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 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.

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 methods for the determination of azaspiracids in shellfish, using ion-trap multiple tandem MS (Lehane et al., 2002). Azaspiracids were determined using LC-MS with the target parent and fragment ion combinations as follows: [M+H]+ → [M+H-H2O]+ → [M+H-2H2O]+; 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...
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, 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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References


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 (C₃₉H₆₀O₈) is similar in structure to the hemibrevetoxin B, (C₂₈H₄₂O₇) (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).

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, λ = 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 1 phenyl-hexyl column (0.4 × 25 cm, 90% MeOH, 1.4 mL/min, λ = 215) was used. Separation of the toxins was performed using a traditional C₁₈ column (0.4 × 25 cm, 85% MeOH, 1.4 mL/min, λ = 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.

Figure 1 Structure of the new brevetoxin antagonist AJB6.0P (brevenal).
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 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 1H, 13C, DEPT, and HSQC NMR spectra showed that AJB6.0P contains 1 doublet CH1, 5 singlet CH’s, 11 aliphatic methylenes, 1 aliphatic methine, 3
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).

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**References**

W.M. Abraham, A. Ahmed, A.J. Bourdelais and D.G. Baden, this Proceedings.


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**Table 1** Fish bioassay.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>N</th>
<th>Time to Death (min ± s.e.m.)</th>
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<tbody>
<tr>
<td>Control</td>
<td>20</td>
<td>No deaths after 24 hrs</td>
</tr>
<tr>
<td>AJB 6.0P 1 mg/mL</td>
<td>10</td>
<td>No deaths after 24 hrs</td>
</tr>
<tr>
<td>AJB 6.0P 2 mg/mL</td>
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<td>No deaths after 24 hrs</td>
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<tr>
<td>PbTx-2 1 mg/mL</td>
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<td>7.5 ± 1.06</td>
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<tr>
<td>PbTx-2 1 mg/mL + AJB 6.0P 1 mg/mL</td>
<td>10</td>
<td>17.0 ± 2.84</td>
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</table>
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 CO2 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. 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.
**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). Iso-
cratic elution was performed with 65% B, where eluent A was H2O and eluent B was 95% CH3CN/H2O, both con-
taining 3.5 mM formic acid and 2 mM ammonium formate. The flow rate was 0.2 mL/min and a sample injection vol-
ume 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% CH3CN. 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 seri-
ally 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 ex-
pected 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. Sig-
nificant 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 re-
tention times matching those of standards. The identitiies

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![Figure 1](image-url)

**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.
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**

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**References**


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

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<th>Analysis Method</th>
<th>Extraction Method</th>
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<tr>
<td>LC-MS #1</td>
<td>#1: Methanol (TTX method)</td>
<td>20,000</td>
</tr>
<tr>
<td>LC-MS #2</td>
<td>#2: Acetic acid</td>
<td>36,000</td>
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Lipophylic Toxins of Different Strains of Ostreopsidaceae and Gonyaulaceae

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Abstract

Production of lipophylic toxins of the yessottoxicos 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 hemolytic 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 macarenensis (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 Acetonitrile/Acetic acid 1% (3:7) and was chromatographed on Vydac 201TP54 C18 column at 30°C, resuspended in Acetonitrile/Acetic acid 1% and was partitioned with 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 CaCl2 and 1

Table 1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Species</th>
<th>Location</th>
</tr>
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<tbody>
<tr>
<td>OS06BR</td>
<td>Ostreopsis cf. ovata</td>
<td>SW Atlantic</td>
</tr>
<tr>
<td>OS2V</td>
<td>Ostreopsis cf. siamensis</td>
<td>Mediterranean</td>
</tr>
<tr>
<td>GG1AM</td>
<td>Protoceratium reticulatum</td>
<td>NE Atlantic</td>
</tr>
<tr>
<td>CCMP1720, CCMP1721</td>
<td>Protoceratium reticulatum</td>
<td>NW Atlantic</td>
</tr>
<tr>
<td>CCMP1889, CCMP404</td>
<td>Protoceratium reticulatum</td>
<td>NW Pacific</td>
</tr>
<tr>
<td>LP3A, LP4V, LP5V, LP6V, LP7V, LP8V, LP9V, LP10V</td>
<td>Lingulodinium polyedrum</td>
<td>NE Atlantic</td>
</tr>
<tr>
<td>CM1V, CM2V, CM3V, CM4V, CM5V, CM6V</td>
<td>Coolia monotis</td>
<td>NE Atlantic</td>
</tr>
<tr>
<td>CCMP305, CCMP1345, CCMP1744</td>
<td>Coolia monotis</td>
<td>NW Atlantic</td>
</tr>
</tbody>
</table>

mM H$_3$BO$_3$. 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 yezotoxins 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$_2$Cl$_2$. 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 possessing 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-*
Ostreopsis 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 hemolytic 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 Coelola, although it was detected in an Australian strain (Holmes, 1995). Palytoxins look to be restricted to the family Ostreopsisidae and YTX to Gonyaulacaceae.

Acknowledgements
Prof. T. Yasumoto for continuous advice, E. Cacho for mice bioassays, A. Miguez for MS-Analysis and Luz Mamán, Sergio O. Lourenço and Yolanda Pazos for providing samples. Funded through projects CCVIEO and LIPOTOX (Xunta de Galicia; PR-404G PROY 99-1); CLIPOTOX (MCYT;REN2001-2959-C04-01/MAR).

References
**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 –20°C. 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 deSTX provided by the Community Bureau of Reference of the EU for an inter-comparison 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: \( U = K \times \text{RSD} \times c \) (\( U = \text{expanded uncertainty} \); \( K = 2 \); \( \text{RSD} = \text{relative standard deviation}; \ c = \text{concentration} \).

**Stability Study**

Once the homogeneity of the material was verified, the stability at –20°C was checked at three time intervals (zero, nine and twelve months) by determining the
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 (t0.95 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 lines were calculated. The slope of the regression lines did not differ significantly from zero (t0.95 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.

**Acknowledgements**

We thank the Director and the Deputy Director of the Centro Nacional de Alimentación for their support in this study. We are also grateful to “Conservas Ubago, S.L.” for its contribution; to Gabriel Jiménez, photographer of the ISCIII; to Josefa Pérez, Section’s Chief of the Animal Department, for her technical assistance; and to Rafael Cabanillas and Isabel García León for their collaboration in this work.
References
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 novaeezelandiae*), 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 Micro-algae 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 Na2SiO3.5H2O, 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 CuSO4.5H2O 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...
or filtrate (50 mL) were acidified (pH 2.5) 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 CA WB 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 CuSO₄·5H₂O 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...
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. multis-triata 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 (\(1.0 \times 10^{-4}\) 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.

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**References**


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 CH₂Cl₂. 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 CH₃CN/MeOH/H₂O 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 yessotoxin (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.
from the hepatopancreas of mussels of the Adriatic sea, such as adriatoxin (Ciminiello et al., 1998), carboxyyessotoxin (Ciminiello et al., 2000a), carboxyhomoyoessotoxin (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 ‘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-Hep-tanor-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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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...
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 (ThermoFinnigan, 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^n mode, AN produced a major fragment ion due to ammonia loss at m/z 149 [166-NH,=H]^+; MS^n yielded the fragment ions for AN at m/z 131 [166-NH,OH=H]^+, m/z 121, 107, 105, 91, 81 and 79.
Similarly, for HMAN the MS2 gave major fragment ions at m/z 163 \([180-\text{NH}_3+\text{H}]^+\), m/z 145 \([180-\text{NH}_3-\text{H}_2\text{O}+\text{H}]^+\), m/z 135, 120, 107, 91. MS3 spectra were obtained by trapping and fragmenting the ion at m/z 163. The ions at m/z 107 \([\text{M}-\text{NH}_3-\text{COCHR}]^+\) and m/z 91 \([\text{C}_7\text{H}_7]^+\) were observed for both toxins in the MS3 spectra. Linear correlations were obtained for AN in each MS mode and sensitivity increased when using LC-MS2 and LC-MS3 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) MS2 (AN), c) MS3 (HMAN), d) MS2 (AN), e) MS3 (HMAN). The selected ions were m/z 149 and m/z 163 in MS2 with m/z 131 and m/z 145 selected in the MS3 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-MS2 and LC-MS3 modes. The relative standard deviation (\% RSD, n = 5) values were \(\leq 2\) at 5.0 \(\mu\)g/mL and \(\leq 7.0\) at 0.10 \(\mu\)g/mL, respectively. The detection limit (S/N = 3) was 0.6 \(\mu\)g/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 MS2 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 homoaatoxin-a degradation products which have not yet been found in nature.

Acknowledgements
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References
Genotoxicity Investigation of Chlorinated Degradation Products of a Cyanobacterial Toxin, Cylindrospermopsis

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Abstract

Cylindrospermopsis (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. Cylindrospermopsis (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) (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 and lungs of each mouse were put into 10 mL fixative solution (2.5% glutaradehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate trihydrate, 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. 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 pur-
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 5CIUra 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 5CIUra 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 deoxycyclindospermopsin (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 considerably 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 5CIUra 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 5CIUra, 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 periacinetic 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 5CIUra 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 5CIUra. A significant decrease in MML value was observed 6 hours post dosing, suggesting an impact on DNA integrity due to exposure to 5CIUra (Fig. 1). There is, therefore, some evidence that 5CIUra 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 5CIUra 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 5CIUra 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-
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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References
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.

**Table 1** Growth parameters from isolates maintained under identical conditions.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Clone ID</th>
<th>Isolation Date</th>
<th>Max Biomass (cells/mL)</th>
<th>Mean growth rate (d⁻¹)</th>
<th>S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. australis</em></td>
<td>Au-211M</td>
<td>Feb-02</td>
<td>15727</td>
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<td>0.26</td>
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<td>16167</td>
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<td>0.18</td>
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<td>0.16</td>
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<td>26776</td>
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cylindrical chemostat vessels were maintained under 24 h light (ca. 100 µmol photons m⁻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 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...
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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**References**


**Abstract**

In July 2000, a bloom of *Pseudo-nitzschia australis*, identified by Transmission Electron Microscopy (TEM), reached an abundance of $2.8 \times 10^4$ 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), homogenization 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. 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 $2.8 \times 10^4$ 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), homogenization 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.

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

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 (\textit{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).

**Results and Discussion**

Increasing cell concentrations of one species of \textit{Pseudo-nitzschia}, later identified by TEM as \textit{P. australis} (Fig. 2), were recorded from May to July throughout the surveyed period. The occurrence of \textit{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 \(\times\) 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 \textit{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 large extent of the coastal area, the width of the Argentine continental shelf, and the scarce human population along the coast.

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. jacobus* 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
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-

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

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

HP: hepatopancreas; M: adductor muscle; FG: female gonads; MG: male gonads; G: gills; and MT: mantle. nd: not detected.
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” (QCAII/med.4/PROPESCA Program) also supported this work.

References
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 al.* 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-l (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 10 µE m−2s−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 × 105 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 µE m−2s−1 illumination, in Gulf Stream, USA, water diluted to 15 psu. Each of these four isolates were grown in parallel either autotrophically, by adding f2-Si nutrient mixture, or mixotrophically, by periodically adding *Rhodomonas* (CCMP 767) as the sole food source. All were harvested at concentrations of 3–5 × 105 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 × 106 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 × 104 cells mL−1 *K. micrum*. Isolate...
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.

<table>
<thead>
<tr>
<th>Map Code</th>
<th>Isolate / Sample</th>
<th>Collection Location</th>
<th>Isolation Date</th>
<th>Salinity (PSU)</th>
<th>Main Toxin</th>
<th>Map Code</th>
<th>Isolate / Sample</th>
<th>Collection Location</th>
<th>Isolation Date</th>
<th>Salinity (PSU)</th>
<th>Main Toxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CCMP 1974</td>
<td>Chesapeake Bay, MD</td>
<td>5/95</td>
<td>12</td>
<td>KmTx1</td>
<td>9</td>
<td>Cell J</td>
<td>Neuse River, NC</td>
<td>7/99</td>
<td>15</td>
<td>KmTx2</td>
</tr>
<tr>
<td>2</td>
<td>CCMP 1975</td>
<td>HyRock Farm, MD</td>
<td>7/96</td>
<td>12</td>
<td>KmTx1</td>
<td>10</td>
<td>Water Sample</td>
<td>NCSU Field Lab</td>
<td>6/02</td>
<td>6</td>
<td>KmTx2</td>
</tr>
<tr>
<td>3</td>
<td>MD5CR-0599</td>
<td>Choptank River, MD</td>
<td>5/99</td>
<td>12</td>
<td>KmTx1</td>
<td>11</td>
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<td>15</td>
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</table>

*Mixed fish kill, 4 × 10⁴ cells mL⁻¹ *K. micrum*. †Routine sampling, 7 × 10³ cells mL⁻¹ *K. micrum*. ‡Suspect area, no fish kill, 1.6 × 10⁴ cells mL⁻¹ *K. micrum*. §Fish kill (3600 silversides), 5.7 × 10⁴ cells mL⁻¹ *K. micrum*. †Two samples each, one autotrophic, one mixotrophic. *Genbank numbers AF352365, AF352367 (Litaker et al., 2003). ‣Sampled five days after kill, 3.5 × 10⁴ cells mL⁻¹ *K. micrum* during kill. ††Mixed fish kill, 7 × 10⁴ cells mL⁻¹ *K. micrum*. Isolated from fish kill (h), kill described in (Kempton et al., 2002).
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**

The authors wish to thank members of the MD Departments of Natural Resources and the Environment for providing *K. micrum* bloom samples and fish kill data, and A. Garber and C. Couch (NCSU) for providing samples and observations from the NCSU aquaculture kill. This research was funded by grants from NOAA ECOHAB (NA860PO492), and NIEHS (PO1-ES9563). This is contribution #04-608 from the Center of Marine Biotechnology. This is contribution #92 from the ECOHAB program.

**References**


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-ichthyotoxic 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.

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**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).
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 Coulter Multisizer II counter. From the remaining 20 mL, an aliquot containing 10⁶ 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 HP1100 HPLC coupled to an HP UV diode array detector. The mobile phase was a linear MeOH:H₂O 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-ichthyotoxic 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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**References**

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 than the parent algal metabolite.

Figure 1 Structure of domoic acid and its various isomers.

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 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.

**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.
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.

References

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 were found to increase when samples were subjected to solar radiation. Four of the photochemical by-products were found to decrease while photochemical by-products represented ~75% of the total material in the sample. Thus, brevetoxin (PbTx2 spike) concentrations 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 represented ~75% of the total material in the sample after 24 hrs of exposure. Most (19 of 21) of the by-product masses detected did not correlate to any 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,
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 photo-products 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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**References**


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.

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...
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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**References**


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 sp² character of its double bond. Therefore, introduction of more rotational freedom on the A-ring carbonyl 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,
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-
directly 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 triated 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**

This work was supported by the National Institute of Environmental and Health Science (ES05853-07). We also thank Susan Campbell, Jody Lamberto and Allison Weidner for the production and purification of PbTx-3 and PbTx-2.

**References**


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 spiromine 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 spirolime 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, Phenomenex 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...
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 $0.77 \pm 0.18 \mu g \text{GYM/mL}$ 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 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.
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 fraction 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 µm³. 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**

Thanks to Tracy Neil and Andy Selwood, Cawthron Institute for assistance with the LC-MS analyses and Dr. Chris Miles Ag-Research for providing the purified gymnodimine reference material. This research was funded by the New Zealand Foundation for Research Science and Technology under contract CAWX0201.

**References**


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.

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
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 Cl2 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 Na2S2O3 (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 Cl2 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.

<table>
<thead>
<tr>
<th>Sample</th>
<th>C1, C2</th>
<th>dcSTX</th>
<th>GTX2, 3</th>
<th>GTX5</th>
<th>STX</th>
<th>Initial Cl2 Dose µg/L</th>
<th>Initial Toxin Concentration</th>
<th>Residual Cl2 Level after 30 min µg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.6</td>
<td>121 (46)</td>
<td>0.03 (95)</td>
<td>11.4 (58)</td>
<td>1.2 (93)</td>
<td>1.2 (78)</td>
<td>7.2</td>
<td>226</td>
<td>0.28</td>
</tr>
<tr>
<td>pH 8.2</td>
<td>85 (62)</td>
<td>0.04 (93)</td>
<td>8.4 (69)</td>
<td>0.9 (95)</td>
<td>0.8 (85)</td>
<td>7.2</td>
<td>265</td>
<td>0.29</td>
</tr>
<tr>
<td>pH 8.6</td>
<td>58 (74)</td>
<td>0.04 (93)</td>
<td>6.1 (77)</td>
<td>0.7 (96)</td>
<td>0.6 (89)</td>
<td>7.2</td>
<td>20.0</td>
<td>0.31</td>
</tr>
<tr>
<td>pH 9.0</td>
<td>47 (79)</td>
<td>0.03 (93)</td>
<td>4.6 (85)</td>
<td>0.6 (96)</td>
<td>0.3 (95)</td>
<td>7.2</td>
<td>29.0</td>
<td>0.35</td>
</tr>
<tr>
<td>pH 9.3</td>
<td>39 (83)</td>
<td>0.01 (98)</td>
<td>3.1 (88)</td>
<td>0.4 (98)</td>
<td>0.2 (96)</td>
<td>7.2</td>
<td>5.5</td>
<td>0.85</td>
</tr>
<tr>
<td>pH 9.6</td>
<td>29 (87)</td>
<td>0.00 (100)</td>
<td>1.7 (92)</td>
<td>0.4 (98)</td>
<td>0.1 (98)</td>
<td>7.2</td>
<td>5.7</td>
<td>0.58</td>
</tr>
</tbody>
</table>

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

**References**


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, deSTX 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 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.

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 (pH 9) and high temperature (30°C) 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

![Figure 1](image1.png)

![Figure 2](image2.png)

![Figure 3](image3.png)

![Figure 4](image4.png)

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

<table>
<thead>
<tr>
<th>Incubation condition</th>
<th>Total PST</th>
<th>GTX5</th>
<th>NeoSTX</th>
<th>dcSTX</th>
<th>STX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile / deionized water</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20°C pH7</td>
<td>0.0107 [65]</td>
<td>0.0092 [75]</td>
<td>0.0301 [23]</td>
<td>–</td>
<td>0.0139 [50]</td>
</tr>
<tr>
<td>20°C pH9</td>
<td>0.0113 [61]</td>
<td>0.0213 [33]</td>
<td>0.0302 [23]</td>
<td>–</td>
<td>0.0077 [90]</td>
</tr>
<tr>
<td>30°C pH7</td>
<td>0.0218 [32]</td>
<td>0.0322 [22]</td>
<td>0.073 [9]</td>
<td>–</td>
<td>0.0208 [33]</td>
</tr>
<tr>
<td>Culture medium</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
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, dcSTX) 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 conditions, 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 microcystsins 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 deproteination of culture medium supports this hypothesis, but further studies are needed.

References


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 µM, 11.7 µM, 117 µM, or 1170 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-
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 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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**References**


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).

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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 toxicity 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...
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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References

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. Possible individual variation in the PSP toxicity of molluscs from the same area 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 80 µg STX equiv./100 g meat. Possible individual variation in the PSP toxicity of molluscs from the same area 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.

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).
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

![Figure 3 Foot of *A. tuberculatum*.](image)

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

<table>
<thead>
<tr>
<th>Toxicity range</th>
<th>Distal Portion</th>
<th>Middle Portion</th>
<th>Proximal Portion</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg STX equiv./100 g meat</td>
<td>M</td>
<td>S (n¹)</td>
<td>CV (%)</td>
</tr>
<tr>
<td>69–200</td>
<td>12</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>200–400</td>
<td>14</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>400–700</td>
<td>13</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>700–1324</td>
<td>10</td>
<td>1</td>
<td>–</td>
</tr>
<tr>
<td>69–1324</td>
<td>49</td>
<td>1</td>
<td>–</td>
</tr>
</tbody>
</table>

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 sub-samples 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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References
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>
<thead>
<tr>
<th>Year</th>
<th>Date (month/day)</th>
<th>Alexandrium spp. (cells/liter) × 10³</th>
</tr>
</thead>
<tbody>
<tr>
<td>1999</td>
<td>3/23</td>
<td>N.D.*</td>
</tr>
<tr>
<td></td>
<td>4/5</td>
<td>2.46</td>
</tr>
<tr>
<td></td>
<td>4/19</td>
<td>1.07</td>
</tr>
<tr>
<td></td>
<td>4/26</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>5/10</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>5/19</td>
<td>N.D.</td>
</tr>
<tr>
<td>2000</td>
<td>4/3</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>4/17</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>5/8</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>5/15</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>5/22</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

*N.D.: Not detected*
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 × 10³ cells/L on 5 April, whereas in 2000, the concentrations detected were 0.01 × 10³ 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 to 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).

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.

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

<table>
<thead>
<tr>
<th>No.</th>
<th>Species</th>
<th>Date (month/day)</th>
<th>Toxicity (MU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oh-1</td>
<td>T. acutidens</td>
<td>4/19</td>
<td>80.0</td>
</tr>
<tr>
<td>Oh-2</td>
<td>T. acutidens</td>
<td>4/19</td>
<td>30.0</td>
</tr>
<tr>
<td>Oh-3</td>
<td>M. galloprovincialis</td>
<td>4/21</td>
<td>9.6</td>
</tr>
<tr>
<td>Oh-4</td>
<td>C. gigas</td>
<td>4/21</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Oh-5</td>
<td>M. galloprovincialis</td>
<td>4/25</td>
<td>&lt;2.0</td>
</tr>
<tr>
<td>Oh-6</td>
<td>C. farreri nipponensis</td>
<td>4/25</td>
<td>5.2</td>
</tr>
<tr>
<td>Oh-7</td>
<td>C. farreri nipponensis</td>
<td>4/25</td>
<td>10.0</td>
</tr>
<tr>
<td>Oh-8</td>
<td>C. farreri nipponensis</td>
<td>4/25</td>
<td>10.2</td>
</tr>
</tbody>
</table>

*Two specimens, *three specimens combined for analysis. ‘over 15 min until mice died.

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.
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**

We express our gratitude to Dr. Oshima of Tohoku University, Dr. Noguchi of former Nagasaki University, and the Fisheries Agency of Japan for providing the PSP toxin standards.

<table>
<thead>
<tr>
<th>Species</th>
<th>Date</th>
<th>GTX1</th>
<th>GTX2</th>
<th>GTX3</th>
<th>GTX4</th>
<th>C1</th>
<th>C2</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. acutidens</em></td>
<td>4/17</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>T. acutidens</em></td>
<td>4/17</td>
<td>–</td>
<td>Tr.</td>
<td>–</td>
<td>–</td>
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<td><em>T. acutidens</em></td>
<td>4/17</td>
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<td>–</td>
<td>–</td>
<td>Tr.</td>
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<tr>
<td><em>T. acutidens</em></td>
<td>4/17</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Tr.</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td><em>T. acutidens</em></td>
<td>4/17</td>
<td>–</td>
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<td>–</td>
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<tr>
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<td>5/11</td>
<td>0.16</td>
<td>Tr.</td>
<td>0.15</td>
<td>0.17</td>
<td>Tr.</td>
<td>0.68</td>
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<td>–</td>
<td>Tr.</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td><em>C. japonica</em></td>
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<td>–</td>
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<td>Tr.</td>
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<tr>
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<td>5/11</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Tr.</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>Tr.</td>
<td>–</td>
<td>–</td>
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<tr>
<td><em>C. japonica</em></td>
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<tr>
<td><em>C. japonica</em></td>
<td>5/11</td>
<td>–</td>
<td>Tr.</td>
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<td>–</td>
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<td>5/11</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>–</td>
</tr>
</tbody>
</table>

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

Toxin (nmole/g)

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

**References**


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

Luis A. O. Proença, Marcio S. Tamanaha, and Charrid Resgalla Jr.

Centro de Ciências Tecnológicas da Terra e do Mar – CTTMar, Universidade do Vale do Itajaí, Rua Uruguaí, 458, Centro, 88302-202, Itajaí, SC, Brazil

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 Mytilidae *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/m²/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 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. (=Proto- gonyaulax) 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 · m² · 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 × 10⁶ 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 $1.7 \times 10^6$ cells $\cdot$ 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 1% respectively. For the invertebrate tests, a negative control was performed using a culture of the diatom _Chaetoceros 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 ous extract from strain 2. No peaks were identified within the elution times for the tested standards. Different from

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 $\cdot$ 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 $\cdot$ 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.

**Acknowledgements**

This work was founded by The Universidade do Vale do Itajaí, UNIVALI. We thank Clarisse Odebrecht for helping with the determination of the studied species.

**References**


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


Institute for Marine Biosciences, National Research Council of Canada, 1411 Oxford St., Halifax, NS, Canada

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-proromoiety, which has been found elsewhere only in the marine.

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 ionization 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 C18 purification using a Vydac 201TP510 C18 HPLC column which was eluted isocratically with 70% A (50 mM formic acid, 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 CD3OD at 500.13 MHz (H) and 125.7 MHz (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.
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 (1H, 13C 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 1H and 13C 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 13C 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 13C shifts at 118 (C15) and 110 (C18) ppm which were characteristic of 5,5 and 5,6-spiroketal carbons, respectively. The two remaining quaternary 13C 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 1 LC-MS analysis of methanol extract of *A. ostenfeldii* strains LF37 and LF38.

Figure 2 Structure of 13-desmethyl spirolide C.

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.

Acknowledgements
The authors thank Cathy Lyons from the RCMP Forensic Laboratory, Halifax, N.S. for the FTIR spectra and Øjvind Moestrup (Copenhagen) for the isolates.

References


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 1H and 13C 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 D2O/0.1M CD3COOD, pH 2.0 solution and in 9/1 H2O/D2O/0.1M CD3COOD, pH 3.9 solution. 1H and 13C NMR spectra were recorded.

Figure 1 Chemical structures of known and new PSP toxins.

Results and Discussion

Table 1 NMR data ($\delta_C$, $\delta_H$, $J_{HH}$) for PSP compounds M1$^\beta$ and M3.

<table>
<thead>
<tr>
<th>Pos.</th>
<th>$\delta_C$</th>
<th>$\delta_H$, $J_{HH}$</th>
<th>$\delta_C$</th>
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<tr>
<td>4</td>
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<tr>
<td>5</td>
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<tr>
<td>6</td>
<td>53.29</td>
<td>3.83, ddd 5.1, 9.9, 1.0</td>
<td>53.30</td>
<td>3.85, ddd 5.1, 9.7, 0.7</td>
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<td>154.19</td>
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<tr>
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<td>3.26, dd 6.8, 10.5</td>
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<td></td>
<td></td>
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<tr>
<td>19</td>
<td>158.17</td>
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*Multiplicity s = singlet, d = doublet, $J_{HH}$ in Hz (error ± 0.3 Hz). nd = not detected, * interchangeable. *Sample dissolved in D$_2$O/DCI, pH 2.0. *Sample in 9/1 H$_2$O/D$_2$O/0.1M CD$_3$COOD, 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, \( \Delta = 1.6 \) 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 SO3), m/z 314 (loss of SO3 + H2O), m/z 289 (loss of SO3 + NHCO), m/z 271 (loss of HO,SNHCOOH), and m/z 253 (loss of HO,SNHCOOH + H2O). Additional ions not present in the spectrum of M1β were observed at m/z 296 (loss of SO3 + 2H2O) and m/z 235 (loss of HO,SNHCOOH + 2H2O), 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/13C HSQC NMR spectra of M3 (Table 1) showed again two 1H spin systems, one (\( \delta_H 4.83, 3.85, 4.11, 4.43 \)) corresponding closely in \( \delta_H, J_{HH}, \) and \( \delta_C \) of directly-bonded carbons, to system A in M1, the other (\( \delta_H 3.64, 3.88 \)) 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. 13C NMR results completely supported that M3 was 11,11-dihydroxy-N-sulfo-carbamoyl 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 \( C_{10}H_{17}N_7O_7 \) ([M+H]+ 332.1325, calc. 332.1319, \( \Delta = 0.6 \) 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).

Acknowledgements
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References
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, ichthyotoxic 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·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 (24:24:1) 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, 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 ichthyotoxicity, 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/CH₃CN). Details of the purification are to be published elsewhere.

Bioassays  Extracts and fractions were evaluated for ichthyotoxicity (i.e., toxicity to fish), antibacterial activity
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 1](image1.png)

**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).

![Figure 2](image2.png)

**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, cytotoxic 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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References
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 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 triquerta, 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 triquerta (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.
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.

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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**References**
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.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Isolation location (month)</th>
<th>Cells/mL (estuary)</th>
<th>Cells × 10⁶/mL (assays)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chattonella subsalsa (SC)</td>
<td>Hilton Head, SC (April)</td>
<td>4 × 10²</td>
<td>0.7–1.2</td>
</tr>
<tr>
<td>C. subsalsa (NC)</td>
<td>Neuse River, NC (July)</td>
<td>2 × 10²</td>
<td>0.6–2.9</td>
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<tr>
<td>Fibrocapsa japonica (SC)</td>
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<td>Heterosigma akashiwo (SC)</td>
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<td>2.00 × 10⁶</td>
<td>4.8–14</td>
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<tr>
<td>H. akashiwo (NC)</td>
<td>Neuse River, NC (June)</td>
<td>1.0 × 10³</td>
<td>2.9–7.1</td>
</tr>
<tr>
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<td>2.0 × 10³</td>
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<tr>
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<td>San Mateo Bay, Canada (July)</td>
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<td>4.9–8.7</td>
</tr>
<tr>
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<td>n.a.</td>
<td>5.3–7.7</td>
</tr>
<tr>
<td>C. marina (NIES-14)**</td>
<td>Harima-Nada, Japan (Feb.)</td>
<td>n.a.</td>
<td>0.6–0.9</td>
</tr>
</tbody>
</table>

* 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.
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 × 10⁷ 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 H₂O₂ analysis, cultures were diluted 1:1 with natural ultra-filtered seawater at the appropriate salinity, and production was quantified fluorometrically utilizing H₂O₂-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 H₂O₂ with varying production rates (Fig. 1A). *C. marina* had the highest production rate, followed by *C. subsalsa*, *F. japonica*, and *H. akashiwo*. Maximum H₂O₂ 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
We thank Dr. J.N.C. Whyte for providing the Canadian H. akashiwo strain. Funding support was provided by NSF (OCE-99-12089) and the NC General Assembly.

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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 (Quiliciam 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.
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, \( R_t = 9.23 \) min, 380 ng/g), 1157.5 (45-OHYTX, \( R_t = 8.60 \) min, 270 ng/g), 1171.5 (45-OHhomoYTX, \( R_t = 8.65 \) min, 15 ng/g), 1173.5 (carboxyYTX, \( R_t = 8.25 \) min, 265 ng/g), 1187.5 (carboxyhomoYTX, \( R_t = 8.30 \) min, 30 ng/g), 1047.5 (noroxoYTX, \( R_t = 8.00 \) 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 \([M-H]^-\) at \( m/z \) 1141.5 (YTX, \( R_t = 9.23 \) min, 380 ng/g), 1157.5 (45-OHYTX, \( R_t = 8.60 \) min, 270 ng/g), 1171.5 (45-OHhomoYTX, \( R_t = 8.65 \) min, 15 ng/g), 1173.5 (carboxyYTX, \( R_t = 8.25 \) min, 265 ng/g), 1187.5 (carboxyhomoYTX, \( R_t = 8.30 \) min, 30 ng/g), 1047.5 (noroxoYTX, \( R_t = 8.00 \) min, 205 ng/g). \([M-H]^-\) and \([M-80-H]^-\) ions were used as precursor ions in LC-MS at a collision energy (CE) of 35% and 45%, respectively. 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 SO3 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 \( CH_2 \equiv C \equiv O \) 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
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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References


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^n 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) (Brañña 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^n 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.
Results and Discussion

For azaspiracids, the most sensitive LC-MS3 method involves trapping and fragmentation of the parent ion, [M+H]+, and the product ion, [M+H-H2O]+, and trapping the [M+H-2H2O]+ 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 MS3 and MS stages being more dramatic than the decline in analyte signal (Biancotto et al., 1997). Through the implementation of this LC-MS3 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 MS3 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% (MS3) and 35% (MS) 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 R1 and R4 leaving the ion containing the remainder of the azaspiracid structure with R2 and R3. 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 R2 and R4 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-MS3. The selected ion combinations for the LC-MS3 of the new toxins are: AZA6

Table 1 The variable substituents, R1, R2, R3, R4, in azaspiracids.

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<tr>
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<th>R1</th>
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</tr>
<tr>
<td>AZA11</td>
<td>CH3</td>
<td>CH3</td>
<td>OH</td>
<td>H</td>
</tr>
</tbody>
</table>
(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 acetoniitrile: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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References
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 · m⁻² · 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 × 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 × 10⁶ cells · 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 × 10⁶ 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 1 mL completely lysed erythrocytes. After 24 hrs incubation, reaction vessels were centrifuged at 1500 × 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...
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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References


