *m/z* 396 and an $[M-H]^-$ ion at *m/z* 394, indicating a molecular weight of 395 for the free base. High-resolution ESI-MS data were consistent with an elemental composition of $C_{10}H_{17}N_7O_8$ ($[M+H]^+$ 396.0940, calc. 396.0938, $\Delta = 0.5$ ppm), confirming M1 to be an isomer of GTX-2 and -3. The MS/MS fragment ion spectrum of the $[M+H]^+$ ion showed prominent ions at *m/z* 316 (loss of SO_3), *m/z* 298 (loss of $SO_3 + H_2O$), *m/z* 273 (loss of SO_3 + NHCO), *m/z* 255 (loss of $HO_3SNHCOOH$) and *m/z* 237 (loss of $HO_3SNHCOOH + H_2O$). This fragmentation pattern was similar to that observed for GTX5 (B1), strongly indicating the presence of an N21-sulfocarbamate group. The MS/MS spectrum of the positive fragment ion *m/z* 316 from M1 was identical with that of M2β (11β-OH-STX), suggesting that M1 was the N-sulfocarbamoyl derivative of 11-hydroxysaxitoxin. ^{1}H COSY, TOCSY and ^{1}H/^{13}C HSQC NMR spectra for M1 (Table 1) showed two isolated ^{1}H spin systems (A: δ_H 4.79, 3.83, 4.11, 4.40 and B: δ_H 3.26, 3.99, 4.42) corresponding to CH-CH-CH_2 and CH_2-CH moieties, respectively. Comparison of ^{1}H and ^{13}C chemical shifts and ^{1}H-^{1}H coupling constants of M1 with literature data for GTX5, 11α- and 11β-OH STX (Hall, 1982; Fix

Table 1 NMR data (δ_C, δ_H, J_{HH}) for PSP compounds M1β and M3.

	M1β[a, b]		M3[a, c]	
Pos.	δ_C	δ_H, J_{HH}	δ_C	δ_H, J_{HH}
2	156.14		156.49	
4	82.34		81.93	
5	58.07	4.79, d *1.0*	58.54	4.83, d *0.7*
6	53.29	3.83, ddd *5.1, 9.9, 1.0*	53.30	3.85, ddd *5.1, 9.7, 0.7*
8	154.27		154.19	
10a	48.99	3.26, dd *6.8, 10.5*	55.10	3.64, d *10.4*
10b		3.99, dd *8.2, 10.5*		3.88, d *10.4*
11	71.03	4.42, dd *8.2, 6.8*	97.06*	
12	98.16		97.27*	
17a	64.14	4.11, dd, *5.1, 11.8*	64.15	4.11, dd, *5.1, 11.8*
17b		4.40, dd *9.9, 11.8*		4.43, dd, *9.7, 11.8*
19	158.17		158.19	

[a]Multiplicity s = singlet, d = doublet, J_{HH} in Hz (error ± 0.3 Hz). nd = not detected, * interchangeable. [b]Sample dissolved in D_2O/DCl, pH 2.0. [c]Sample in 9/1 H_2O/D_2O/0.1M CD_3COOD, pH 3.9.

Wichmann *et al.,* 1981) as well as with NMR data of authentic samples of 11β-OH STX and GTX5 in the same experimental conditions, indicated that M1 was 11β-hydroxy-N-sulfo-carbamoyl saxitoxin (thereafter coded as M1β). This was further substantiated by an acid hydrolysis of M1, which resulted in the formation of M2β.

ESI mass spectra of M3 showed an $[M+H]^+$ ion at *m/z* 412 and an $[M-H]^-$ at *m/z* 410, indicating a molecular weight of 411 amu. High-resolution ESI-MS data were consistent with an elemental composition $C_{10}H_{17}N_7O_9S$ ($[M+H]^+$ 412.0893, calc. 412.0887, Δ = 1.6 ppm). The MS/MS fragment ion spectrum of the $[M+H]^+$ ion of M3 paralleled that of M1β, showing prominent ions at *m/z* 332 (loss of SO_3), *m/z* 314 (loss of $SO_3 + H_2O$), *m/z* 289 (loss of SO_3 + NHCO), *m/z* 271 (loss of $HO_3SNHCOOH$), and *m/z* 253 (loss of $HO_3SNHCOOH + H_2O$). Additional ions not present in the spectrum of M1β were observed at *m/z* 296 (loss of $SO_3 + 2H_2O$) and *m/z* 235 (loss of $HO_3SNHCOOH + 2H_2O$), in the MS/MS spectrum of the positive fragment ion *m/z* 332 from M3, indicating an additional hydroxyl function in M3 compared with M1β. ^1H COSY, TOCSY and $^1H/^{13}C$ HSQC NMR spectra of M3 (Table 1) showed again two ^1H spin systems, one (δ_H 4.83, 3.85, 4.11, 4.43) corresponding closely in δ_H, J_{HH}, and δ_C of directly-bonded carbons, to system A in M1, the other (δ_H 3.64, 3.88) consisting of a geminal pair. Absence of an ^1H resonance for H11 and the lack of vicinal couplings for H10a,b, taken with the unequivocal molecular formula above, provided strong evidence that C11 in M3 bore two OH groups. ^{13}C NMR results completely supported that M3 was 11,11-dihydroxy-N-sulfocarbamoyl saxitoxin. The M3 structure contains a very unusual vicinal di-gem-diols moiety, which is unique in marine natural products chemistry.

The ESI mass spectrum of M4 showed an $[M+H]^+$ at *m/z* 332, indicating a molecular weight of 331 for the free base. High-resolution ESI-MS data were consistent with an elemental composition of $C_{10}H_{17}N_7O_7$ ($[M+H]^+$ 332.1325, calc. 332.1319, Δ = *0.6 ppm*). The MS/MS fragment ion spectrum of the $[M+H]^+$ ion was identical to that of the positive fragment ion *m/z* 332 from M3. This suggested that M4 was the carbamate analogue of M3. NMR data (not shown) were consistent with the assignment of M4 as 11,11-dihydroxy saxitoxin. This assignment was further substantiated by acid hydrolysis of M3, which resulted in the formation of M4.

Quantities of M5 were insufficient for NMR investigation but mass spectra were quite different than those of M1β, suggesting a significant structure modification. Work is continuing on this compound. The novel M1β, M3 and M4 structures represent significant additions to the PSP toxins class. These new compounds are not produced by plankton but appear to be metabolites formed in shellfish. This sheds new light on the fate of PSP toxins as they enter the food web. The very limited amounts of pure M1β, M3, and M4 isolated from mussels prevented us from evaluation of their toxicological properties. However, it is expected that their specific toxicities will be relatively low, based on reported data for other saxitoxin analogues (Oshima, 1995).

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Isolation of Bioactive Metabolites from a *Lyngbya* Species Isolated from Periphyton of the Florida Everglades

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Abstract

The filamentous Cyanobacterial genus *Lyngbya* is being found to be a rich source of toxic and otherwise bioactive metabolites. A cyanobacterial isolate from the periphyton of a floating mat in the Everglades was identified based on morphology as a species of *Lyngbya,* and subsequent sequencing and phylogenetic analysis of the 16s rDNA suggest it may be a new species. The Lyngbya isolate was furthermore identified in bioactivity screening to produce antimicrobial, icthyotoxic and cytotoxic constituents. Bioassay-guided fractionation of constituents from the isolate has purified a cytotoxic compound with a large molecular weight. Data on the continuing characterization and structural elucidation of the toxin are presented.

Introduction

The Florida Everglades is an oligotrophic marsh containing very productive microbial communities, specifically organized into either benthic or "floating mats" of periphyton. In particular, these microbial communities are characterized by a diversity of cyanobacteria (*i.e.,* "blue-green algae") which are known to produce an array of toxic or otherwise bioactive metabolites (Gerwick *et al.,* 2001). A cyanobacterial isolate from the Everglades was identified as a species of the cosmopolitan genus, *Lyngbya.* Recognized as a genus to produce a diversity of bioactive compounds, this *Lyngbya* isolate has, indeed, shown particular promise as a source of such compounds. Evaluation of bioactive metabolites from this Everglades isolate, and the current status of the characterization and structure elucidation of these metabolites, is presented.

Materials and Methods

Isolation of* Lyngbya *sp. strain 15-2 *Lyngbya* sp. strain 15-2 was isolated from the floating periphyton mat in the Florida Everglades, specifically next to the C-111 canal levee (southeast Miami-Dade County). A sample of the mat was homogenized, diluted and filtered through 0.45 micron membrane filter, which was then placed onto BG11 medium (Rippka *et al.,* 1979) plate. Individual filaments were picked up and grown into a unialgal, non-axenic culture. The organism was maintained and cultured in BG11 medium, buffered with MES, pH 7.2, at 24°C and constant light (20 microEm^{-1}s^{-1}).

Sequencing of the 16s Gene Cells (0.25 g) were ground in liquid nitrogen, suspended in a fivefold volume of digestion solution (50 mM Tris, 50 mM EDTA, pH 8.0, 200 mM NaCl, 1% SDS, 0.1% proteinase K) and incubated overnight at 55∞C. DNA was purified by extraction with phenol:chloroform: isoamyl alcohol (25:24:1) followed by chloroform: isoamyl (24:1), precipitated with isopropanol, washed with 70% ethanol and resuspended in TE (pH 8). The SSU rRNA gene was amplified using primers (CY106F and CYA781R) and conditions described previously (Nübel *et al.,* 1997). The amplicon of the anticipated size (675 bp) from the PCR reaction was purified from a 0.8% low-melt agarose gel and ligated into the T/A site of the pCR2.1 plasmid vector (Invitrogen) with T4 DNA ligase. Competent *E. coli* (INVαF') were transformed according to the manufacturer's instructions. Two clones were selected at random for sequencing. The sequences were obtained using a Li-Cor automated sequencer Long Readir 4200 using the manufacturers protocol. The M13 F and M13 R primers were used for the sequencing reactions.

Extraction and Bioassay-Guided Fractionation Samples of biomass (12.8 g dry weight in total) were collected from cultures of *Lyngbya* 15-2 and extracted with 80% MeOH. Extracts were filtered, taken to dryness by flash evaporation and lyophilization, and re-taken in water (ca. 10 mL). Water-soluble constituents were separated by centrifugation, and loaded onto MaxiClean™ (Alltech, Deerfield, IL) C-18 solid-phase extraction (SPE) cartridges (600 mg), pre-conditioned sequentially with MeOH and 20% MeOH (in water). In addition to the aqueous "loading eluate," fractions were eluted sequentially with 20% MeOH, 80% MeOH and 100% MeOH. Each fraction was evaluated for icthytoxicity, cytotoxicity and antibacterial activity (see *Bioassays,* below). A cytotoxic compound was purified from the 80% MeOH eluate by bioassay-guided fractionation using solvent partitioning, size-exclusion chromatography (G-10 Sephadex), column chromatography (Si gel) and preparative HPLC (Zorbak SB-C18, 9.4 mm × 25 cm; 2 mL/min, 70:30 20 mM ammonium acetate/CH_3CN). Details of the purification are to be published elsewhere.

Bioassays Extracts and fractions were evaluated for ichthyotoxicity (*i.e.,* toxicity to fish), antibacterial activity

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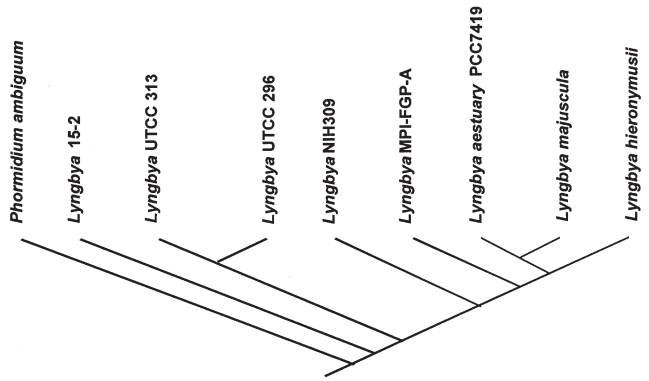

Figure 1 Phylogenetic tree showing the relationship of *Lyngbya* sp. 15-2 and other previously published 16S rDNA sequences of genus *Lyngbya.* According to BLAST analysis, *Phormidium* had the highest degree of similarity (97%), and was therefore used to root the tree. The phylogenetic analysis was carried out using Maximum Parsimony and Minimum Evolution. Sequences used for phylogenetic analysis are from those deposited in GenBank, and specifically include sequences from freshwater species *Phormidium ambiguum* (AB003167), *Lyngbya* UTCC 313 (AF218369), *Lyngbya* UTCC 296 (AF218377) and *L. hieronymusii* (AB045906), and marine representatives *Lyngbya* NIH309 (AY049752), *Lyngbya* MPI-FGP-A (AJ272599), *Lyngbya* PCC7419 (AJ000714), and *L. majuscula* (AF368300).

and cytotoxicity. Ichthyotoxicity was evaluated using "mosquitofish" (*Gambusia holbrookii*) in 6-well plates (two fish per well) as described previously (*e.g.,* Berry *et al.,* 2002a). Antibacterial activity was evaluated against *Bacillus megaterium, Escherichia coli, Proteus mirabilis, Staphylococcus aureus* and *Streptococcus mitis,* cultured on nutrient-agar (Becton Dickinson, Sparks, MD) at 37°C, using a "disc-diffusion assay" (Lennette, 1985). Cytotoxicity against a rat neuroblastoma line (B50) was assessed using microdilution method as has been described (*e.g.,* Berry *et al.,* 2002b).

Results and Discussion

The Florida Everglades is characterized by a rich, microbial community comprised of cyanobacteria and related microalgae. An isolate of cyanobacteria (15-2) from the Everglades was identified, based on morphological characteristics, as a species of *Lyngbya.* Specifically, the organism has unbranched, non-heterocystous ensheathed filaments composed of discoid cells with a cell width of 23 μm and cell length of 5 μm. Phylogenetic analysis, based on the 16s rDNA sequence (Fig. 1), suggests that *Lyngbya* 15-2 is not closely related to other *Lyngbya* species, but rather most closely related to the genus *Phormidium.* Given this, it is proposed that *Lyngbya* 15-2 may represent a new species.

Preliminary fractionation of the extracted constituents from *Lyngbya* 15-2 suggests that metabolites from this iso-

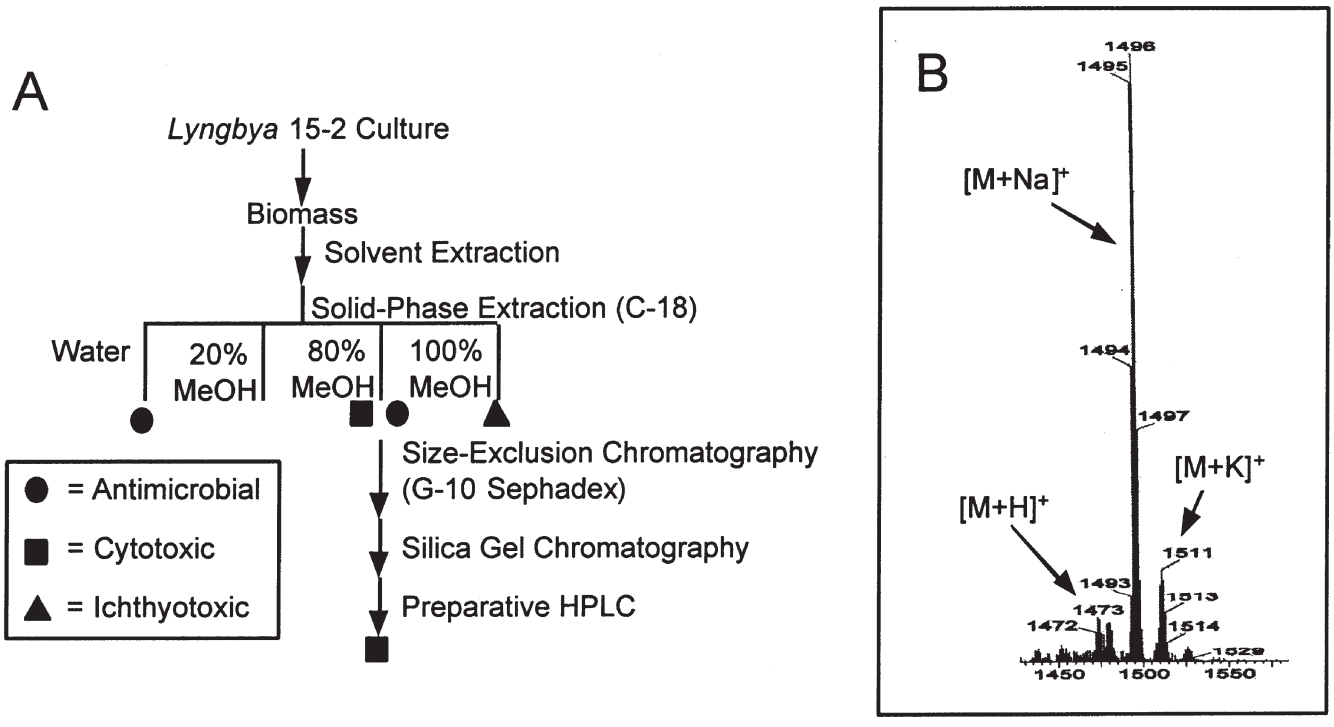

Figure 2 Identification of bioactive metabolites from *Lyngbya* 15-2, and purification of a cytotoxic constituent. The isolation scheme **A** shows bioactivities of fractions, including antimicrobial (●), cytotoxic (■) and ichthyotoxic (▲) fractions, from solvent extraction and preliminary fractionation of *Lyngbya* 15-2 biomass, as well as further bioassay-guided isolation of a cytotoxic compound. Also shown is the FAB-MS of the purified cytotoxic compound **B**, specifically showing the *m/z* of the $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ ions.

late may include as many as 3 or 4 separate compounds with various bioactivities, including antimicrobial, cytototoxic and ichthyotoxic constituents (Fig. 2). Indeed, *Lyngbya* has emerged, through the work of numerous investigators (see review by Gerwick *et al.,* 2001), as one of the most diverse genera of cyanobacteria in terms of bioactive secondary metabolites. To date, however, most of the research on biologically active compounds from *Lyngbya* has focused on the cosmopolitan, marine species, *L. majuscula.* Though there have been a few reports on bioactive compounds from freshwater *Lyngbya* (Carmichael *et al.,* 1997; Onodera *et al.,* 1997; Teneva *et al.,* 2003), the present report is the first (to the authors' knowledge) to focus on freshwater *Lyngbya* from the Everglades.

A cytotoxic constituent from *Lyngbya* 15-2 was further purified using solvent partitioning, size-exclusion chromatography, silica gel chromatography and preparative HPLC. Mass spectrometry, including fast atom bombardment (FAB), matrix assisted laser absorption/time-of-flight (MALDI-TOF) and electrospray ionization (ESI), indicates a large molecule of 1473 MW ($[M+H]^+$), along with corresponding $[M+Na]^+$and $[M+K]^+$ ions (1496 and 1511 MW, respectively), as shown in Fig. 2. Little or no fragmentation, however, was observed as evidenced by the lack of additional ions (data not shown). The apparent molecular weight of the cytotoxic compound suggests that this is, indeed, a novel molecule from *Lyngbya,* as none of the many, previously identified compounds are this large. We are continuing to purify sufficient amounts of the compound for NMR and other spectroscopic analyses in order to elucidate the structure of this molecule.

Acknowledgements

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Hemolytic Toxin of the Dinoflagellate *Heterocapsa circularisquama* as a Possible Causative Factor Responsible for Shellfish Kill

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Abstract

We found that *H. circularisquama* produces species-specific hemolytic activity toward mammalian erythrocytes and among the species tested, rabbit erythrocytes showed the highest sensitivity. Seven strains of *H. circularisquama* isolated from various localities in Japan showed differing hemolytic activities toward rabbit erythrocytes. The strains which are known to be highly toxic to bivalves tended to show stronger hemolytic activities and vice versa, suggesting that the hemolytic activity was paralleled with the shellfish toxicity. Since the culture supernatant of *H. circularisquama* also showed a weak but significant hemolytic activity, a part of the hemolytic toxin may be released from the flagellate cells into the medium during active growth.

Introduction

Heterocapsa circularisquama is a recently identified red tide dinoflagellate that has caused mass mortalities of bivalve molluscs in embayments of western Japan since 1988 (Yamamoto and Tanaka, 1990; Horiguchi, 1995; Matsuyama *et al.,* 1996). Blooms of *H. circularisquama* can kill more than 12 bivalve species, but no harmful effects on wild fish populations, cultured fish, or on public health in general have been reported so far (Yamamoto and Tanaka, 1990; Matsuyama *et al.,* 1995). Since other harmful dinoflagellates such as *Gyrodinium aureolum,* for instance, are known to kill not only shellfish but also finfish and crustacean species, the species-specific toxicity of *H. circularisquama* is one of the characteristic features of this alga (Tangen, 1977; Lesser and Shumway, 1993). Recently, it has been reported that pearl oysters exposed to *H. circularisquama* (5,000 to 10,000 of cells/mL) showed vigorous clapping and shrinkage of their mantle edges and gills, and about 50% of the pearl oysters subsequently underwent cardiac arrest and eventually died after 48 h (Nagai *et al.,* 1996). Regarding the mechanism of shellfish toxicity of this dinoflagellate, Matsuyama *et al.* have shown several lines of evidence supporting the idea that unstable toxic substances located on the surface of *H. circularisquama* cells may be responsible for the toxicity to bivalves (Matsuyama *et al.,* 1997). Furthermore, Matsuyama has observed that an influx of Ca2+ was induced in trochophore larva of the short-necked clams (*Ruditapes philippinarum*) after exposure to *H. circularisquama* (Matsuyama, 1999a). Some marine toxins such as palytoxin (Habermann, 1989) and maitotoxin (Igarashi *et al.,* 1999) are known to induce Ca2+ influx into mammalian erythrocytes and eventually cause hemolysis. Recently, we have found that *H. circularisquama* produces hemolytic activity toward rabbit erythrocytes in a cell density-dependent manner (Oda *et al.,* 2001). These findings suggest that *H. circularisquama* may kill bivalves through certain hemolytic toxins.

Since 1998, 26 cases of *H. circularisquama* red tide have been recorded in 14 locations of western Japan and several strains of *H. circularisquama* have been isolated in some of these areas (Matsuyama, 1999b). Interestingly, it has been known that the potency of *H. circularisquama* to produce toxic effects on bivalve species is dependent upon the strains, and it appears that there are toxic and less-toxic strains (unpublished observation) (Matsuyama, 2001). Therefore, this study was undertaken to gain insight into the relationship between hemolytic activity and shellfish toxicity of *H. circularisquama*. We compared the hemolytic activities of seven strains of *H. circularisquama* isolated in different localities in Japan to those of *Heterocapsa triquetra*, which is morphologically similar to *H. circularisquama* but is not toxic to bivalves.

Materials and Methods

Seven strains of *Heterocapsa circularisquama*—HAG9408A, HU9433, HHR9509A, HMZ98, HOB9708A, HI9425, and HBZ97—were isolated in Ago Bay, Uranouchi Bay, Hiroshima Bay, Maizuru Bay, Obama Bay, Imari Bay, and Buzen Bay, respectively. *Heterocapsa triquetra* (HT94-1) was isolated in Hiroshima Bay. Clonal cultures of all these flagellate strains were obtained by repeated washings using capillary pipettes. These algae were cultured at 26°C in sterilized Erd-Schreiber modified (ESM) medium (pH 8.2) under illumination from a fluorescent lamp (30 mE/m2/S) with a 12:12 light-dark cycle. Flagellates in the exponential growing phase were used throughout the experiments. All cultivations were done using sterilized instruments. Cells were counted with a hemocytometer.

Mortality tests were done at 23–24°C in the dark using blue mussel, as described previously (Nagai *et al.,* 1996; Matsuyama, 2001). In brief, juvenile mussels, *Mytilus galloprovincialis* (shell height; 4.81 ± 0.43 mm), collected from Hiroshima Bay were exposed to *H. circularisquama* (10,000 cells/mL) in filtered seawater, and the dead individuals were counted. Death was judged from the lack of both byssus production and closing valves response even if the mantle edge was stimulated with a needle. Hemolytic assay was done as described previously (Oda *et al.,* 2001) using rabbit erythrocytes.

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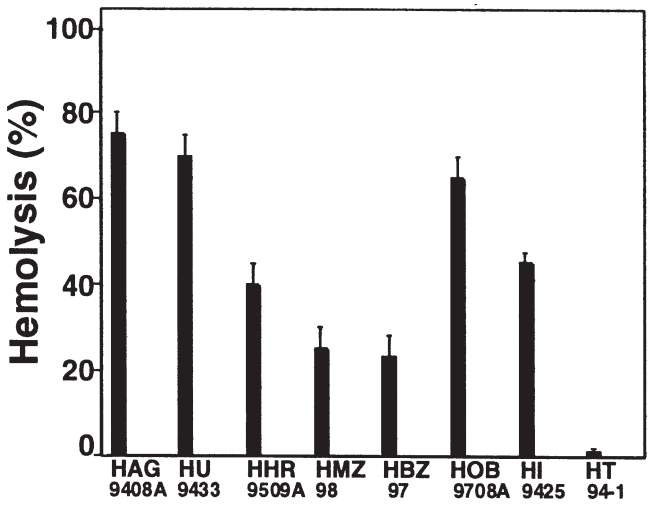

Figure 1 Hemolytic activities of seven strains of *H. circularisquama* isolated in different localities in Japan (HAG9408A, HU9433, HHR9509A, HMZ98, HBZ97, HOB9708A, and HI9425) and *H. triquetra* (HT94-1). Rabbit erythrocytes were mixed with each strain of dinoflagellate (final concentration, 5×10^4 cells/mL) and incubated for 5 h at 26°C in the dark. The extents of hemolysis were measured as described previously (Oda *et al.,* 2001).

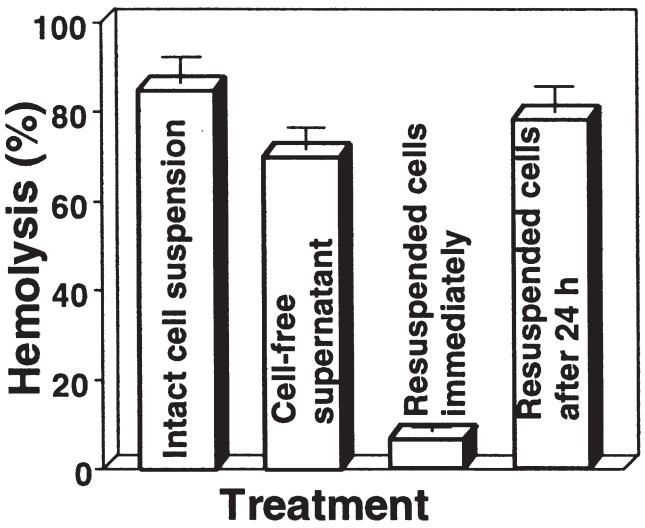

Figure 2 Hemolytic activities of intact cell suspension, culture supernatant, and ≥24 h cultured cells prepared from pelleted *H. circularisquama* cells (HAG9408A) (1×10^5 cells/mL).

Results and Discussion

In contrast to their morphological similarity, these 7 strains of *H. circularisquama* showed marked differences in their hemolytic activity toward rabbit erythrocytes (Fig. 1). HAG9408A and HU9433 strains, which are known to be highly toxic to bivalves (Matsuyama, 2001), had the most potent hemolytic activities, but HMZ98, HBZ97, HHR9509A, and HI9425 strains, which are known to be less toxic to bivalves (unpublished observation; Matsuyama, 2001), showed lower hemolytic activities. Mortality tests using blue mussel also confirmed the differences in shellfish toxicities between a toxic (HAG9408A) and a less-toxic strain (HHR9509A) of *H. circularisquama* (Table 1). Furthermore, non-toxic dinoflagellate species *H. triquetra* (HT94-1) showed no hemolytic activity (Fig. 1). These results suggest that the hemolytic activities of *H. circularisquama* strains correlate with their potency to shellfish.

Table 1 Mortalities of blue mussel (*Mytilus galloprovincialis*) after exposure to toxic (HAG0408A) or less-toxic strains (HHR95094) of *H. circularisquama*. Individuals (n = 18) were exposed to each strain of *H. circularisquama* (10,000 cells/mL). No mortality was observed in control filtered seawater.

	Mortality (%)	
Days after exposure	Toxic strain (HAG9408A)	Less-toxic strain (HHR9509A)
1	6	0
3	17	0
6	50	22
9	72	22

To obtain a clue as to the location of hemolytic toxin in *H. circularisquama* cells, the hemolytic activities of the culture supernatant and the cell pellet resuspended in fresh ESM medium, which were prepared from *H. circularisquama* at exponential growth phase by centrifugation (1,000 g at 4°C for 10 min), were examined. Pelleted cells were resuspended in fresh ESM medium and subjected to the hemolytic assay immediately or after 24 h. As shown in Fig. 2, the supernatant showed slightly weak but significant hemolytic activity, while the resuspended cell pellet showed almost no hemolytic activity. The pelleted cells were morphologically changed, and discharge of the body plate in some cells were observed. However, after 24 h of incubation under the usual culture conditions, normal morphological features of these cells were recovered together with the hemolytic activity. Thus, it seems likely that hemolytic toxin may be loosely attached to the surface of *H. circularisquama* cells, and it can be easily detached from the cell surface by physical stimulation such as centrifugation. This notion may be supported by the finding that shellfish toxicity disappeared after the treatment with detergent without affecting the integrity of flagellate cells (Matsuyama *et al.,* 1997; Matsuyama, 1999b). Since hemolytic activity in the culture supernatant decreased rapidly, such hemolytic toxin may be unstable in the medium after once released from the cells in which such toxin may be continuously produced during cell growth (data not shown). In addition to the unstable hemolytic toxin, our recent study has demonstrated the presence of an ethanol-soluble, relatively stable, hemolytic agent in *H. circularisquama* cells, although the involvement of such a hemolytic agent in shellfish toxicity still remains to be clarified (Sato *et al.,* 2002).

In conclusion, we found that *H. circularisquama* strains

isolated in different localities in Japan showed species-specific hemolytic activities, and the potency of each strain was quite different. Since the hemolytic activity of each strain tends to correlate with the shellfish toxicity, the hemolytic agent may be a factor responsible for the shellfish toxicity of *H. circularisquama.*

Acknowledgements

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Evaluation of Toxicity in Nine Raphidophyte Strains Isolated from Different Geographic Regions

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Abstract

We evaluated the ichthyotoxic potential of nine clonal cultures of raphidophytes, including potentially toxic *Heterosigma akashiwo, Chattonella subsalsa, Chattonella marina,* and *Fibrocapsa japonica,* isolated from estuaries and brackish ponds of the eastern U.S.A., western Canada, and western Japan. Clonal cultures were grown using standardized culturing techniques and evaluated for production of brevetoxins, reactive oxygen species (hydrogen peroxide, superoxide), and toxicity to fish. HPLC/MS analysis and receptor binding assays yielded no detectable brevetoxins, and repeated acute toxicity microassays with sheepshead minnows (*Cyprinodon variegates*) showed no toxicity of these raphidophytes to test fish within 24 hr. Reactive oxygen species were detected from some isolates, but at levels too low to induce fish stress or mortality. Thus under the culture conditions used, these isolates had low potential to cause physiological stress in this fish species. In ongoing research we are evaluating the effects of nutrient availability, light regime, fatty acid synergism, and other factors on toxic activity by raphidophytes.

Introduction

Estuarine and marine raphidophytes (Raphidophyceae) have been associated with pen-reared fish mortality and major economic losses in Europe, Asia, and Australia since the early 1970s (Black *et al.,* 1991; Yang *et al.,* 1995; Smayda, 1998; Munday and Hallegraeff, 1998), and may have been involved in an estuarine fish kill in the U.S.A. (Bourdelais *et al.,* 2002). In Japan, economic losses exceeding 20 billion yen (ca. $190 million U.S.) have been linked to raphidophyte blooms involving *Chattonella* spp. and *Heterosigma* spp (Okaichi 1989). Mass death of cultured fish has been associated with *Heterosigma akashiwo* (formerly *H. carterae*) or *Chattonella marina* in South Australian waters, western Canada and Washington state in the U.S. (Horner *et al.,* 1991; Munday and Hallegraeff, 1998; Whyte *et al.,* 1999). The mechanism(s) by which raphidophytes kill fish is unclear (Twiner *et al.,* 2001). One hypothesized mechanism involves production of brevetoxins (Endo *et al.,* 1992; Khan *et al.,* 1997; Ono *et al.,* 2000), which may cause cardiac disorders and gill damage in fish. Alternatively, fish mortality has been shown to result from excessive production by some raphidophyte strains of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals (Yang *et al.,* 1995; Oda *et al.,* 1997). Other researchers have suggested involvement of lectin-like polysaccharides (Nakamura *et al.,* 1998; Oda *et al.,* 1998; Smayda, 1998), co-occurring heterotrophic bacteria, and unsaturated fatty acids (Carrasquero-Verde, 1999; Marshall *et al.,* 2002) in raphidophyte-associated fish mortality. However, all of this supporting research has been based on few strains (*e.g.,* 1 each for *C. antiqua* and *C. subsalsa,* 3 for *Fibrocapsa japonica,* 5 for *C. marina,* 7 for *H. akashiwo;* Khan *et al.,* 1996, 1997; Yang *et al.,* 1995; Oda *et al.,* 1997; Kim *et al.,* 1999; Twiner *et al.,* 2001; Marshall *et al.,* 2002, 2003).

Table 1 Raphidophyte clonal cultures evaluated in this study.

Strain	Isolation location (month)	Cells/mL (estuary)	Cells × 10^4/mL (assays)
Chattonella subsalsa (SC)	Hilton Head, SC (April)	4×10^2	0.7–1.2
C. subsalsa (NC)	Neuse River, NC (July)	2×10^2	0.6–2.9
Fibrocapsa japonica (SC)	Kiawah Island, SC (May)	1.41×10^5	0.5–2.4
Heterosigma akashiwo (SC)	Hilton Head, SC (April)	2.00×10^5	4.8–14
H. akashiwo (NC)	Neuse River, NC (June)	1.0×10^3	2.9–7.1
H. akashiwo (DE)	Torquay Canal, DE (Oct.)	2.0×10^3	6.6–10
H. akashiwo (CAN)*	San Mateo Bay, Canada (July)	2.00×10^5	4.9–8.7
H. akashiwo (NIES-6)**	Osaka Bay, Japan (Aug.)	n.a.	5.3–7.7
C. marina (NIES-14)**	Harima-Nada, Japan (Feb.)	n.a.	0.6–0.9

* From Dr. J.N.C. Whyte, Pacific Biological Station, Fisheries and Oceans, Canada; n.a. = not available. ** From the Microbial Culture Collection, National Institute for Environmental Studies, Japan.

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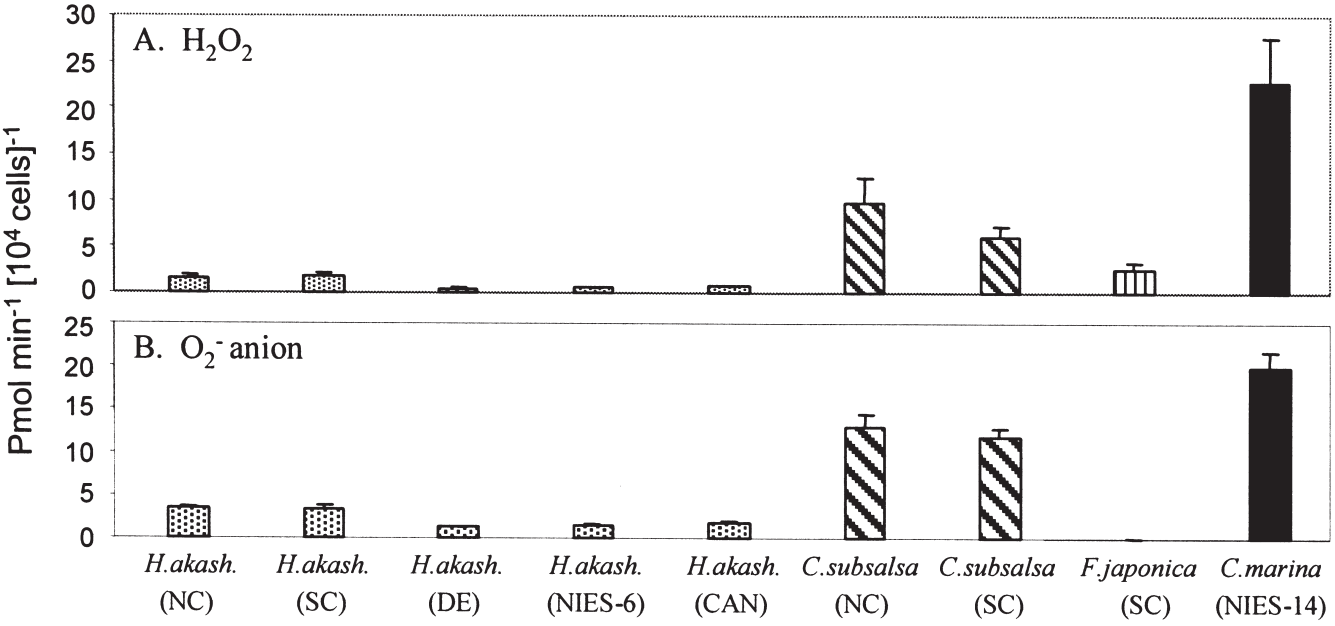

Figure 1 Production rates of bioactive substances by these raphidophyte clones, including **A** hydrogen peroxide and **B** superoxide anion.

Within the past decade, there has been increasing recognition of high variability in toxicity among strains in many species of potentially toxic algae, ranging from negligible toxicity to highly toxic (see review in Burkholder *et al.,* 2001). Here we tested four raphidophyte species for production of brevetoxins and reactive oxygen species, and for measurable ichthyotoxic effects. The data, along with published literature, were used to test the hypothesis that potentially toxic raphidophyte species show a range in production of toxin(s) and other bioactive substances.

Materials and Methods

Nine non-axenic, clonal raphidophyte cultures (Table 1) were grown at 23°C in f/2-Si medium or L-1-Si medium under a 16-h:8-h, light:dark cycle (80 µmol photons m^{-2} sec^{-1}) and salinity matching that of the respective collection sites (15 to 30 psu). Cultures in late logarithmic growth phase were used for all evaluations. Assays were conducted for fish toxicity, brevetoxins, and reactive oxygen species (hydrogen peroxide, superoxide anions; Table 1). Acute toxicity micro-assays (Burkholder *et al.,* 2001) with larval sheepshead minnows (*Cyprinodon variegatus,* age 25 days, length ~1 cm) were conducted for 24 h in a 6-well plate for each treatment (1 fish/well, 8 mL of clonal raphidophyte culture). Control (without raphidophytes) and test fish were in f/2-Si medium; fish were fed prior to assay, and were monitored for mortality or signs of distress. Results were compared to data for actively toxic *Pfiesteria shumwayae* (clone CAAE270A2, capable of killing fish ± physical contact as in Gordon *et al.,* 2002; 8×10^3 flagellated cells/mL). For brevetoxin analysis, cultures were filtered (2 L; 0.45 µm-porosity filters), extracted using 100% methanol, sonicated for 10 min to ensure complete lysis, and stored at –80°C prior to analysis (within 4 weeks). Brevetoxins (PbTx-2, PbTx-3) were analyzed using receptor binding assays (Van Dolah *et al.,* 1994) and mass spectroscopy (SCIEX API-III triple quadruple mass spectrometer; HPLC-MS/MS mode) (Fairey *et al.,* 2001). For H_2O_2 analysis, cultures were diluted 1:1 with natural ultra-filtered seawater at the appropriate salinity, and production was quantified fluorometrically utilizing H_2O_2-dependent oxidization of scopoletin (7-hydroxy-6-methoxy-2H-1-benzopyran-2-1) (Twiner *et al.,* 2001). Superoxide anion generation was measured spectrophotometrically based on superoxide dismutase inhibitable reduction of ferricytochrome *c* (Johnston *et al.,* 1978).

Results and Discussion

The larval fish micro-assays yielded no mortality in any of the treatments or controls, and no noticeable difference in fish behavior; in contrast, all fish in micro-assays with toxic *P. shumwayae* died within 5 h. The assays were extended for 96 h past the standard 24-h duration. Although fish activity decreased in a similar manner across all treatments and controls over that period, no mortality was observed. All isolates produced H_2O_2 with varying production rates (Fig. 1A). *C. marina* had the highest production rate, followed by *C. subsalsa, F. japonica,* and *H. akashiwo.* Maximum H_2O_2 concentrations ranged from 0.16 to 1.57 µM. All isolates except *F. japonica* also produced measurable superoxide anion. The production rate of *Chattonella marina* was highest among all strains (mean ± 1 standard error, 19.87 ± 1.07 pmol min^{-1}, followed by *C. subsalsa* and *H. akashiwo* (12.31 ± 0.60 and 2.28 ± 0.47 pmol min^{-1}, respectively; Fig. 1B). Brevetoxins (PbTx-2, PbTx-3) were not detected from these raphidophyte isolates, based on both receptor binding assays and HPLC-MS/MS. Thus, under the experimental conditions described, these raphidophyte isolates presented little potential to cause physiological stress or mortality in

fish. Although most strains produced reactive oxygen species, production rates were much lower than literature reports for toxic strains (*e.g.,* Oda *et al.,* 1997). The concentrations of hydrogen peroxide measured were at least 100- to 1,000-fold lower than published lethal concentrations for fish and a marine invertebrate (Twiner *et al.,* 2001) and for *C. marina,* ca. 1,000-fold lower than in another strain that was ichthyotoxic (Marshall *et al.,* 2002, 2003).

There is increasing recognition of variability in toxicity of raphidophyte strains (*e.g.,* Kahn *et al.,* 1995 vs. Marshall *et al.,* 2003), as described for many other toxic algae from cyanobacteria to chrysophytes and dinoflagellates (reviewed in Burkholder *et al.,* 2001). Additional research is needed to assess the extent to which potentially toxic raphidophyte species produce benign strains, and molecular controls on synthesis of bioactive substances. Marshall *et al.* (2003) reported that for ichthyotoxicity in *C. marina,* synergy involving reactive oxygen species and certain free fatty acids is important. In ongoing research we are comparing interactive effects of bioactive substances and cofactors that are produced by strains that do, versus strains that do not, express ichthyotoxicity.

Acknowledgments

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Determination of Known and New Yessotoxins from Adriatic Shellfish by Liquid Chromatography-Mass Spectrometry

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Abstract

A liquid chromatography-mass spectrometry (LC-MS) method is proposed for direct detection of various yessotoxins. The method was tried out by analyzing toxic Adriatic mussels. Along with known yessotoxins (**1**, **3**–**6**), a new derivative, **7**, was detected and its structure deduced on the basis of MS/MS evidence.

Introduction

Since 1995, yessotoxin (YTX) and its analogues (Fig. 1) have been shown to be the main phycotoxins contaminating Adriatic shellfish (Ciminiello *et al.,* 2001; Satake *et al.,* 1997). Routine monitoring for YTX is generally carried out using mouse bioassay for Diarrhetic Shellfish Poisoning (DSP) toxins which may lead to overestimation of risk of DSP in consumers. In fact, the lethal dose of YTX for intraperitoneal injection is the lowest amongst all the DSP toxins, whereas YTX effects on humans have not been proven. Instrumental methods are, therefore, required to identify causative toxin. Fluorescence labeling followed by HPLC analysis (Yasumoto *et al.,* 1997) is the most common method for specific detection of YTXs. Unfortunately, it is not reliable for detection of those derivatives which lack of a conjugated diene functionality in the molecule. A more comprehensive approach is provided by liquid chromatography combined with ion spray mass spectrometry (LC-MS). In this paper we have examined the suitability of the LC-MS method for detection of lipophilic toxins (Quilliam *et al.,* 2001) to unequivocally detect all yessotoxins (Ciminiello *et al.,* 2002). Standard solutions of YTX and its various analogues as well as samples of *Mytilus galloprovincialis* were employed. Along with known derivatives the method allowed us to highlight the presence of a new analogue, noroxoYTX (**7**) whose structure was deduced by means of MS/MS experiments.

Materials and Methods

Yessotoxin was purchased from the Institute of Environmental Science and Research Limited (Wellington Science Center, NZ). YTX analogues (**2**–**6**, **8**) were obtained from Italian contaminated mussels in the period 1995–1999. Toxic mussels samples were collected along Cesenatico coasts (Emilia Romagna, Italy) in June 2001. Digestive glands (20 g) were homogenized and extracted with an acetonitrile/water (8:2) 0.1% formic acid solution. Clean-up was accomplished by partitioning the crude extract with hexane and subsequently chloroform. An aliquot of the chloroformic extract was dissolved in 3 mL of 20 mM ammonium formate buffer-methanol (7:3) and loaded on a Sep-Pak C-18 plus cartridge (Waters, Milford, USA). The column was washed with 10 mL of methanol-water (3:7) and eluted with 10 mL of propanol-water (2:8). The eluate was redissolved in 0.5 mL of methanol and injected into the LC-MS system. A LCQ MAT ion trap mass spectrometer coupled to a high-pressure pump SP model P 4000 (Thermo, San Jose, USA) was used. Separations were performed on a Hypersil C8 BDS, 50 × 2.00 mm, 3 µm column (Phenomenex, Torrance, USA), eluted with 10% to

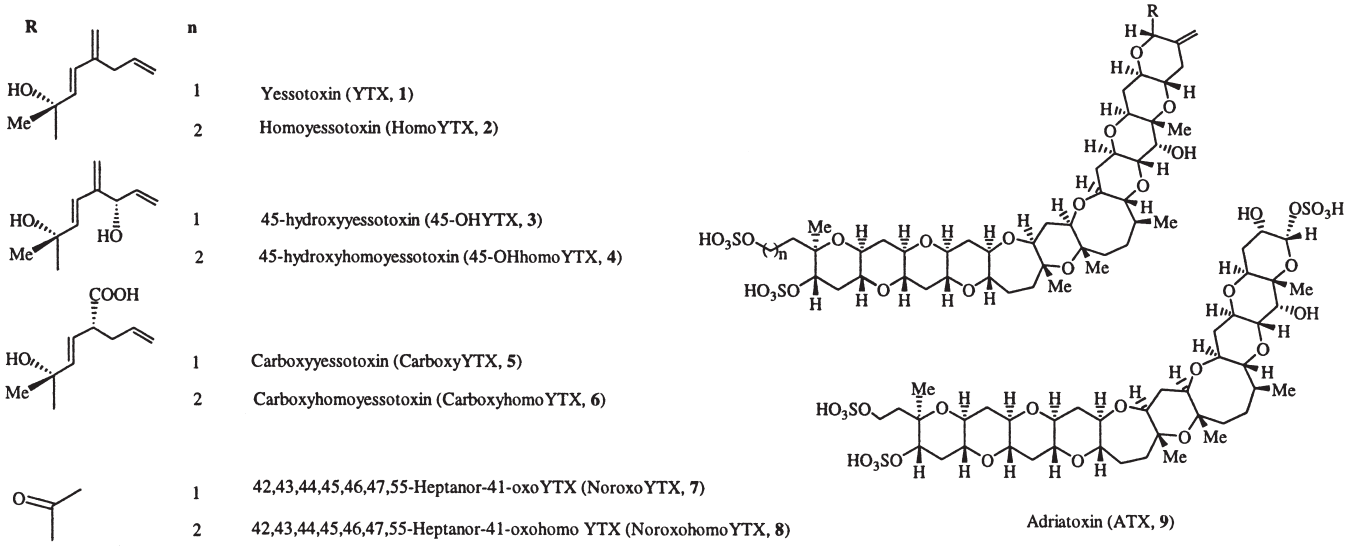

Figure 1 Structures of various yessotoxins occurring in Adriatic mussels.

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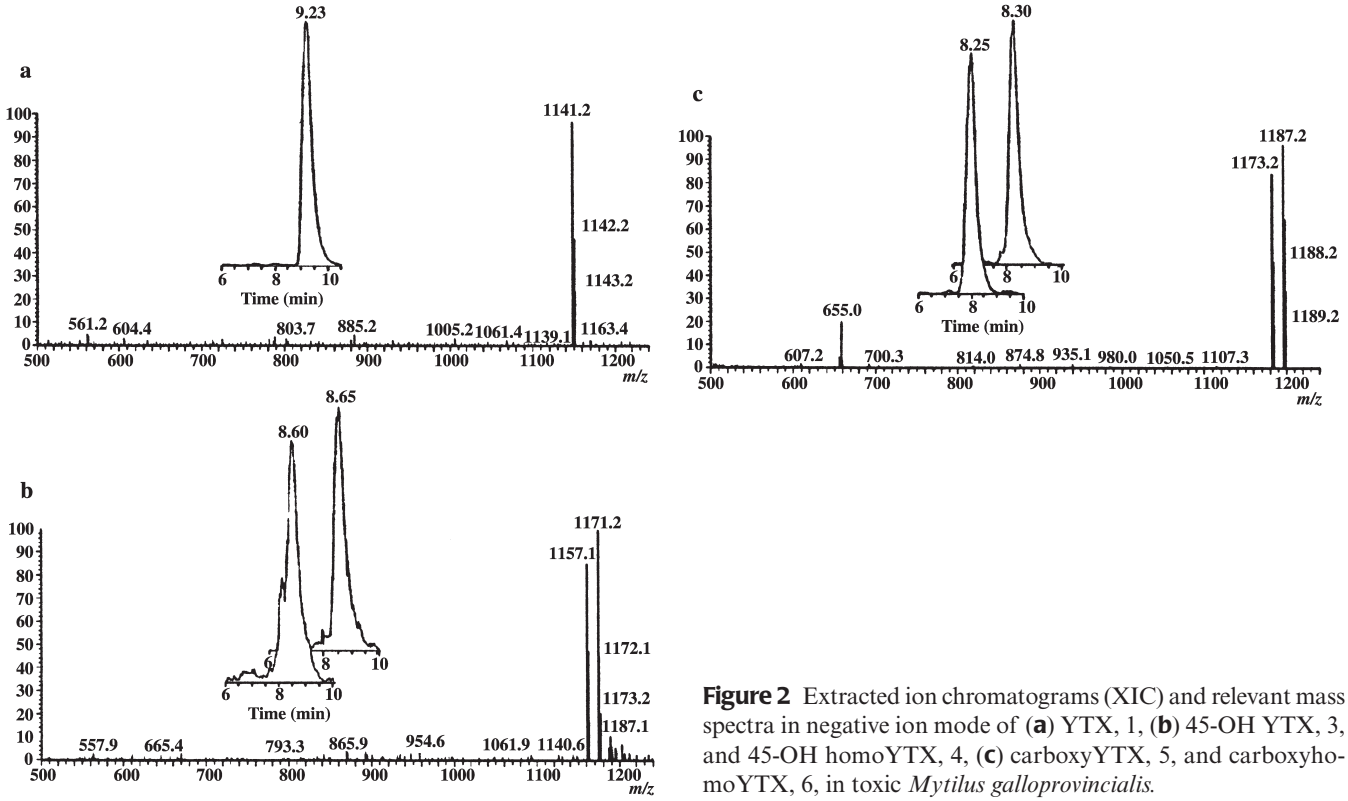

Figure 2 Extracted ion chromatograms (XIC) and relevant mass spectra in negative ion mode of (**a**) YTX, 1, (**b**) 45-OH YTX, 3, and 45-OH homoYTX, 4, (**c**) carboxyYTX, 5, and carboxyhomoYTX, 6, in toxic *Mytilus galloprovincialis*.

100% B in 10 minutes then 100% B for 15 minutes. Eluent A was water and B was a 95% acetonitrile/water solution, both eluents containing 3.5 mM ammonium formate and 50 mM formic acid. The flow rate was 200 µL/min. Full scan spectra (negative ion mode) were collected from *m/z* 500 to 1500. Extracted ion chromatograms (XIC) were obtained selecting ions $[M-H]^-$ at *m/z* 1141.5 (YTX, **1**, Rt = 9.23 min, 380 ng/g), 1157.5 (45-OHYTX, **3**, Rt = 8.60 min, 270 ng/g), 1171.5 (45-OHhomoYTX, **4**, Rt = 8.65 min, 15 ng/g), 1173.5 (carboxyYTX, **5**, Rt = 8.25 min, 265 ng/g), 1187.5 (carboxyhomoYTX, **6**, Rt = 8.30 min, 30 ng/g), 1047.5 (noroxoYTX, **7**, Rt = 8.00 min, 205 ng/g). $[M-H]^-$ and $[M-80-H]^-$ ions were used as precursor ions in LC-MS/MS at a collision energy (CE) of 35% and 45%, respectively.

Results and Discussion

Flow injection analysis experiments (FIA-MS) were first carried out on the individual standard solution of YTX to optimize all source parameters. The obtained full scan spectra showed the exclusive presence of pseudomolecular ion at *m/z* 1141.5 due to $[M-H]^-$. The negative ion LC-MS was then implemented by using a reversed phase column and a mobile phase containing a volatile buffer as suggested by Quilliam *et al.* for analysis of DSP toxins. The minimum detection level for matrix-free toxin on column was 70 pg (S/N = 3) with good linearity ($r^2 > 0.998$). On the basis of the good detection limit for YTX, it was considered worthwhile to test the suitability of the method for detection of all YTXs isolated so far. A number of YTX analogues could be easily detected, although most of them overlapped. Toxins with different molecular masses, however, were monitored by XIC of the $[M-H]^-$ ions, thus allowing their unambiguous identification. The developed method was finally tried out by analyzing the toxic extract of *M. galloprovincialis* collected in June 2001. In Fig. 2, XIC obtained for each known YTX derivative contained in the propanol-water eluate of the SPE are shown.

The retention times and mass spectra of each of the above peaks were compared with those of individual reference samples, injected in the same experimental conditions, and resulted perfectly coincident thus confirming their assignment. Besides these known yessotoxins, the total ion current (TIC) chromatogram showed a significant chromatographic peak at 8.00 min thus revealing the presence of a potentially new analogue (Fig. 3a). The associated full scan mass spectrum displayed a signal at *m/z* 1047.1 which couldn't be associated to any of the already known YTXs. The MS/MS spectrum of **7**, (*m/z* 1047.5, CE 35%), contained an intense peak at *m/z* 967.5 $[M-H-80]^-$ due to loss of one SO_3 molecule. This suggested the presence of at least two sulfate functionalities in **7**. The MS/MS spectrum of the negative fragment ion at *m/z* 967.5 was definitely more informative (Fig. 3b), as it contained product ions which exactly matched with the characteristic fragmentations of the polycyclic backbone skeleton of yessotoxin. Furthermore, the loss of 42 mass units from the $[M-H-SO_3]^-$ ion, which originates ion peak at *m/z* 925.3, was indicative of a $CH_2 = C = O$ neutral loss, which suggested that the eastern side chain in **7** was constituted by an acetyl moiety. The emerging structural features were suggestive of **7** being the

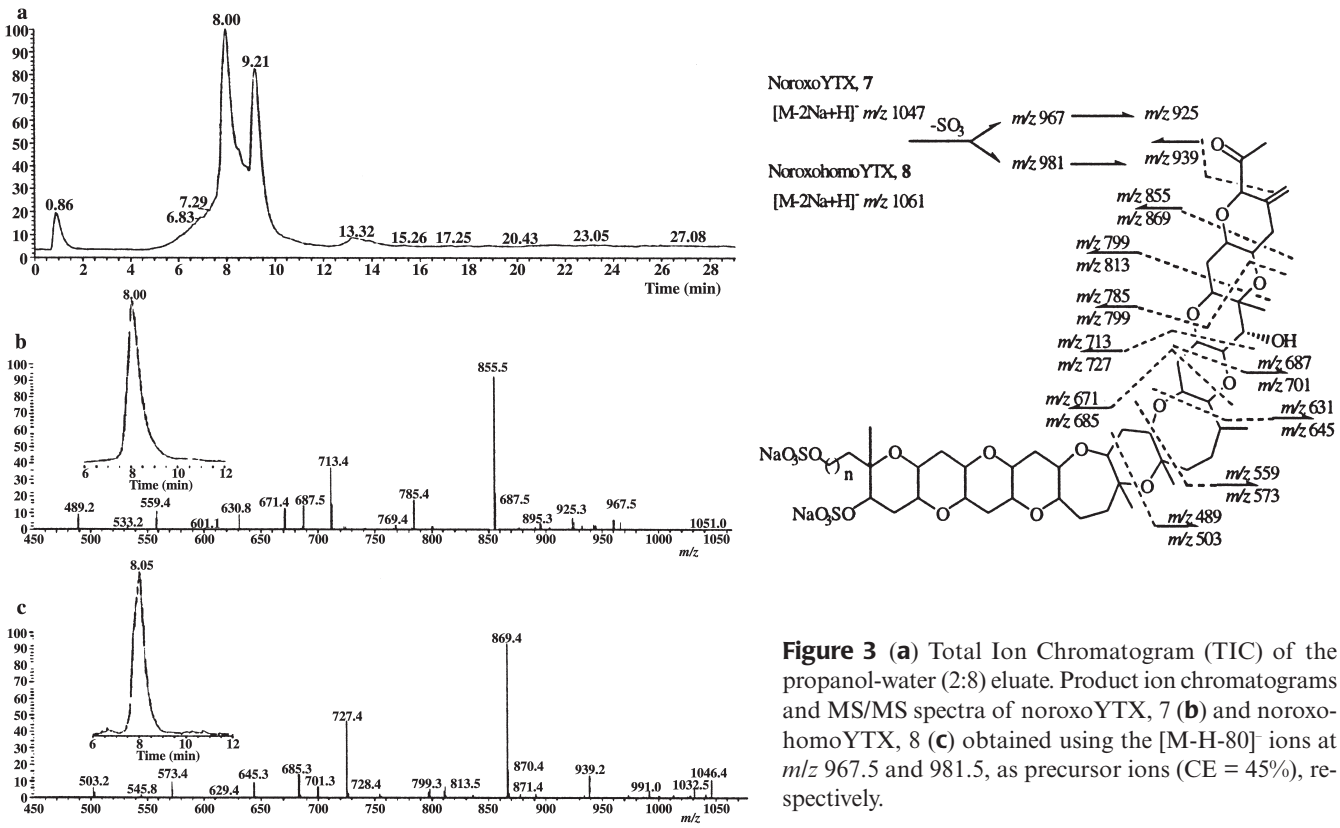

Figure 3 (**a**) Total Ion Chromatogram (TIC) of the propanol-water (2:8) eluate. Product ion chromatograms and MS/MS spectra of noroxoYTX, 7 (**b**) and noroxohomoYTX, 8 (**c**) obtained using the $[M-H-80]^-$ ions at *m/z* 967.5 and 981.5, as precursor ions (CE = 45%), respectively.

homologue in the YTX series of the noroxohomoYTX (**8**), that we have recently isolated and fully characterized. This hypothesis was supported by a comparison of the chromatographic and mass spectral properties of the involved compounds: **7** and **8** eluted at almost the same retention time and their MS/MS spectra appeared to be almost superimposable, as long as they were shifted of 14 mass units (Fig. 3c).

The power of LC-MS technique to highlight the presence of a new toxin, undetectable by more common instrumental methods such as LC-FLD, have been unequivocally demonstrated. This technique can be usefully employed for structure elucidation of new toxins whenever great structural analogies occur between toxins under investigation and known compounds. Thus, effective structural hypothesis can be advanced even when full structure elucidation of new toxins by NMR spectroscopy is hampered by the limited amount of available material.

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Detection of Six New Azaspiracids in Shellfish Using Liquid Chromatography with Multiple Tandem Mass Spectrometry

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Abstract

The polyether dinoflagellate toxins, azaspiracids, are responsible for azaspiracid poisoning (AZP), a new human toxic syndrome arising from the consumption of shellfish. Using recently developed sensitive analytical methods, involving liquid chromatography with multiple tandem ion-trap mass spectrometry (LC/MS^n), six new azaspiracids AZA6-AZA11 have been identified in contaminated mussels (*Mytilus edulis*). AZA6 is a positional isomer of AZA1 and four of the new compounds are their hydroxylated analogs, AZA7-AZA10. AZA11 is the hydroxylated analog of AZA2. The separation of all azaspiracids was achieved using isocratic reversed phase liquid chromatography with a combination of eluent additives, trifluoroacetic acid and ammonium acetate, and a long elution time. The ion-trap MS experiments, with electrospray ionization, were carried out in positive mode using optimized collision energies at each stage. A unique parent-fragment ion combination was identified in each azaspiracid that permitted the analysis of each toxin without the need for full chromatographic separation and this led to the development of a rapid LC-MS^3 method.

Introduction

Azaspiracids were identified for the first time in Irish mussels that induced human intoxications in The Netherlands in 1995 (Satake *et al.,* 1998a, 1998b). Following several poisoning incidents throughout Europe, a new toxic syndrome, Azaspiracid Poisoning (AZP), was declared (Ofuji *et al.,* 1999). AZA1 was the first of these toxins to be discovered and is usually the predominant toxin in shellfish. AZA2 and AZA3 are the 8-methyl and 22-demethyl analogs of AZA1, respectively (Ofuji *et al.,* 1999). AZA4 and AZA5 are the 3- and 23-hydroxy analogs of AZA3 (Ofuji *et al.,* 2001) and are found in low abundance in shellfish. The potential widespread distribution of AZP toxins has been confirmed by the detection of these toxins in shellfish from Norway and the U.K. (James *et al.,* 2002a). Azaspiracids accumulate in filter-feeding bivalve molluscs, including mussels (*Mytilus edulis*) (James *et al.,* 2002b) and scallops (*Pecten maximus*) (Braña Magdalena *et al.,* 2003). AZP toxins may be potentially more dangerous than DSP toxins since recent acute and chronic toxicological studies showed that azaspiracids caused widespread organ damage and induced tumors in mice (Ito *et al.,* 2000), (Ito *et al.,* 2002). Until recently, regulatory control of AZP in Europe relied exclusively on live animal bioassays that were developed to monitor DSP toxicity in shellfish but this repeatedly failed to prevent acute human intoxications due to AZP (James *et al.,* 2002a). Liquid chromatography tandem mass spectrometry (LC-MS/MS) was first used for the determination of AZA1 in shellfish (Draisci *et al.,* 2000). Recently, we reported robust and sensitive analytical methods for the determination of AZA1-AZA5 using ion-trap multiple tandem MS (Furey *et al.,* 2002, Lehane *et al.,* 2002). New hydroxyl analogs of azaspiracids have now been detected and probable structures are proposed for these new compounds.

Materials and Methods

A Waters 2690 Alliance LC (Waters Corporation, Milford, MA, USA) was linked to a Finnigan MAT LCQ Classic ion-trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). Isocratic chromatography was performed using acetonitrile-water (46:54) containing 0.05% trifluoroacetic acid (TFA) and 0.5 mM ammonium acetate, at a flow rate of 200 µL/min, with a reversed phase column (Luna-2 C18, 3 µm, 150 × 2.0 mm, Phenomenex, Macclesfield, UK) at 40°C. Full details have been reported previously (Furey *et al.,* 2002, Lehane *et al.,* 2002). Multiple tandem MS produced collision-induced dissociation (CID) spectra and were obtained using the protonated molecule for each toxin which fragmented similarly giving major ions due to the sequential loss of water molecules. Azaspiracids were determined using LC-MS^3 by targeting parent and water-loss ions to produce spectra that contained unique ions due to the fragmentation of the A-ring of each toxin (Fig. 1).

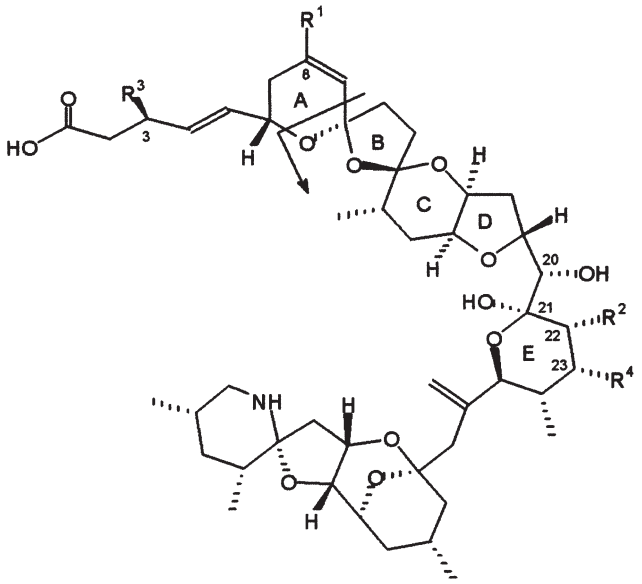

Figure 1 Structures of azaspiracids.

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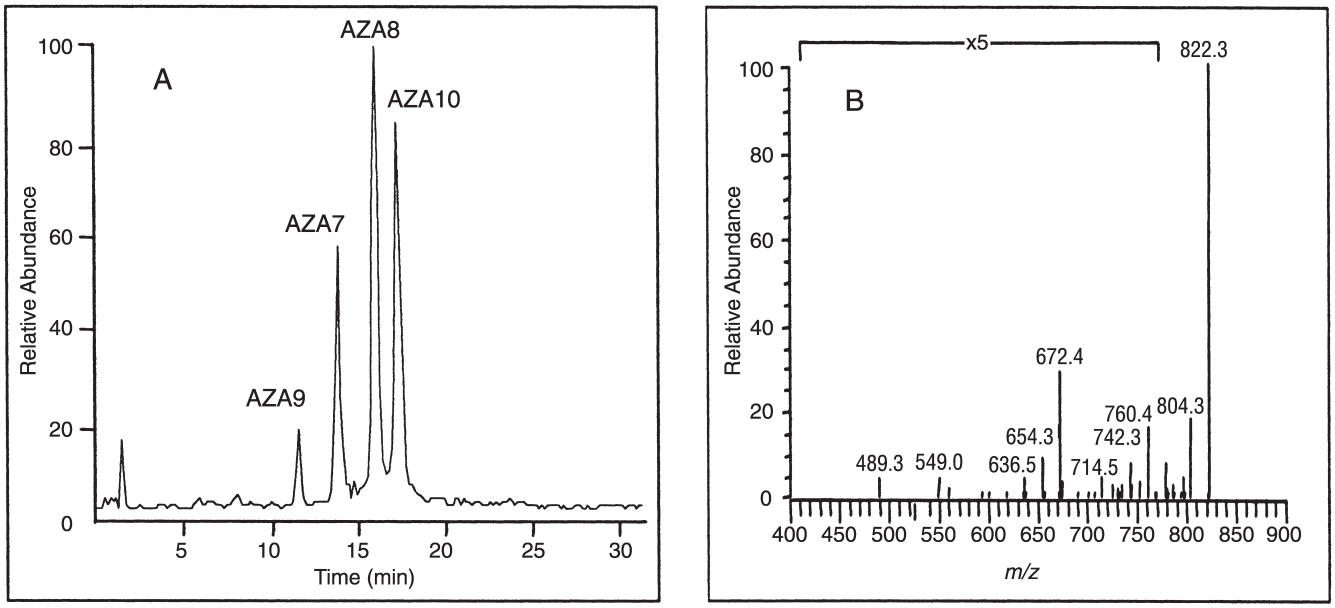

Figure 2 **A** Chromatogram of the isomeric azaspiracids, AZA7–AZA10, determined by LC-MS^3 using ion-trap MS and **B** the spectrum of AZA7.

Results and Discussion

For azaspiracids, the most sensitive LC-MS^3 method involves trapping and fragmentation of the parent ion, $[M+H]^+$, and the product ion, $[M+H-H_2O]^+$, and trapping the $[M+H-2H_2O]^+$ ion. A feature, typical of ion-trap MS, is that there is an improvement in detection sensitivity in multiple MS modes. This is attributed to the reduction in background noise in MS^2 and MS^3 stages being more dramatic than the decline in analyte signal (Biancotto *et al.,* 1997). Through the implementation of this LC-MS^3 method, the separation of the ten azaspiracids, AZA1–AZA11, was achieved in 60 min. The chromatographic conditions presented here were developed primarily to separate the four hydroxy isomers, AZA7–AZA10, as the molecular masses do not distinguish between these compounds (Fig. 2).

The mass spectrum obtained from the MS^3 of AZA7 is shown in Fig. 2B. The spectrum of azaspiracids typically showed ions derived from multiple water losses from the protonated molecule ion, together with ions due to fragmentation of the azaspiracid backbone. These fragmentation processes include the A-ring, C-ring, C19-C20 and E-ring. For example, AZA7 gave the same ion, m/z = 672, as produced by the A-ring fragmentation of AZA1, showing that the hydroxyl substituent was in the C1-C9 fragment that was lost. Thus, the fragment loss in AZA7 was 168 Da, a difference of 16 Da from the corresponding loss of 152 Da in AZA1.

The relative collision energies (% RCE) were 80% (MS^2) and 35% (MS^3) and were selected to produce optimum ions from the fragmentation of the A-ring. Each azaspiracid produced unique mass spectra. For example, the fragmentation at the A-ring (Fig. 1) causes the loss of a fragment containing R^1 and R^3 leaving the ion containing the remainder of the azaspiracid structure with R^2 and R^4. There are three groups of isomers: AZA1, AZA6; AZA4, AZA5; AZA7 – AZA10. The minor toxin, AZA11, eluted at 13.6 min (not shown). The optimized chromatographic conditions separated all the azaspiracids but the most difficult separation was the AZA7 – AZA10 group (Fig. 2). These isomers are 16 Da larger than AZA1 and AZA6. They have similar structures to the latter, apart from an additional hydroxyl moiety. Table 1 shows that the R^2 and R^4 combination is different in AZA7–AZA10, and the important consequence of this in multiple tandem MS is that each of the isomers AZA7–AZA10 produces an ion with a different mass following A-ring fragmentation. Thus, it is possible to differentiate between these toxins without chromatographic separation using LC-MS^3. The selected ion combinations for the LC-MS^3 of the new toxins are: AZA6

Table 1 The variable substituents, R^1, R^2, R^3, R^4, in azaspiracids.

	R^1	R^2	R^3	R^4
AZA1	H	CH_3	H	H
AZA2	CH_3	CH_3	H	H
AZA3	H	H	H	H
AZA4	H	H	OH	H
AZA5	H	H	H	OH
AZA6	CH_3	H	H	H
AZA7	H	CH_3	OH	H
AZA8	H	CH_3	H	OH
AZA9	CH_3	H	OH	H
AZA10	CH_3	H	H	OH
AZA11	CH_3	CH_3	OH	H

(m/z = 842.5 → 824.5 → 658.4); AZA7 (m/z = 858.5 → 840.5 → 672.4); AZA8 (m/z = 858.5 → 840.5 → 688.4); AZA9 (m/z = 858.5 → 840.5 → 658.4); AZA10 (m/z = 858.5 → 840.5 → 674.4); AZA11 (m/z = 872.5 → 854.5 → 672.4). A reduced chromatographic run-time of 12 min was achieved using acetonitrile:water (65:35) and toxins were distinguished by their characteristic parent and product ion combinations. Azaspiracids with hydroxyl substituents at the C3 or C24 positions were detected only in *M. edulis.* The toxin profiles in *P. maximus* were less complex with usually only AZA1 and AZA2 detected.

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Hemolytic Compounds from *Fibrocapsa japonica* (Raphidophyceae)

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Abstract

The hemolytic effect of *Fibrocapsa japonica* extracts is reported here for the first time. Three hemolytic compounds were separated by HPLC in extracts of *Fibrocapsa japonica* isolated from the German Wadden Sea. Hemolytic effects of three toxic compounds isolated by HPLC were determined by erythrocyte lysis assay at concentrations of 10 µg/mL. Preliminary mass spectrometric analysis showed that these compounds are not brevetoxins previously reported in *F. japonica* extracts.

Introduction

The raphidophyte microalgae *Chattonella* spp., *Fibrocapsa japonica* and *Heterosigma akashiwo,* produce red tide blooms that induce mass mortality of cultured fish in southeast Asian coastal waters (Toriumi and Takano, 1973; Nakamura, 1983). The species *F. japonica* has caused significant damage to coastal fisheries of Japan (Toriumi and Takano, 1973; Yoshimatsu, 1987; Montani *et al.,* 1995). From the beginning of the 1990s, *F. japonica* was observed in Europe in French and Dutch coastal waters (Billard, 1992; Vrieling *et al.,* 1995), and in 1996 it was isolated from the German Wadden Sea as well (Rademaker *et al.,* 1998). Both molecular and physiological data on *F. japonica* strains suggest recent range expansion of this species (Kooistra *et al.,* 2001; de Boer *et al.,* 2002). The mechanism behind the toxicity of *F. japonica* is still under debate. Both the production of radicals (Oda *et al.,* 1997) and of toxins (Khan *et al.,* 1996) have been reported. The fish-killing toxins were identified as brevetoxins based on their chromatographic behavior only. In this note we report on the purification and the hemolytic nature of toxins isolated from *F. japonica.*

Materials and Methods

The clonal culture used (strain CCRuG_Cl3, culture collection University of Groningen, The Netherlands) was started in 1997 by taking one cell from a culture that had been initiated from a sample of a monospecific bloom in Büsum Harbor, Germany, in 1995 (U. Tillmann, pers. comm.). Algae were cultured in f/2-Si medium (Guillard 1975) at 20°C, an irradiance of ca. 67 µmol photons $\cdot$ m^{-2} $\cdot$ s^{-1} and a 12-hour light-dark cycle. The seawater used for preparing the f/2 medium came from the Jade Bay (29–30 PSU) and was filtered through cotton and then autoclaved at 120°C for 20 minutes. All culture work was carried out in a Gallenkamp Orbital Incubator INR-401. Algal cell numbers were determined by nephelometry (DRT-15CE Portable Turbidimeter).

Cells from a batch culture were harvested by filtration (GF/C 47 mm Ø, Whatman), extracted with methanol and stored at –18°C. Solid phase extraction (SPE) was performed on subsamples of 3 mL which were loaded on 3 mL cartridges (C-18, Supelco, Inc.) using a vacuum manifold. The cartridge was eluted with a gradient of aqueous methanol, 0% to 100% in steps of 10% and a volume of 2 mL. The different fractions obtained were evaporated to dryness at 40°C and redissolved in 2 mL assay buffer (Eschbach *et al.,* 2001) for the erythrocyte lysis assay in duplicate.

For detection of hemolytic compounds we used an erythrocyte lysis assay (ELA) method which is based on photometrical determination of the released hemoglobin from the lysis of erythrocytes caused by hemolytic compounds (Yariv *et al.,* 1961). We used human erythrocytes (Group A) obtained from the Blood Donation Service, Oldenburg, Germany, and stored at 4°C. Assay buffer was made according to the recipe of Eschbach *et al.* (2001). The erythrocytes were washed with this assay buffer two times at 1500 $\times$ *g* for 5 min at 15°C, and resuspended. Cell numbers were determined by microscopy in a blood counting chamber. To scan for the maximal absorption of human blood cells, 1.5 mL (7×10^6 cells $\cdot$ mL^{-1}) was disintegrated completely with an ultrasonic disintegrator (Soniprep 150) under ice-cold conditions. The lysed erythrocytes solution was scanned from 350 to 700 nm with a photometer UVIKON 930 to confirm the maximum absorption wavelength of human hemoglobin at 414 nm. A calibration curve was routinely made by serial dilution of lysed erythrocytes (steps of 20%). For the analysis of the SPE fractions, 12 mL centrifuge tubes were used as reaction vessels. SPE fractions of 1 mL volume were incubated separately with 1 mL erythrocyte suspension (final concentration 4.5×10^6 cells/mL) at 15°C for 24 hrs. One mL ELA buffer together with 1 mL erythrocytes was used as the negative control; the positive control was of 1 mL ELA buffer and 1mL completely lysed erythrocytes. After 24 hrs incubation, reaction vessels were centrifuged at 1500 $\times$ *g* for 5 min at 15°C, and 1.2 mL of each supernatant was measured at the maximum absorption wavelength.

High performance liquid chromatography (HPLC) was used for separation and detection of compounds present in the SPE fractions. The HPLC system consisted of the following parts: a degassing unit, a Constametric 4100 pump and an AS 100 autosampler (all Thermo Separation Products). Detection of separated components was accomplished

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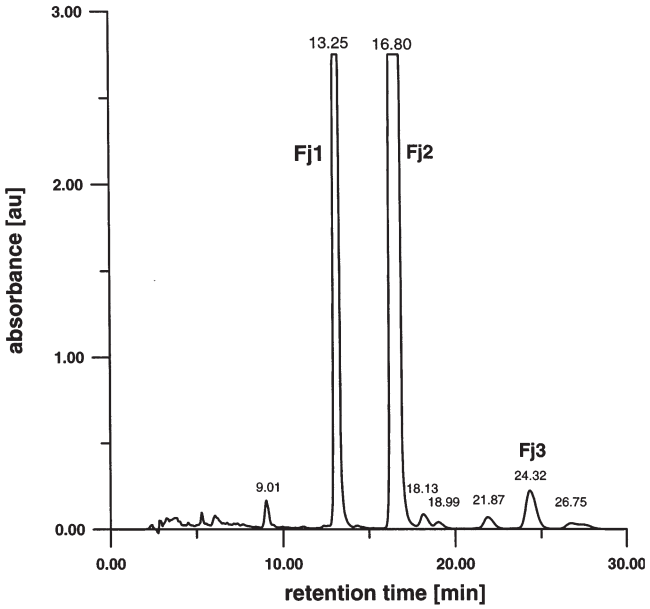

Figure 1 HPLC chromatogram of hemolytic compounds (detection wavelength 210 nm) from an extract of *Fibrocapsa japonica.*

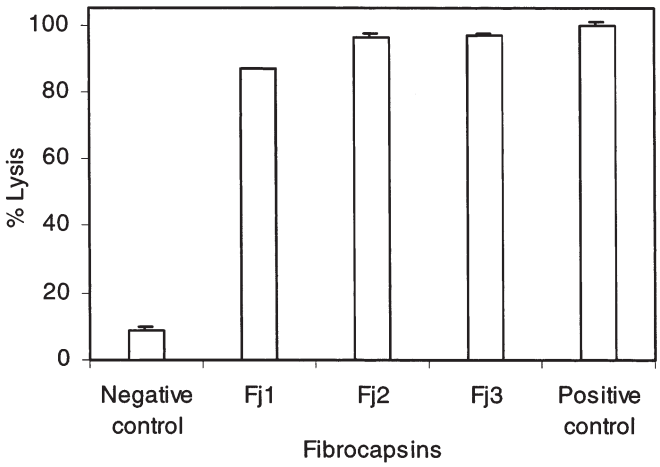

Figure 2 Hemolytic effects of fibrocapsins (10 µg/mL each) after 24 hrs incubation. Negative control: 1 mL ELA buffer + 1 mL erythrocytes; positive control: 1 mL ELA buffer + 1 mL completely lysed erythrocytes.

by a photodiode array detector (Merck PDA L7450) that measured absorption from 200 to 500 nm. A Phenomenex column was used (Aqua, 5 µm, C18, 250 × 4.6 mm). Chromatograms were evaluated by D-7000 HPLC-System Manager software (Merck). All analyses were carried out at room temperature. Isocratic elution was employed using 21% water containing 0.05% (v/v) trifluoroacetic acid and 79% acetonitrile. The flow rate was 1.0 mL · min^{-1} and the injection volume was 20 µL. The optimal absorption wavelengths were fixed at 210 nm.

Concentrated SPE toxic fractions identified by ELA were mixed and subjected to HPLC. The elution was repeated to collect the three major peaks, respectively, according to their retention times. These were named fibrocapsins 1 to 3 (Fj1, Fj2, Fj3). Each collected compound was redissolved in assay buffer for the erythrocyte lysis assay in duplicate after drying. Each compound was also rechromatographed individually to verify its purity. The assay method was the same as described above, with the final concentration of each compound being 10 µg/mL in the test solution.

Results and Discussion

Methanol extracts from *Fibrocapsa japonica* that were evaporated and taken up in seawater were toxic both to the brine shrimp *Artemia salina* and to the luminescent marine bacterium *Vibrio fischeri,* indicating general toxicity of the strain used (data not shown). In preliminary mass spectrometric investigations of bulk extracts there were no peaks at a molecular mass around 900 Dalton, which would be expected for brevetoxins, and none after the purification procedures described both by Kahn *et al.* (1996) and Hua *et al.* (1996). Therefore, we used an assay to detect hemolytic compounds and found strong activity after solid phase extraction (SPE) with 60%, 70% and 80% aqueous methanol. The toxic SPE fractions were pooled and then subjected to high performance liquid chromatography. Three main peaks were separated and named tentatively fibrocapsins 1, 2 and 3 (Fj1, Fj2, Fj3; Fig. 1).The human erythrocyte lysis assay of these compounds again displayed strong hemolytic effects (Fig. 2).

To our knowledge this is the first report of hemolytic compounds produced by *F. japonica* and the second for the Raphidophyceae. For *Chattonella marina,* three toxic fractions being neurotoxic, hemolytic and hemagglutinative have been described before, all of them ichthyotoxic to juvenile red seabream (Onoue and Nozawa, 1989).

Khan *et al.* (1996) detected five toxins of *F. japonica* using HPLC in a strain that was isolated from the same area as ours. These toxins had the same HPLC chromatographic behavior as brevetoxins PbTx 1, 2, 3, 9 and oxidized PbTx-2. We have not cross-checked our purified samples with brevetoxin standards, but it seems unlikely that they are brevetoxins. No compounds with a brevetoxin-matching molecular mass were detected in the crude and concentrated extracts and the major peaks in our methanol extract were hemolytic, a characteristic not expected for neurotoxins. The brevetoxin-producing *Karenia brevis* was found to contain hemolytic as well as organo-phosphous cardiotoxic compounds also (Mazumder, 1997), but a hemolytic action of brevetoxins themselves has not been described.

There could be a number of explanations why Khan's and our observations are seemingly contradictory. Firstly, different strains could actually produce different toxins. Secondly, the production of certain toxins could be controlled by environmental conditions that were probably different in the different laboratories. Another explanation could be that the peaks observed in Khan's study were actually the same as ours but eluted with a similar behavior as brevetoxins. The purified fibrocapsins of this study are currently being investigated for their chemical nature.

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