DETECTION AND ANALYTICAL TECHNIQUES SESSIONS

Comparison of Four Assays for the Detection of Microcystins

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Abstract

Four different methods were compared for their ability to detect toxic levels of microcystins in a water sample. The protein phosphatase inhibition assay (PPIA) and a commercial ELISA assay were the most similar (97%), followed by ELISA and LCMS (89%), PDA and LCMS (86%), and PPIA and LCMS (85%). The amount of microcystin in the sample affected the similarity index; however, the ELISA and PPIA assays showed excellent agreement over a wide range (0.001–1000 μg L^{-1}) of microcystin concentrations. These results suggest that choice of analytical technique is important for studies involving the measurement of microcystins in natural waters.

Introduction

Cyanobacterial (blue-green algal) blooms occur worldwide and toxins produced by these algae are responsible for adverse health effects to humans and domestic animals. There are at least five different classes of cyanobacterial toxins. One of the more common and widely distributed are the microcystins, a large family of closely related hepatopeptides with both hepatotoxic and carcinogenic activity (Falconer *et al.,* 1999). A 1996 microcystin outbreak in Caruaru, Brazil, resulted in over 50 human fatalities (Jochimsen *et al.,* 1998; Azevedo *et al.,* 2002) and led to a WHO provisional guideline limit of 1 μg L^{-1} microcystin-LR equivalents for drinking water (Falconer *et al.,* 1999). While over 60 different microcystins have been identified (Sivonen and Jones, 1999), the primary structural variation occurs at just 2 amino acid positions. The resultant effect of this variation on toxicity is profound. This inherent variation in structure and toxicity makes this family of toxins difficult to quantitate. Structural-based assays such as ELISA can overestimate the total toxicity by detecting nontoxic analogues. Activity-based assays such as the enzyme inhibition assays can underestimate the amount of lower toxicity analogues. Full chemical analysis using HPLC is often limited by the availability of standards for the less common congeners. Thus a combination of structural-based assays, activity-based assays, and chemical analysis is often needed to fully understand the total toxin profile of a given sample. Here we report on the comparison between two different assays, ELISA and PPIA, and HPLC using two different detection techniques, for the measurement of microcystins in water samples.

Materials and Methods

Sample Collection and Extraction Water samples were collected from 305 sites in and around New York State during the summer and fall of 2001 by filtration onto Whatman 934AH glass fiber filters. These filters were extracted in 50% acidified methanol using ultrasound and analyzed using the protein phosphatase inhibition assay (PPIA) as described below. Of the initial 305 samples, a subset of 129 were chosen for further analysis using an antibody assay (ELISA), by liquid chromatography mass spectroscopy (LCMS) and by liquid chromatography with photodiode array detection (PDA) as described below. Samples were considered toxic if they contained greater than 0.2 μg L^{-1} microcystin-LR equivalents (PPIA and ELISA), if they contained molecular ions that matched the weights of published microcystin variants and had appropriate retention times (LCMS), or if they contained compounds with a UV max of 239 nm and had appropriate retention times (PDA). The 0.2 ug L^{-1} level was chosen since it serves as an early indicator of a possible toxic event.

PPIA Assays were run in 96-well plates containing 0.1 mU enzyme (recombinant protein phosphatase 1, catalytic subunit, Roche Applied Science), 1.05 mg *p*-nitrophenyl phosphate (Sigma) and 10 μL of sample or microcystin-LR (Sigma Biochemical) using the method of Carmichael and An (1999). The rate of phosphate hydrolysis was calculated from the change in absorbance at 405 nm over 1 hr and compared to the control (no added microcystin-LR) and standards containing between 6 and 40 μg L^{-1} microcystin-LR. Blanks (no enzyme, no toxin), unknowns, standards, and controls were all run in duplicate.

ELISA Samples (20 μL) were assayed using EnviroLogix's (Portland, ME) microcystin plate kit following the manufacturers instruction with the exception that microcystin-LR standards were prepared in 50% acidified methanol and 50% acidified methanol was used for the negative control. Unknowns, standards (containing 0.16, 0.5 and 1.6 μg microcystin-LR L^{-1}), blanks and controls were all run in duplicate.

HPLC Samples were separated using a Dupont Ace 4.6 × 250 mm 5μ C_{18} column and a two-step linear gradient of 30 to 70% acidified acetonitrile to acidified water at 0.8 mL/min. Detection was either mass selective using electrospray ionization (LCMS, Agilent 1100 series MSD) or by UV absorbance using a Water's model 996 photodiode array detector (PDA) between 210 and 300 nm. For LCMS, all ions between 900 and 1250 amu were combined to form the total ion chromatograph and potential microcystins identified on the basis of their molecular ions and retention times.

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Table 1 A comparison of the four different techniques based on their ability to distinguish the samples as toxin containing (>0.2 μg L^{-1}) or non-toxic as defined in the Materials and Methods. "X" indicates which methods are being compared with the method in the left column.

	ELISA	LCMS	PDA	n	Percent Similarity[a]
PPIA	X	X	X	76	78%
PPIA	X	X		98	87%
PPIA	X		X	77	82%
PPIA		X	X	85	72%
PPIA	X			99	97%
PPIA		X		118	85%
PPIA			X	96	80%
ELISA		X	X	76	80%
ELISA		X		98	89%
ELISA			X	77	84%
LCMS			X	85	86%

[a]%S = (number of samples classified the same) × 100/n

For PDA detection, potential microcystins were identified on the basis of having UV absorbance maximum at 239 nm and on their retention times.

Table 2 A comparison of the ELISA and PPIA methods for classifying samples into different toxin level categories.

Category (μg L^{-1})	PPIA	ELISA	Both	Similarity Index (%)[a]
Less than 0.2	42	45	42	97%
0.2–0.6	19	20	16	82%
0.6–1.0	8	3	1	23%
1.0–5.0	16	17	12	73%
5.0–10.0	1	1	1	100%
10.0–100	4	4	4	100%
Greater than 100	9	9	9	100%
Total	99	99	85	86%

[a]SI was calculated as $SI = (n_{ab}/2) \times (1/n_a + 1/n_b)$ where n_{ab} is the number of samples classified the same by both ELISA and PPIA, and n_a is the number classified by PPIA, and n_b the number classified by ELISA.

Results and Discussion

Because of time constraints, only 76 of the 129 samples were analyzed by each of the 4 methods. Different results were obtained depending on the assay or analysis used. Of the 76 samples analyzed using all four techniques, 56 samples (74%) were determined to contain toxin using the activity-based PPIA assay whereas only 53 samples (70%) were determined to contain toxin using the structure-based

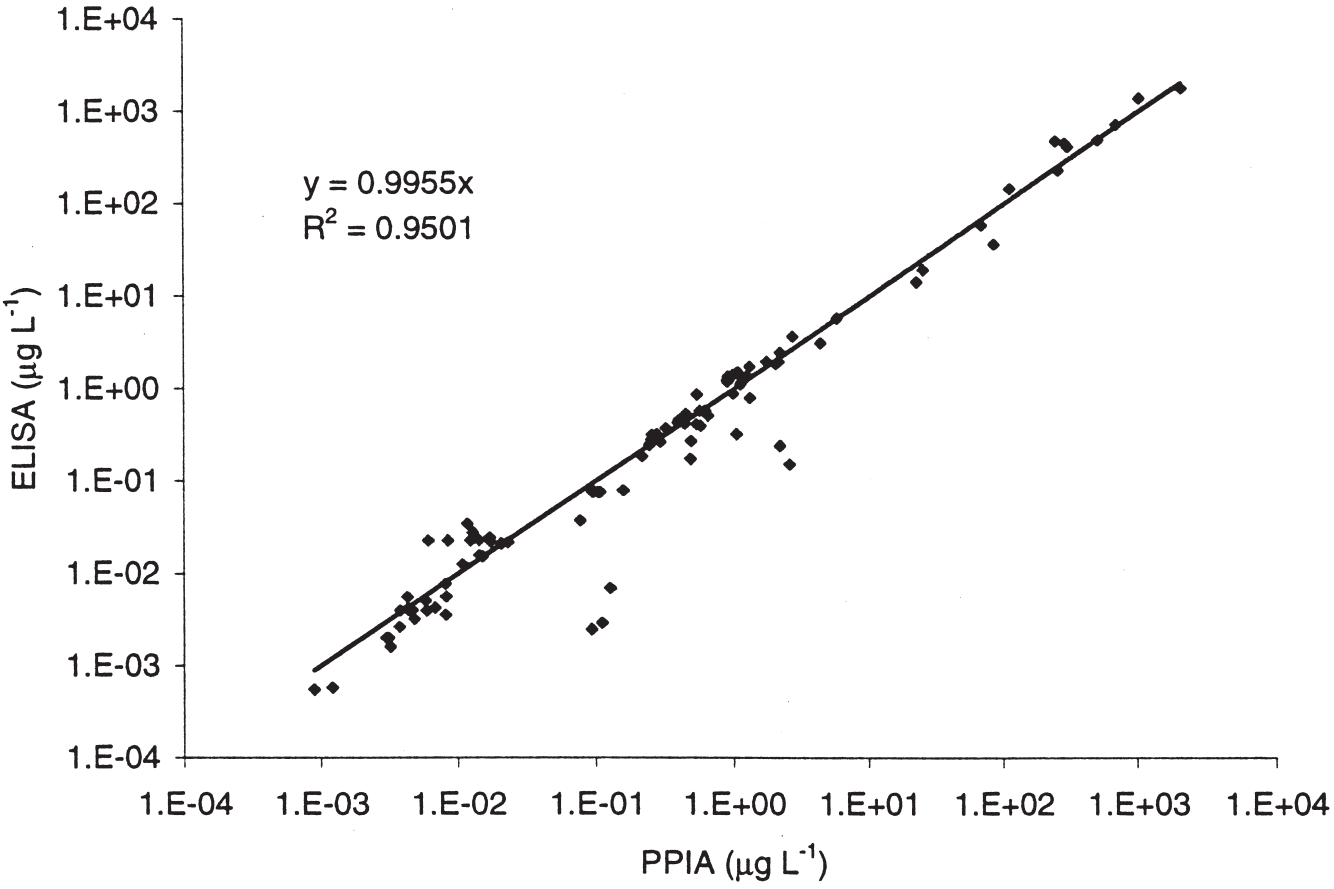

Figure 1 A comparison on the quantitative results obtained by the PPIA and ELISA assays (n = 99).

ELISA assay. Chemical analysis using HPLC gave even lower results. HPLC using PDA detection determined that 50 samples (66%) contained toxin while LCMS found microcystins in only 48 of 76 samples (63%). Similarity comparisons of all possible combinations of the four different techniques are shown in Table 1.

The highest similarity was obtained between the PPIA and ELISA assays, with 97% of the samples classified the same in regards to toxin level, as defined as >0.2 μg L^{-1} (Table 1) and 91% of the samples classified the same in regards to toxin level, as defined as >1.0 μg L^{-1} (data not shown). The lowest similarity (72%) was obtained when the PPIA assay was compared to the two HPLC techniques. The two HPLC techniques (LCMS and PDA) classified the samples the same 86% of the time. This increased variability can be explained in part by the higher limit of detection for the HPLC analysis (50–125 ng on-column for LCMS and 10–25 ng on-column for PDA) when compared to PPIA (60 pg $assay^{-1}$) and ELISA (~ 3 pg $assay^{-1}$). While the two biological-based assays were more sensitive and gave an integrated value for total microcystin content, the HPLC-based techniques were essential for identification of the individual toxin congeners.

To further compare the PPIA and ELISA methods, the samples were divided into different categories based on their measured toxin level of microcystin-LR equivalents, and similarity indices calculated for each category. These results are shown in Table 2. A graphical comparison of the quantitative results for the PPIA and ELISA assays is shown in Fig. 1. These assays were extremely robust when used with environmental samples and showed excellent agreement ($R^2 > 0.95$) when comparing microcystin-LR concentrations spanning across 6 orders of magnitude.

Acknowledgements

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Certified Reference Materials for Marine Algal Toxins

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Abstract

The Certified Reference Materials Program at the National Research Council's Institute for Marine Biosciences produces and certifies calibration standards and tissue reference materials for use in regulatory programs and scientific research. Certified reference materials (CRMs) are available for a variety of toxins of marine algal origin, and an expanded suite of marine phycotoxin standards and reference materials is now in preparation through a collaboration of several laboratories. The procedures involved in the production and certification of CRMs are presented, along with plans for future materials.

Introduction

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

An ambitious program was launched in 2001 to expand the suite of marine phycotoxin CRMs (Table 1). This work is being done in collaboration with a number of other organizations through funding from the Trade and Investment Liberalisation and Facilitation fund of the Asia-Pacific Economic Cooperation (APEC). Our collaborating organizations provide in-kind contributions such as plankton or shellfish tissues, purified toxin or technical expertise. Some organizations receive part of the final product in return for their contributions. Preparation of CRMs requires careful attention to toxin purity and stability. Accurate quantitation involves a cross-comparison of results from different procedures, including gravimetry, nuclear magnetic resonance (NMR) spectroscopy, and separation methods, such as liquid chromatography (LC) and capillary electrophoresis (CE) coupled with diverse detection systems such as ultra-violet (UVD), fluorescence (FLD), mass spectrometry (MS) and chemiluminescence (CLND). Periodic checks and eventual replenishment of CRMs are part of our program.

Results and Discussion

Production and Purification of Toxins Toxic strains of algae are grown in large quantities to produce the biomass needed to isolate the toxins for the calibration solutions. In

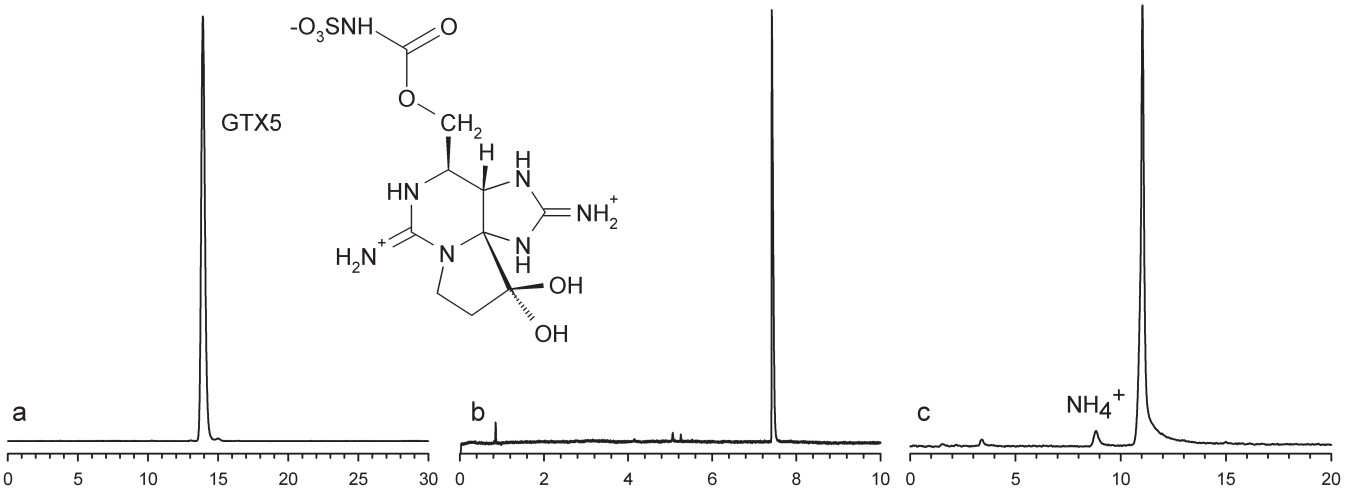

Figure 1 Purity analyses of GTX 5 using (**a**) LC-fluorescence with post column oxidation (Oshima, 1995); (**b**) capillary electrophoresis with diode-array detection (Thibault *et al.,* 1991); and (**c**) LC with chemiluminescence nitrogen detection (Quilliam *et al.,* manuscript in preparation).

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

Available at present:	
Amnesic Shellfish Poisoning (ASP) Toxins	
NRC CRM-DA-d	domoic acid calibration solution CRM
NRC CRM-ASP-Mus-b	mussel tissue CRM for ASP toxins
Diarrhetic Shellfish Poisoning (DSP) Toxins	
NRC CRM-OA-b	okadaic acid calibration solution CRM
NRC-CRM-DSP-Mus	mussel tissue CRM for DSP toxins
Paralytic Shellfish Poisoning (PSP) Toxins	
NRC CRM-STXdiAc	saxitoxin calibration solution CRM
NRC CRM-STX-d	saxitoxin dihydrochloride calibration solution CRM
NRC CRM-dcSTX	decarbamoylsaxitoxin calibration solution CRM
NRC CRM-NEO-b	neosaxitoxin calibration solution CRM
NRC CRM-GTX1&4-b	gonyautoxin-1 and -4 calibration solution CRM
NRC CRM-GTX2&3-b	gonyautoxin-2 and -3 calibration solution CRM
NRC CRM-GTX5	gonyautoxin-5 calibration solution CRM
Under development:	
Paralytic Shellfish Poisoning (PSP) Toxins	
NRC CRM-dcGTX2&3	decarbamoylgonyautoxin-2 and -3 calibration solution CRM
NRC CRM-dcGTX1&4	decarbamoylgonyautoxin-1 and -4 calibration solution CRM
NRC CRM-C1&2	N-sulfocarbamoylgonyautoxin-2 and -3 calibration solution CRM
NRC CRM-C3&4	N-sulfocarbamoylgonyautoxin-1 and -4 calibration solution CRM
NRC CRM-GTX6	gonyautoxin-6 calibration solution CRM
NRC CRM-dcNEO	decarbamoylneosaxitoxin calibration solution CRM
NRC CRM-PSP-Mus	mussel tissue CRM for PSP toxins
Other Toxins	
NRC RM-PTX2	pectenotoxin-2 calibration solution RM
NRC RM-PTX2sa	pectenotoxin-2 seco acid calibration solution RM
NRC RM-SPX1	spirolide (13-desmethyl-C) calibration solution RM
NRC RM-GYM	gymnodimine calibration solution RM
NRC RM-YTX	yessotoxin calibration solution RM
NRC RM-AZA1	azaspiracid calibration solution RM

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

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

Stability Studies Stability studies are an important part of CRM preparation. Valuable quantities of toxins are used in the production of a product, and more importantly, we need to be confident of the stability of the final product during storage (which may be up to 5 years) and

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

	CRM-STX-d	CRM-NEO-b	CRM-dcSTX
NMR	65.9 ± 2.7	63.8 ± 0.9	62.1 ± 0.8
LC-CLND	64.6 ± 1.2	65.7 ± 0.5	62.5 ± 0.5
Certified value	65.0 ± 3	65.0 ± 2	62.0 ± 2
6-month check (LC-CLND)	64.6 ± 1.2	65.0 ± 2.5	61.9 ± 3.1

shipment to ensure the certified value is reliable over time. Parameters investigated include type of solvent and sensitivity to oxygen, light, pH, and temperature.

Quantitation For many toxins, which may be isolated in only 10–100 mg quantities, it is difficult to determine the amounts of salts, counter-ions, or water of hydration associated with the compound. PSP toxins are particularly difficult because they cannot be crystallized easily, are hygroscopic and are in the form of salts. Quantitation of a CRM is done using at least two independent methods. Molar responsive systems are used to determine the concentration of the toxin in a stock solution, which is then diluted accurately. In Table 2, three PSP toxin calibration solution CRMs were quantitated using NMR (Walter *et al.,* manuscript in preparation) and LC-CLND (Quilliam *et al.,* manuscript in preparation). A routine check 6 months later revealed good agreement with previous measurements.

Production of Standards When a solution has been prepared using the purified toxin, it is then placed into ampoules using an automated ampouling machine. Each ampoule is pre-purged with argon, filled with a small amount of solution, then immediately flame-sealed to prevent any evaporation. After the ampouling process is completed, each individual ampoule is inspected for proper volume and labeled with a number that indicates order of filling. A representative number of ampoules from those produced are selected to test for homogeneity of the analyte concentration throughout the whole set. Each product is then stored under specific conditions that are continuously monitored. Careful planning is needed to ensure each product is replaced when stocks are depleted. As well, concentrations must be checked on a regular basis to ensure the certified values hold true.

Production of Tissue CRMs For any analytical method, it is important to test the whole method for accuracy with a tissue CRM. To produce such a CRM, large amounts of naturally incurred tissue homogenates are treated with antioxidant, de-aerated under vacuum, and dispensed into small bottles. These are sealed and taken through a steam retort process for sterilization. After cooling, the seals are inspected and cap closures installed. After receiving a unique number according to the order of filling, each bottle is individually heat-sealed in trilaminate pouches.

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

Conclusions

The National Research Council's CRM program is addressing the lack of accurate calibration standards for algal toxins by expanding the product line of calibration solution and shellfish tissue CRMs. Particular care is taken to ensure accuracy of concentration and to investigate the stability of the CRMs. CRMP is now the world's primary distributor of toxin CRMs.

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Detection of *Dinophysis* Cell Toxicity Using Parthenogenetic and Clonal *Artemia*

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Abstract

Bioassays with parthenogenetic *Artemia* obtained from the M. Embolon salt works (Thessaloniki) were used to detect *Dinophysis* cf. *acuminata* toxicity. Thirty-two-hour-old nauplii (instar III), and ~25-day-old adults from the salt works and from an isolated clone (ME1) were tested. Nauplii mortality was not related to the consumption of *Dinophysis* cells, whereas the mortality of 25-day-old individuals from the salt works was correlated. Mortality of 25-day-old individuals reached 50% when *Dinophysis* consumption was approximately 500 cells, whereas the mortality was above 80% when *Dinophysis* consumption exceeded 1,000 cells. The mortality of clonal *Artemia* was similarly related to *Dinophysis* consumption. Furthermore, the use of clonal *Artemia* revealed that the toxicity of *Dinophysis* cells increased during the decline phase of the bloom. Mortalities were compared and analyzed using non-parametric statistical tests. These preliminary results indicated that both field and clonal *Artemia* adults are suitable assay material for the short-term detection of *Dinophysis* toxicity. Further experimentation is needed to develop detailed and reliable protocols for future tests.

Introduction

Dinophysis species are associated with diarrhetic shellfish poisoning (DSP), a severe gastrointestinal illness in humans, resulting from the consumption of shellfish contaminated by diarrhetic shellfish toxins (Yasumoto *et al.,* 1980; Lee *et al.,* 1989). The first *Dinophysis* outbreak in Greek coastal waters was recorded in the winter of 2000 (Koukaras and Nikolaidis, 2001) and since then, *D.* cf. *acuminata* has been responsible for three consecutive DSP episodes in the area.

The development of fast and easy methods for the determination of microalgae toxicity is necessary; otherwise, the reliable prediction of toxic species may not be accurate. Such methods are short-term biotoxicity tests with zooplankton (Wells, 1999), where ingestion or close contact with whole toxic algal cells can generate lethal effects (Landsberg, 2002). The use of brine shrimp (*Artemia* spp.) has been used to investigate toxicity in different phytoplankton, such as *Alexandrium* species (Demaret *et al.,* 1995; Lush and Hallegraeff, 1996), *Prorocentrum lima* (Demaret *et al.,* 1995; Heredia-Tapia *et al.,* 2002), *Chrysochromulina* species (Simonsen and Moestrup, 1997) and cyanobacteria (Kiviranta *et al.,* 1991; Lawton *et al.,* 1994). There are no previous reports using *Artemia* to test toxicity of *Dinophysis* species. The aim of this study is to present the preliminary results on the detection of *D.* cf. *acuminata* toxicity using parthenogenetic and clonal *Artemia* in short-term tests.

Materials and Methods

Bioassays for the detection of *D.* cf. *acuminata* toxicity from three different blooms in Thermaikos Gulf during 2002 were carried out with parthenogenetic *Artemia* obtained from the M. Embolon salt works (Thessaloniki). Cells of *D.* cf. *acuminata* were collected using a 20 μm-mesh plankton net. The abundance of this species represented more than 98% of the genus *Dinophysis.* The plankton samples were sieved with a net mesh size of 100 μm to give a final abundance of *D.* cf. *acuminata* ranging 55–65% of the total phytoplankton biomass. The length of *D.* cf. *acuminata* cells ranged 34–52 μm and their width 20–38 μm. The duration of the bioassays was 24 hours. The toxic effects of *D.* cf. *acuminata* were tested on the following:

- *Artemia* nauplii of 32 hours (instar III). Four consumption levels were examined: 100, 300, 500 and 1,000 *Dinophysis* cells. Ten nauplii in 5 mL test water per Petri dish were tested (five replicates for each consumption level and for control).
- ~25-day-old females collected from the salt works. Six consumption levels were used: 100, 300, 500, 1,000, 3,000 and 5,000 *Dinophysis* cells. Twenty-five individuals (one individual per tube in 40 mL test water) were used (three replicates for each consumption level and for control).
- ~25-day-old females of the same age from an isolated clone (cultured in the laboratory) from the M. Embolon salt works population (ME_1) (Abatzopoulos *et al.,* 2003). The ME_1 clone was used to exclude possible different genotypic responses to the toxicity (several genotypes/clones exist in the M. Embolon *Artemia* population). Three consumption levels were used: 100, 200 and 1,000 *Dinophysis* cells. Twenty-five individuals (one individual per tube in 40 mL test water) were used (three replicates for each consumption level and for control).

Individuals used as controls were fed with 75% formulated yeast feed (LPZ, INVE International) and 25% *Dunaliella tertiolecta.* Salinity was 35 psu for the experiments. The consumption was calculated from the difference between the initial and the final (after 24h) *Dinophysis* abundance in the water of the test tubes or petri dishes, as counted by scanning the whole bottom of the chamber using an inverted microscope by the Utermöhl (1958) method. All the remaining volume of water was counted for *Dinophysis* cells, and dead cells were not taken into account. Since ANOVA assumptions

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Table 1 Mortalities of *Artemia* mature females (a) and clonal *Artemia* (b) related to the consumption level of *D.* cf. *acuminata* cells. Mortalities that share the same letter are not statistically different (Kruskal-Wallis and Mann-Whitney tests).

a		b	
Consumption (cells) (Mean±SD)	Mortality (%) (Mean±SD)	Consumption (cells) (Mean±SD)	Mortality (%) (Mean±SD)
100 (±15)	30.57[a] (±2.89)	100 (± 18)	27.62[a] (± 14.56)
500 (±22)	58.54[b] (±12.87)	200 (± 27)	44.76[a] (± 13.27)
1,000 (±27)	85.77[c] (±11.49)	1,000 (± 48)	60.95[b] (± 5.88)
5,000 (±68)	100.00[c]		
3,000 (±47)	87.91[c] (±13.69)		

had not been met (even after transformation of the initial values—*i.e.,* arcsine, log or square root), mortalities were compared and analyzed using non-parametric statistical tests (Kruskal-Wallis and Mann-Whitney tests, $P < 0.05$).

Results and Discussion

Nauplii The results showed no statistical difference ($P > 0.05$) in mortalities among the nauplii used as controls and the nauplii used for experiments during the three *Dinophysis* blooms. In some cases, the mortality of control individuals was greater than for those used for the test experiments. Additionally, nauplii mortality was variable and not related to *Dinophysis* consumption. This variability may be attributed to the inability of nauplii to feed on *Dinophysis* cells because of their size limitation. Nauplii seemed to be unsuitable material for use in short-term biotoxicity tests for the detection of *Dinophysis* toxicity.

Artemia Adult Females from Field Mortality of *Artemia* adults from the salt works was correlated with *Dinophysis* cells consumption. Differences among the three consumption (per individual) levels (100, 500 and >1,000 cells) were found to be statistically significant ($P < 0.05$, Table 1a). Mortality reached 50% when consumption was approximately 500 *Dinophysis* cells, whereas the mortality was above 80% when *Dinophysis* consumption exceeded 1,000 cells.

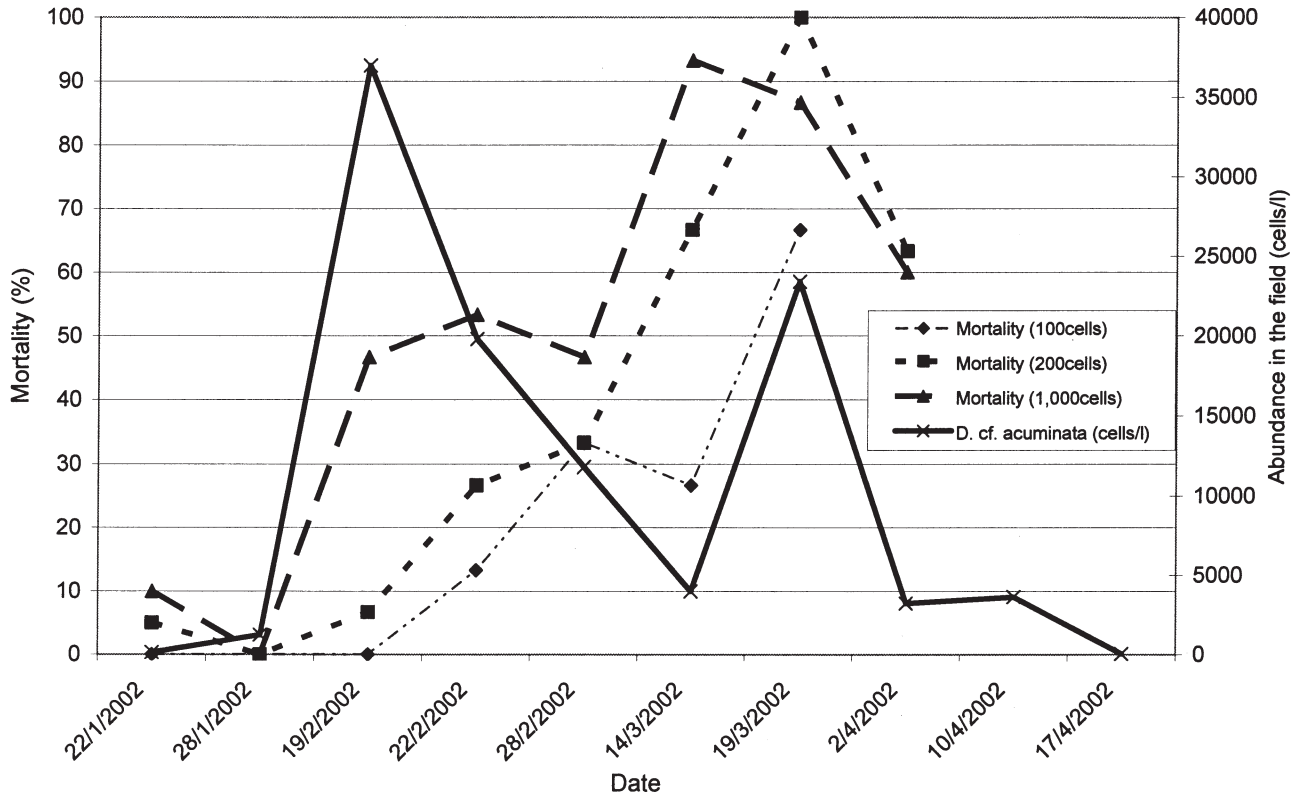

Figure 1 Mortality of clonal *Artemia* at three different consumption levels and the development of the *Dinophysis* outbreak in Thermaikos Gulf (2002).

Clonal* Artemia *Individuals Using clonal *Artemia* as a test organism, mortality was correlated with *Dinophysis* cells consumption (Table 1b), giving similar results to the previous experiment. Even though there was no statistical difference in mortality at 100 or 200 *Dinophysis* cells consumption, there was a difference between these two levels and 1,000 *Dinophysis* cells consumption (Table 1b).

Mortalities of clonal *Artemia* individuals at each of the consumption levels were variable during the *Dinophysis* bloom in 2002 (Fig. 1). The identical genotype of *Artemia* individuals allowed us to assume that mortality was affected by differences in *Dinophysis* toxicity. Mortality values were higher in the last stages of the *Dinophysis* bloom at consumption levels of 100, 200 and 1,000 *Dinophysis* cells, probably due to an increase in toxin concentration within the *Dinophysis* cells. This variation in toxicity of *D. acuminata* (different toxins and variable production of toxins) during bloom development has also been observed by other researchers (Andersen *et al.,* 1996; Sato *et al.,* 1996).

The aforementioned preliminary results demonstrate a significant lethal effect on both field and clonal *Artemia* adults due to the ingestion of *Dinophysis* cells. This indicates that adult *Artemia* is suitable material for short-term biotoxicity tests for the detection of *Dinophysis* toxicity, although the variability in production of toxins by *Dinophysis* cells may induce variability in the bioassay. Therefore, additional experiments are required in order to develop detailed and reliable protocols for future testing practices.

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Brevetoxin Metabolism and Elimination in the Eastern Oyster: Implications for Methods Development

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Abstract

Rapid metabolism of brevetoxins (PbTx) was demonstrated in the Eastern oyster (*Crassostrea virginica*) through controlled laboratory exposures to pure PbTx and to *Karenia brevis* culture. Parent PbTx and their metabolites in oyster were determined by LC/MS and *in vitro* assay. Waterborne PbTx-3 was rapidly accumulated, but not metabolized, in the oyster. In oysters exposed to pure PbTx-2, metabolites included the reduction product PbTx-3 and a cysteine adduct, consistent with the metabolite profile in *K. brevis*-exposed oysters (field and laboratory). PbTx-3 in oysters, whether derived through PbTx-3 exposure or PbTx-2 metabolism, was largely eliminated within two weeks after dosing. The cysteine adduct was measurable for at least 8 weeks after dosing and is a reliable indicator (marker) of PbTx exposure via *K. brevis* blooms.

Introduction

The marine dinoflagellate *Karenia brevis* produces brevetoxins (PbTx) that are accumulated in shellfish during coastal blooms of this organism. Consumption of PbTx-contaminated shellfish causes human illness, known as neurotoxic shellfish poisoning (NSP). Prevention of NSP relies upon environmental monitoring for the causative organism and closure of affected shellfish harvesting areas. Re-opening of shellfish resources after a *K. brevis* bloom is contingent upon results of the mouse bioassay of shellfish extracts. Efforts to replace the mouse bioassay with *in vitro* assay or instrumental methods are complicated by the

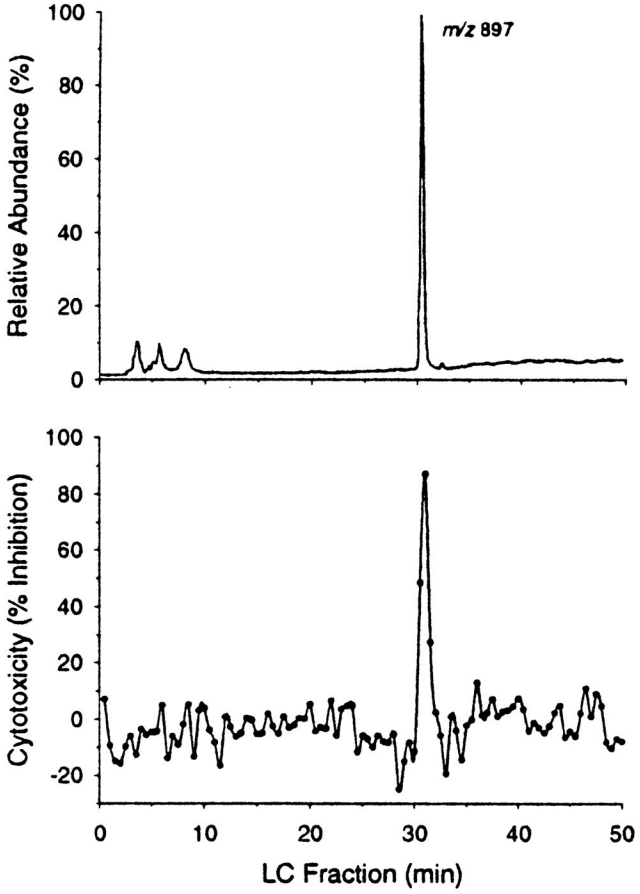

Figure 1 Chromatograms from oysters exposed to PbTx-3. Top: LC/MS composite of selected ions monitored. Bottom: Cytotoxicity of LC fractions.

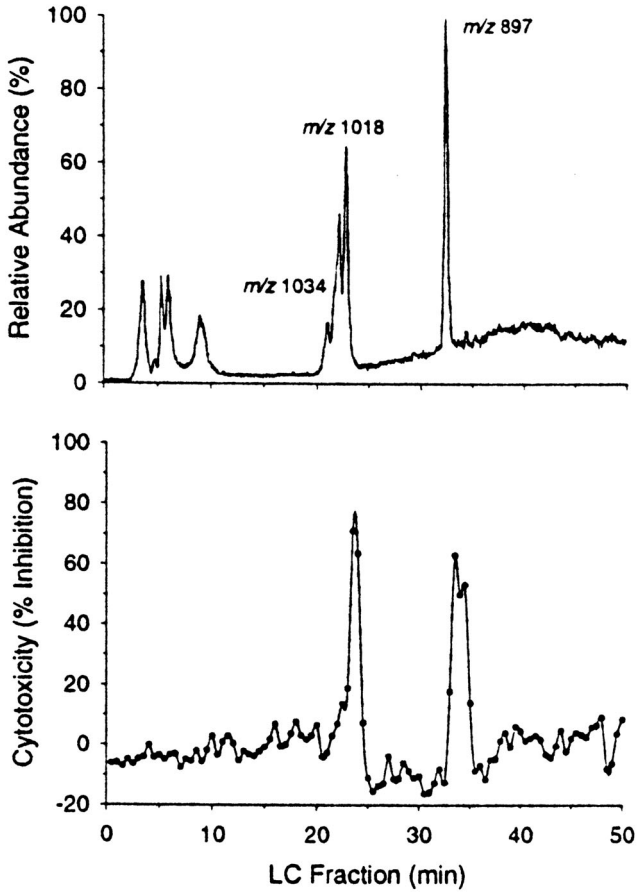

Figure 2 Chromatograms from oysters exposed to PbTx-2. Top: LC/MS composite of selected ions monitored. Bottom: Cytotoxicity of LC fractions.

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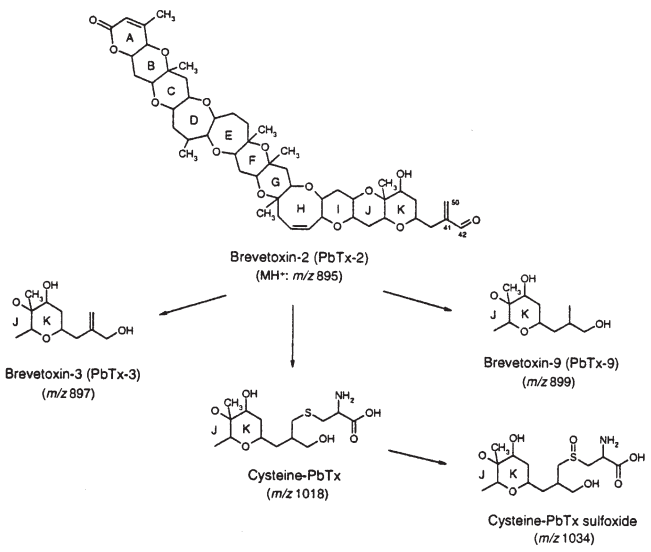

Figure 3 Metabolism of PbTx-2 in the Eastern oyster.

extensive metabolism of PbTx in shellfish. We examined the metabolism and elimination of PbTx in the Eastern oyster (*Crassostrea virginica*) under controlled laboratory conditions and describe its implications for methods development and regulatory monitoring.

Materials and Methods

Oysters were individually exposed to pure PbTx-2 or -3, ^{3}H-labeled PbTx-3, or *K. brevis* cultures and sampled at various intervals over an 8-week elimination period. Oyster tissue homogenates (1 g portions) were extracted with acetone (2 × 2 mL), extracts were evaporated under nitrogen, and residues were re-solubilized in 80% methanol. Methanolic solutions were defatted with hexanes, evaporated, and re-solubilized in 25% methanol. Defatted extracts were cleaned-up on C_{18} solid-phase extraction (SPE) columns by washing with 25% methanol and eluting with 100% methanol. PbTx residues were determined by LC/MS using a Hewlett-Packard Model 1100 LC system and Thermo Finnigan Navigator MS with electrospray ionization (ESI^+). LC separations were performed on a YMC J'sphere ODS-L80 S-4, 2.0 × 250 mm column under gradient conditions. Data were acquired by selected ion monitoring of the *K. brevis* parent PbTx (MH^+) of *m/z* 867 (PbTx-1), 869 (PbTx-7), 895 (PbTx-2), 897 (PbTx-3), 899 (PbTx-9), and for the oyster metabolites of *m/z* 1018 and 1034 (Dickey *et al.,* 1999). Minor metabolites of *m/z* 990 and 1006 were also identified. All data were expressed in PbTx-3 equivalents/g. Metabolite structures were identified by accurate mass measurements and LC/MS/MS using a Micromass Q-Tof II hybrid quadrupole/orthogonal time-of-flight mass spectrometer. Sodium channel binding activity in whole extracts and LC fractions were determined by sodium channel-specific (mouse neuroblastoma) cytotoxicity assay (Manger *et al.,* 1993; Dickey *et al.,* 1999) or sodium channel receptor (rat brain synapsomal) binding assay (Trainer and Poli, 2000). Radiometric analyses of extracts and LC fractions were determined by Packard Tricarb liquid scintillation analyzer.

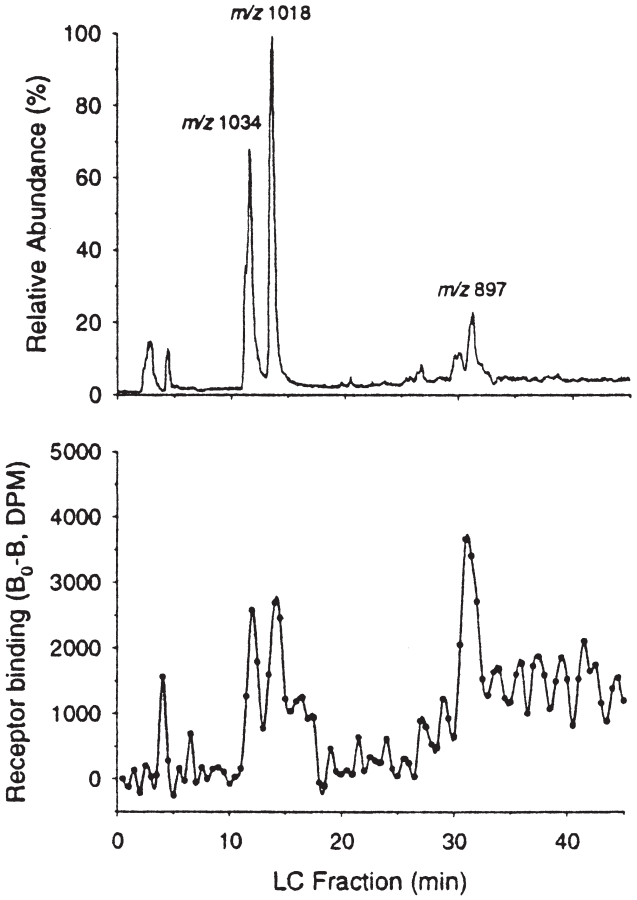

Figure 4 Responsiveness of receptor binding assay to brevetoxin metabolites. Top: LC/MS composite of selected ions. Bottom: Receptor binding assay.

Results and Discussion

Rapid metabolism of PbTx in Eastern oyster, first observed after natural exposure to *K. brevis* blooms (Dickey *et al.,* 1999), was confirmed in controlled laboratory exposures to *K. brevis* culture. As in the field studies, prominent metabolites of *m/z* 1018 and 1034 (MH^+) were recognized. These molecules were structurally identified by accurate mass measurements and MS/MS analysis as a cysteine-PbTx conjugate and its sulfoxide (Plakas *et al.,* 2002). By exposure of oysters to pure toxins, we further elucidated the pathway for formation of these, and other, metabolites.

In oysters exposed to pure PbTx-3, we found no metabolites by LC/MS or *in vitro* assay (Fig. 1); furthermore, no metabolites were found in oysters exposed to ^{3}H-PbTx-3, by radiometric analysis. In oysters exposed to pure PbTx-2, metabolites included the reduction product PbTx-3 (MH^+: *m/z* 897), and the cysteine adducts (*m/z* 1018, 1034), consistent with the metabolite profile in *K. brevis*-exposed oysters (Fig. 2). Little or no parent PbTx-2 was found in

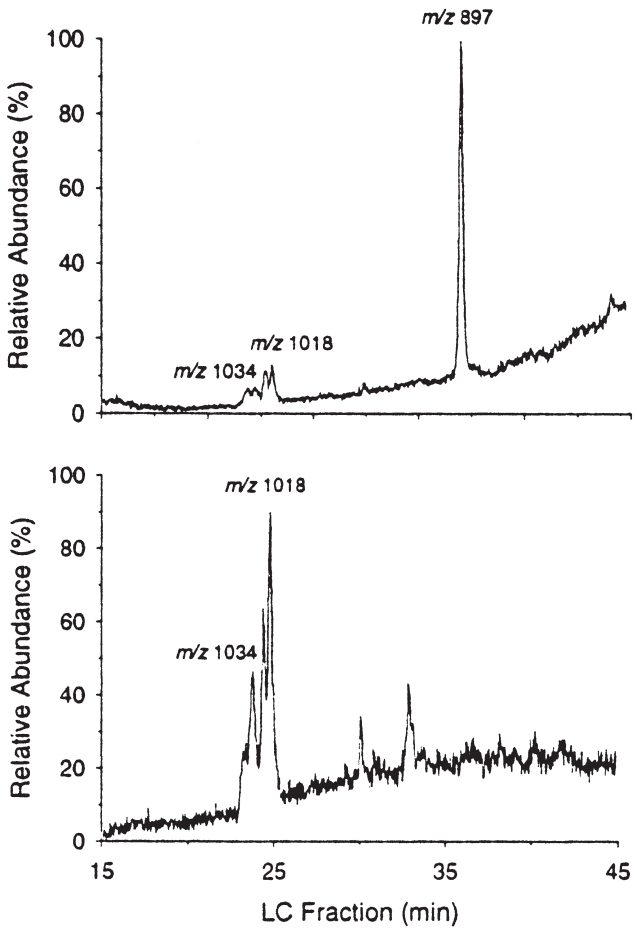

Figure 5 Persistence of PbTx metabolites (*m/z* 1018,1034) in oysters exposed to PbTx-2. Top: Immediately after exposure. Bottom: After 8 weeks of depuration.

PbTx-2- or *K. brevis*-exposed oysters.

Our results with pure toxins indicate PbTx-2 metabolic pathways in oysters as shown in Fig. 3.

Cysteine-PbTx sulfoxide in the Eastern oyster is the same compound as the metabolite BTX-B2 isolated from greenshell mussels (*Perna canaliculus*) by Murata *et al.* (1998). They found BTX-B2 lethal to mice when dosed i.p. at 306 µg/kg, with sodium channel-activating potency (cytotoxicity assay) one-third that of PbTx-3. We found that cysteine-PbTx sulfoxide is also formed from cysteine-PbTx as an artifact during sample preparation and storage. In addition to their cytotoxic activity, the cysteine adducts also appear responsive in the receptor binding assay (Fig. 4).

PbTx-3 in oysters, whether derived through PbTx-3 exposure or through PbTx-2 metabolism, was largely eliminated within 2 weeks after dosing (*i.e.,* 25 µg/L, 72-hr exposure), while the cysteine adduct was measurable for at least 8 weeks (Fig. 5).

We found additional cytotoxic compounds of apparent metabolic origin in *K. brevis*-exposed oysters. Two of these were associated with peaks in the mass chromatograms of *m/z* 990 and 1006, with relatively low area. These may be cysteine adducts of PbTx-1. PbTx-9 is also present in oysters as a parent toxin from *K. brevis* and as a metabolite of PbTx-2. Other less polar metabolites remain unidentified.

In oyster homogenates spiked with PbTx-2 and immediately extracted, recovery of intact PbTx-2 was very low, and none of the above metabolites were recovered. This may be due to covalent or non-covalent interactions with matrix constituents. The extent to which this occurs in live oysters is unknown.

Cysteine-PbTx and its sulfoxide, as measured by LC/MS, are reliable indicators (markers) of oyster exposure to PbTx. In field-exposed oysters, the cysteine adduct content (based on area response by LC/MS) also correlates well with toxicity by mouse bioassay (Pierce *et al.,* 2002). Further study may prove the cysteine adduct to be a useful marker for composite toxicity of PbTx-contaminated shellfish. However, the application of LC/MS for evaluating toxic shellfish is hindered by the lack of authentic standards for PbTx metabolites.

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Detection of *Karenia brevis* by a Microtiter Plate Assay

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Abstract

A microtiter plate assay was adapted to detect toxic dinoflagellates in coastal waters. The assay was designed to detect multiple organisms simultaneously. Detection of *Karenia brevis* and *Amphidinium carterae* was achieved using a single set of hybridization conditions. Extracted DNA was amplified using universal biotin-labeled primers, and amplicons were hybridized to species-specific probes immobilized on the wells of a microtiter plate. This method yielded quick, colorimetric results and conserved DNA by using only one amplification step. Species-specific PCR and DNA cloning and sequencing verified detection of *K. brevis* in environmental samples. This assay is a promising method for simultaneous screening of multiple dinoflagellate species.

Introduction

Coastal water quality is critical to human and ecosystem health as well as to fishing, aquaculture, and tourism industries. Harmful algal blooms (HABs) threaten many coastal states and can impact large geographic areas. Blooms are associated with fish kills, neurotoxic shellfish poisoning, and eye and respiratory irritation in marine animals and humans (Blum *et al.,* 2000). Fisheries and beaches impacted by HABs are closed to protect human health. However, management decisions are based on time- and labor-intensive assays that often require considerable taxonomic expertise. In addition, sampling is often restricted spatially and temporally, giving a limited view of the extent and dynamics of a bloom. Improved assays are needed to better protect health and economic interests and to aid ecological studies.

Species identification is vital to coastal zone managers working to protect humans from toxins in shellfish and/or in recreational waters. Development of nucleic acid-based methods can provide rapid detection of problem organisms. Such methods could allow more widespread screening of waters, thus protecting health while avoiding blanket closures of coastal waters (Tyrrell *et al.,* 1997). In New Zealand, incorporation of RNA probes into a marine biotoxin monitoring led to a decrease in shellfish bed closures because the probes distinguished between toxic and non-toxic strains of *Pseudo-nitzschia* (Rhodes *et al.,* 1998). Ribosomal RNA and DNA (rRNA, rDNA) sequences are widely used to distinguish organisms at the species level. The D1/D2 region of the large subunit (LSU, 28S) has been particularly useful for distinguishing species and strains among dinoflagellates (Scholin *et al.,* 1994). This report describes preliminary results for a DNA hybridization assay in microtiter plate format. The assay is designed to provide rapid, colorimetric detection of multiple species in coastal waters.

Materials and Methods

Samples were obtained from cultures of *K. brevis, A. carterae,* and from the Rookery Bay National Estuarine Research Reserve (NERR) during a red tide event in February and March of 2002. Cultures were grown under the following conditions: L1 medium (Guillard and Hargraves, 1993) with no added silicate, 19°C, 14:10 hr light:dark at 1,220–2,030 lux, and transferred to new medium between 14–22 days. Rookery Bay NERR samples were collected from the laboratory dock on February 23 and from Big Marco Pass, Caxambas Pass, Fakahatchee Bay, Hendersens Creek, and the 951 ramp on February 27. On March 13, 2002, samples were collected from Big Marco Pass, Caxambas Pass, Goodland, and the 951 ramp. Water was collected in whirlpacks and shipped overnight in a cooler containing ice packs. Samples were passed through 64-µm mesh to remove large particles and organisms. Samples were gently vacuum filtered (~1 drop per second) onto sterile, 5-µm, 47-mm mixed cellulose ester filters using sterilized filter holders and forceps. Filters were stored at –20°C until DNA extraction.

DNA was extracted from filters using spin kits (BIO 101) following manufacturer's directions. Amplification of the LSU rDNA D1/D2 domain using biotinylated PCR primers D1R (forward) (Scholin *et al.,* 1994) and R635 (reverse) (Fell *et al.,* 2000; Kiesling *et al.,* 2002a) yielded amplicons approximately 700 bp in length. D1R (5'-ACCCGC TGAATTTAAGCATA-3') is a general dinoflagellate primer and R-635 (5'-GGTCCGTGTTTTCAAGACGG-3') is a universal eukaryote primer. PCR reactions typically consisted of 5 µL 10× buffer (1.5 mM $MgCl_2$, final), 0.2 mM dNTPs, 50 pmol of each universal biotinylated primer, 2.5 µL genomic DNA, 1 unit of polymerase (Finnzyme), and autoclaved Milli-Q water to bring the reaction volume to 50 µL. Amplification was carried out using a thermal cycler (MJ Research PTC-100 Hotbonnet) with the following program: 94°C for 1 min, 29 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 1 min), 70°C for 8 min, 4°C hold.

Species-specific probes were designed within the D1R/R-635 amplicons. Probes and primers were designed based on the existing GenBank sequences for dinoflagellates, including those for *K. brevis* (accession number U92248) and *A. carterae* (accession number AF260380). Dinoflagellate sequences were aligned using Megalign (DNAstar) software.

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Probe and primer sequences were checked for hairpin, duplex and dimer formation and compatible melting temperatures using MacVector and Oligo 4.0 software. Sequences were blasted against the GenBank database to test the specificity of primers and probes for their target. The sequence for *K. brevis* was termed Karenia (5'-CTAGGGACATGGTAATTTGCTTC-3') and the sequence for *A. carterae* was called Amp (5'-TTT GCCCAAGGAGGATTACG-3'). Direct amplification using Karenia or Amp and R-635 (reverse) yielded amplicons of 206 bp or 338 bp, respectively.

Species-specific probes were T-tailed and immobilized to 96-well microtiter plates (polystyrene, amine surface; Costar). The following reagents were combined to make T-tailed probe for 25 plates: 50 µL of 5X terminal transferase (Tdt) buffer, 100 µL sterile dH_2O, 10 µL 100 mM dTTP (Roche Molecular), 25 µL of 100 pM probe, 20 µL terminal transferase (25 U/µL; Roche Molecular), 5 µL of 10 mg/mL pyrophosphatase (Sigma). The mixture was incubated in a 37°C water bath for 4 hours. Afterward, 42.5 µL EDTA (0.5 M, pH 8.0) and 2.25 mL 1× TE (pH 7.4) were added. For each plate, 100 µL of T-tailed probe was mixed with 10 mL TE (pH 7.4), and the mixture was added (100 µL per well) to yield 1 pmol T-tailed probe per well. Plates were dried in an oven (37°–50°C) overnight or until dry. Blocking buffer (200 µL, 5× Denhardt's solution and 1× phosphate buffered saline) was added to each well. Plates were incubated at room temperature for 1–4 hr. Buffer was dumped, and plates were dried upside down at room temp until completely dry (~1–2 hr). Plates were sealed and stored in a plastic or foil bags containing desiccant at 4°C until use.

DNA hybridization between biotin-labeled amplicons and species-specific probes occurred at 55°C. A positive reaction produced a yellow color by an enzyme catalyzed reaction, as described in Kiesling *et al.,* (2002 a,b). Color was detected visually and spectrophotometrically (absorbance at 450 nm). Negative controls used sterile dH_2O in place of DNA.

Direct amplification of genomic DNA with species-specific primers was used to verify microtiter plate assay results. The amplification protocol was the same as that given above, except primers were not biotinylated. Cloning and sequencing of DNA extracted from environmental samples provided additional verification of results. DNA extracted from Rookery Bay dock water and fromMarch samples from Big Marco Pass, Goodland, and from the 951 ramp was amplified and cloned using the PCR2.1 vector (Invitrogen) according to manufacturer's instructions. Amplification in these cases used Taq polymerase to create T-A overhangs. DNA was amplified with D1R/R635 (reverse) or D1R/Karenia (reverse) primer sets. Reactions consisted of 5 µL of 10× buffer, 2 mM $MgCl_2$, 0.1 mM dNTPs, 25 pmol of each primer, 2 µL DNA, 2.5 units of Taq (Promega), and autoclaved Milli-Q water to bring the reaction volume to 50 µL. At least twenty clones were selected from each sample for sequencing. Sequencing primers were for the M13 region of the cloning vector. Sequencing was performed on an automated sequencer (Li-Cor Long Readir 4200) using e-Seq 1.2.1 and AlignIRv1.2 software. Sequences were obtained using the standard Li-Cor protocol with IRD800 and IRD700 conjugate primers and the Thermo Sequenase Primer Cycle Sequencing Kit with 7-deaza dGTP (Amersham).

Table 1 Comparison of microplate assay results to other means of detection.

Sample	Microplate Assay	FMRI Counts[a]	Species-Specific PCR
K. brevis culture	+	N/A	+
Negative control	–	N/A	–
February 2002			
Rookery Bay dock a	+	N/A	+
Rookery Bay dock b	+		+
Rookery Bay dock c	+		+
Big Marco Pass a	+	low	+
Big Marco Pass b	+		+
Caxambas Pass	+	low	+
Fakahatchee Bay	–	negative	–
Hendersens Creek	+	very low (a)	+
951 ramp	+	low	+
March 2002			
Big Marco Pass	+	low	+
Caxambas Pass a	–	negative	–
Caxambas Pass b	–	negative	–
Goodland	–	negative	½+(b)
951 ramp	–	negative	–

[a]FMRI samples collected Feb. 27, 2002, ours Feb. 29. In March, both collected March 13, 2002. low = >10,000 to <100,000 cells/L; very low (a) = >1,000 to <5,000 cells/L. N/A = data not available.
[b]Very faint band on an agarose gel.

Results and Discussion

A colorimetric, microplate assay (Kiesling *et al.,* 2002 a,b) was adapted to detect toxic dinoflagellates in coastal waters. The assay was designed to simultaneously detect multiple organisms. This was achieved, in part, by using universal primers to amplify the extracted DNA, allowing identification of multiple species while requiring minimal sample DNA. The dinoflagellates *A. carterae* and *K. brevis* were used as model organisms in this study. *A. carterae* was chosen because it is an unarmored, toxic dinoflagellate (Jeong *et al.,* 2001) that is easy to grow in the laboratory, thus yielding an ample supply of DNA for experiments. *K. brevis* was chosen because it is an unarmored, toxic dinoflagellate that causes recurrent red tides in Florida, including the Rookery Bay NERR study site.

Microplate assay results were consistent with microscopic detection of *K. brevis* determined by the Florida Marine Research Institute (FMRI). Assay sensitivity was sufficient to detect *K. brevis* in samples containing "low" amounts of the organism. Sensitivity appeared sufficient to

detect "very low" amounts of *K. brevis,* although in this case FMRI counts occurred two days earlier than our sample collection (Table 1). Species-specific PCR was consistent with microplate assay, showing slightly increased sensitivity in only one case (Table 1).

The microplate assay detected *K. brevis* from pure cultures, enrichment cultures, and mixtures of DNA. In addition, the assay successfully detected *K. brevis* in water samples collected from the Rookery Bay NERR during a red tide event (Table 1).

Sequencing of cloned rDNA from the water samples yielded sequences identical to *K. brevis* (GenBank entry U92248). In addition, sequences with 1–4 bp differences from U92248 were frequently observed, which may represent intraspecific variability. Overall, cloning and sequencing of environmental DNA was consistent with microplate assay results, but appeared more sensitive than other methods of detection because *K. brevis* clones were identified in March samples collected from Goodland and the 951 ramp locations.

The microplate assay successfully detected *A. carterae* from pure cultures and mixtures of DNA. *A. carterae* and *K. brevis* were detected simultaneously, using a single set of DNA hybridization conditions. All Rookery Bay NERR samples were negative for *A. carterae* by microplate assay and species-specific PCR methods, indicating that this organism was not present in those samples. Finally, results indicate that the microtiter plate assay can be further developed to provide simultaneous detection of a variety of dinoflagellate species and strains.

Acknowledgements

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An Alternative Method for Domoic Acid Determination in Seawater Particulates: A Receptor Binding Assay Using Glutamate Dehydrogenase

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Abstract

The receptor binding assay (RBA) is a high-throughput method for detection of domoic acid in extracts of seawater and shellfish. The RBA quantifies domoic acid (DA) activity by the competitive displacement of radiolabeled kainic acid from a cloned glutamate receptor by DA in a sample. Prior to the analysis, an enzyme digestion step is required to remove glutamate from each sample due to the interfering binding of glutamate to the cloned receptor. In the past, glutamate decarboxylase (GLDC) has been used to digest endogenous glutamate. However, suppliers have recently discontinued production of GLDC due to difficulty in expressing the enzyme. Here we propose an alternative method for digestion of glutamate in seawater particulates using glutamate dehydrogenase (GLDH). The reaction is forced in the forward direction (digestion of glutamate) using hydrazine and a basic pH. Remaining hydrazine is removed by inclusion of 1% bovine serum albumin in the RBA. The maximum glutamate concentration that can be successfully digested using GLDH is 700 µM, which is in excess of the maximum glutamate concentration measured in seawater particulates. DA concentrations determined via the RBA using GLDH were tightly correlated with DA levels quantified by high performance liquid chromatography ($r^2 = 0.94$), suggesting that using GLDH for digestion of endogenous glutamate is an effective replacement for the standard RBA that uses GLDC.

Introduction

The receptor binding assay (RBA) is a commonly used method for detection of domoic acid (DA) in seawater and shellfish. Due to its sensitivity and ability to adapt to high throughput format, the RBA is often chosen for research purposes over high performance liquid chromatography (HPLC) and the mouse bioassay. The RBA measures DA "activity" in a sample through the displacement of a radiolabeled kainic acid derivative from a glutamate receptor. Glutamate receptors (GluR6) used in the assay are cloned into a viral cell line, expressed in insect cell lines, and then purified (Taverna *et al.,* 1994). Prior to the RBA, a glutamate digestion step is required to remove glutamate from the sample to prevent interference in the DA-kainic acid competition. Previously, glutamate decarboxylase (GLDC) was the enzyme used to remove glutamate (Van Dolah *et al.,* 1997). However, enzyme suppliers have recently discontinued supplying GLDC. Here we describe the glutamate dehydrogenase (GLDH) digestion assay as an alternative method of glutamate digestion of seawater particulate samples prior to the domoic acid RBA.

Materials and Methods

Glutamate Determination To determine endogenous glutamate levels in the field, free glutamate was quantified in 9 seawater particulate samples. To account for varying glutamate levels that may be associated with samples from various oceanic environs, particulate seawater samples were collected from in shore to 28 km off the coast of Washington State, and at depths ranging 0–20 meters. One liter samples were collected in Niskin bottles, filtered, and processed as described in Adams *et al.* (2000). Glutamate concentrations were determined by the ninhydrin reaction with appropriate solid phase extracts (Spackman *et al.,* 1958).

Glutamate Digestion Step GLDH removes hydrogen from glutamate forming α-ketoglutarate and is accompanied by the reduction of NAD^+ to NADH. The enzymatic digestion of glutamate by GLDH is shown by the following reaction:

$$\text{L-glutamate} + NAD^+ + H_2O \overset{GLDH}{\rightleftharpoons} \alpha\text{-ketoglutarate} + NH_4 + NADH$$

The equilibrium of the GLDH reaction favors glutamate formation. To overcome the unfavorable kinetics, a high concentration of NAD^+, low proton concentration (pH 9), and hydrazine hydrate (a trapping agent for α-ketoglutarate hydrazine hydrate) are used in the assay. ADP is included to activate GLDH and to decrease its reactivity with other amino acids (Bergmeyer *et al.,* 1983). To reduce activity of hydrazine with the glutamate receptor, the buffer used in the RBA is prepared with BSA.

For removal of glutamate prior to DA determination by the RBA, the samples were treated with GLDH. Briefly, seawater particulate sample (178 µL) was added to 20 µL of 30 mM ß-nicotinamide adenine dinucleotide, 2 µL of 0.1 M adenosine 5-diphosphate, 4 µL GLDH (Sigma, 2626) and 200 µL of buffer containing 0.1 M Trizma base, 0.002 M EDTA and 3.2% hydrazine at pH 9.0. Thereafter, the samples were vortexed and incubated at room temperature for one hour to ensure complete digestion of glutamate.

Maximum Glutamate Concentration Digested by RBA The RBA was performed as in Adams *et al.* (2000) using the same buffers and reagents except where noted. The maximum glutamate concentration that could be successfully digested using GLDH was determined by measuring the displacement of [^{3}H] kainic acid (PerkinElmer Life Sciences, NET875) with increasing concentrations of enzyme

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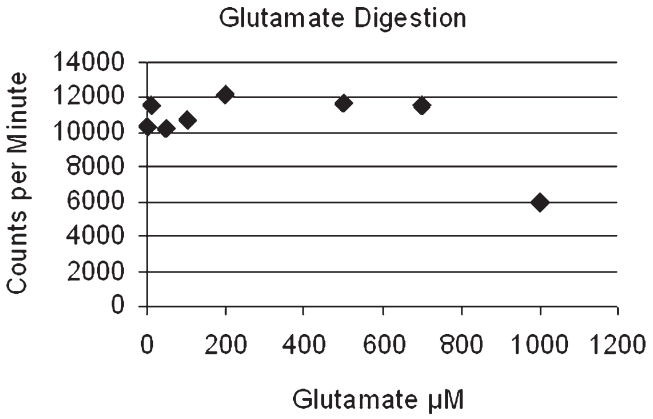

Figure 1 Competitive [^{3}H] kainic acid binding at GluR6 receptor with increasing glutamate concentrations determined by RBA.

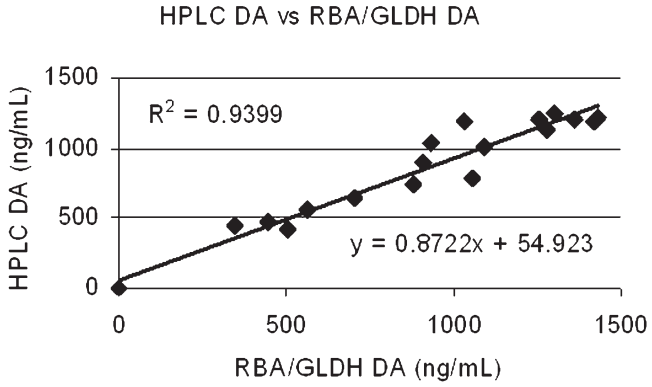

Figure 2 Comparison of DA of values determined by HPLC and RBA of 9 field collected seawater particulate samples from various oceanic environments and distilled water samples spiked with DA and glutamate.

treated glutamate (0, 10, 50, 100, 200, 500, 700, 1000 µM). GluR6 receptors were allowed to incubate on ice for 30 min. in the presence of 50 µL GLDH treated standard, 50 µL of 0.02 µM [^{3}H] kainic acid in 50 mM Tris buffer with 1% BSA, and 100 µL of GluR6 receptors in buffer. The GluR6 receptors with bound [^{3}H] kainic acid were collected onto UniFilter-96 GF/B filters, to which 30 µL scintillation fluid were added and counts per minute of bound [^{3}H] kainic acid were determined by scintillation spectroscopy (Van Dolah *et al.,* 1997).

Comparison of DA Quantified by HPLC and RBA/GLDH in Seawater Particulate Samples DA levels determined by RBA in samples treated with GLDH for the removal of glutamate were compared to those determined by HPLC (Hatfield *et al.,* 1994). For determination of DA by the RBA, GLDH treated DA standards (0–1000 ng/mL; National Research Council, Canada) and GLDH treated seawater particulate samples (n = 9) were analyzed as described above.

In order to compare the two techniques, seawater particulate samples were spiked with DA to yield a final concentration of at least 400 ng/mL to ensure that DA levels of seawater particulate samples were above the HPLC detection limit (400 ng/mL). To remove material that might interfere with HPLC analysis, samples were processed through a 0.45 µM micron filter. In addition to the above sample set, 9 additional samples were prepared by spiking distilled water with 100 µM glutamate and DA, yielding final concentrations of 0 and 400 to 1000 ng/mL.

Results and Discussion

Glutamate Determination in Seawater Particulates Intracellular dissolved free glutamate concentrations up to 50 µM were measured in the 9 seawater samples collected off the coast of Washington. Total cellular glutamate levels of 0.08 µM have been reported previously in seawater sampled from the north Pacific Ocean (Goes *et al.,* 1995). Other estimates of free glutamate levels in coastal waters range from 0.02–0.12 µM (Jason Smith, pers. comm.).

Maximum Concentration of Glutamate Digested Determined by RBA To demonstrate that GLDH could digest the maximum concentration of glutamate detected in seawater particulate samples, GLDH digestion was performed on glutamate standards (0, 10, 50, 100, 200, 500, 700, 1000 µM). Digested glutamate samples up to 700 µM showed similar binding affinity as samples with no glutamate, indicating that the enzyme is capable of complete digestion of glutamate at concentrations up to 700 µM (Fig. 1). This is in excess of the maximum glutamate levels quantified in the 9 seawater particulate samples analyzed in this study as well as those reported in other studies (Goes *et al.,* 1995). Due to expected high levels of glutamate in shellfish, this enzyme may not be suitable for shellfish extracts.

Comparison of DA Values Determined by RBA/GLDH and HPLC DA levels determined by RBA on samples treated with GLDH for the removal of glutamate were tightly correlated with DA levels quantified by HPLC (Fig. 2).

Conclusion

Glutamate dehydrogenase (GLDH) can successfully digest glutamate concentrations up to 700 µM, which is in excess of maximum glutamate levels measured in seawater particulate samples (50 µM) collected from a variety of coastal oceanic environments. A tight correlation of DA levels determined by the RBA and HPLC indicates that GLDH does not interfere with the DA RBA assay. In conclusion, GLDH is a suitable replacement of GLDC for the removal of glutamate from seawater particulate samples prior to the DA RBA analysis.

Acknowledgements

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Development of Real-Time PCR Assays for the Detection of *Chattonella* Species in Culture and Environmental Samples

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Abstract

Raphidophytes have been associated with fish kill events in Japanese, European and U.S. coastal waters, and many produce toxins that can pose a threat to human health. The recognition of raphidophytes as HAB species in mid-Atlantic estuarine waters led us to initiate molecular phylogenetic analyses of these taxa and to develop real-time PCR assays for rapid detection of important species. The molecular studies and PCR detection methods will enhance ongoing taxonomic, toxicologic and ecological assessment of these organisms and will be a useful tool in HAB monitoring programs.

Introduction

Chattonella subsalsa (Raphidophyceae) was first described by Biechler (1936) and since that description, several other *Chattonella* species have been identified: *C. antiqua* (Ono *et al.,* 1980), *C. globosa* (Hara *et al.,* 1994), *C. ovata* (Hara *et al.,* 1994), *C. marina* (Hara and Chihara, 1982) and *C. verruculosa* (Hara *et al.,* 1994). Other notable species in the family Raphidophyceae include *Fibrocapsa japonica* (Toriumi and Takano, 1973) and *Heterosigma akashiwo* (Hada, 1967). Effects of raphidophyte species on fish health vary (Schimada *et al.,* 1983, Onoue *et al.,* 1989, Chang *et al.,* 1990, Tanaka *et al.,* 1994, Ahmed et al,. 1995). Fish kills attributed to *Chattonella* spp. have been reported from inland seas of Japan (Okaichi, 1983), and in 1990, fish kills in New Zealand, Chile and British Columbia were associated with *Heterosigma* spp. (Chang *et al.,* 1990). Blooms of raphidophyte species have also occurred in the coastal waters of Florida (Tomas, 1998) and along the eastern seaboard. Microscopic methods have been traditionally utilized for identifying raphidophyte species in environmental water samples, however, these cells do not preserve well and may alter their morphology when disturbed by discharging ejectosomes, trichocysts and mucocysts (Hallegraeff *et al.,* 1995). Vrieling *et al.* (1995) developed antibodies against Japanese strains of *Chattonella,* while Murayama-Kayano *et al.* (1998) detected differences between species and strains of cultured *Chattonella* spp. using the random amplified polymorphic DNA technique. More recently, Tyrrell *et al.* (2001) adapted a sandwich hybridization assay for detecting *H. akashiwo* and *F. japonica,* and Connell (2000, 2002) designed primers targeting the ITS region of the raphidophyte genome.

We utilized Taqman® PCR technology (Wittwer *et al.,* 1997, Bowers *et al.,* 2001) which involves labeling a probe complimentary to the PCR-amplified target sequences. Previous assays developed by our laboratory were based on this technology and have proven successful in detecting other harmful algal species in culture and environmental samples (Bowers *et al.,* 2000, Tengs et al,. 2001, Jakobsen *et al.,* 2002). PCR-based assays, coupled with morphological descriptions, are strong tools for identifying organisms in culture and assessing diversity in environmental samples. Furthermore, they can aid in determining whether closely related species are actually the same organism or if strain variation is apparent at the genetic level.

Materials and Methods

Samples and DNA Extraction A commercially available kit (Puregene, DNA Isolation Kit; Gentra Systems) was used for extraction of total DNA from an environmental sample collected from a *Chattonella* bloom in Arnell Creek, and other raphidophyte cultures (Table 1).

PCR-Primer Design, Cloning and Sequencing A matrix was generated (MacClade 4.04; Sinauer Associates, Inc.) from raphidophyte 18S sequence data downloaded from GenBank: *Chattonella subsalsa* (U41649), *Heterosigma carterae* (L42529), *Heterosigma akashiwo* (AB001287) and Heterokont/Stramenopile 18S sequence data downloaded from an SSU rRNA database (www.rrna.uia.ac.be). Raphidophyte group-specific primers were designed and used with eukaryotic-specific primers designed by others (Medlin *et al.,* 1988) and our laboratory (unpublished) to obtain sequence data from four CCMP cultures (*C. marina* 2049, *C. antiqua* 2050, *C.* sp. 218, *F. japonica* 1661), five *C. subsalsa* cultures, a *C. marina* culture, three *C. verruculosa* cultures, and the environmental sample. 50 µL PCR reactions were performed, gel purification was utilized on target bands using a commercially available kit (MinElute™ Qiagen), and the TOPO TA Cloning® kit (Invitrogen) was used for ligation and transformation. PCR products were further purified using a Performa™ DTR gel filtration column (Edge Biosystems) and sequenced using the DYEnamic™ ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech, Inc.).

Phylogenetic Analysis An alignment was made using ClustalX (Thompson *et al.,* 1997), and phylogenetic analyses were performed using PAUP* (Swofford, 2002; Figure 1). A minimum evolution (ME) tree was made using Kimura 2-parameter (K2P) distances and random addition of the

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Table 1 Specificity testing of various raphidophyte real-time PCR assays. Species-specific real-time PCR assays were developed for *C.* "Delaware" (based on a novel raphidophyte sequence derived from an environmental sample), *C.* cf. *verruculosa* (C. Tomas isolate), *Chattonella verruculosa* (strain KAGAWA 111), and *C. subsalsa* (CCMP 217). The assay developed for *C. subsalsa* does not differentiate between *C. subsalsa, C. marina, C. antiqua* and *C.* sp., and has been designated as a "*Chattonella* species" (*C.* sp.) assay. *18S sequence data from these cultures used to design real-time PCR assays.

			Real-Time PCR Results			
Culture/Source	Morphological ID	Location	*C.* "Delaware"	*C.* cf. *verr.*	*C.* sp.	*C. verr.*
CCMP 2049	*C. marina*	North Pacific, Japan	NEG	NEG	**POS**	NEG
S. Yoshimatsu	*C. marina*	Japan	NEG	NEG	**POS**	NEG
CCMP 2050	*C. antiqua*	North Pacific, Japan	NEG	NEG	**POS**	NEG
CCMP 2052	*C. antiqua*	Mikawa Bay, Japan	NEG	NEG	**POS**	NEG
S. Yoshimatsu	*C. antiqua*	Japan	NEG	NEG	**POS**	NEG
CCMP 216	*C.* cf. *ovata*	North Pacific, Japan	NEG	NEG	**POS**	NEG
CCMP 218	*C.* sp.	North Pacific, Japan	NEG	NEG	**POS**	NEG
CCMP 1596	*H. akashiwo*	Narragansett Bay, RI	NEG	NEG	NEG	NEG
CCMP 1680	*H.* cf. *akashiwo*	Sandy Hook Bay, NJ	NEG	NEG	NEG	NEG
CCMP 1870	*H. akashiwo*	Los Angeles River, CA	NEG	NEG	NEG	NEG
CCMP 1912	*H. akashiwo*	Kalalich, WA	NEG	NEG	NEG	NEG
CCMP 302	*H. akashiwo*	Milford Sound, New Zealand	NEG	NEG	NEG	NEG
CCMP 452	*H. carterae*	Long Island Sound	NEG	NEG	NEG	NEG
CCMP 1661	*F. japonica*	Australia	NEG	NEG	NEG	NEG
C. Ono	*C. verruculosa*	Japan	NEG	NEG	NEG	**POS**
J. Goebel	*C. verruculosa*	Japan	NEG	NEG	NEG	**POS**
C. Ono/S. Yoshimatsu	**C. verruculosa*	Japan/KAGAWA 111	NEG	NEG	NEG	**POS**
C. Tomas	**C.* cf. *verruculosa*	Delaware	NEG	**POS**	NEG	NEG
CCMP 217	**C. subsalsa*	Gulf of Mexico	NEG	NEG	**POS**	NEG
C. Tomas	*C. subsalsa*	Rehoboth Bay, DE	NEG	NEG	**POS**	NEG
C. Tomas	*C. subsalsa*	Salton Sea, CA	NEG	NEG	**POS**	NEG
C. Tomas	*C. subsalsa*	Corpus Christi, TX	NEG	NEG	**POS**	NEG
C. Ono	*C. subsalsa*	Japan (NIES culture)	NEG	NEG	**POS**	NEG
M. Holms	*C. subsalsa*	Singapore	NEG	NEG	**POS**	NEG
Environmental sample	N/A	Arnell Creek, Delaware	**POS**	N/A	N/A	N/A

sequences using tree-bisection-reconnection branch swapping. Ten random additions were done, and the same topology was found every time. Maximum likelihood values for a general-time reversible substitution matrix, gamma distribution (4 rate categories), base frequencies and proportion of invariable sites were estimated simultaneously using the K2P topology, and a new ME tree was made using maximum likelihood distances and these parameters. Ten random additions of sequences were done using TBR branch swapping, and the same tree was found in all searches. Bootstrapping was done (100 pseudo replicates) using the same model.

Real-Time PCR Design and Validation Based on the sequence matrix, group-specific primers were designed to be used in conjunction with species-specific Taqman® probes for *C.* "Delaware" (based on the novel sequence derived from the environmental sample), *C.* cf. *verruculosa* (C. Tomas isolate), *C. subsalsa* (CCMP 217) and *C. verruculosa* (strain KAGAWA 111).

Results and Discussion

Raphidophyte blooms in US coastal waters with associated fish kills prompted our laboratory to design PCR-based assays to rapidly and specifically detect these species, avoiding problems associated with distortion of cells by fixatives. Our strategy involved developing common primers and unique probes for each species based on real-time Taqman® PCR technology (see Bowers *et al.,* 2000 and Tengs *et al.,* 2001). Using all available raphidophyte cultures for validation, we found the *C. verruculosa* and *C.* cf. *verruculosa* assays to be highly specific, and validation will be ongoing as more isolates become available. Since there were no morphological data or culture isolates derived from the environmental sample collected from the Arnell Creek bloom, we will need to continue to screen cultures with the *C.* "Delaware" assay to identify the source organism. Based on the cross-reactivity of the assay designed for *C. subsalsa* and subsequent sequence data, 18S SSU rRNA is not a feasible target for differentiating *C. subsalsa* (not known to produce toxins) and *C. marina* (a toxin producer), which differ by only 0.01% across the 18S SSU sequence and interestingly are morphologically difficult to distinguish. Preliminary data suggest that ITS1 and ITS2 may be variable enough between these two species to permit using this region for assay development (data not shown). This approach was utilized by Connell (2002) to identify raphidophyte species in fresh and archived material. Additionally, we have generated plastid 16S sequence data from the majority of these cultures and believe that this locus has sufficient heterogeneity to allow development of species-specific assays (see Tengs *et al.,* 2001). Our assay, designed to be specific for *C. subsalsa,* also cross-reacted with three cultures of *C. antiqua* and an isolate identified as *C.* sp. deposited at CCMP. Sequencing of the 18S region revealed that these sequences are 100% identical to *C. marina,* and further supports the need for targeting the ITS region for assay development. The genetic observations presented here clearly illustrate the need for coupling molecular assays with morphological descriptions to verify whether organisms that are difficult to distinguish are in fact two separate species or are demonstrating strain variation. Once

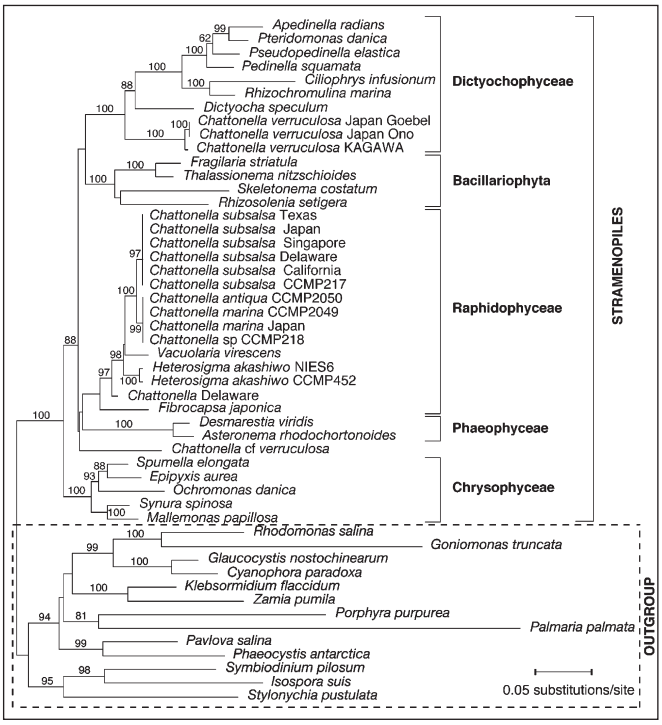

Figure 1 Phylogenetic distance analyses of SSU rRNA sequences from raphidophytes and other protists. Bootstrap values above 75% are indicated.

validated, these assays can be utilized on archived samples to better understand temporal and spatial distributions (*i.e.,* bloom dynamics and consequences) of these species. Interestingly, the phylogenetic analyses suggest that *C. verruculosa* is more likely a member of the Dictyochophyceae. We acknowledge Dr. Daiske Honda for first bringing the Dictyochophyceae character of *C. verruculosa* to our attention, based on his own detailed morphologic and sequencing studies (Honda, pers. comm.) and await his publication of this work with great interest. Finally, we note that the novel brevetoxin-producing *Chattonella*-like organism isolated by C. Tomas (labeled *C.* cf. *verruculosa* in Fig. 1) remains phylogenetically ambiguous. In BLAST searches, the 18S sequence of this organism is most closely related to the freshwater raphidophyte *Vacuolaria virescens.* However, placement of the sequence in phylogenetic analysis is unstable, pointing to the need for further morphologic analysis and greater taxon sampling.

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Analysis of Cyanobacterial Toxins by Hydrophilic Interaction Liquid Chromatography-Mass Spectrometry

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Abstract

Hydrophilic interaction liquid chromatography coupled with electrospray mass spectrometry (HILIC-MS) has been investigated for the analysis of assorted cyanobacterial toxins. Field samples and laboratory cultures of *Anabaena circinalis* and *Cylindrospermopsis raciborskii* were examined. The method proved to be sensitive and robust, with similar results obtained in two different laboratories.

Introduction

Cyanobacteria are found in fresh and brackish water throughout the world and are known to produce a range of toxins (Fig. 1), including neurotoxins (saxitoxins and anatoxin-a) and hepatotoxins (cylindrospermopsin and microcystins). The monitoring of drinking water supplies for the presence of these toxins is of critical importance for the assessment of environmental and health risks. Thus, analytical methods for unambiguous and simultaneous determination of cyanotoxins are highly desirable. Hydrophilic interaction liquid chromatography-mass spectrometry (HILIC-MS) is proposed herein for the analysis of assorted cyanotoxins. Standard solutions of **1**–**23**, as well as field and cultured samples of *Cylindrospermopsis raciborskii* and *Anabaena circinalis,* were employed.

Materials and Methods

Standard solutions of saxitoxins and cylindrospermopsin (CYN) were provided by the Marine Analytical Chemistry Standard Program (NRC, Halifax, CA) and by Queensland Health Scientific Services laboratory (Australia), respectively. Anatoxin-a (ATX-a) and microcystins were purchased from Calbiochem (La Jolla, CA, USA) and Sigma-Aldrich Corporation (St. Louis, MO, USA), respectively. Field samples of *A. circinalis* were collected in October 1997 South East Queensland (Australia). *C. raciborskii* (culture strain AWT 205/1) was provided by Australian Water Technologies (Peter Hawkins, PO Box 73, West Ryde, NSW 211, Australia). Cells (20 mg, dry weight) were extracted thrice with 125 µL of acetonitrile/water/formic acid (80:19.9:0.1) and centrifuge filtered. The crude extracts (total volume = 0.5 mL) were analyzed directly by HILIC-MS. LC-MS experiments were performed either at the Institute for Marine Biosciences using a PE SCIEX API-III+ triple quadrupole mass spectrometer (Thornhill, ON, Canada) coupled to an HP1090 liquid chromatograph (Agilent Technologies, CA, USA), or at the Queensland Health Scientific Services using a PE SCIEX API-300 mass spectrometer coupled to a PE200 series HPLC system (Perkin Elmer, Norwalk, CT, USA). A 5-µm TSK gel, Amide-80 (2 × 250 mm) column (Tosohaas, PA, USA) and the following eluting systems were used: (a) 65% B isocratic for **1**–**18**; (b) 75% B isocratic for **19**, **20**, **21**; (c) gradient 1 (90–65% B over T = 13 min and hold 5 min) for **22**, **23**; (d) gradient (75% B for 5 min,

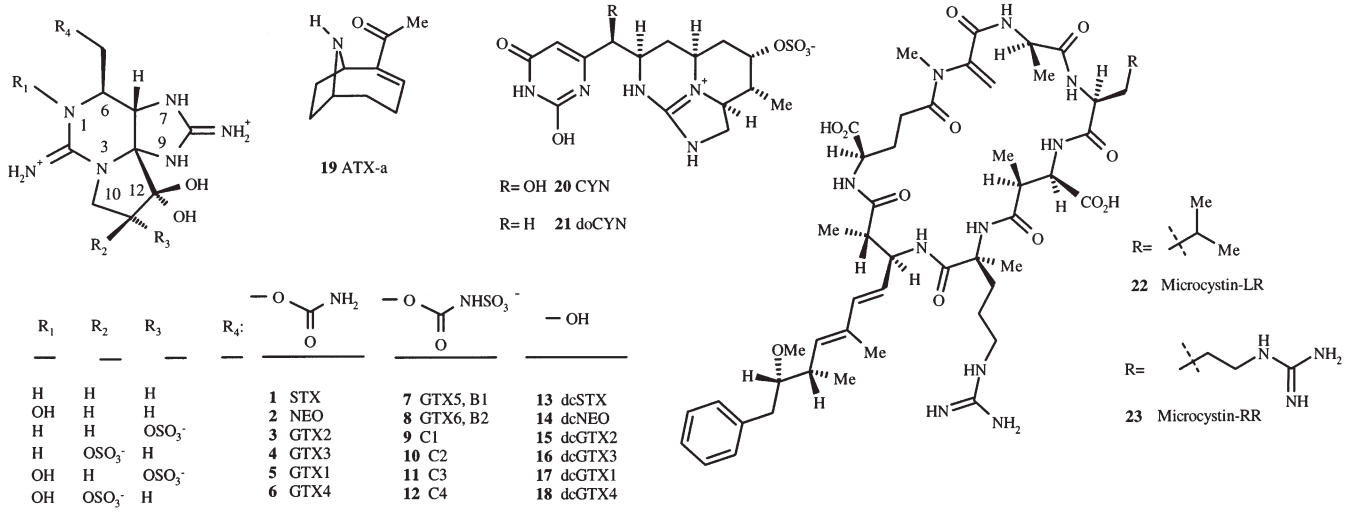

Figure 1 Structures of some toxins produced by cyanobacteria.

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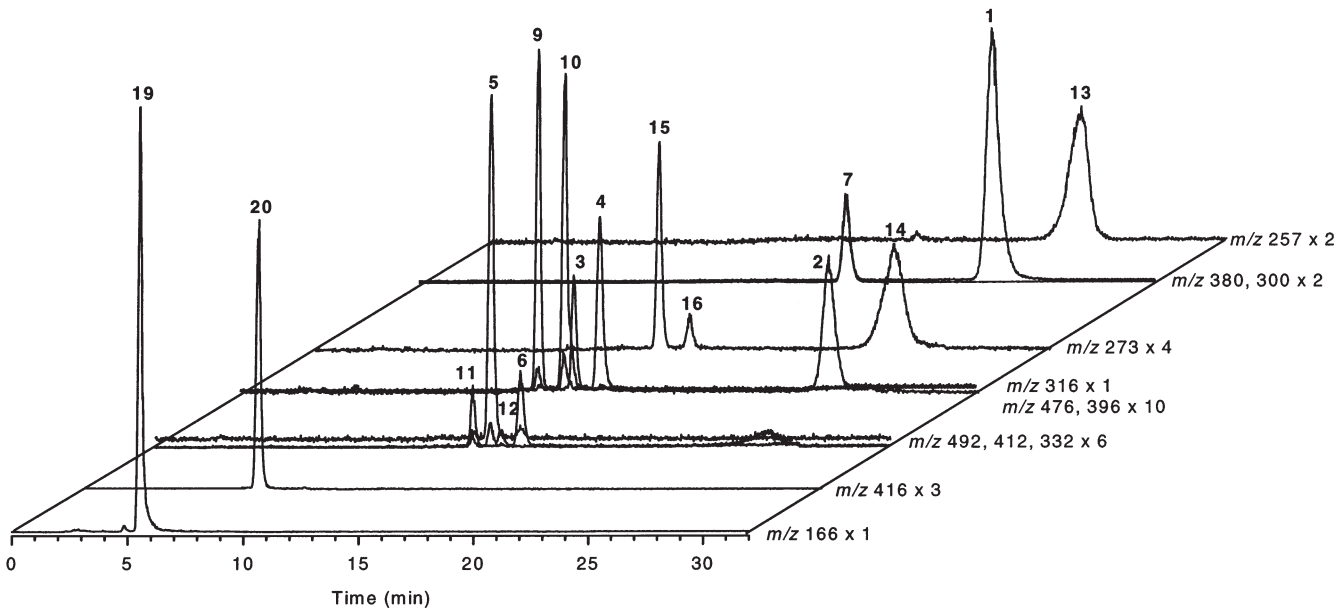

Figure 2 HILIC-MS analysis in SIM mode of a standard mixture containing saxitoxins, ATX-a, and CYN.

75–65% B over 1 min, hold 13 min, 65–45% B over 4 min, hold 10 min) for multiple toxins (**1**–**21**). Eluent A was water and B was a 95% acetonitrile/water solution, both containing 2 mM ammonium formate and 3.6 mM formic acid (pH 3.5). The flow rate was 0.2 mL/min. MS detection was carried out in the selected ion monitoring (SIM) and selected reaction monitoring (SRM) positive ion modes.

Results and Discussion

Electrospray ionization-mass spectrometry (ESI-MS) is a good technique for detection of all of the toxins of concern (Quilliam *et al.,* 1989; Kondo *et al.,* 1996), which are quite basic and therefore give strong $[M+H]^+$ ions. The LC-MS challenge lies in the chromatographic separation method. In fact, many of the very polar cyanotoxins require ion-pairing agents (Quilliam, 1997) which are either incompatible with ESI or lead to reduced ionization efficiency. HILIC overcomes these drawbacks, the basis of the separation being the combination of an amide bonded stationary phase and a mobile phase containing high percentage organic and low concentration of volatile buffers. Such features facilitate high sensitivity for MS detection.

Isocratic conditions (65% B) are normally used for sensitive and selective determination of saxitoxins (Quilliam *et al.,* 2001). A higher percentage organic was required for effective retention of CYN and ATX-a. A gradient was developed for the analysis of all toxins in one run and the results are shown in Fig. 2 for a mixture of standard compounds. This holds great promise for multi-toxin screening. A gradient elution was also required for analysis of microcystin-LR and -RR, which eluted at 7.9 and 13.5 min, respectively. The peak shapes were not satisfactory, however, and existing LC-MS methods (Pietsch *et al.,* 2001) are probably a better tool for analysis of microcystins.

A cultured sample of *C. raciborskii* containing about 2 mg/g of CYN and doCYN was acquired for testing. HILIC-MS analysis of the crude extract provided sufficient selectivity and sensitivity in both SIM (Fig. 3) and SRM modes with detection limits for CYN in the range 0.4–0.9 μM and 0.04–0.2 μM, respectively. A field sample of *A. circinalis* was acquired for testing. A preliminary LC-FLD analysis had shown several saxitoxins (**1**, **3**, **4**, **9**, **10** and **13**) to be present in the sample at concentrations of 75–1000 μg/g. A simple extraction method was used and HILIC-MS analysis was carried out on the crude extract (API-III+ system) in SIM and SRM modes. SIM trace was quite confusing due to extra peaks from other components in the extract, to the high background signal, and to a hump at about 20 min associated with the sample matrix. A clean-up step by solid phase extraction (SPE) could help to solve such problems (Bire *et al.,* 2003), but to the best of our knowledge, there is no SPE method that works for all cyanobacterial toxins. Thus instrumental solutions were investigated. SRM mode (Fig. 4) proved to be useful, allowing the confirmation of all toxins detected by LC-FLD as well as **7**, **15**, and **16**. It also highlighted the presence of two additional peaks at 7.5 and 9.7 min in the *m/z* 380>300 trace, whose identification requires further investigation. The SRM method is recommended since it is highly selective, sensitive and presents almost zero background signal. Different field and cultured samples of *A. circinalis* were analyzed using the API300 system in SRM mode. Sensitivity varied with the different toxins but was generally excellent (60–600 fmol injected).

The developed HILIC-MS method has been shown to be sensitive, straightforward and readily automated. For saxitoxin and its analogues, SRM is the preferred method due to its higher selectivity. Indeed, a clean-up step is needed

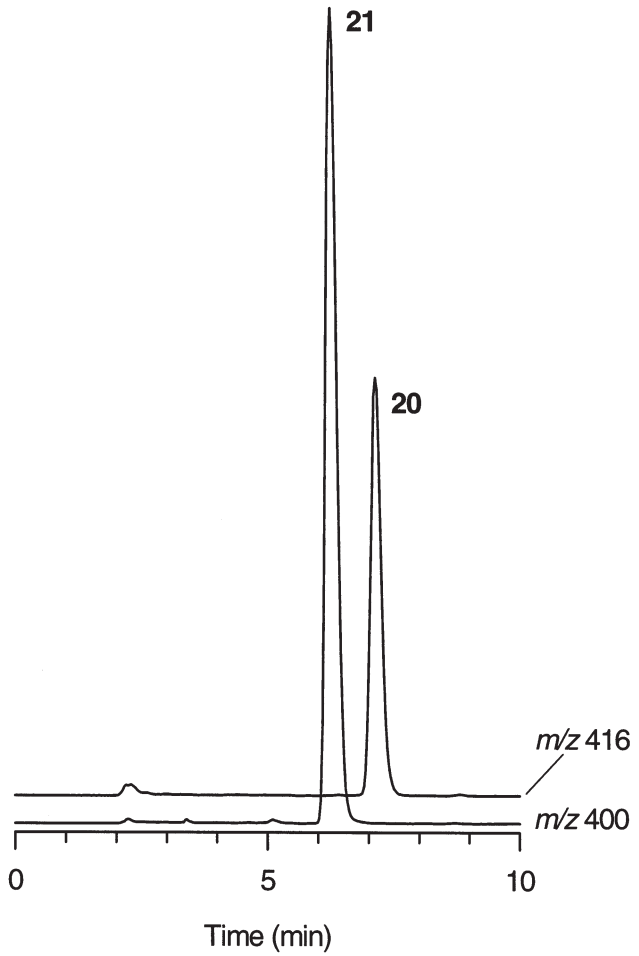

Figure 3 HILIC-MS analyses in SIM mode of a *Cylindrospermopsis raciborskii* extract.

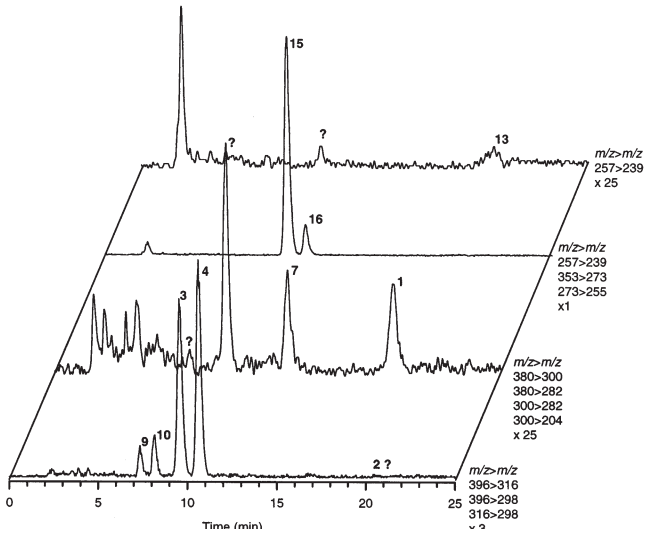

Figure 4 HILIC-MS analyses in SRM mode of *Anabaena circinalis* extract containing saxitoxins.

if SIM is the only acquisition mode available. For CYN and ATX-a, the SIM method is adequate, but SRM can provide additional selectivity for confirmatory analyses. The use of SPE (Harada *et al.,* 1984, 1994, 1999) may enhance sensitivity, making this method suitable for lower levels of the toxins in water or tissue samples. The method proved to be quite robust, with similar results obtained in two different laboratories. Multi-toxin determination is possible, thus allowing the rapid, simultaneous screening of an entire range of toxins.

Acknowledgements

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HPLC/MS/MS Determination of Nodularin Levels in Fish, Mussels and Prawns During a Bloom of *Nodularia spumigena* in the Gippsland Lakes, Victoria, Australia

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Abstract

Since 1996, the Gippsland Lakes have experienced six major blooms of *Nodularia spumigena.* During the bloom in January and February 2002, samples of water and seafood were collected by the Victorian Department of Natural Resources and Environment and tested for the toxin nodularin using HPLC/MS/MS. A simple direct extraction with 80% methanol/water and analysis by HPLC coupled to a tandem mass spectrometer run in the multiple reaction monitoring mode gave good recoveries, with minimal interference and detection limits equating to 10 mg/kg of seafood.

Introduction

The Gippsland Lakes are a series of shallow, interconnected coastal lagoons about 200 km east of Melbourne, Victoria, Australia. The Lakes, which run parallel with the Ninety-Mile Beach of Bass Strait, are almost 70 km long and 10 km wide at the widest point. Since the 1860s, land use and other changes have altered the quality and quantity of catchment inflows to the Lakes. The construction and maintenance of a navigable channel at Lakes Entrance since 1889 has allowed the intrusion of seawater into the Lakes. These changes have transformed what was once a freshwater lake and marsh system to a saline, high nutrient environment. During the summer/autumn period when nitrogen is limiting, cyanobacterial blooms typically occur. Major blooms have been recorded in the Gippsland Lakes in 1965, 1971, 1974, 1987–88, 1989–90, 1995–96, 1996–97, 1999, 2001 and 2002.

In the most recent blooms, the eastern waters of Lake Victoria, all of Lake King and all the waters through to Lakes Entrance were affected by *Nodularia spumigena.* Routine monitoring typically tested for cyanobacterial cell numbers in lake waters using classical microscopic techniques. Cyanotoxins also were monitored in lake water and in the flesh and viscera of a range of fish species, prawns and mussels using HPLC/MS/MS. Sample sites for collection of fish included all waters affected by blooms. Mussels were collected from the tidal waters in the far-eastern areas and prawns were collected from Lake King through to Lakes Entrance and for at least 10 km to sea from the entrance.

Materials and Methods

Mussel samples, fish liver samples and prawn viscera (liquid contents of the prawn heads) were extracted by macerating 1 to 4 grams with 15 mL of 80% methanol using an Ultra-Turrax T25 macerator at 11,000 rpm. Fish and prawn flesh samples were extracted by a similar method to the mussel and viscera samples using 5 g of sample with 15 mL of 80% methanol. Using this method, spiked standard recovery was in the range of 70 to 106% for all sample types.

All extracts were centrifuged at 3,000 rpm and the su-

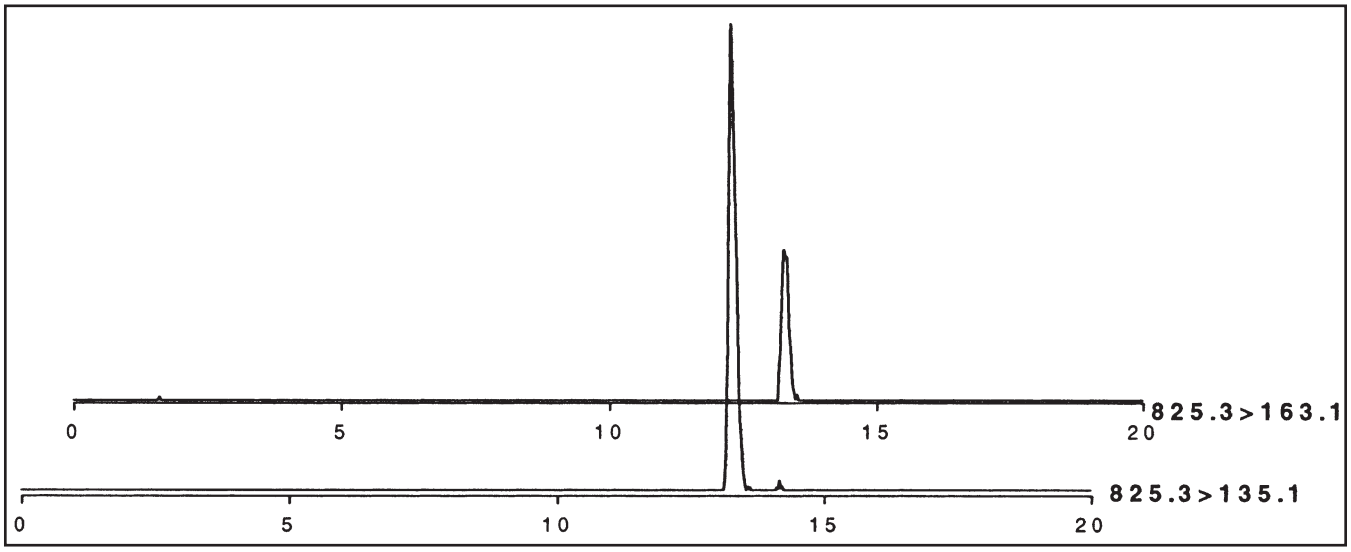

Figure 1 Mass chromatograms from a prawn viscera sample (concentration 5mg/kg).

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pernatant filtered using a 0.45 micron syringe filter (Millex-HV, Millipore) The filtered sample was analysed using a Perkin Elmer Series 200 HPLC system coupled via a Turbo ionspray source to a Sciex API300 mass spectrometer. Separation was achieved using a gradient (30% B for 1 minute to 75% B in 10 minutes, held for 5 minutes then re-equilibrated, A was 10% methanol B was 95% methanol, both containing 5 mM ammonium acetate) on an Alltima C18 column (Alltech, Australia). The retention time for nodularin using this system was 14 minutes. The flow rate through the column was 800 µL/min with a post column split such that approximately 250 µL/min were directed to the mass spectrometer. The mass spectrometer was operated in the multiple reaction monitoring mode using nitrogen as collision gas with the transition 825.3>135.1 being used for quantitation and 25.3>163.1 as confirmation. The orifice and ring voltages were 40 and 290 volts respectively and collision energy of 68 volts was used. Under these conditions and using a 20 µL injection volume, the limit of detection was 3 ng/µL of extract at a signal to noise of 3:1. Nodularin levels were compared to a standard obtained from Calbiochem (95% pure by HPLC).

Results and Discussion

Results of testing are displayed in Tables 1 to 3. These results are for free nodularin only. Any conjugated nodularin would not have been detected, thus total levels may be underestimated. It has been reported however that nodularin does not bind covalently to protein phosphatase unlike the closely related microcystin toxins (Bagu *et al.,* 1997). Limits of reporting using the direct extraction method were 10 µg/kg for flesh samples and 50 µg/kg for viscera samples. A matrix effect was noted with prawn viscera samples (signal enhancement of up to 130%) which could be countered by standard addition. Spike recoveries were excellent (70–106%) over all sample types. Levels of nodularin were high in prawn viscera and mussel flesh and moderate levels were detected in some fish liver samples. Flesh samples of prawns and fish were generally much lower. Lower levels were still detectable in mussel and prawn viscera samples taken 1 month after the bloom had dispersed.

This method was used to determine nodularin levels in a number of marine species. Multiple reaction monitoring using two characteristic transitions enabled the unequivocal identification of nodularin in difficult matrices. As

Table 1 Prawns (*Melicertus plebejus*). Sites A and B are inside the lakes system, Site C is in Bass Strait, 6–10 km outside the lakes entrance.

		Free Nodularin (µg/kg)		Water
Site	Date	Viscera	Flesh	(mg/L)
A (2 samples)	1-Feb-2002	4,300–10,160	ND–27	4–83
B (2 samples)	1-Feb-2002	14,040–22,430	55–65	7
B (2 samples)	6-Feb-2002	4,560–5600	31–52	2
C (6 samples)	7-Feb-2002	3,670–8,190	ND–33	
B	13-Feb-2002	4,000	26	
B	15-Feb-2002	5,870	10	
C (3 samples)	16-Feb-2002	1,120–6,100	ND	
B	18-Feb-2002	8,960	143	
B	24-Feb-2002	6,920	ND	
C	24-Feb-2002	2,720	ND	
B	4-Mar-2002	1,360	ND	
C	4-Mar-2002	1,700	ND	
B	10-Mar-2002	1,100	ND	
C	10-Mar-2002	1,300	ND	
B	17-April-2002	445	ND	
C	17-April-2002	220	ND	

Table 2 Fish samples taken inside the lakes.

		Free Nodularin (µg/kg)		
Fish	Date	Liver	Flesh	Water (µg/L)
Black bream (*Acanthopargus butcheri*)	1-Feb-2002	35–82	ND	4–83
Leatherjacket	1-Feb-2002	60	ND	7
Black bream	15-Feb-2002	450	ND	
Mullet (*Aldrechetta forsteri*)	15-Feb-2002	144	ND	
Trevally (*Pseudocaranx dentex*)	15-Feb-2002	240	ND	

Table 3 Mussel samples taken from the same site inside the lake system.

			Free Nodularin (µg/kg)	
	Site	Date	Whole flesh	Water (mg/L)
Mussels (*Mytilus edulis*)	E	30-Jan-2002	2,725	4
	E	1-Feb-2002	580	1
	E	18-Feb-2002	135	
	E	25-Feb-2002	185	
	E	17-April-2002	45	

minimum sample preparation was involved, timely results were produced which enabled a prompt response to the public health impacts of the bloom. Nodularin levels detected aided in the issuing of warnings regarding recreational and commercial usage of the Lakes and consumption of marine species by referring to previously derived health alert levels of 250 µg/kg for fish, 1,100 µg/kg for prawns and 1,500 µg/kg for mussels (Van Buynder *et al.,* 2001.

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Developing an LC/MS Method to Separate the US EPA Priority Listed Algal Toxins

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Abstract

The United States Environmental Protection Agency final Drinking Water Contaminant Candidate List contains freshwater algae including cyanobacteria and their toxins. Current drinking water treatment techniques may be inadequate in controlling pathogenic algae because the current Surface Water Treatment Rule and the Enhanced Surface Water Treatment Rule focus on fecal coliform removal, not algae removal. In order for the US EPA to determine if algal toxins need to be regulated in drinking water, they need to determine the health risk, occurrence, and susceptibility to treatment. It is crucial that standard toxin concentrations, sample preparations, and analytic methods be developed, verified and validated. None of the published analytical procedures produced by academic researchers have been rigorously tested or approved by USEPA. At present, this is the weakest link. A recent meeting held by USEPA created a priority list for the algal toxins. The highest priority list contained microcystins (LR, YR, RR, and LA), anatoxin-a and cylindrospermopsin. Our research goal is to develop a liquid chromatography/mass spectroscopy (LC/MS) quantitative analytical method for the separation of microcystin LR, RR, LA and YR, anatoxin-a, and cylindrospermopsin. Linear calibration with duplicate injections of 7 levels (0.5–250 ppb) by LC/MS for microcystin LR, YR, and RR, and anatoxin-a, was achieved with no sample preparation. A sample from a lake in Kentucky analyzed with no sample preparation contained 42.5 ppb, 10.5 ppb, 26.2 ppb and 3.2 ppb of microcystin LR, YR, RR and anatoxin-a, respectively.

Introduction

The USEPA final Drinking Water Contaminant Candidate List (CCL) contains cyanobacteria, freshwater algae, and their toxins (Federal Register, 1998). Increased taste and odor complaints have been seen throughout the world. Current World Health Organization (WHO) specification for one cyanobacterial toxin, microcystin LR, is 1 ppb in drinking water. Interest in a selective and relatively fast analysis for all of the main toxins (microsystin LR, YR, RR, nodularin, anatoxin-a, and saxitoxin) in surface and drinking water has been a goal of many researchers. Current analysis methods, such as ELISA (Enzyme-linked Immunosorbent Assay), provide a means to see if any microcystins are present but does not provide any specificity. The tests also suffer from false positives, especially true in very turbid samples, since the results are read spectrophotometrically. High performance liquid chromatography (HPLC) offers a powerful separation tool to separate specific toxins; however, the typical detection technique (UV) lacks the sensitivity and specificity of LC/MS without extensive sample preparation or enrichment prior to analysis. GC/MS offers good specificity but may require off-line derivatization and cannot be used for the higher molecular weight, nonvolatile algal toxins such as microcystin. LC/MS has previously been shown to provide valuable molecular weight information and some of the target compounds have been previously analyzed using electrospray ionization. (Fujii, K., *et al.,* 1997; Kondo, F, 1995) Given the different molecular weights of the various toxins, LC/MS offers the advantage of monitoring for only those specific masses of interest as well as verification of the identification by retention times. The work presented in this paper focused on trying to develop a simple and selective LC/MS method to analyze for as many of the target toxins as possible, using a single method. A challenge to monitoring these toxins is that very few of them are available as standards. For this reason, five of the toxins, the only ones commercially available at the time, were analyzed. One of the toxins analyzed included microcystin LR, the more common toxin found in water samples and the one specifically listed in the WHO guidelines.

Materials and Methods

Pure standards of the various microcystins and nodularin were purchased from Calbiochem (San Diego, CA, USA) and anatoxin-a was purchased from Sigma (St. Louis, MO, USA). All standards were initially dissolved in methanol and kept in a –4°C freezer when not being used to prepare standards. Working standards for quantitation were prepared in high purity water and kept at 10°C in a refrigerated autosampler for analysis. All solvents were of HPLC grade or better and high purity water was generated from a Milli-Q system (Millipore, Bedford, MA, USA). Any mobile phase additives were of ACS grade or better. A YMC Pro C18 column (2.1 × 150 mm)(Waters, Milford, MA, USA) was used for analysis. Water samples, collected from a lake near the university that had recently had an algae bloom and had tested positive for microcystin by ELISA, were frozen until analysis. A control sample was collected from a lake that had tested negative for microcystins by ELISA. Water samples were stored frozen until analyzed.

Equipment and Procedure The LC/MS system we used consisted of a 2695 solvent and sample manager, a 996 photodiode array (PDA) detector and a ZQ single quadrapole mass spectrometer (Waters Corporation, Milford, MA,

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Table 1 Compound names, retention time, masses monitored, and calibration curve information for each component.

Compound	Retention Time (min)	Mass Monitored	Correlation Coefficient (r^2)
Saxitoxin	1.6	191.1 (fragment ion)	0.9977
Anatoxin-a	2.5	166.2 $(M+H)^+$	0.9938
RR	12.2	520.0 $(M+2H)^+$	0.9979
Nodularin	14.9	825.5 $(M+H)^+$	0.9980
YR	16.6	1045.7 $(M+H)^+$	0.9981
LR	16.9	995.2 $(M+H)^+$	0.9973

USA). An electrospray probe operating in the positive mode was used for analysis. The cone voltages and masses monitored for analysis were optimized for each individual component studied. Selected ion monitoring (SIM) was done for the specific masses of interest. The mobile phase consisted of a binary gradient employing a third solvent (1% Formic acid) that was added at 10% during the entire run to allow for consistent pH throughout the run. The two other solvents (water and acetonitrile) were mixed at 88% and 2% initially for 4 minutes, followed by a step change over 1 minute to 65% and 25% of each, respectively. After that, a linear gradient until 29 minutes was done for a final concentration of 33% and 55% of each.

As mentioned previously, standards were prepared by diluting the concentrated standards into water. Seven different levels from 0.5 ppb to 250 ppb were prepared. Duplicate injections were made and an external, linear calibration done with a correlation coefficient (r^2) of 0.997 or greater obtained. Table 1 summarizes the masses monitored and retention times of each component. Prior to analysis, standards and

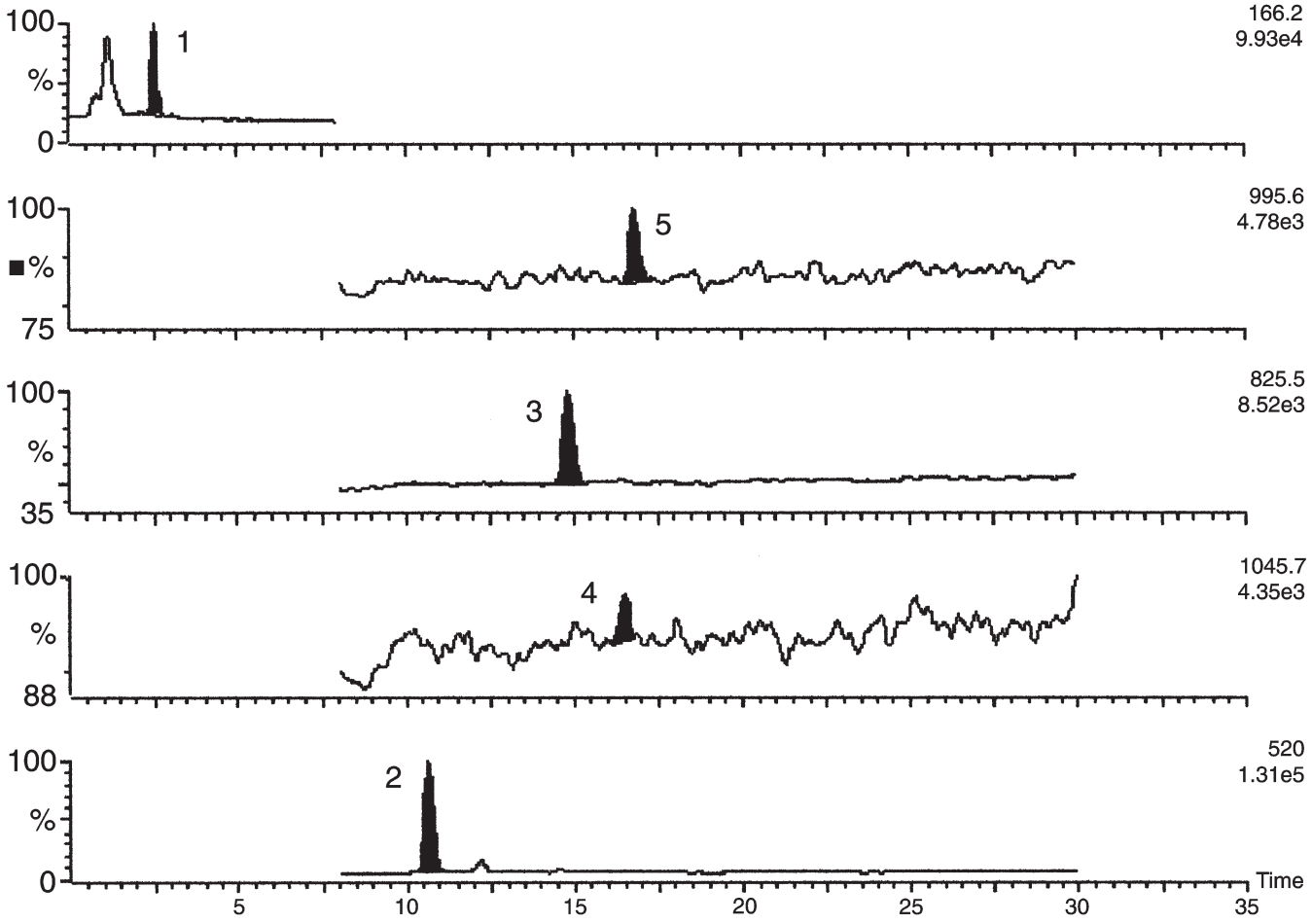

Figure 1 Selected ion recording (SIR) for each component for a 20 µL injection of a 2.5 ppb standard. Masses monitored and relative intensities are given for each in the upper right hand portion of each trace. Peaks identified; 1 = anatoxin-a, 2 = microcystin RR, 3 = nodularin, 4 = microcystin YR, and 5 = microcystin LR.

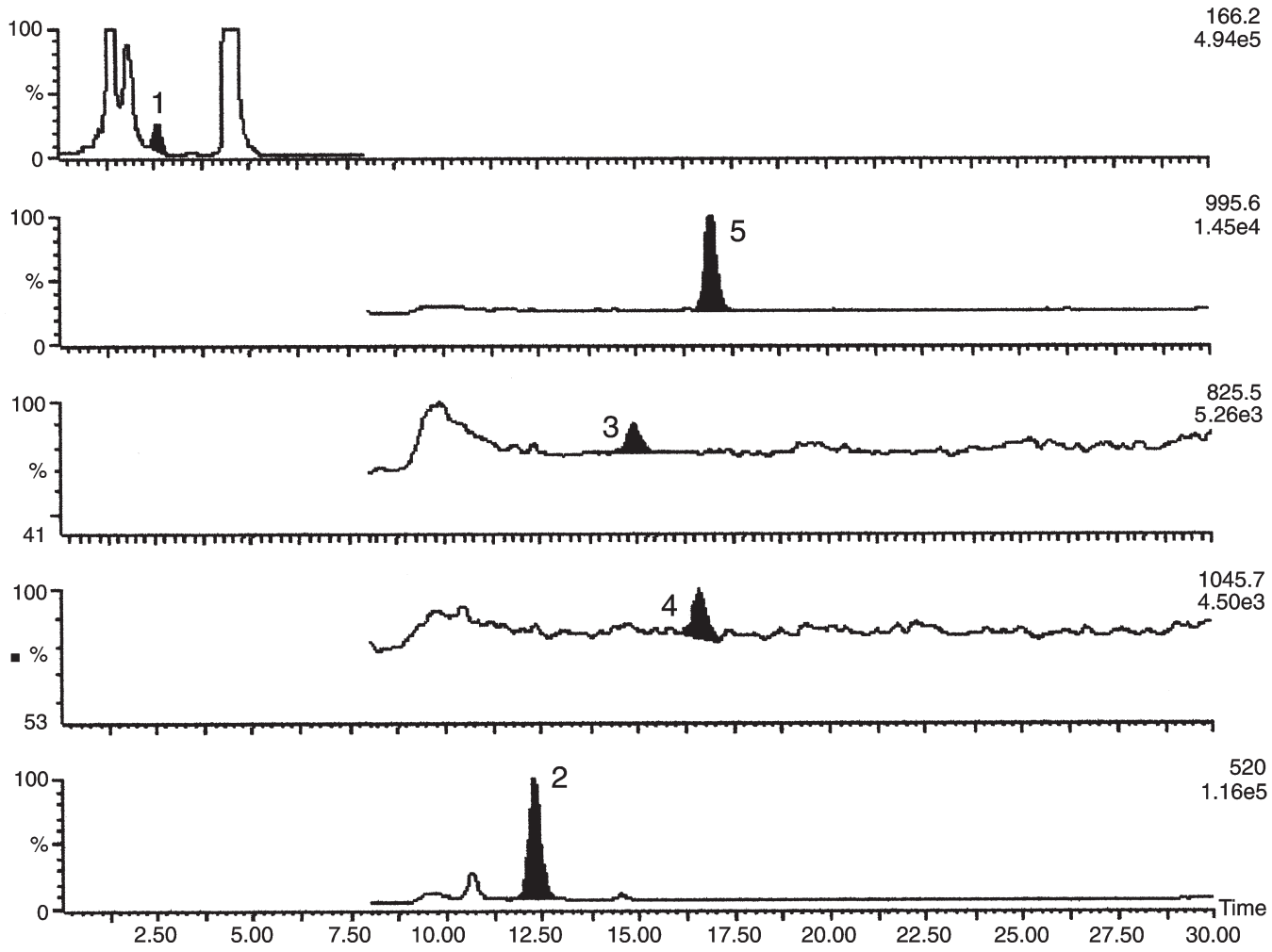

Figure 2 SIR channels for the analysis of a Kentucky lake water sample (20 µL injected). Peaks identified; 1 = anatoxin-a (3.2 ppb), 2 = microcystin RR (26.2 ppb), 3 = nodularin (<0.5 ppb), 4 = microcystin YR (10.4 ppb), and 5 = microcystin LR (42.5 ppb).

samples were filtered with a 0.45 µm GHP filter (Waters Corporation, Milford, MA).

Results and Discussion

Figure 1 shows selected ion chromatograms for the various toxins at 2.5 ppb. All of the toxins could easily be seen at a level of 2.5 ppb without any sample preparation. At the lower levels (0.5 ppb and 1 ppb), all could be detected with the exception of microcystin YR, which was not detected at 0.5ppb. This method was used to analyze a lake water sample, which had tested positive for the presence of microcystin by ELISA. Figure 2 contains the selected ion chromatograms for the lake water sample showing the presence of the various microcystins, especially LR and anatoxin-a.

Preliminary work has been accomplished on improvements in the retention of saxitoxin. In addition, lower detection limits can be determined by using solid phase extraction, and the inclusion of cylindrospermopsin has been done and discussed in a recent paper (Oehrle *et al.,* 2003). Further improvements in retention of saxitoxin and inclusion of cylindrospermopsin allow for the possibility of LC/MS to be used as a screening tool. In addition the preliminary data for SPE of water samples may lower the detection limit even more. Further work will continue in this laboratory to investigate these issues.

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Ion-Trap Mass Spectrometry for the Determination of Yessotoxins in Shellfish

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Abstract

A new, sensitive LC-MS method was developed for the determination of yessotoxin (YTX) and 45-hydroxy-yessotoxin (45-OH-YTX) in shellfish and marine phytoplankton. In the negative mode, the molecular related ion species at *m/z* 1141 $[M-2Na+H]^-$ was used as the parent ion for multiple MS experiments. For example, MS^{2-3} gave major fragment ions at *m/z* 1061 and *m/z* 925. At the MS^4 stage, two major fragmentation ions, *m/z* 855 and 713, are due to fragmentation of the polyether ring backbone of yessotoxins. A reversed phase amide C16 column was used to separate YTX and its analog, 45-OH-YTX, which were found in mussels (*Mytilus edulis*) from the Sognefjord in the west coast of Norway. Mussels were sampled from 3 different locations from different depths, and variation in the levels of YTX and 45-OH-YTX in cultured mussels with depth were observed.

Introduction

This study involved developing MS methods, quadrupole ion-trap and quadrupole time-of-flight (QqTOF), to analyse yessotoxin (YTX) and its analogs with minimal sample pre-treatment. Yessotoxins have been reported in shellfish in Japan (Murata *et al.,* 1987), Norway (Lee *et al.,* 1988), Italy (Ciminiello *et al.,* 1997) and New Zealand where the dinoflagellate *Protoceratium reticulatum* was identified as the biogenetic origin of YTX (Satake *et al.,* 1997a). YTX was originally classified among the DSP toxin group due to its polyether structure but YTX does not induce diarrhea. However, cardiotoxic effects have been demonstrated in mice after intraperitoneal injection of YTX (Ogino *et al.,* 1997). YTX is highly toxic towards mice following i.p. injection and this can lead to an overestimation of DSP when using the mouse bioassay for monitoring. A sensitive LC-MS^2 method was recently developed for the determination of YTX and 45-hydroxy-YTX (45-OHYTX) (Draisci 1998). LC-MS has been applied to the determination of yessotoxin in shellfish; matrix interferences present problems (Goto *et al.,* 2001), but multiple tandem MS (MS^n) overcomes these difficulties.

Materials and Methods

YTX and 45-OHYTX standards were prepared from contaminated mussels harvested from Norway using procedures that have been described previously (Satake *et al.,* 1997b). HPLC-grade solvents were purchased from Labscan (Dublin, Ireland). Contaminated blue mussels (*Mytilus edulis*) were harvested in Skjer, Sognefjord, at the south-west coast of Norway. Mussels were sampled from 3 different locations in the fjord and at three depths (2–4 m, 6–8 m and

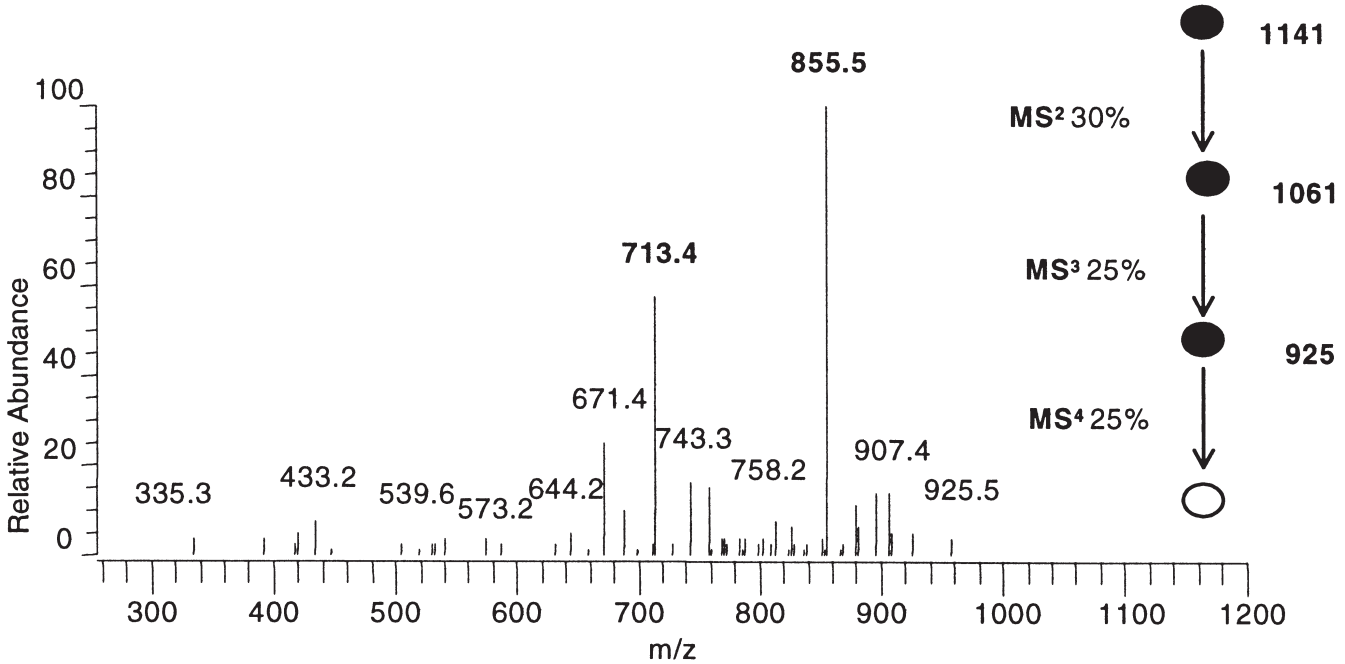

Figure 1 Spectrum of YTX in shellfish, obtained by $LC-MS^4$ using and ion-trap mass spectrometer.

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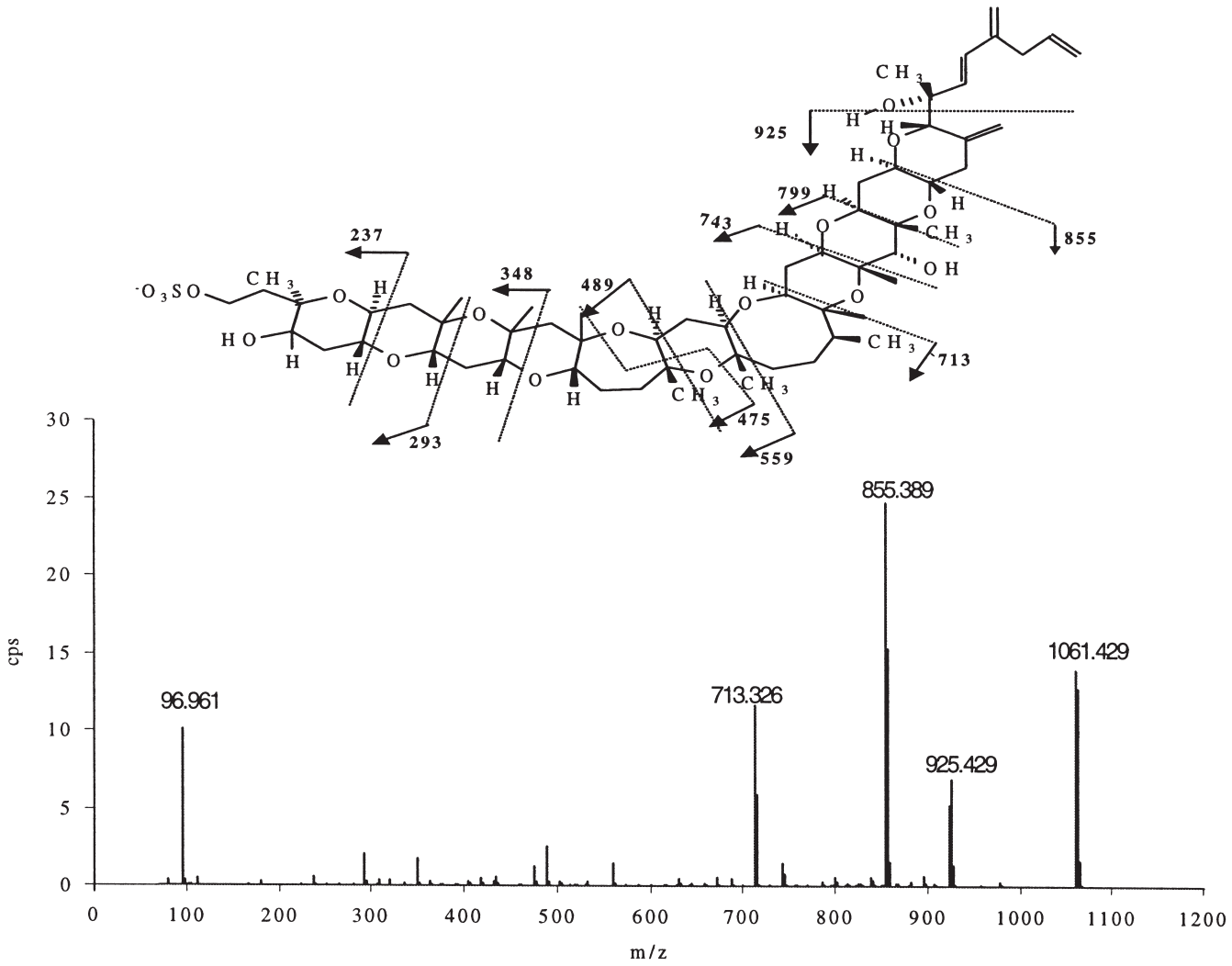

Figure 2 Charge-remote fragmentation of YTX observed using nano ESI QqTOF MS.

10–12 m). Mussel tissues (50 g) were homogenized for 1 min. The homogenate (1 g) was accurately weighed into a centrifuge tube (50 mL), 80% methanol (9 mL) was added and the mixture was homogenized for 1 min followed by centrifugation at 3000 g for 3 min. The supernatant was evaporated to dryness and the residue was reconstituted with methanol (500 µL), syringe filtered (0.45 µm) and an aliquot (3–5 µL) was analyzed. 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 (60:40) containing 0.5 mM ammonium acetate, at a flow rate of 500 µL/min. The LC column was a Discovery RP Amide C_{16}, 150 × 4.6 mm, 5 µm (Supelco, Dublin, Ireland). Nano electrospray (ESI) quadrupole hybrid time-of-flight (QqTOF) MS was obtained using a QSTAR spectrometer (Applied Biosystems).

Results and Discussion

$MS^{2\text{-}3}$ gave major fragment ions at *m/z* 1061 $[1141–SO_3H]^-$ and *m/z* 925 $[1061–C_9H_{12}O]^-$. The latter is a useful diagnostic ion for YTX and its analogs because most of these toxins differ by substitution on the side chain, which is lost at the MS^3 stage. Negative ion fast atom bombardment (FAB) MS^2 is a useful method for providing valuable structural information for molecules containing fused ether rings or repeating structural units. The spectra derived from charge-remote fragmentations are informative by providing data on the composition of each ring of YTX (Naoki *et al.,* 1993).

A similar fragmentation pathway was observed using nano ESI QqTOF. In addition, high mass accuracy MS (<10 ppm) was achieved to confirm the identities of 12 fragment ions. Using the ion-trap MS (LCQ), the fragmentation of the polyether backbone is apparent at the MS^4 stage. Good chromatographic resolution was obtained for YTX and 45OH-YTX (Fernández *et al.,* 2002). Good calibration data were obtained using LC-MS^3: 0.25–5.0 µg/mL (r^2 = 0.999). Using shellfish extracts spiked with YTX, the detection limit using LC-MS^3 was 50 pg on-column (equivalent to 5 ng/g). Relative standard deviation (RSD) values were

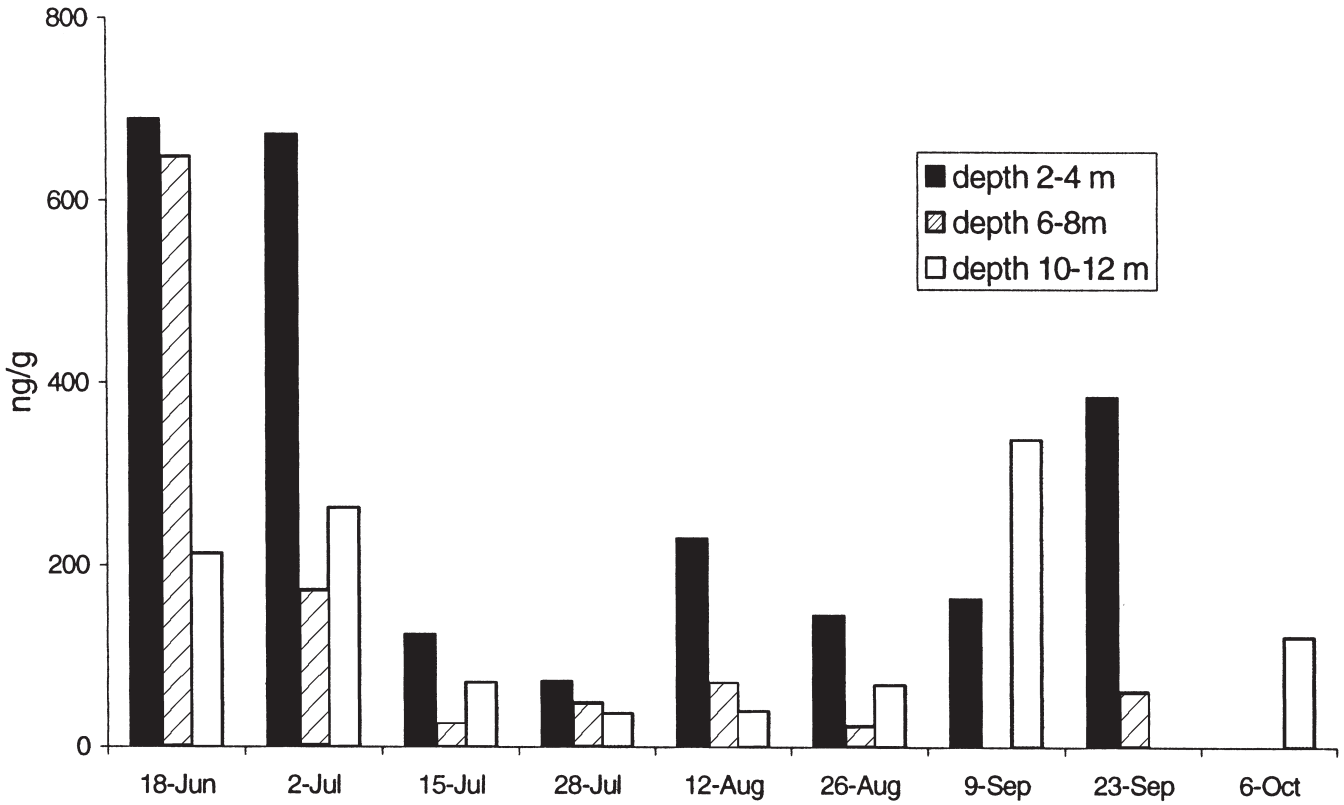

Figure 3 Variation of YTX with cultivation depth in rope cultured mussels (*M. edulis*), Norway

less than 6.3% (0.25 µg YTX/mL, n = 5). Multiple tandem MS displays a dramatic reduction in background noise.

A study of the variation of YTXs with depth of mussel cultivation in Skjer, Norway, is shown in Fig. 3.

It has been found that the toxicity not only varied with the season (Ramstad *et al.,* 2001) but mussels were more toxic in the surface sea water and toxicity decreased with depth. This corroborates a recent New Zealand study (MacKenzie *et al.,* 2002) where it has been shown that *P. reticulatum* producing YTX was most abundant near the surface, between 0–3 m. However, this phytoplankton has not been confirmed as the source of YTX in Norway.

Acknowledgements.

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Ion-Trap and Nanoelectrospray Quadrupole-TOF Mass Spectrometry for the Determination of Polyether Marine Toxins in Phytoplankton

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Abstract

Polyether marine toxins belonging to the diarrhetic shellfish poisoning (DSP) class were detected by LC-MS operating in the positive ion mode. LC-MS^n experiments resulted in fragmentation patterns for each toxin found in phytoplankton samples, and was supplemented with data from high-resolution mass spectra obtained using a QqTOF mass spectrometer. The combination of this instrumentation permits toxinology studies on very small samples, including picked phytoplankton cells that cannot be cultured. This approach revealed a complex polyether toxin profile in picked cells of *Dinophysis acuta* collected in the southern coastal region of Ireland. The toxins identified included okadaic acid, dinophysistoxin-2 (DTX2), pectenotoxin-2 (PTX2) and pectenotoxin-2 seco acids (PTX2SAs) and were quantified.

Introduction

Polyether toxins that contaminate shellfish (mussels, oysters, clams, scallops) cause acute human illness characterised by severe gastrointestinal disturbance. These toxins are responsible for the syndromes Diarrhetic Shellfish Poisoning (DSP) and Azaspiracid Poisoning (AZP). Three classes of DSP toxins have been identified in shellfish, a) okadaic acid (OA) and dinophysistoxins (DTXs) (Murata *et al.,* 1982), (Kumagai *et al.,* 1986). b) pectenotoxins (PTXs) (Yasumoto *et al.,* 1984) and c) yessotoxins (YTXs) (Murata *et al.,* 1987), (Naoki *et al.,* 1993). This study involved the use of two MS methods, quadrupole ion-trap and a QqTOF to analyse trace amounts of toxins with minimal sample pretreatment. The co-occurrence of multiple toxins from different classes in the same shellfish sample is common, and together with the lack of availability of reference standard toxins, present difficulties for chemical analysis. Therefore, the development of ultra-sensitive LC-MS methods is a prerequisite when only small amounts of toxin standards are available (Quilliam, 1995), (James *et al.,* 1997), (Draisci *et al.,* 1998), (Draisci *et al.,* 1999), (Suzuki and Yasumoto, 2000). *Dinophysis* spp. cannot be cultured and the field acquisition of bulk phytoplankton samples proved a valuable source of both DTX2 and PTX standard toxins (Daiguji *et al.,* 1998), (James *et al.,* 1999a), (James *et al.,* 1999b).

Materials and Methods

Collection of high concentrations of *Dinophysis* spp. was achieved using a custom-made double phytoplankton net (4×1 m) and seawater pumped from 5–15 m depths at 600 L/min. An inner net (44 μm) and outer net (108 μm) were used to accumulate phytoplankton with enrichment up to 80% of one species, *Dinophysis acuta.* Cells were manually collected from microscope slides and batches of 200 cells were extracted with methanol. Solvent was evaporated and the residue was reconstituted in acetonitrile. Separation of okadaic acid, DTX2, PTX2 and PTX2 seco acid isomers was achieved using liquid chromatography (LC) on C_{18} reversed phases (Luna-2, 150×2.1 mm, 5 μm, Phenomenex, Macclesfield, UK) operated at 35°C using acetonitrile–ammonium acetate (1 mM) using gradient elution, starting at 40% acetonitrile and stepped to 75% acetonitrile over 12 min. The LC was coupled, via an electrospray ionization (ESI) source at atmospheric pressure, to an ion-trap mass spectrometer (Finnigan LCQ). The toxins were determined using positive LC-MS trapping the $[M+NH_4]^+$ ions. The MS was tuned using an okadaic standard solution (OA, 10 μg/mL), which was infused at 3 μL/min with monitoring of the $[M+NH_4]^+$ ion at *m/z* 822. Using an automated sequence, the eluent flow was diverted to waste for 1 min after sample injection and MS detection was carried out between 1–13 min of the chromatography run. Nano electrospray (ESI) quadrupole hybrid time-of-flight (QqTOF) MS was obtained using a QSTAR spectrometer (Applied Biosystems).

Results and Discussion

Sample collection times of 30 min produced a concentrate

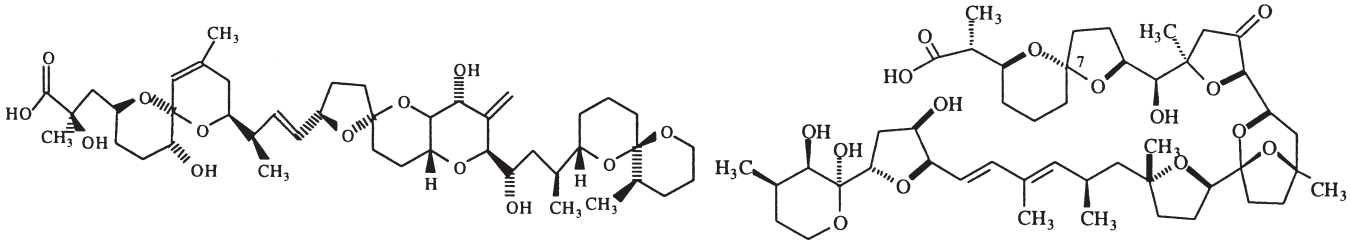

Figure 1 Structures of okadaic acid, DTX2 and pectenotoxin-2 seco acid.

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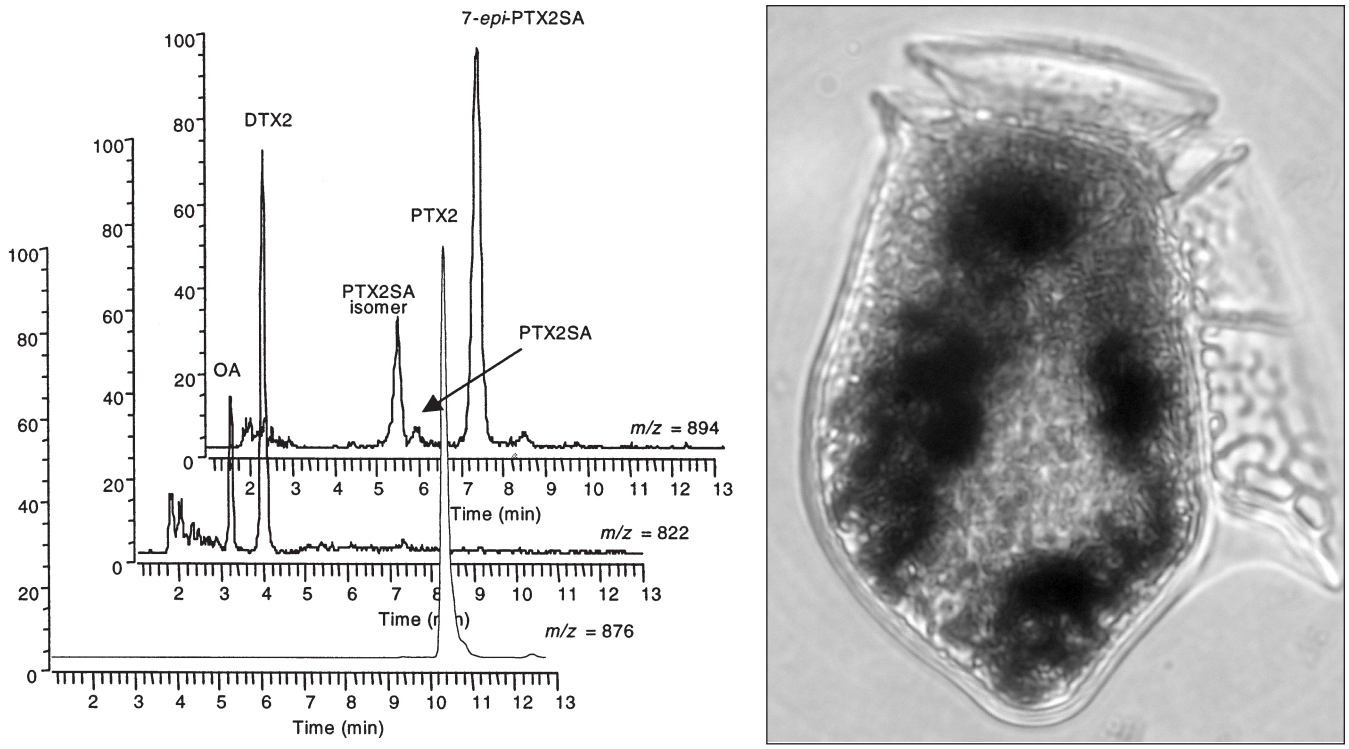

Figure 2 Chromatograms from the analysis of DSP toxins in *Dinophysis acuta* (inset) using positive ESI ion-trap mass spectrometry.

containing *ca.* 70,000,000 cells. Total polyether toxin content was estimated as 100 pg (DTXs+PTXs)/cell. Samples were collected 1–5 km off the southwest coast of Ireland between Aug and Oct 2002. Ion-trap mass spectrometry was applied to identify the isomers, OA and DTX2, PTX2 and the three PTX2 seco acid isomers in marine phytoplankton (*Dinophysis acuta*). Under positive electrospray ionization (ESI) conditions, the DSP compounds produced the adduct ions $[M+NH_4]^+$ exclusively. Good chromatographic resolution was required to separate the groups of isomeric

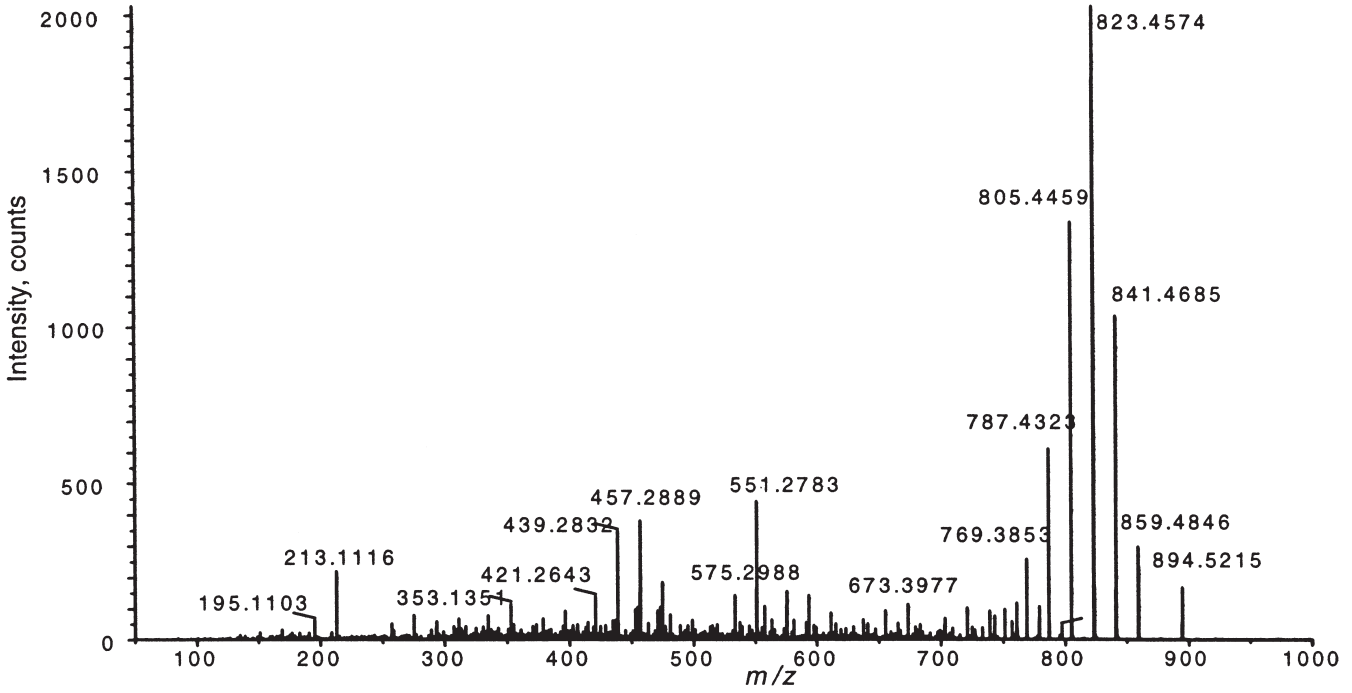

Figure 3 Nano ESI mass spectrum of 7-*epi*-pectenotoxin-2 seco acid determined using a high mass accuracy quadrupole–TOF spectrometer.

toxins (Fig. 2), including OA, DTX-2 and PTXs, using reversed-phase chromatography and gradient elution. Ammonium acetate (1 mM) was used as eluent buffer and the LC was linked with a quadrupole ion-trap mass spectrometer.

The major advantage of this instrumentation is the ability to determine polyether toxin profiles using very small samples, especially picked phytoplankton cells. A multiple toxin analysis ESI-LC-MS method was developed for a mixture of DSP toxins, including OA, DTX-2 and PTXs, using reversed phase chromatography linked with an LCQ quadrupole ion-trap mass spectrometer and this was applied to marine phytoplankton (*Dinophysis acuta*). Under positive electrospray ionisation (ESI) conditions, the DSP compounds produced the adduct ions $[M+NH_4]^+$ exclusively. Fragment ions resulting from the loss of water molecules from the polyether skeletons, which constitute the backbone of these compounds, were apparent in the full scan spectrum without the application of any collision energy. For the DSP compounds water losses are facile and may be induced in the heated capillary. Good chromatographic resolution was essential to separate the isomeric toxins as they have identical mass spectra. The composition of the bulk phytoplankton samples was OA (600 µg/L), DTX-2 (980 µg/L), PTX2 (500 µg/L), PTX2SA isomer (10 µg/L), PTX2SA (1 µg/L) and 7-epi-PTX2SA (5 µg/L).

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Sample Preparation Methods for Analysis of Brevetoxins in Oysters by LC/MS

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Abstract

Analysis of brevetoxins (PbTx) in oysters is complicated by matrix interference and metabolism. Various solvent extraction and clean-up techniques, and analyte derivatization, were investigated for the determination of PbTx in oysters by liquid chromatography/mass spectrometry (LC/MS). Acetone was highly efficient for extraction of PbTx. Recovery of PbTx was improved by removal of neutral lipids in hexane and clean-up by C_{18} solid phase extraction (SPE), coupled with reduction of sample load onto the LC column. Further purification of the oyster extracts by silica (Si) SPE removes additional matrix interferences and improves the apparent recovery of parent PbTx; however, analysis time is increased.

Introduction

The consumption of brevetoxin (PbTx)-contaminated oysters by humans can result in neurotoxic shellfish poisoning (NSP). The mouse bioassay for monitoring toxic shellfish is an important component of NSP prevention strategies, although efforts are underway to replace it. In the Eastern oyster (*Crassostrea virginica*), PbTx-2 (the principal algal PbTx) is rapidly metabolized, which complicates its determination by alternative methods. Previously identified metabolites include two cysteine adducts and the reduction product PbTx-3; several other metabolites remain unidentified (Plakas *et al.,* 2002). The toxicity of shellfish metabolites relative to algal PbTx is not well established and their significance to NSP is not known. In addition, the oyster matrix interferes with the separation and detection of PbTx. Some components of the oyster matrix (*e.g.,* pigments, proteins, and lipids) tend to bind the toxins by covalent and non-covalent interactions. We used LC/MS to evaluate various extraction and clean-up techniques for analysis of PbTx and metabolites in oysters.

Materials and Methods

Extraction Oyster homogenates (1 g) were extracted with acetone (2 × 2 mL) by sonicating and vortex mixing for 1 min and centrifuging at 3500 × g for 5 min at 5°C. Supernatants were combined and evaporated at 40°C under nitrogen. Acetonitrile, methanol, and tetrahydrofuran (THF) were compared with acetone for extraction efficiency.

Purification The first step in clean-up was removal of neutral lipids. The dried oyster extract was re-solubilized in 2 mL 80% methanol and washed with 95% n-hexane (2 × 2 mL) by vortex mixing and centrifugation. The methanolic layer was evaporated and residues re-solubilized in 25% methanol by sonicating and vortex mixing. The solution was applied to a C_{18} SPE column (Varian Bond Elut, 500 mg, 10 mL) previously conditioned with methanol and water (10 mL each). The column was washed with 25% methanol and analytes were eluted with 100% methanol. The eluant was evaporated and residues were re-solubilized in methanol for LC/MS analysis. For further sample purification, the methanol eluant from C_{18} SPE was evaporated, residues re-solubilized in chloroform, and applied to an Si SPE column (Varian Bond Elut, 500 mg, 3 mL) previously conditioned with hexane and chloroform. The column was washed with 4 mL of chloroform:ether (8:2) and parent toxins (PbTx-2, -3, and -9) were eluted with 5 mL of chloroform:acetone (7:3). To recover the previously identified polar metabolites (Dickey *et al.,* 1999; Plakas *et al.,* 2002), the column was washed with 5 mL of chloroform:methanol (7:3) and metabolites were eluted with 5 mL of chloroform:methanol (1:9) and 5 mL of chloroform:methanol:acetic acid (1:8.7:0.3). All analyte fractions were evaporated under nitrogen and re-solubilized in methanol for LC/MS analysis. Liquid-liquid partition with chloroform was also examined as an alternative to C_{18} SPE, prior to Si SPE purification. After the hexane wash step, as described above, the 80% methanolic solution was evaporated to ~0.4 mL and back-extracted with chloroform. The chloroform extract was evaporated under nitrogen and further purified by Si SPE.

Reduction of PbTx-2 PbTx-2 is poorly recovered from spiked oyster homogenate. We examined chemical reduction as a means to improve the recovery of PbTx-2. PbTx-2 was reduced to PbTx-3 by treatment of raw oyster homogenate with $NaBH_4$ (dissolved in acetonitrile) in THF reaction solvent. PbTx-2 was reduced to PbTx-9 by treatment of freeze-dried oyster homogenate with $NaBH_4$ in acetonitrile. After reaction of excess $NaBH_4$ by acetone or acetic acid in acetonitrile, purification followed the same procedure described above.

Liquid Chromatography/Mass Spectrometry (LC/MS) LC separation was performed on either a Phenosphere 3 µ ODS(2), 2.0 × 50 mm column or YMC J'sphere 4 µ ODS-L80 2.0 × 250 mm column using HP1100 LC system. The mobile phase was water (A) and acetonitrile (B) binary system with 0.1% acetic acid additive. For Phenosphere column, the gradient was: 2 min of 35% B, linear gradient to 80% B at 25 min, then to 100% B at 27 min, hold for 6 min and return to initial conditions at 36 min and hold for 7 min. Gradient conditions for the YMC column were similar to that reported previously (Plakas *et al.,* 2002). The

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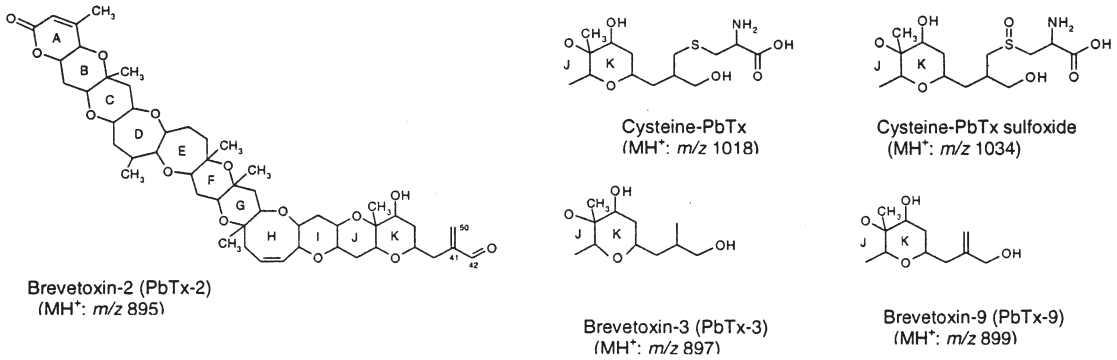

Figure 1 Structures of PbTx-2, PbTx-3, PbTx-9, Cysteine-PbTx, and Cysteine-PbTx sulfoxide.

Table 1 The effect of LC column loading on recovery of PbTx-3.

Sample*	Injection Volume (µL)	Peak Area (10^6)	Recovery
Standard	10	1.03	–
Oyster spike	10	0.57	55%
Standard	5	0.38	–
Oyster spike	5	0.31	82%

*Samples were spiked oyster extract and corresponding standard at a concentration of 0.32 ppm. The extract was diluted to 0.4 g oyster equiv./mL.

analytes were detected using Thermo Finnigan Navigator AQA MS with ESI interface (positive ion mode). Tune settings were: capillary voltage, 4 kV; probe temperature, 300°C; source voltage, 40 V; Rf lens voltage, 0.3 V.

Results and Discussion

Several physicochemical properties of PbTx challenge their analytical determination. PbTx contain an ester group in their A ring (Fig. 1), and consequently are prone to decomposition by hydrolysis of the A ring at high or low pH (pH < 2), particularly in alkaline aqueous solution. The α,β-unsaturated aldehyde group of PbTx-2 and the sulfide group of the oyster metabolite cysteine-PbTx are highly reactive and unstable in oyster matrix (Fig. 1).

Because only PbTx-2 and PbTx-3 are commercially available in high purity, we first examined recovery of these compounds in spiked oyster homogenates. After extraction and clean-up by C_{18} SPE, the mean recovery of PbTx-2 in spiked (≤1.0 µg/g) oyster homogenate was less than 5%; recovery of PbTx-3 varied with the amount of oyster extract loaded onto the LC column (Table 1). The attenuated PbTx-3 signal with higher column loading was caused by co-eluting oyster matrix components which suppress ionization at the ESI interface. Possibly, other interactions (non-covalent binding) with oyster matrix occur as well. These matrix effects varied with the season and harvest site of the oysters.

Si SPE reduced the level of the co-eluting matrix components and improved recovery of PbTx-3 (to ≥90%) in spiked oyster homogenate, while recovery of PbTx-2 remained low (Fig. 2). For PbTx-2 and -3, results were the same when chloroform back-extraction replaced C_{18} SPE clean-up, prior to Si SPE purification. However, for the higher polarity metabolite fraction, carry-over problems occurred which interfered with the LC/MS signal (Fig. 3).

The reactive α,β-unsaturated aldehyde group of PbTx-2 is probably responsible for its poor recovery from the spiked oyster matrix. We examined chemical reduction of

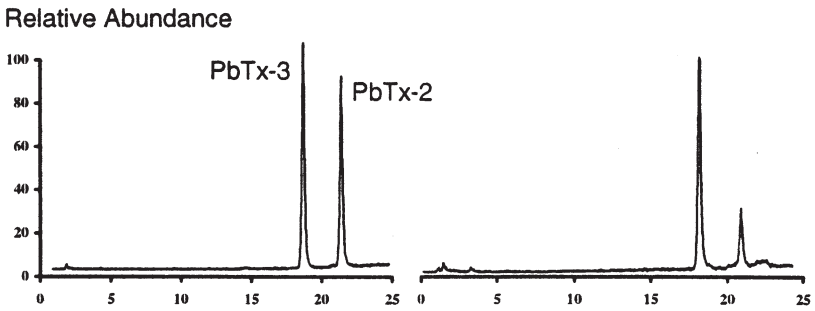

Figure 2 Total ion chromatogram (TIC) of PbTx-2 and PbTx-3 in: standard solution, 0.8 mg/mL (left); oyster spike, 0.4mg/g, 2 g equiv./mL extract (right).

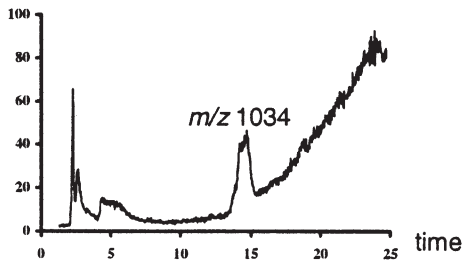

Figure 3 TIC of selected ion monitored of the metabolite fraction after C18/Si SPE clean-up.

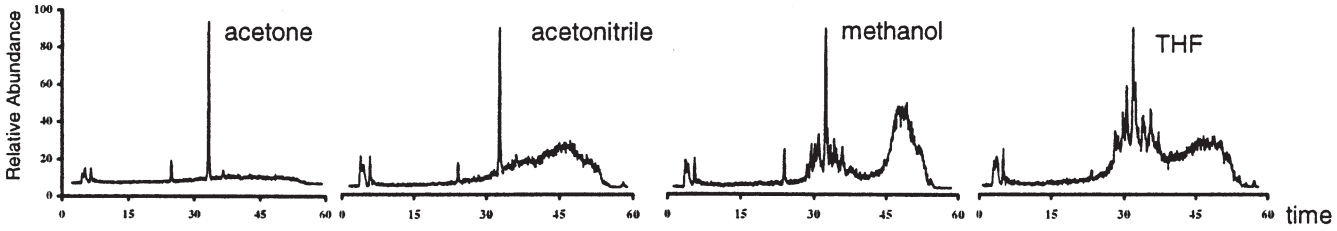

Figure 4 Reconstructed PbTx-3 ion chromatograms comparing various solvents for extraction of PbTx-3 in spiked oyster homogenates using C_{18} clean-up.

Table 2 Change in metabolite ratio in a field-exposed oyster extract during sample clean-up.

Sample	Sum of peak area ($\times 10^7$) *m/z* 1018+1034	Peak area ratio *m/z* 1018/1034
C_{18} SPE clean-up (70% methanol wash)*	5.02	0.4
C_{18} SPE clean-up (25% methanol wash)	5.65	1.9

*Used to examine the elution of *m/z* 1018 and 1034.

Table 3 Comparison of peak areas in a field sample with different sample clean-up (4g/mL. 5 µL injection).

	Ion peak area (10^6)				
Method (SPE)	PbTx-2	PbTx-3	PbTx-9	Cysteine-PbTx	Sulfoxide product
C_{18}	0.02	0.35	0.04	34	18
C_{18}/Si	0.02	0.69	0.09	30	26

PbTx-2 within the oyster matrix as a means to improve recovery. Recovery of PbTx-2 as the reduction product PbTx-3 was 50%, and recovery as PbTx-9 was 39%, at a spiking level of 2.5 µg/0.3 g oyster. At lower spiking levels, recovery by reduction was not improved compared with C_{18}/Si SPE clean-up.

The relative extraction efficiency of the parent PbTx by various solvents, using C_{18} SPE clean-up, was: acetone > acetonitrile > methanol > THF (Fig. 4). For C_{18}/Si SPE clean-up, the relative extraction efficiency was, typically: acetonitrile ≥ acetone > methanol. In some samples, acetonitrile changes the physical property of the oyster, and results in lower recovery.

We applied the two SPE methods, C_{18} and C_{18}/Si, to a field-exposed oyster sample. With C_{18}/Si SPE clean-up, the matrix interference for parent PbTx was reduced, and recovery increased, compared with C_{18} SPE clean-up. For metabolites, conversion of the cysteine-PbTx conjugate (MH^+: *m/z* 1018) to sulfoxide product (*m/z* 1034) can occur during sample preparation. For example, drying the extract without nitrogen or varying the wash conditions of C_{18} SPE changed the ratio (Table 2). Factors contributing to this conversion within oyster matrix are not well understood. High amounts of peroxide in ether will cause partial conversion. Cysteine-PbTx was also converted to some unknown products after standing in ether. By eliminating the ether step, C_{18}/Si SPE can separate the intact algal parent toxins from the oyster metabolites. Further study showed the two step elution of polar metabolites in Si SPE clean-up could be replaced by a single step elution with 5.5 mL of chloroform:methanol:water (1:8:1). These fractions can be used to study their contribution to overall toxicity of shellfish extracts. Major red pigments in metabolite fractions are removed by Si SPE clean-up, and metabolites can be further purified for studying their properties.

Other sorbent types of SPE (*i.e.,* cyano, diol, alumina, ion exchange) were less successful for the clean-up of PbTx in oyster matrix.

In summary, C_{18} SPE clean-up of oyster extracts removes salts and high polarity interfering components for LC/MS analysis of PbTx. Reducing the amount of oyster extract loaded onto the LC column is effective and convenient in improving apparent recovery of PbTx after C_{18} SPE clean-up. Si SPE further purifies the oyster extracts and improves the recovery of parent toxins; however, analysis time is increased.

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Confirmation of Azaspiracids, Okadaic Acid and Dinophysistoxins in Phytoplankton Samples from the West Coast of Ireland by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

During a cruise in the coastal and shelf waters off the west coast of Ireland in July/August 2001, a total of 70 phytoplankton samples were collected by vertical net hauls or from the ship's clean surface water supply. The filtered samples were extracted with 80:20 methanol:water and pure methanol and analysed by liquid chromatography coupled to tandem mass spectrometry for okadaic acid (OA), dinophysistoxins (DTXs) and azaspiracids (AZAs). All compounds were determined with a multitoxin method, using a gradient HPLC run and multiple reaction monitoring or scanning in MS. OA and DTXs eluted from 6.5–8 min and azaspiracids eluted from 10–12 min. Samples with high concentrations were also analysed in scanning mode, and fragmentation spectra were obtained. The toxins present were confirmed to be OA, DTX-2, AZA-1 and AZA-3. While OA itself was mostly found at concentrations of 25-50% of the total OA-equivalents, the concentration of OA was higher than DTX-2 in only one sample. AZA-2 was not detected whereas AZA-3 was detected only at low levels (<5% of the total AZA). Highest AZA concentrations were found in different locations than the highest concentrations of OA and DTX-2. These findings demonstrate that azaspiracids are produced by a different organism than OA and DTXs. They also indicate that AZA-1 is the main toxin produced by the causative organism, with AZA-2 and –3 possibly being metabolic products.

Introduction

Previous studies related to phytoplankton in the coastal and shelf waters off the west coast of Ireland have largely focused on the relationship between physical oceanographic processes and the distribution and occurrence of phytoplankton, including toxic or harmful species (Raine *et al.,* 1993; Raine and McMahon, 1998; McMahon *et al.,*1998; Raine *et al.,* 2002). These studies have yielded invaluable information on advective transport pathways and the occurrence of toxic phytoplankton species in shellfish production areas in coastal embayments. The occurrence of toxic phytoplankton species in these bays has resulted in the accumulation of toxins in shellfish, principally Diarrhetic Shellfish Poisoning (DSP) and Azaspiracid Shellfish Poisoning (AZP) toxins, that in turn has led to often prolonged closures of production areas. Since the mid-1980s a national monitoring programme has been in place in Ireland to detect the presence of toxins in shellfish, but to date, few data are available on the distribution of individual toxins in the plankton. In spring 2001, liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) testing was added to the suite of assays used on a routine basis in the national biotoxin monitoring programme, and in July/August 2001 bulk phytoplankton samples for chemical analysis were collected in the coastal and shelf waters west of Ireland. Here we report the results of the analysis of these phytoplankton samples, which provides the first reports of the geographical distribution of individual toxins and their relative concentrations. Such data, when compared with data on the distribution of these toxins in bivalve mollusks from shellfish production areas along the west coast of Ireland, provide insights into the factors determining their accumulation in commercially important shellfish species.

Materials and Methods

During a cruise aboard the RV *Celtic Voyager* from 28/07/01–03/08/01 in the coastal and shelf waters on the west coast of Ireland, a total of 70 phytoplankton samples were collected. Water samples were filtered onto glass-fibre filters and stored frozen in petri dishes. For the first extraction cycle, filters were cut and 20 mL of 80% aqueous methanol was added. The sample was vortex-mixed for 1 min and sonicated for 10 min prior to centrifuging and filtration of supernatant using 0.2 µm syringe filters. For the second extraction cycle, 10 mL methanol were added to the pellet and vortex-mixed for 1 min prior to sonication for 10 min. The sample was again centrifuged and filtered using 0.2 µm syringe filters. The filtrates were combined and evaporated to dryness using a centrifugal evaporator. After evaporation to dryness, 1.5 mL of 80% methanol was added prior to vortex-mixing for 1 min and sonication for 10 min. Finally, all extracts were analysed using LC-MS-MS.

LC-MS-MS methodology was developed during early 2001 for reference materials and contaminated shellfish, using a Quattro Ultima (triple quadrupole mass spectrometer). Collision energy was optimised to 50 eV to obtain fragments different from the loss of water. The LC-method is based on a gradient method similar to that of Quilliam *et al.,* 2001. The development of the method has been described by Hess *et al.,* 2001 and 2003. Typical performance characteristics for shellfish analysis showed greater than 90% recovery from reference materials, ratios of fragment ions that had <20% CV and between-batch reproducibility of reference materials of <12.5% CV. The quantities of phytoplankton collected on the cruise in 2001 did not allow for an evaluation of the recovery from phytoplankton, therefore, large extraction volumes relative to the sample were chosen for the analysis of the extracts of glass-fibre filters.

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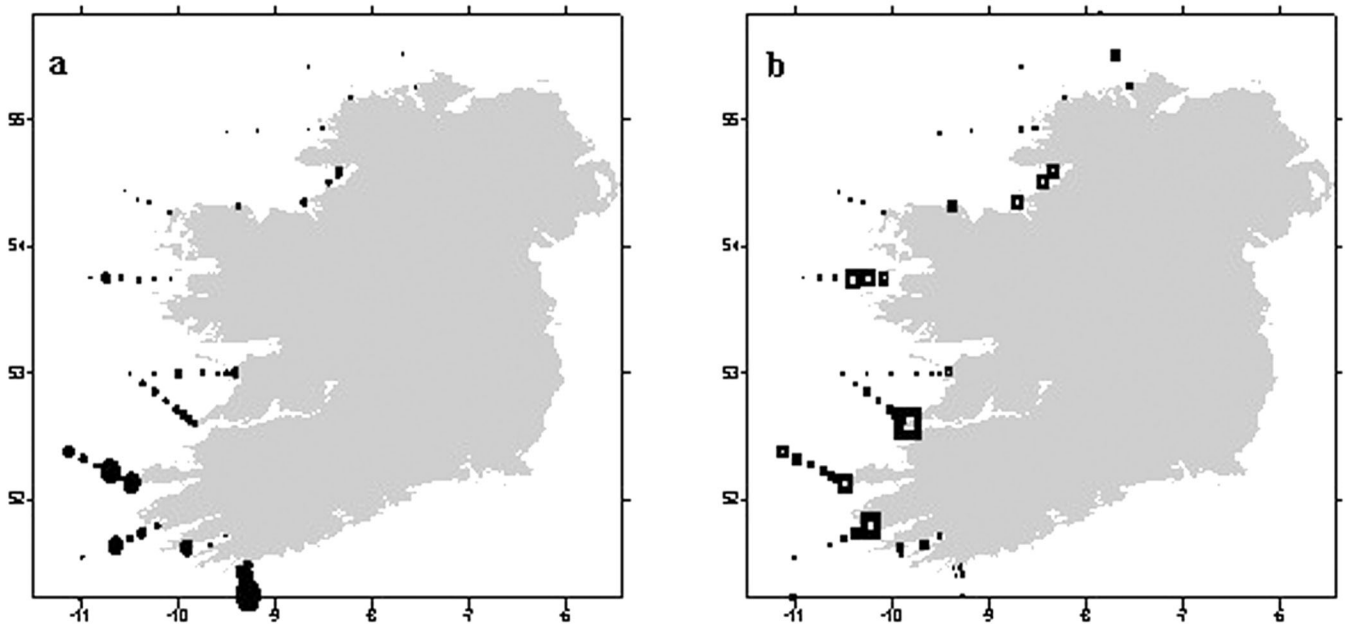

Figure 1 Relative concentrations found in phytoplankton net-haul samples for (**a**) DTX-2 (max conc. 1970 ng/m^3) and (**b**) AZA-1 (max conc. 42 ng/m^3).

Results

The LC-MS method was shown to detect relevant levels of lipophilic algal toxins in phytoplankton samples concentrated onto glass-fibre filters from several cubic metres of seawater, even though cell concentrations did not typically discolour the water. Both AZP and DSP toxins showed a wide distribution and were detected in 100% and 94% of the samples, respectively. Dinophysistoxin-2 (DTX2) was the dominant DSP toxin detected and, with the exception of one sample, was present in higher concentrations than okadaic acid. Azaspiracid-1 was the dominant AZP toxin and was detected in all samples. AZA-3 was only detected in 20% of the samples, while AZA-2 was not detected. The maximum concentration of DTX2 measured was 1970 ng/m^3 while the maximum concentration of AZA-1 measured was 42 ng/m^3. Overall, OA-equivalents (ΣOA+DTX2) were up to 65 times as concentrated in the phytoplankton samples as AZA-equivalents. Although most of the samples contained both groups of compounds, the samples with the highest concentrations of azaspiracids (42 ng/m^3) only contained intermediate levels of dinophysistoxins (330 ng/m^3) (compare Fig. 1a and b). Some differences in the geographical distribution of both toxin groups were noted. The highest concentrations of OA-equivalents typically occurred in samples from the southwest coast at sampling stations south of 52°N, while the highest concentrations of AZA-1 typically occurred in nearshore samples taken at sampling stations north of 52°N. The phytoplankton samples from the deepest net hauls yielded the extracts with the highest concentrations, thereby suggesting that the sample volume estimated from the diameter of the phytoplankton net and the haul depth can be used as a normalising factor for comparison of different sites. This comparison, however, is only considered semi-quantitative due to the bias that may be introduced by clogging of the phytoplankton net, which is difficult to quantify.

Discussion

This survey would not have been possible without the use of LC-MS-based methods, which have only emerged over the past decade as routine tools for the analysis of algal toxins. The results demonstrate that the LC-MS-MS method used in our routine monitoring is an excellent tool for the analysis of algal toxins in a variety of matrices, including phytoplankton isolated from seawater.

The overall dominance of OA analogues over azaspiracids in the phytoplankton was also reflected in the concentrations found in shellfish during the Irish national biotoxin monitoring programme in 2001, where the maximum concentration of OA-equivalents in whole flesh was ca. 48 µg/g, while the maximum concentration of AZA-equivalents was ca. 2 µg/g (Hess *et al.,* 2001). Similarly, the detection of DTX2 as the dominant DSP toxin in the plankton is also reflected in the detection of this toxin at higher concentrations than okadaic acid in shellfish samples typically found in Ireland in August (McMahon *et al.,* 1996: McMahon and Silke, 1998: unpublished monitoring data), associated with the seasonal succession of *Dinophysis acuminata* and *Dinophysis acuta.*

The differences in toxin patterns from different locations clearly show that the causative organism of azaspiracids is not directly related to the causative organism of OA or DTX-2. This insight was not evident from the shellfish monitoring during 2001, since DTX-2 and AZAs co-occured in a number of shellfish harvest sites (Hess *et al.,* 2001). More recent information also confirms these results, since in 2002

and 2003, OA and DTX-2 have also occurred in shellfish without azaspiracids being present (unpublished monitoring data).

Available data on the azaspiracid toxin profile in shellfish shows that typically AZA-1, AZA-2 and AZA-3 co-occur, with AZA-2 ranging from 16–34% of the total azaspricid concentration (Hess *et al.,* 2003; James *et al.,* 2002; unpublished monitoring data). It is interesting therefore to note the absence of AZA-2 in any of the phytoplankton samples analysed in this study. This result could be interpreted as evidence for this homologue being formed in shellfish rather than in phytoplankton, but James *et al.* (2003) have reported the presence of AZA-2 in *Protoperidinium crassipes* isolated from a bulk phytoplankton sample taken off the southwest coast of Ireland in 1999. Further studies on the AZA profiles in phytoplankton are necessary to describe the factors leading to the production of different AZA homologues.

Although the concentrations of toxins, expressed in ng/m^3 seawater, generally increased with closeness to the coastline, it remains difficult to interpret whether toxic phytoplankton are advected into the bays or if the toxic microalgae-producing azaspiracids develop *in situ* within a bay when local environmental conditions are favourable for their growth. Future research will focus on the analysis of bulk harvests of phytoplankton from yet larger phytoplankton nets and on the analysis of laboratory cultures of potentially AZA-producing organisms.

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Characterization of Lectin Binding Profiles for *Pfiesteria* spp. and Other Dinoflagellates

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Abstract

Previous research has indicated that lectin binding assays (LBAs) can be an effective method to distinguish among dinoflagellate species. Here, 18 fluorescein isothiocyanate (FITC)-lectin probes, conjugated to carbohydrate-binding lectins, were tested as a technique to differentiate among eleven species of dinoflagellates in clonal cultures, based on their demonstrated affinities for simple and complex moieties of chitobiose, glucose, galactose, fucose, lactose, and mannose. Three lectins were useful in distinguishing between *Pfiesteria piscicida* and *Pfiesteria shumwayae* actively toxic (fish-fed) versus nontoxic algal-fed strains. LBPs were also useful in evaluation of *Karenia* spp. isolates. *K. brevis* showed the highest diversity of surface-expressed polysaccharides. A *K. brevis* isolate with low brevetoxin (PbTx-2) production exhibited a LBP that differed significantly from that of another isolate with higher PbTx-2 production. *K. mikimotoi* was distinct from *K. brevis* in that it did not bind to GSLI or SBA. Overall, LBAs showed promise as a technique to screen for *Pfiesteria* spp. and *Karenia* spp. populations, and may have potential in discerning highly toxic strains from strains with low or negligible toxicity.

Introduction

Fluorochrome labeled cell-surface lectins have been widely used to identify bacteria and protists including algae (Slifkin and Doyle, 1990); more specifically, fluorescein isothiocyanate (FITC)-labeled lectins have been useful in analysis of cell-surface-expressed glycan moieties of dinoflagellates (Costas and Lopez Rodas, 1994; Costas *et al.,* 1995; Rhodes *et al.,* 1995; Lopez-Rodas and Costas, 1997; Aguilera and Gonzalez-Gil, 2001). Lectin binding patterns have been effective in distinguishing among dinoflagellate species within the same genus (Costas and Rodas, 1994; Myklestad, 1995; Aguilera and Gonzalez-Gil, 2001); among strains within a given dinoflagellate species (Lopez-Rodas and Costas, 1997; Cho 2001); and between nontoxic and toxic dinoflagellate species (Rhodes *et al.,* 1995).

In this study we tested lectin binding profiles (LBPs) for use in rapidly detecting dinoflagellate species such as *Pfiesteria* spp. and *Karenia brevis,* and in gaining insights about the physiological ecology of these species. We screened for the presence of cell-surface glycoconjugate components and unique LBPs in *Pfiesteria* spp. strains with different prey history (algae versus fish), and in several *Karenia brevis* strains, two of which are known brevetoxin producers. We hypothesized that some species would show unique lectin binding patterns, suggesting the potential for use of LBPs as rapid, inexpensive, microscopy-based assays for detecting these dinoflagellates.

Materials and Methods

The taxa used for this study were maintained at 22°C in natural seawater diluted to an appropriate salinity for each species (with Milli-Q® analytical grade water; then sequentially filtered through 25, 10, 5, 1 µm, activated carbon filters followed by ultra-filtration through 0.22 µm-porosity filters in a laminar flow hood). This technique produced water free of microbial contaminants before the addition of culture material, which was then enriched through the addition of Guillard's f/2-Si (Guillard 1975; Sigma Chemical Co.). The following clonal cultures of heterotrophic dinoflagellates were maintained at a salinity of 15 psu. *Pfiesteria piscicida* (clones CAAE1020C, CAAE1121C; non-inducible or NON-IND functional type of Burkholder *et al.,* 2001a,b) had been grown with algal prey (*Cryptomonas* HP9101, 30,000 cells mL^{-1} initially) for 9 months. *Pfiesteria shumwayae* (clones CAAE786T2, CAAE 270A2; long-term TOX-B functional type of Burkholder *et al.,* 2001a,b) had been grown with *Cryptomonas* prey for 14 months. Ichthyotoxic *P. piscicida* (clone CAAE101701) and *Pfiesteria shumwayae* (clone CAAE101642) were maintained in standardized fish bioassays (Burkholder *et al.,* 2001c) with live fish (juvenile tilapia, *Oreochromis niloticus*).

Two clones of the photosynthetic dinoflagellate, *Karenia brevis* (Wilson strain provided by D. Kamykowski, NCSU; and NOAA-1 strain, National Ocean Service [NOS], Charleston) had previously been known brevetoxin (PbTx-2, PbTx-3) producers. One clone of *Karenia mikimotoi* (strain 1651X, St. Petersburg, FL, isolated by C. Zheng, NCSU) was also analyzed, and had not previously produced detectable toxin (Dr. P. Moeller, NOS-Charleston, unpublished data). *Karenia* spp. strains were maintained at a salinity of 33 psu. The *Pfiesteria* spp. and *K. brevis* strains were tested for toxin (a water-soluble *Pfiesteria* toxin, PfTx, Moeller *et al.,* 2001; and PbTx-2, respectively) by NOS, Charleston, SC.

LBPs were determined in triplicate using 18 commercially available FITC-conjugated lectins (abbreviated here by their acronyms; Lectin Kit FLK-2100, FLK-3100, and FLK-4100; Vector Laboratories Inc., Burlingame, CA). The full names and additional information concerning the binding affinities of these lectins can be found at http://www.vectorlabs.com. Samples were assayed during late log-growth phase and fixed with 5% v/v formalin. For each assay, a 1-mL aliquot was removed and concentrated into a pellet by centrifugation (5,000 rpm for 5 min). The

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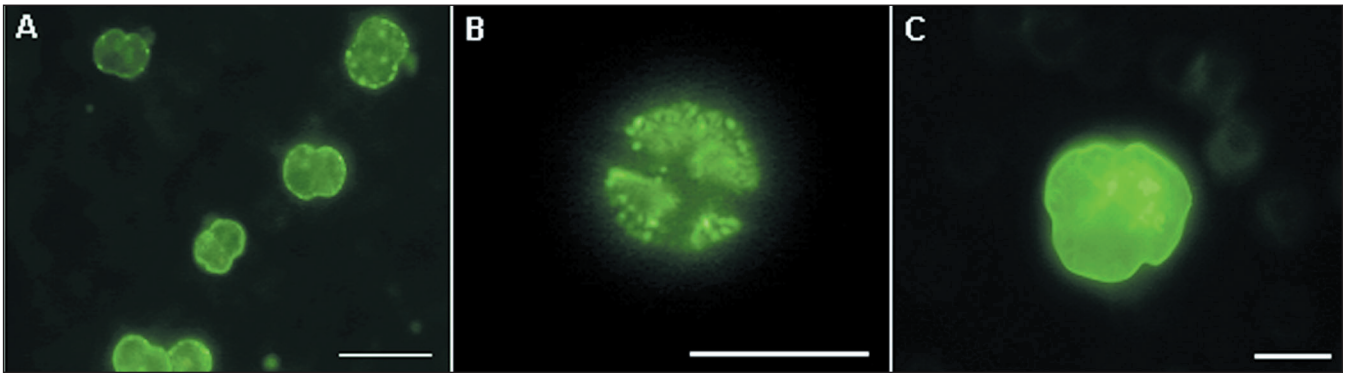

Figure 1 **A** *Pfiesteria piscicida* (fish-fed) flagellated cell bound with PNA lectin, **B** *Pfiesteria shumwayae* flagellated cell bound with WGA; **C** *Karenia brevis* flagellated cell (Wilson isolate) labeled with CON-A. Scale bars = 10 µm.

pellet was rinsed by resuspension in 500 µL of ultra-filtered (0.2-µm) phosphate-buffered saline (PBS), recentrifuged (5,000 rpm for 5 min), and resuspended in phosphate buffered saline (PBS). Sufficient lectin stock solution was added to effect a 50 µg mL^{-1} reaction concentration. Cells were collected on black polycarbonate membrane filters (1.0 µm-porosity; Operon Technologies) by vacuum filtration (250 mm Hg), rinsed twice with 15-mL PBS, and gently placed onto slides. Each filter was coated with Vectashield® Mounting Media (H-1000; Vector Laboratories Inc.), which prevented photo-bleaching and allowed for extended observations.

Cellular reactivity with the FITC-lectin probes was observed with an Olympus AX70 microscope equipped with a BX-FLA fluorescence attachment and a triple band filter cube (DAPI/ FITC/Texas Red-61002; Chroma Technologies Corp.). For all assays, the first ≥100 cells on each slide were qualitatively evaluated for lectin labeling intensity and localization (modified from Aguilera and Gonzalez-Gil, 2001). Labeling intensity (n = 3) was rated as: (++) strongly labeled cells with bright fluorescence from more than 80% of the cells evaluated; (+) weakly reactive cells with less then 80% labeling or a discernible lack of fluorescence intensity; or (–) non-labeled cells showing no fluorescence.

Table 1 Summary of lectin binding characteristics for *Pfiesteria* spp. FITC-conjugated DBA bound to *P. shumwayae,* but not to *P. piscicida*. GSLII and ECL bound preferentially to fish-fed *Pfiesteria* spp., but not to algal-fed *P. piscicida* or *P. shumwayae*.

	P. piscicida		*P. shumwayae*	
Lectin	on algae	on fish	on algae	on fish
DBA	–	–	+	+
PNA	–	+	+	+
GSLII	–	+	–	+
ECL	–	+	–	+
VVA	–	+	+	+

Results and Discussion

Pfiesteria piscicida exhibited LBPs that indicated the presence of chitobiose, simple and complex glucose moieties, mannose, sialic acid and/or N-acetyl-D-glucosamine (positive binding with CON-A, ECL, GSLII, LCA, PNA, PSA, VVA, WGA) (Fig. 1). In contrast, simple and complex galactose residues and N-acetyl- D-glucosamine residues were not detected in *P. piscicida* (negative for binding with DBA, SBA, LEL, STL). Nontoxic algal-fed *P. piscicida* cultures differed from fish-fed *P. piscicida* in lack of affinity toward PNA, GSLII, ECL and VVA (Table 1).

Actively toxic and nontoxic *Pfiesteria shumwayae* strains were differentiated from *P. piscicida* by affinity for DBA (Table 1). *P. shumwayae* expressed simple glucose and mannose moieties, complex glucose moieties, sialic acid and/or N-acetyl-D-glucosamine residues (positive for CON-A, Jacalin, LCA, WGA). PNA and VVA (α-lactose, α- or β-linked terminal N-acetylgalactosamine) consistently produced the strongest fluorescence intensities, and were considered the most promising for detecting/screening for *P. shumwayae*. Nontoxic *P. shumwayae* differed from toxic *P. shumwayae* in lack of affinity for ECL and GSLII (specific toward galactosyl [β-1,4] N-acetylglucosamine, α- or β- linked N-acetylglucosamine).

Table 2 Lectin binding signatures of *Karenia* spp. (5 of 18 lectins shown). A low toxin-producing clone (<2 pg/cell) of the Wilson strain exhibited diverse binding activity, whereas another strain with a relatively high rate of brevetoxin production (NOAA-1, >11 pg/cell) bound to only two lectins (GSL1, SBA).

Lectin	*Karenia brevis* NOAA Isolate	*Karenia brevis* Wilson Isolate	*Karenia mikimotoi* 1651X isolate
ECL	–	–	+
GSLI	+	+	–
SBA	+	+	–
Succinylated WGA	–	+	+
VVA	–	+	–

Karenia brevis expressed high affinity for most lectins tested, indicating complex carbohydrate expression at the cell surface. Simple and complex glucose and mannose moieties, simple and complex galactose moieties, sialic acid and/or N-acetyl-D-glucose amine, and fucose moieties were all detected (positive for CON-A, GSLI, Jacalin, LCA, PSA, SBA,WGA). *K. brevis* was the only species tested that demonstrated strong affinity for PHA-E (specific toward a complex polysaccharide, N-acetylgalactosamine). Differences in lectin binding were apparent among the two *K. brevis* isolates (Table 2). The NOAA-1 isolate, with relatively high brevetoxin production (11 pg cell $^{-1}$), bound to significantly fewer lectin conjugates then did an isolate of the Wilson strain with relatively low brevetoxin production (<2 pg cell $^{-1}$). The *K. mikimotoi* isolate did not bind to GSLI or SBA, whereas the two PbTx-2 producing *K. brevis* isolates bound strongly to those lectins.

LBPs showed promise as a technique for rapidly differentiating the dinoflagellate species examined in this study. Functional types of the two *Pfiesteria* spp. were distinguished by ECL and GSLII binding, and *P. shumwayae* was discerned from *P. piscicida* especially by its strong affinity for DBA. *Karenia brevis* and *K. mikimotoi* could be differentiated by GSLI and SBA binding. We plan to expand our analyses to examine potential links between toxin production and lectin binding activity by testing additional toxic and nontoxic strains of these and other dinoflagellate species.

Acknowledgements

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Multiple Tandem Mass Spectrometry Methods for the Determination of Toxic Cyclic Heptapeptides, Microcystins

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Abstract

Microcystins are chemically a very diverse group of heptapeptide toxins, with over 65 characterised to-date. Microcystins are specific inhibitors of protein phosphatases, PP1 and PP2A, and they can also act as potent tumour promoters. The challenges facing scientists in the detection of these toxins include the lack of commercially available toxin standards, poor recoveries from clean-up steps, interfering matrix compounds and inconclusive identification of unknowns. Here, we report the application of liquid chromatography-mass spectrometry (LC-MS) techniques for the trace detection and structural characterization of these microcystins. The MS techniques applied included electrospray ion-trap mass spectrometry and quadrupole time-of-flight (QqTOF) mass spectrometry. These techniques complement each other and allow the rapid identification and quantitation of trace cyanobacterial toxins in algae and drinking water samples.

Introduction

Toxic cyanobacteria waterblooms in lakes, rivers and reservoirs have become a common occurence in many countries (Skulberg *et al.,* 1984). These toxic waterblooms have caused the death of wild and domestic animals, plus illness and death in humans (Chorus and Bartram, 1999), (Pouria *et al.,* 1999). The hepatotoxins produced by cyanobacteria include the cyclic peptide toxins microcystins and nodularins. At least 65 microcystin analogs are known (Carmichael, 1997). Microcystins (Nishiwaki-Matsushima *et al.,* 1992) are liver tumour promoters (Ohta *et al.,* 1994). Epidemiological studies of certain areas in China show positive correlation between the presence of microcystins in water supplies and the incidence of human primary liver cancer (Yu, 1989). Thus, both acute and chronic exposure to cyanobacterial hepatotoxins may be significant human health risks. Electrospray ionization mass spectrometry (ESIMS) coupled with tandem mass spectrometry (MS/MS) is capable of detecting microcystins (Zweigenbaum *et al.,* 2000). Low-energy collision-induced dissociation (CID) mass spectra of microcystin –LR and –RR (Edwards *et al.,* 1993) and electrospray MS/CID of microcystins (Yuan *et al.,* 1999), (Robillot *et al.,* 2000) have been described. In this paper, an electrospray ion-trap mass spectrometry method (MS/MS) was developed for the detection and characterization of microcystins in water samples. Full characterization of microcystins was achieved using an orthogonal hybrid quadrupole time-of-flight (QqTOF) mass spectrometer.

Materials and Methods

Purchased chemicals included Microcystin-RR, Microcystin-YR, Microcystin-LR, Microcystin-LA (Calbiochem, Nottingham, UK); Microcystin-LW, Microcystin-LF (Alexis Corporation, Nottingham, UK). LC-spectrograde trifluoroacetic acid (TFA) was purchased (Sigma-Aldrich, Dorset, UK). LC-grade solvents were purchased from Labscan (Dublin, Ireland). Lakewater/algae samples (50 mL) were repeatedly freeze-thawed. The samples were then filtered and applied to a solid phase extraction (SPE) column. The SPE method utilised Bakerbond C_{18} Polarplus cartridges and was similar to a previously developed procedure (Keshavanath *et al.,* 1994). The cartridge was conditioned with methanol and water. A filtered lakewater sample (50 mL) was applied and the cartridge was washed with 10 mL portions of 5%, 15% and 25% methanol/water. Toxins were eluted using methanol (6 mL) containing 0.1% trifluoroacetic acid (TFA) and the eluent was evaporated to dryness and reconstituted in water (1 mL) for analysis. The LC system was a Waters 2690 Alliance (Waters Corporation, Milford, MA, USA) that was linked to a Finnigan MAT LCQ ion-trap mass spectrometer (Thermo-Finnigan, San Jose, CA, USA). Gradient chromatography was performed using acetonitrile-water (30:70 to 100:0) containing 0.05% TFA, over 42 minutes at a flow rate of 200 µL/min. The analytical column (Luna (2) C_{18}, 150 × 2.0 mm, 3 µm, Phenomenex, Macclesfield, UK) was operated at 35°C. Mass spectrometric analysis was carried out at atmospheric pressure using an electrospray ionisation (ESI) source and data were acquired in positive mode. MS/MS experiments were obtained by trapping the $[M+H]^+$ ion for each toxin, for subsequent fragmentation experiments to produce characteristic spectra for each toxin. Nano electrospray (ESI) quadrupole hybrid time-of-flight (QqTOF) MS was obtained using a QSTAR spectrometer (Applied Biosystems).

Results and Discussion

For method development, a mixture of six commercially available standard MCs and the pentapeptide, nodularin-R were used. The MS/MS spectrum for each standard was obtained. The optimized relative collision energies (RCE) were 35% for MC-RR, 35% for nodularin, 35% for MC-YR, 37% for MC-LR, 27% for MC-LA, 36% for MC-LW and 25% for MC-LF. The microcystins were determined using

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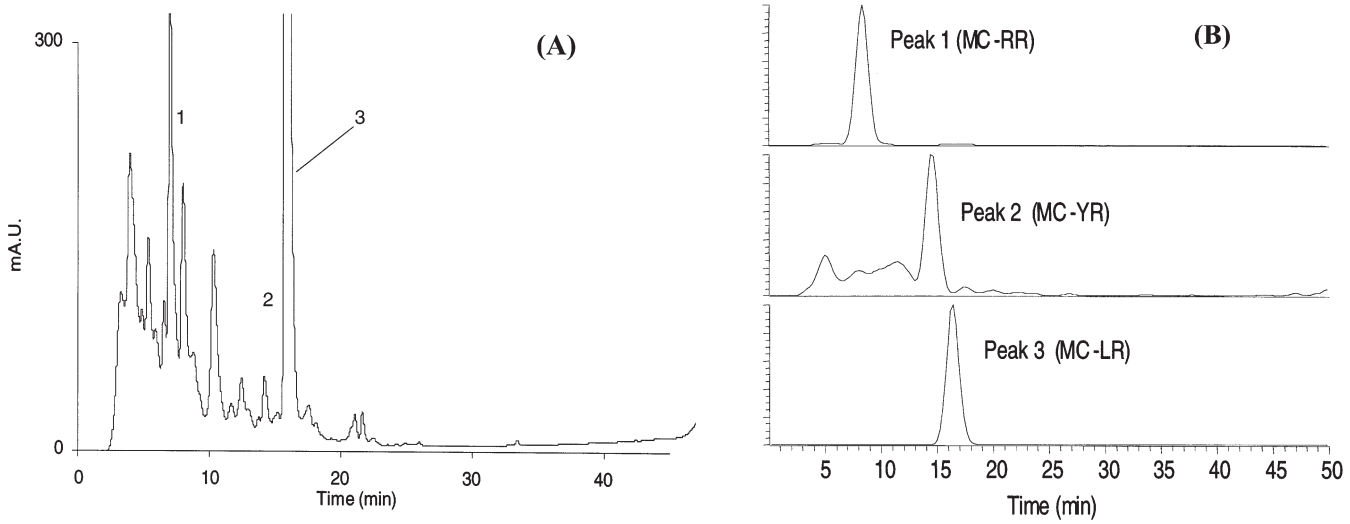

Figure 1 **A** LC-UV diode-array chromatogram of a freshwater sample, Drumiona Lake, Ireland, after SPE clean-up; Peaks 1) MC-RR, 2) MC-YR, 3) MC-LR. **B** Corresponding selected mass ion chromatogram LC-MS^2 chromatograms obtained on a quadrupole ion trap MS. The parent-product ion combinations used are: Peaks 1) MC-RR: *m/z* 1038 ⇒ 1021, 1020, 996 and 909, 2) MC-YR: *m/z* 1045 ⇒ 1028, 1027, 1017, 916 and 599, 3) MC-LR: *m/z* 995 ⇒ 978, 977, 967, 866 and 599.

the following target parent and fragment ion combinations in the mass spectrometer: MC-RR: *m/z* 1038 ⇒ 1021, 1020, 996 and 909; Nodularin: *m/z* 825 ⇒ 808, 807, 781, 776 and 674; MC-YR: *m/z* 1045 ⇒ 1028, 1027, 1017, 916 and 599; MC-LR: *m/z* 995 ⇒ 978, 977, 967, 866 and 599; MC-LA: *m/z* 910 ⇒ 893, 892, 878, 776 and 759; MC-LW: *m/z* 1024 ⇒ 1007, 1006, 992 and 962; MC-LF: *m/z* 986 ⇒ 969, 968, 954, 852 and 835. MC-LW and MC-LF were not completely chromatographically resolved but this did not hinder their determination as these toxins have different parent and fragment ion combinations which allowed their facile discrimination. Figure 1A shows the LC-UV diode array chromatogram for a cyanobacterial sample from Drumiona Lake, Co. Cavan. MC-RR, MC-YR and MC-LR were tentatively identified in this sample but confirmation was difficult due to the presence of several matrix peaks. Multiple tandem MS results in an improved signal/noise (S/N) ratio when compared with single stage MS and a 100-fold improvement in the S/N ratio was observed for MC-RR using LC-MS/MS mode compared with single stage MS in

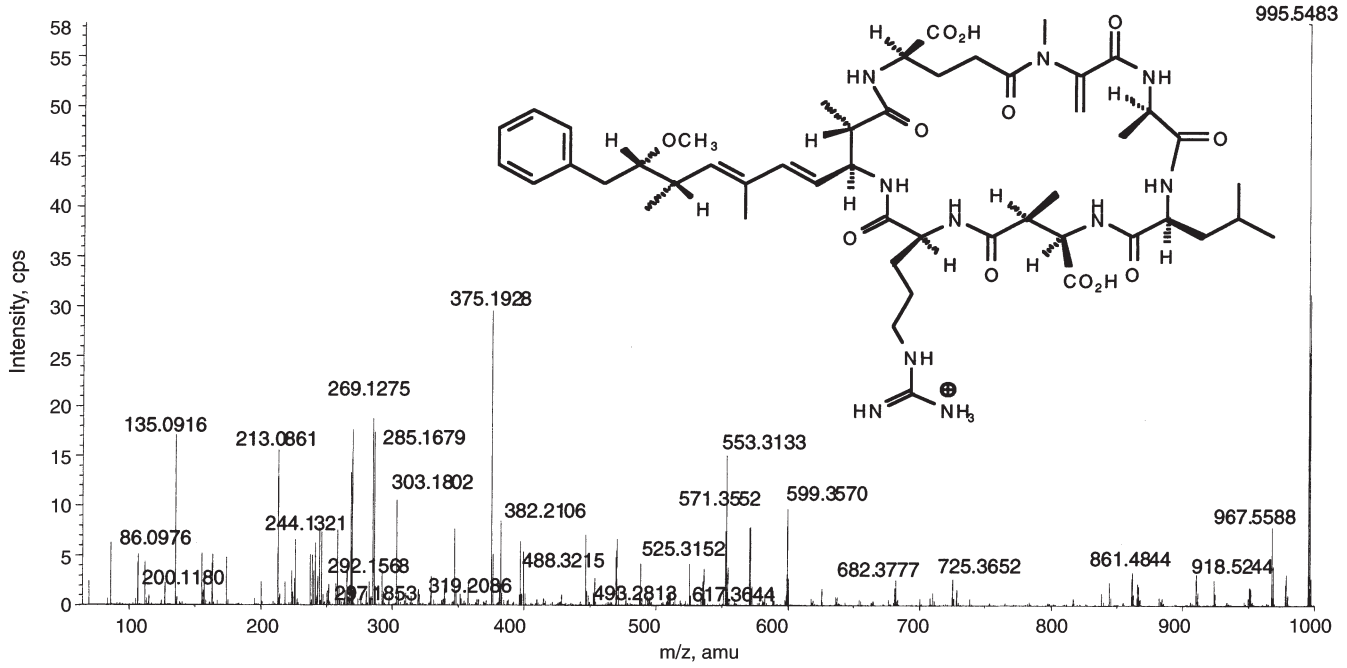

Figure 2 High mass accuracy spectrum (MC-LR) using a nano ESI source coupled with a QqTOF MS.

the quadrupole ion trap MS. Fig. 1B illustrated the power of LC-MS^2 analysis of cyanobacterial samples. Using MC-LR as the reference compound, optimum calibration and reproducibility data were obtained using LC-MS^2; 0.1 µg/mL – 5.0 µg/mL, $r^2 = 0.992$ (n = 3); % RSD ≤ 7.3 at 0.25 µg MC-LR/mL (n = 3). The detection limit (S/N = 3) was better than 0.1 ng.

In the full scan spectra, generated using the QqTOF MS, many single clusters were observed at *m/z* 995.5566 $[M+H]^+$, *m/z* 1017.5884 $[M+Na]^+$, *m/z* 1039.5807 $[M+2Na–H]^+$. Also some double charge ions are found at *m/z* 509.2853 $[M+H+Na]^{2+}$, *m/z* 520.2869 $[M+2Na]^{2+}$, *m/z* 531.2836 $[M+3Na–H]^{2+}$ and *m/z* 542.2797 $[M+4Na–2H]^{2+}$. QqTOF MS/MS studies on MC-LR were carried out to determine all possible fragmentation ions. This was achieved by ramping the collision energy from 20–90% over the product ion scan time of 40 min and accumulating all scans over that period. Predominant fragment ions were noted to occur in the lower mass range < *m/z* 400 (Fig. 2) at *m/z* 135, corresponding to the typical Adda group cleavage after the methoxy substituent, $[C_9H_{11}O]+$; at *m/z* 155 [Mdha+Ala+H]$^+$; at *m/z* 163 $[C_{11}H_{15}O]+$; at *m/z* 213 [Glu+Mdha+H]$^+$; and at *m/z* 375 $[C_{11}H_{15}O$+Glu+Mdha]$^+$. These five ions are common to all microcystins and are a valuable indicator in the detection of unknown microcystins (Zweigenbaum *et al.,* 2000). Less intense common microcystin ions were at *m/z* 284 [Ala+Mdha+Glu+H]$^+$; *m/z* 446 $[C_{11}H_{15}O$+Glu+Mdha+Ala]$^+$. Characteristic fragment ion generated by the loss of small molecules from MC-LR $[M+H]^+$ were also noted at *m/z* 967 $[M+H-CO]^+$, *m/z* 978 $[M+H-NH_3]^+$, *m/z* 977 $[M+H-H_2O]^+$ and *m/z* 951 $[M+H-CO_2]^+$. Other predominant fragment ions for MC-LR were at *m/z* 553 [Mdha+Ala+Leu+MeAsp+Arg+H]$^+$, at *m/z* 525 [Mdha+Ala+Leu+MeAsp+Arg+H-CO]$^+$ which was due to the loss of CO from *m/z* 553 and at *m/z* 682 [Glu+Mdha+Ala+Leu+MeAsp+Arg+H]$^+$. Since the amino acid arginine is present in MC-LR, this produces a number of distinguishable fragment ions at *m/z* 599 [Arg+Adda+Glu+H]$^+$, *m/z* 571 [Arg+Adda+Glu+H-CO]$^+$, the loss of CO, *m/z* 174 $[Arg+NH_3+H]^+$ and at *m/z* 286 [MeAsp+Arg+H]$^+$. New identified characteristic fragment ions found for MC-LR include the ions at *m/z* 127 [Mdha+Ala+H-CO]$^+$ are due to the loss of CO from the *m/z* 155 ion; *m/z* 157 [Arg+H]$^+$; *m/z* 195 [Glu+Mdha+H-H_2O]$^+$ the loss of H_2O from the *m/z* 213 ion; *m/z* 200 $[Arg+NH_2+CO]^+$; *m/z* 268 [Mdha+Ala+Leu+H]$^+$; *m/z* 269 $[MeAsp+Arg+H-NH_3]^+$ the loss of ammonia from the *m/z* 286; at *m/z* 292 $[C_{11}H_{15}O$+Glu]$^+$; at *m/z* 347 $[C_{11}H_{15}O$+Glu+Mdha-CO]$^+$ the loss of CO from the *m/z* 375 ion and at *m/z* 570 [Mdha+Ala+Leu+MeAsp+Arg+NH_2+2H]$^+$.

Acknowledgements

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Real-Time RT-PCR Study of Differential Expression of Two Genes in *Alexandrium tamarense* (Lebour) Balech, Cultured Under Varying Nitrate/Phosphate Ratios

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Abstract

We studied the differential expression of two genes in *Alexandrium tamarense* cultured under varying N/P ratios. These genes were S-adenosyl homocysteine hydrolase (*Sahh*) and methionine aminopeptidase (*Map*). The study was performed by means of real-time reverse transcription-polymerase chain reaction (RT-PCR). This technique allows us to directly quantify expression, without the use of gel electrophoresis, plus a confirmation technique. We observed changes in gene expression with N/P ratios. *Map* was strongly up-regulated during nitrogen deficiency and down-regulated during phosphorus deficiency. *Sahh* expression pattern was less consistent and showed strong down-regulation only during extreme nitrogen (N) deficiency.

Introduction

Paralytic Shellfish Poisoning (PSP) is caused by some dinoflagellate species of the genus *Alexandrium* which contain saxitoxin (STX), a potent neurotoxin. There are more than 20 known STX derivatives that differ in structure and toxicity (Shimizu 1993). Most studies to date have examined the toxin content of dinoflagellates on time scales of days to weeks using batch cultures. These studies have shown that toxin content varies with nutrient limitation (Boyer *et al.,* 1987; Anderson, 1990).

Taroncher-Oldenburg *et al.* (2000) found some genes in *Alexandrium fundyense* that showed differential display throughout the cell cycle during synchronized circadian growth, among them S-adenosylhomocysteine hydrolase (*Sahh*) and methionine aminopeptidase (*Map*). The first was up-regulated, and the second down-regulated, during G1, the phase at which toxin production was maximal. *Sahh* and *Map* code for important enzymes in the cell protein synthesis. In the present study we aimed at quantifying the expression of the two genes in *Alexandrium tamarense* semicontinuous cultures under five different N/P ratios, ranging from extreme N deficiency to extreme phosphorus (P) deficiency. We performed the RT-PCR technique using quantitative real-time RT-PCR.

Real-time PCR is the most sensitive of the mRNA quantification methods (Wang and Brown, 1999; Bustin, 2000) and is adequate to compare patterns of mRNA expression between different sample populations. It uses fluorescence techniques and combines amplification, detection and quantification. The method is highly accurate because fluorescence development is measured for each individual cycle. The whole process, from RT to final quantification, can be automated.

Table 1 Nutrient concentration in each treatment.

N/P ratio	N (mol L^{-1})	P(mol L^{-1})
1.6/1	2.4×10^{-5}	1.5×10^{-5}
6.4/1	9.6×10^{-5}	1.5×10^{-5}
16/1	24×10^{-5}	1.5×10^{-5}
40/1	2.4×10^{-4}	6.0×10^{-6}
160/1	24×10^{-4}	1.5×10^{-6}

Materials and Methods

Cultures and Growth Conditions A clone of *A. tamarense,* KAC01 (from the University of Kalmar, Sweden culture collection), was grown under semicontinuous culture in Guillard medium. Nitrate and phosphate concentrations and ratios are shown in Table 1.

RNA Extraction Cells were collected by centrifugation at 3200 rpm and 4°C. Total RNA was extracted and purified with the RNeasy mini kit (Qiagen, Venlo, The Netherlands). RNA was eluted in DEPC-water. RNA concentration was determined by ultraviolet (UV) spectrometry. RNA was preserved at –80ºC.

cDNA Synthesis To 0.5–1 µg total RNA we added 2.5 µM poly-T primer, 500 µM dNTPs and DEPC-water. The mixture was heated at 65ºC for 5 min. to denature secondary structure. Then we added buffer ×5 and 40 U RNAse Out and maintained in a bath for 10 min. to facilitate annealing of the hexamers to RNA. Then it was incubated at 42ºC for 2 min. Finally, we added 200 U Superscript II reverse transcriptase and kept the reaction at 42ºC for 50 min. (All reagents used in this step were from Gibco BRL, Life Technologies, Inc., MD).

Real-Time PCR Quantitation of expression of the two genes, *Map* and *Sahh,* was done relative to the housekeeping gene 23S ribosomal RNA, by means of real-time PCR. By means of the software Primer Express, we designed a pair of primers (forward and reverse) specific for each gene and a Taqman probe internal to the fragment amplified by each primer pair. Table 2 shows the primer and probe sequences designed, as well as those for the housekeeping 23S ribosomal gene fragment. As a reporter we used FAM (6-carboxy-fluorescein) in 5' and as quencher, TAMRA (6-carboxy-N,N,N′,N′-tetramethyl-rhodamine) in 3'. As

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Table 2 Sequences of designed primers and probes.

Map
FORWARD: 5'-GGGTGAAGGTGGTGCCTTC-3'
REVERSE: 5'-TCCATGGAGTGCCTCAGGTC-3'
PROBE: 5'-CGAAACAGCGACAATCGAAAGATGA-3'
Sahh
FORWARD: 5'-CTTGATGTTCTCCA-3'
REVERSE: 5'-TGGCCACTTCGACAACGA-3'
PROBE: 5'-CGAAACAGCGACAATCGAAAGATGCA-3'
RNAr23s
FORWARD: 5'-TTCCAATGCCAAGGAGTGTG-3'
REVERSE: 5'-GCGTCAATGAGGGTGAGAATC-3'
PROBE: 5'-TGTTTGTCATGTGCAGCCCTCTGTGC-3'

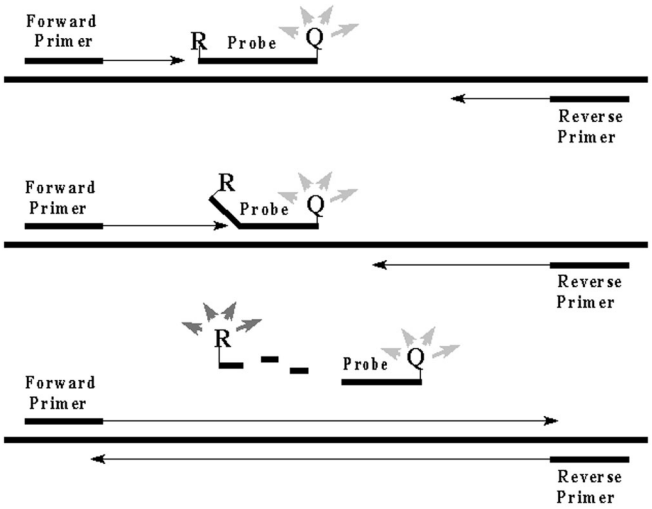


Figure 1 Amplification process of cDNA by means of the primer pairs and the Taqman probe.

an internal reference control we used the fluorochrome ROX (carboxy-X-rhodamine). Figure 1 shows the amplification process. Quantification of gene expression variation, under different environmental conditions, requires its comparison with the expression of a known gene that does not vary under those conditions. To this end, we used the ribosomal 23S gene, after confirming that its expression level remained constant under the five different N/P ratios. Only expression variations that can be assessed against a fixed expression level of a housekeeping gene can be reported as real.

cDNA amplification was performed in the thermocycler ABI Prism™ 7700 Sequence Detector System 1.7 A (Applied Biosystems, Foster City, CA). Technique optimization for reproducible results led to the use of the following product concentrations: 1:4 dilution of cDNA, 300nM primers, 200 nM probe (plus Master Mix) (AmpliTaq Gold, $MgCl_2$, dNTPs, buffer, dUTP, Amperase, Uracil N glycosilase). The amplification conditions were 2 min. at 50°C, 10 min. at 95°C, 1 min. at 60°C, repeated through 45 cycles.

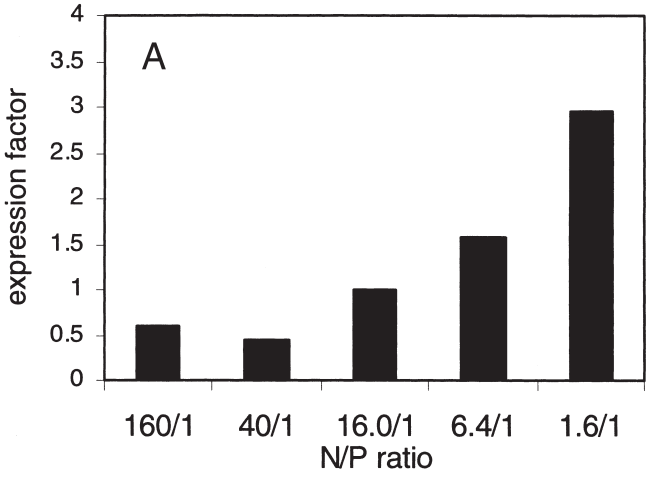
Results and Discussion

Figure 2 shows the expression level of *Map* and *Sahh* under the different culture conditions. Data was normalized to values from the N/P = 16 treatment, which was considered to be the "control."

Map expression increased steadily with N deficiency, showing a value three times higher under extreme N deficiency than under control conditions, and half that value under phosphate deficiency. *Map* was strongly up-regulated under N deficiency, and down-regulated under P deficiency. This gene has been found to be up-regulated in synchronized cultures of *A. fundyense* during the G1 phase of the cell cycle, coincidently with saxitoxin production (Taroncher-Oldenburg *et al.,* 1997; Taroncher-Oldenburg and Anderson, 2000).

Sahh expression trend was not as well defined as that of *Map.* It was down-regulated only during maximum N-deficiency and up-regulated in all the other conditions relative to the control.

Map was down-regulated under P deficiency and N sufficiency, in which conditions saxitoxin production was maximal (data not shown), so that both processes were not simultaneously enhanced. *Map* regulation in the cell is



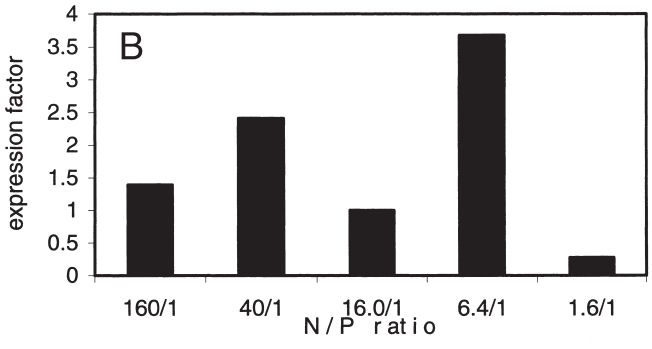


Figure 2 Quantitative differential expression of **A** *Map* and **B** *Sahh* under the varying conditions, relative to control (N/P = 16).

complicated. *Map* enzyme removes methionine from nascent peptides during protein synthesis, contributing to protein maturation and methionine turnover. Nitrogen deprivation in yeast leads to arrest of the cell cycle in G1. (Gallego *et al.,* 1997). If this happened to our cultures, it would explain the up-regulation of *Map* under N-deficiency, since most cells would be in G1, when *Map* is up-regulated (presently under flow cytometry study). This would suggest *Map* regulation through the cell cycle phase, which would, in turn, be induced by cell nutrient status in our experiments.

Sahh was strongly down-regulated only under extreme N deficiency. *Sahh* is a regulator of trans-methylation in general. It is down-regulated in mammal cells under apoptosis, and was down-regulated during the G1 phase of *A. fundyense* cell cycle (Taroncher-Oldenburg and Anderson, 2000). The enzyme is inhibited by adenosine analogs. Disruption of the hydrolase gene in photosynthetic bacteria is related to diminished synthesis of photosynthetic pigments (Aksamit *et al.,* 1995), and expression levels of *Sahh* in our study follow a pattern similar to those of peridinin-chlorophyll *a* binding protein, PCP (Martínez *et al.,* 2000, and data not shown), which suggests a link between *Sahh* activity and pigment synthesis. These results have to be further investigated.

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Using Real-Time PCR to Detect Toxigenic Strains of *Microcystis aeruginosa*

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Abstract

Eleven different strains of *Microcystis aeruginosa,* six of which were previously documented to be toxigenic, were tested with specific primers and probes. As predicted, only the strains with a toxigenic history were positive for the primer and probe set designed to detect the NMT region of the *mcyA* gene. The cycle threshold (Ct) values were significantly correlated with optical density (OD) of the cultures and the dsDNA measured in the extract, $R^2 = 0.86$ and $R^2 = 0.82$, respectively. Using real-time PCR to detect toxigenic strains of *M. aeruginosa* was successful with eleven laboratory strains and indicated the potential for a highly sensitive and precise assay. Further testing to assess the correlation between the presence of this genetic region and toxigenicity of environmental samples is needed.

Introduction

Polymerase Chain Reaction (PCR) used for detection of toxigenic strains of the cyanophyte *Microcystis* has increased the specificity of analytical methods for this organism to a level never possible with microscopy. Real-time PCR, also known as 5'-nuclease PCR, alleviates many more hours of skilled labor; offers greater accuracy for quantitative measurements; and has lower propensity to contaminate the laboratory with amplified DNA than PCR using agarose gels with electrophoresis. Moreover, there is better sensitivity and less bias using real-time PCR instead of gel-type PCR (Becker *et al.,* 2002). We have used polynucleotide sequences within the 16S ribosomal RNA gene and the *mcyA* gene in the microcystin synthetase operon to develop primer/probe sets for a multiplex 5'-nuclease PCR assay. Presumably the primer/probe set developed for the *mcyA* gene will only give positive results with strains that produce microcystins, while the 16S amplification serves as a positive control.

Materials and Methods

Oligonucleotide sequences previously used in PCR assays with electrophoresis to detect the16S gene and the N-methyltransferase (NMT) region of the *mcyA* gene (Kondo *et al.,* 1998; Tillett *et al.,* 2001) were entered into the basic logic alignment search tool, which searches GenBank databases.[1] Nineteen polynucleotide sequences for the *mcyA* gene and four sequences for the 16S gene were found. The 19 sequences for the *mcyA* gene were aligned using Bio Edit©[2] and all were similar with no pair less than 95% identical. All of the 23 sequences (19 for the *mcyA* gene and 4 for the 16S gene) were entered into the Primer Express®[3] software with the following parameter settings for the primers: 1) melting temperature range of 58° to 60°C; 2) GC content between 30% and 60%; 3) 1 residue on the 3' end required to be a G or C; and 4) an optimal length of 20 base pairs (bp). Parameters for a TaqMan® probe included a melting temperature range of 75° to 85°C and a length between 50 and 150 bp. We selected primer/probe sets using two criteria: 1) the lowest probability of meeting the designated parameters by random chance; and 2) the highest number of *M. aeruginosa* strains that contained the selected sequences as found in GenBank for each primer and probe.

Eleven different strains of *M. aeruginosa,* six of which were previously documented to be toxigenic, were obtained from the University of Texas Culture Collection[4] and established in BG-11 media[5]. These strains were originally

Table 1 Cycle threshold (Ct) values generated with multiplex PCR for 11 strains of *M. aeruginosa.*

UTEX Identification	Toxigenic History	Ct, 16S gene	Ct, *mcyA* gene
B 2662	Yes	18.43	18.88
LB 2664	Yes	18.33	19.17
B 2666	Yes	23.21	23.78
B 2667	Yes	18.27	18.40
B 2669	Yes	19.34	19.93
B 2670	Yes	17.44	17.92
LB 2386	No	18.15	Negative
B 2661	No	18.64	Negative
B 2671	No	17.33	Negative
B 2672	No	21.91	Negative
B 2676	No	18.11	Negative
Negative control		Negative	Negative

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Table 2 Comparison of cycle threshold with optical density and DNA content of four toxigenic cultures.

Dilution	Ct, 16S gene	Ct, *mcyA* gene	Culture OD (A_{680})	dsDNA (nG)
None	19.09	20.39	.468	31.8
1:1	20.13	21.00	.224	17
1:10	23.23	24.24	.01	2.1
None	15.81	16.32	1.749	48.4
1:1	16.96	17.33	1.053	32.8
1:10	19.39	19.7	.104	8.1
None	17.19	17.71	.528	60.6
1:1	19.58	20.19	.262	14.3
1:10	22.07	22.73	.025	3.6
None	16.43	16.51	.808	58
1:1	17.19	17.59	.471	36.8
1:10	20.76	21.22	.046	3.9
Negative control	neg	neg	.037	0

isolated from samples collected in Canada, Australia, South Africa, and the United States. The cultures were maintained at 20°C with a light intensity of 1,800 Lux (approximately 22.5 µEinsteins m^{-2} s^{-1} of photosynthetically active radiation). Optical density of the cultures was measured using a Perkin Elmer MBA 2000 spectrophotometer[6] with absorbance at 680 nm (A_{680}). DNA was extracted using the Dneasy™ Tissue Kit[7]. Nucleic acids were quantified by measuring the amount of UV irradiation absorbed at 260 nm following Sambrook and Russell (2001).

The primers and probes were used to test the 11 strains of *M. aeruginosa.* Working dilutions were calculated using the worksheets provided at the Integrated DNA Technologies Inc. website.[8] The Smart Cycler® System[7], with thermal cycler and real-time optical detection, was used with this cycling program: initial hold at 95°C for 70 sec; 40 cycles of 95°C denaturation for 5 sec; 59°C annealing and extension for 55 sec. Each of the two primer/probe sets was optimized individually before multiplex assays were performed. An initial assessment of the quantitative capability of the 5'-nuclease PCR assay was performed using dilutions from 4 toxigenic cultures. PCR products for the 16S gene found in the presence or absence of NMT products served as a positive control to show that PCR inhibitors were not present and that analytical procedures were not compromised.

Results

The Smart Cycler® System produced cycle threshold (Ct) values representing the first cycle in which there was a significant increase in target signal. The target signal is fluorochrome emission when the nucleotide sequence of the probe has been cleaved during the thermocycling reaction. As predicted, only the strains with a toxigenic history were positive for the primer and probe set designed to detect the NMT region of the *mcyA* gene (Table 1). The Ct values were approximately equal for both *mcyA* and 16S genes in the toxigenic strains, indicating a similar number of copies of each gene in the template DNA (Tables 1 and 2). The Ct values had a significant correlation with the optical density (OD) of the cultures (n = 24) and the dsDNA measured in the extract (n = 24), $R^2 = 0.86$ and $R^2 = 0.82$, respectively (Figs. 1 and 2).

Discussion

Using real-time PCR to detect toxigenic strains of *M. aeruginosa* was successful with 11 laboratory strains and in-

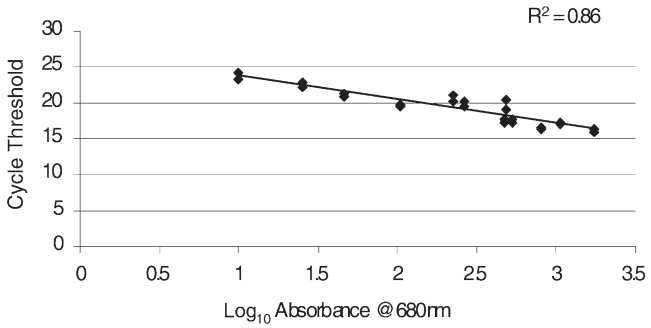

Figure 1 Cycle threshold vs. optical density of the cultures (n = 24).

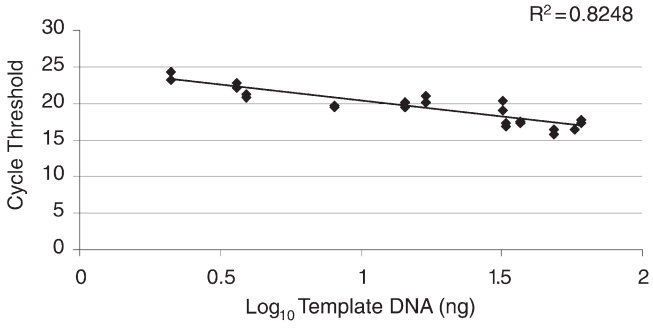

Figure 2 Cycle threshold vs. DNA content (n = 24).

dicated the potential for a highly sensitive and precise assay. To date, the NMT region of the *mcyA* gene has been detected in 18 toxigenic cultures and 2 non-toxigenic cultures, but not in 17 other non-toxigenic cultures (Tillet *et al.,* 2001). Further testing to assess the correlation between the presence of this genetic region and toxigenicity of environmental samples is needed. Moreover, the specificity and interlaboratory precision of the probe and primer sets used in this study will have to be determined. We have cloned the target sequence from the *mcyA* primer/probe set in *E. coli* and are conducting experiments to estimate the number of copies for this sequence in *M. aeruginosa* so that the sensitivity of the assay can be calculated.

Acknowledgements

We would like to acknowledge the logistical assistance of Mary Ann Feige of the Environmental Protection Agency and the support of the Postgraduate Research Participation Program administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the US DOE and the US EPA.

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Footnotes

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[3] Applied Biosytems, 850 Lincoln Centre Dr, Foster City, CA

[4] School of Biological Sciences, The University of Texas at Austin

[5] Sigma-Aldrich Co., PO Box 14508, St. Louis, MO

[6] Perkin-Elmer, 710 Bridgport Ave, Shelton, CT

[7] Qiagen Inc., 28159 Avenue Stanford, Valencia, CA

[8] Integrated DNA Technologies Inc., 1710 Commercial Park, Coralville, IA http://www.idtdna.com/program/dilutioncalc/dilutionDryCalc.asp

Use of *Alexandrium* rRNA Targeted Probes to Predict PSP Events on Kodiak Island, Alaska

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Abstract

Alexandrium catenella abundance was ascertained at a nearshore site on Kodiak Island during 2000 and 2001 using species-specific LSU rRNA targeted oligonucleotide probes in whole cell (WC) and sandwich hybridization (SH) assay formats. Results of the SH assay were calibrated by enumerating *A. catenella* cells collected in samples throughout the two field seasons using the WC assay. *A. catenella* abundance exhibited two distinct peaks (>600 cells L^{-1}) in 2000, one in May/June and a second in August–September, both of which lasted approximately two weeks. *A. catenella* abundance in 2001 was much more sporadic, with 4–5 peaks (400–800 cells L^{-1}), each lasting only 3–7 days. *A. catenella* abundance tracked water column toxicity as determined via a ^{3}H-Saxitoxin receptor-binding assay in 2001. *A. catenella* toxicity averaged 13.7 pg STX equivalents per cell. Importantly, DNA probe data revealed a strong correlation between *A. catenella* abundance and blue mussel (*Mytilus edulis*) toxicity in both 2000 and 2001. The results also demonstrated that increases in *A. catenella* abundance preceded elevated toxin levels in shellfish, suggesting that the DNA probe assays could prove useful as monitoring tools to predict toxic outbreaks prior to shellfish harvest.

Introduction

Alexandrium catenella is the primary species responsible for PSP in Alaska. PSP toxin levels periodically exceed 20,000 mg of saxitoxin per 100 g shellfish (RaLonde, 1996). Monitoring PSP events in Alaska is problematic because of the long, remote coastline. Here we report results of a study using DNA probes to detect and quantify the abundance of *A. catenella* during the summers of 2000 and 2001 on Kodiak Island, Alaska.

Materials and Methods

Water and mussel samples were collected in Trident Basin, Kodiak Island, Alaska, USA (57°47′N, 152°24′W). Trident Basin has an average depth of 27 m and was well- mixed with near-uniform salinity (average 32.5 ‰) and temperature (7°–13°C) during the sampling dates. Water was collected from a dock (water depth = 17 m) every 3–14 days during summer months of 2000 and 2001 during daylight high tide events. A plankton net (20 µm mesh; Aquatic Research Instruments; 20 cm × 80 cm) was towed from a depth of 5 m to the surface in 2000 and cells concentrated to approximately 250 mL. A 5.5-L Niskin bottle was used in 2001 to collect samples from five discreet depths (2× casts from 1,3,5,10, and 17 m). Ten L from each depth were concentrated through 20 µm Nitex mesh, rinsed and concentrated to approximately 100–150 mL. Aliquots for sandwich hybridization (SH) were filtered from each sample in duplicate onto 0.65 µm Durapore (Millipore) filters and stored at –70°C for up to 6 months. Assay protocols followed Scholin *et al.* (1999) using the *Alexandrium* NA1 LSU rRNA targeted capture probe and a signal probe based on that used for *Pseudo-nitzschia* (Scholin, 1999 and unpubl. data). Triplicate OD_{650} readings were averaged for each replicate and results from duplicate samples were averaged. In 2000, a single estimate of *A. catenella* abundance was obtained for each sampling date. Subsequent side-by-side comparisons of plankton net samples and Niskin bottle samples demonstrated that the plankton net underestimated *A. catenella* abundance by a factor of 6.5; the 2000 plankton net data reported here were multiplied by 6.5 for correction. In 2001, water column estimates of *A. catenella* abundance were based on depth-weighted (*i.e.,* integrated) averages from the five discreet sample depths.

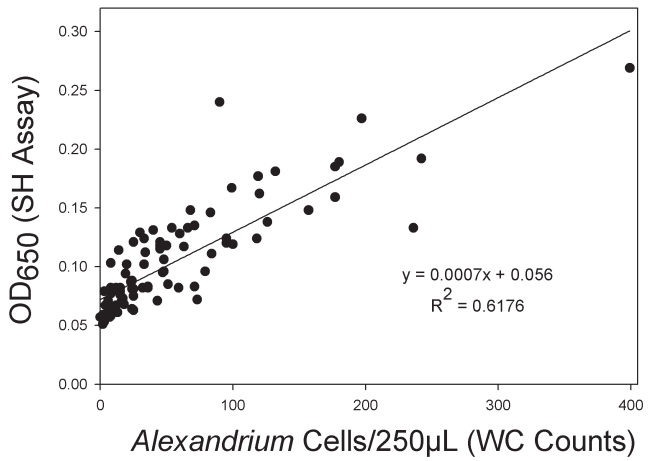

Figure 1 Standard curve derived from 2001 field data.

To calibrate the SH assay, *A. catenella* whole cell (WC) counts were performed 14 times in 2001. Aliquots from each sampling period were filtered in replicate, labeled with the NA1 probe. The entire filters were counted 2 times by epifluorescence microscopy (Miller and Scholin, 1998) and results averaged. For the WC assay, 4–10 filters were collected with various cell densities (to ensure coverage within the standard curve), frozen at –80° C, and analyzed by the SH assay. Data collected for the WC and corresponding SH assays throughout 2001 were combined to generate a stan-

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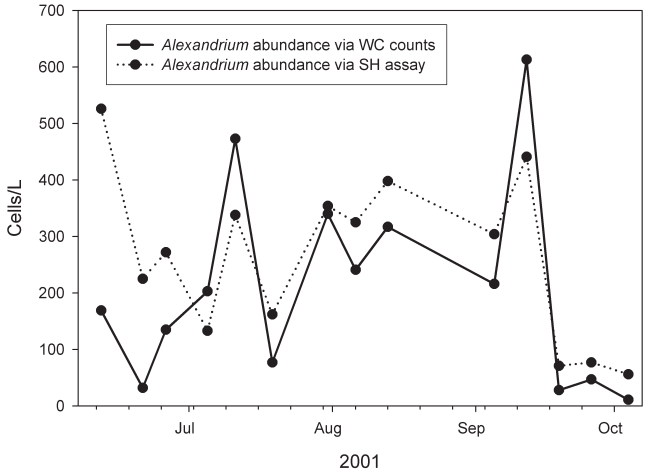

Figure 2 Relationship between *Alexandrium* abundance determined on the same sample at each time point with both the WC and SH assays in 2001.

dard curve via linear regression. *A. catenella* abundance data for 2001 were estimated from this standard curve (Fig. 1); a different curve was used for 2000 (Matweyou, 2003).

Water column toxicity was determined by filtering aliquots of concentrated (through 20 µm Nitex mesh) seawater onto 47 mm, 0.45 µm Millipore HA filters. Mussel toxicity was determined on approximately 40 mussels collected weekly from a beach in close proximity to the water collection site. Mussel toxins were extracted according to AOAC protocol (AOAC 1995). Water column and mussel toxicity were determined by the receptor-binding assay (Trainer and Poli, 2000).

Results and Discussion

Variation between triplicate OD readings for the SH assay was determined from a subset of 174 samples (out of 468 total samples). The average coefficient of variation among these samples was 14% with a range from 0–65%. Of the total 468 field samples processed with the SH assay, 83% of the samples run in triplicate on the assay plate had a co-

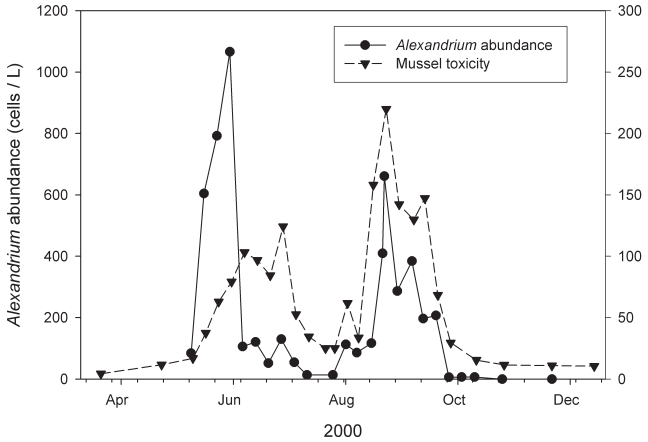

Figure 4 Relationship between *Alexandrium* abundance and shellfish toxicity in 2000.

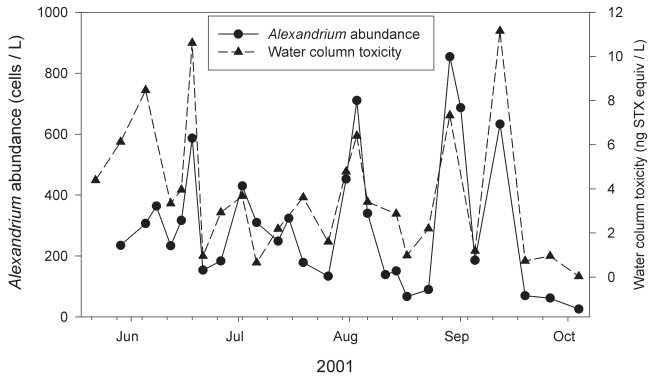

Figure 3 Relationship between *Alexandrium* abundance and water column toxicity in 2001.

efficient of variation equal to or less than 20%, the accepted CV of this study. Neither "fliers" nor "dropout" OD values were discarded in the data reported here. Both "fliers" and "dropouts" were generally, but not always, easy to identify, and represent one problem that needs to be addressed in the future. Both WC and SH assay results are shown for *A. catenella* abundance in 2001 (Fig. 2) using the 14 samples that generated the standard curve (Fig. 1). Results were similar except for three samples in June. The SH assay estimates were approximately twofold greater than those obtained using the WC method (range 0.6 to 6.9 times). Scholin *et al.* (1997) also found a twofold overestimation using the same types of assays with *Pseudo-nitzschia australis.* These over-estimations may be due to environmentally induced variability in the rRNA content of target cells (Anderson *et al.,* 1999) or perhaps grazing if the rRNA of target cells in the gut of zooplankton is intact but cells are unrecognizable using microscopy.

A. catenella abundance as determined by the SH assay was correlated with water column toxicity in 2001 (Fig. 3). *A. catenella* toxicity averaged 13.7 pg STX equivalents per cell (range 2.0–44.8), well within the expected range (Cembella, 1998). Large-scale seasonal patterns in cellular toxicity were not evident. *A. catenella* abundance was also correlated with mussel toxicity in 2000 (Fig. 4) and 2001 (Fig. 5). The correlation between *A. catenella* abundance and shellfish tox-

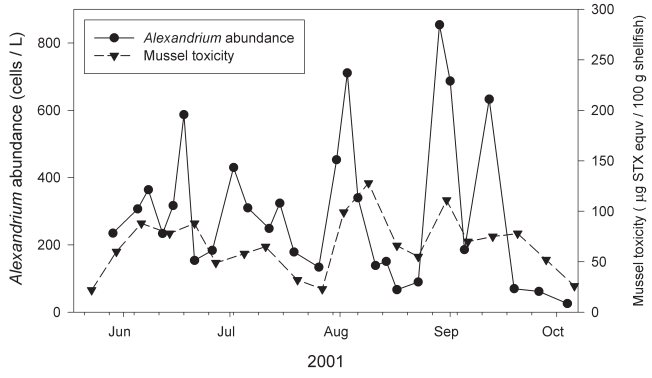

Figure 5 Relationship between *Alexandrium* abundance and shellfish toxicity in 2001.

icity was seen more clearly in 2000 (Fig. 4) with the general trend of increased *A. catenella* abundance followed by, or closely linked to, a rise in mussel toxicity. The *A. catenella* data from 2000 data suffer, however, because they were derived from samples collected with a plankton net, which can clearly lead to arbitrary uncertainties in terms of precision. Regardless, the general trends in *A. catenella* abundance reported in 2000 clearly match those expected based on mussel toxicity (Fig. 4).

We show here that the SH assay can be used in a semi-quantitative manner to estimate *A. catenella* abundance in water samples from Kodiak Island, Alaska. Data from the SH assay was also correlated with water column toxicity (r^2 = 0.7093) and with (after a small lag) shellfish toxicity. The SH assay was rapid, easy to use, and has potential to be an excellent tool in gathering real-time *A. catenella* abundance data and, by default, predicting shellfish toxicity. Throughout most of the 2001 study period, shellfish toxicity remained low, hovering at or below safe consumption levels (80 µg STX equivalents 100 g^{-1}). Three small *A. catenella* blooms with relative densities of 550–800 cells L^{-1} corresponded to increased toxicity above quarantine levels. The 2000 season exhibited two blooms with cell densities between 600–1100 cells L^{-1}. It appears from these data that *A. catenella* cell densities of about 600 cells L^{-1} are reason for concern on Kodiak Island.

A number of problems with the SH assay must be rectified if the test is to be used for routine monitoring. For example, there were occasional problems with "dropout" and "flier" OD readings, variations in triplicate OD readings for individual samples (about 15% of the time), and problems in generating standard curves that convert SH data to cell density (*i.e.,* Should cultures or field-grown cells be used? How should week-to-week variations in rRNA content be handled?). Overall, this report provides compelling evidence that DNA probe chemistry can be used to estimate the abundance of a HAB species and to predict shellfish toxicity.

Acknowledgements

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Variation in Reactivity of rRNA-Targeted Probes Towards *Pseudo-nitzschia multiseries* Grown in Nitrate- and Silicate-Limited Continuous Cultures

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Abstract

Physiologically stressed cells of *Pseudo-nitzschia multiseries* were used to investigate the labeling efficiency of molecular probes designed to detect these diatoms. Cells were grown in continuous culture under either nitrate or silicate limitation and were analyzed using both sandwich (SH) and whole cell (WCH) hybridization assays. Under nitrate limitation, labeling efficiency of both methods was diminished, relative to cells grown under nutrient replete conditions. Cells grown under silicate limitation were found to have an increased signal relative to expectation when analyzed by SH, and showed a fluorescence intensity similar to expectation for nutrient replete cells when analyzed by WCH.

Introduction

To rapidly enumerate harmful algal species, we developed DNA probes as diagnostic tools for quantitative detection of microalgae, a sandwich hybridization (SH) method in which cells are lysed and the resulting cell-free homogenate is processed using an automated robotic processor, and a whole cell fluorescent *in situ* hybridization (WCH) method in which cells are processed intact. Both techniques have been applied to detect toxic and potentially toxic diatoms (*Pseudo-nitzschia*), dinoflagellates (*Alexandrium*) and raphidophytes (*Heterosigma, Fibrocapsa*) in natural samples (Scholin *et al.,* 1999; Tyrrell *et al.,* 2002, and ref therein). In an effort to investigate how cell physiology affects probe reactivity and resultant estimates of *Pseudo-nitzschia multiseries* density, probes were tested against cells grown in continuous culture under a range of nitrate- and silicate-limiting conditions using dilution rates (= specific growth rates) ranging 0.2–1.2 per day. Samples were analyzed with the SH and WCH techniques using a *P. multiseries*-specific probe (Scholin *et al.,* 1999). A receptor binding assay for domoic acid (DA) was also applied to cells and culture media (Van Dolah *et al.,* 1997).

Materials and Methods

Cultured *P. multiseries* used in this study was isolated from Monterey Bay, California, USA, and grown in modified f/2 medium. For nitrate-limited trials, the medium was adjusted to 40 µM nitrate, 120 µM silicate and 40 µM phosphate. For silicate-limited trials, the medium was prepared with 500 µM nitrate, 40 µM silicate and 40 µM phosphate. Cells were harvested and examined using WCH (Miller and Scholin, 1998), SH using 10,000 cells per well (Scholin *et al.,* 1999), and particulate and dissolved DA were measured (Van Dolah *et al.,* 1997). Steady state was determined when cell counts remained constant for several days.

Results

Nitrate Limitation Cell abundance under nitrate limitation ranged 18,600–108,000 cells/mL, with the highest cell

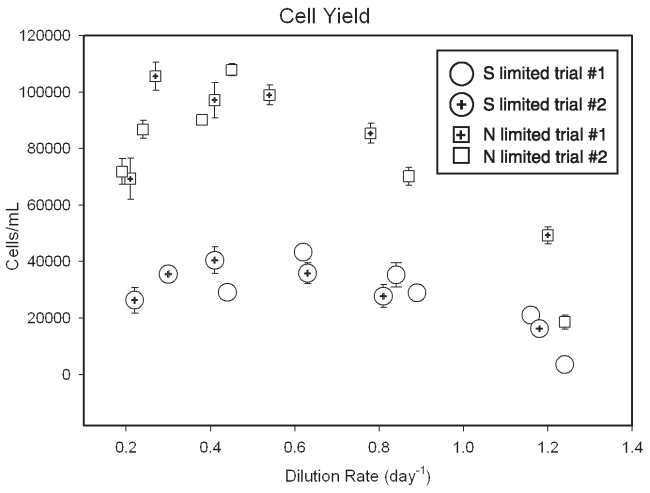

Figure 1 Plots of cell density vs. dilution rate show increasing cell abundance at each rate for N-limited cells compared to those under Si-limitation.

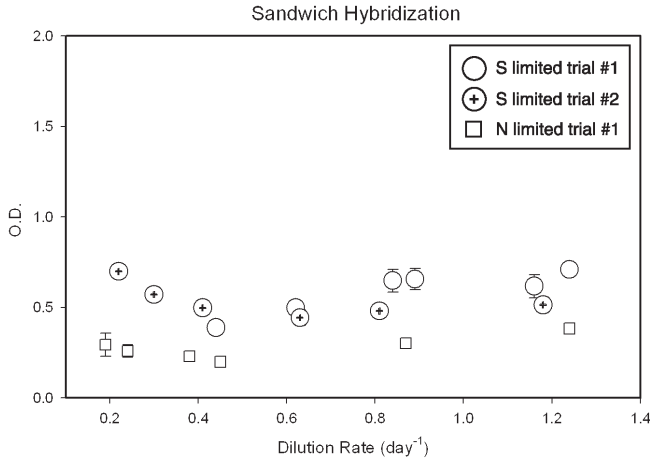

Figure 2 SH results show a slight decrease in signal intensity for N-limited cells compared to expectation for nutrient-replete exponentially growing cells (dashed line). Si-limited cells show an increased signal relative to expectation.

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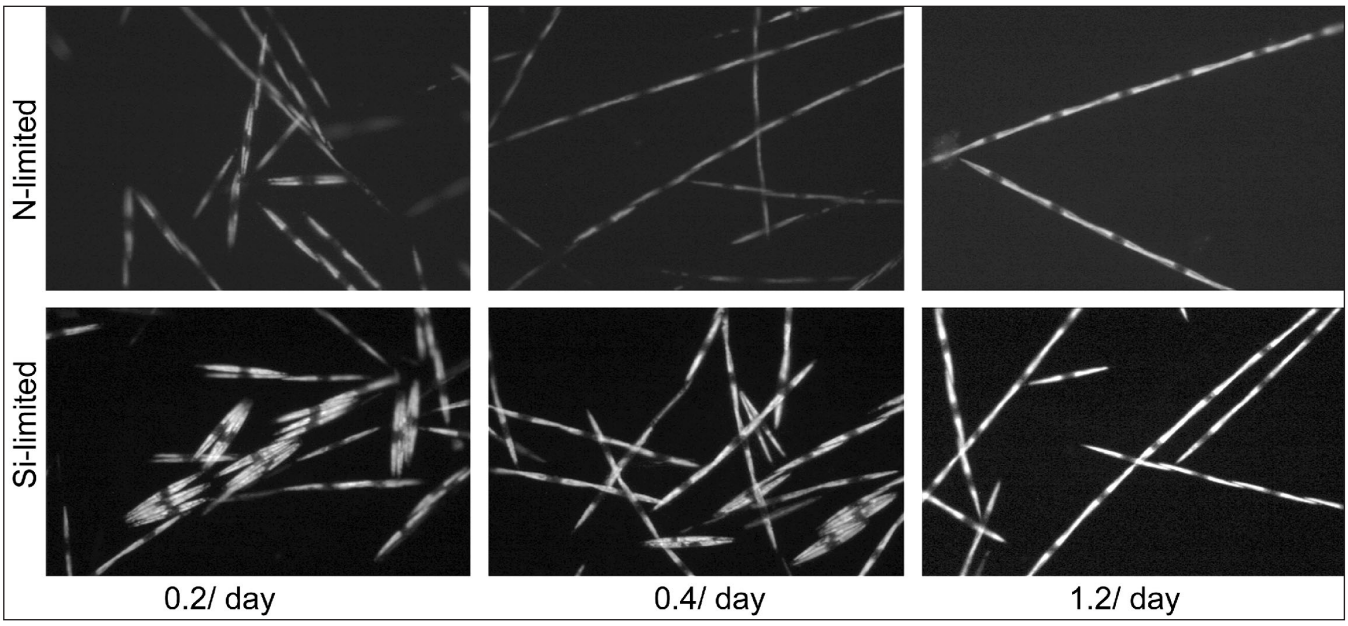

Figure 3 Examples of the type of results obtained from WCH trials. The top panel shows N-limited cells exhibiting a decrease in fluorescence intensity for cells at the lower dilution rates (most nitrate stressed). The Si-limited cells show significantly greater fluorescence intensity at all dilution rates.

numbers occurring at dilution rates between 0.24 and 0.54/day (Fig. 1). For SH, overall values obtained were slightly less than expected, compared to exponential phase cells grown in nutrient-replete batch culture (Fig. 2). Cell density estimates based on those measurements translate to ~70–80% of the number of cells actually collected for analysis. For WCH, a decrease in cell fluorescence was detected at lower dilution rates (slower growth rates) and cells appeared mottled, with contracted cytoplasm (Fig. 3). Particulate (cellular) DA levels increased from undetectable to 291 fg/cell as dilution rate decreased (Fig. 4). Dissolved DA was not detected for all dilution rates (Fig. 5).

Silicate Limitation Cell abundance under silicate limitation ranged 3,600–43,400 cells/mL. The highest cell numbers occurred at dilution rates between 0.4 and 0.6/day (Fig. 1). Results of SH indicated overall values obtained were greater than expected, compared to exponential phase cells grown in nutrient replete batch culture (Fig. 2). Cell density estimates based on those measurements can be twice as high as the number of cells actually collected for analysis. For WCH, there was no significant change in fluorescence intensity over the range of dilution rates examined, and cells appeared uniformly brightly labeled (Fig. 3). Cellular DA levels increased from undetectable to 3,313 fg/cell as dilution rate decreased (Fig. 4). Dissolved DA increased from undetectable to 202 nmol/L as dilution rate decreased (Fig. 5).

Discussion

The SH and WCH methods for species detection are similar in their use of DNA probes that target rRNA, but the details of how these methods are applied differ greatly. With the SH method it is possible to use a relatively large sample (*e.g.,* several hundred mL), which is concentrated onto a filter, prior to rupturing cells with sample lysis buffer to produce a cell-free homogenate. Target rRNA, if present, is detected using a quantitative colorimetric assay that corresponds to the number of target molecules (= "cells") present in the original sample. In contrast, the WCH method uses a much smaller sample (*e.g.,* 10 mL), which is concentrated onto a filter and fixed, thus retaining cell morphology. Detecting cells using this method entails observing fluorescently labeled cells under the microscope, and thus cells must remain intact to be counted. Interestingly, these two methods are not always expected to agree with each other, even when analyzing identical samples (Scholin *et al.,* 1999). This situation can arise as a consequence of the way in which samples are processed. When cells are fragile, such as may be found at the end of a bloom, they may not survive WCH processing and therefore will not be counted. In such cases, the SH method may detect the targeted species and will thus appear to overestimate results relative to WCH. Similarly, we suspect that if cells have been recently ingested by heterotrophic plankton or are enmeshed within a complex matrix of material, they still may be detected using SH but almost certainly will not be visible microscopically using WCH. Finally, the smaller sample volume employed using WCH may impede detection of cells when they are rare, especially for species that are chain-forming, such as *Pseudo-nitzschia* spp., since under conditions of low cell density, the chances of capturing individual chains of cells within a small sample diminishes. Because of these differences, it is important to be aware of the strengths and limitations of the two detection methods and the range of conditions under which each may be used reliably.

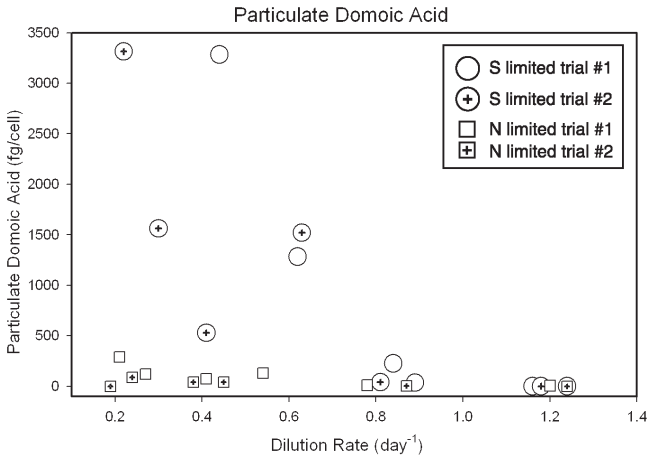

Figure 4 Cellular domoic acid concentration vs. dilution rate for a range of N- and Si-limited cells.

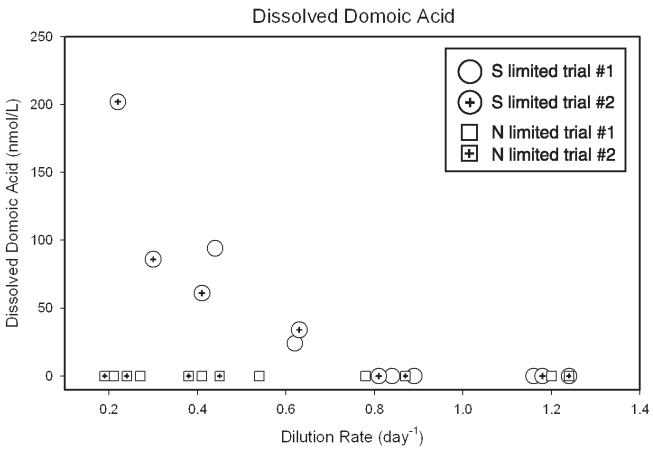

Figure 5 Dissolved domoic acid concentration vs. dilution rate for a range of N- and Si-limited cells.

Results of the tests reported here show that both the WCH and SH methods successfully detected cells under the range of growth rates and nutrient limitations examined. However, under nitrate limitation, both methods showed a decrease in signal strength relative to that expected for nutrient-replete cells. Under such conditions, it can be difficult to see labeled cells using WCH, and use of the SH method could also lead to underestimates of cell density. In contrast, under silicate limitation, all samples showed a strong signal, regardless of growth rate, relative to that expected for nutrient-replete cells. Under these conditions, cells should be highly visible using WCH, and cell densities could be overestimated using the SH method. A physiological basis for these results might be that under nitrate limitation, decreased protein synthesis and therefore decreased ribosomal activity occurs, leading to fewer rRNA target sites to be detected by either SH or WCH methods. In contrast, under silicate limitation, cell growth is inhibited because of insufficient silica for cell wall deposition, but ribosomal activity may continue as usual, and therefore these cells will show strong signal under both SH and WCH assays.

With respect to analysis of natural samples, results of this study suggest that the SH method will likely succeed in detecting cells even under conditions of nutrient stress, whereas the WCH assay may be more problematic, and success in detecting cells will depend significantly on the physical integrity of the cells as well as the background material that cells are found within. If cells are weakly labeled, few in number, and are embedded in a relatively dense matrix, then detection will be more difficult using WCH and the apparent discrepancy between results of the SH analysis will increase. Variation in cellular domoic acid levels with nutrient stress agreed with previous observations (Bates, 1998), showing higher values under silicate- versus nitrogen-limitation or nutrient-replete conditions. The increase in dissolved domoic acid concentrations with increasing silicate limitation is also consistent with earlier findings. Such wide variations in both particulate and dissolved domoic acid emphasize the fact that estimates of cell density do not necessarily reflect the amount of toxin available to the food web. Future studies of toxic *Pseudo-nitzschia* blooms should emphasize application of both cell and DA detection methods.

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DNA Probes for the Detection of *Karenia* Species in New Zealand's Coastal Waters

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Abstract

New Zealand's coastal waters are home to at least five *Karenia* species, most of which produce brevetoxins. The morphological similarity of these species under the light microscope has led to their being reported as *K.* cf. *mikimotoi* for phytoplankton monitoring purposes. The *Karenia* species produce varying concentrations of toxin per cell and may produce additional bioactive compounds, and therefore they pose different toxin risks. Ribosomal RNA-targeted DNA probes (based on fluorescent *in situ* hybridization) have been designed for *K. mikimotoi, K. selliformis, K. brevisulcata,* and *K. papilionacea* and validated against cultured strains. Field trials of the probes were run during a *Karenia* bloom (October 2002) in northeast North Island, New Zealand, to assess their robustness for inclusion in monitoring programs. Treated samples (Lugol's iodine addition to prevent lysis of live cells during transit of untreated cultures to the laboratory) were successfully used with the addition of a decolorizer.

Introduction

Several species of the dinoflagellate genus *Karenia* G. Hansen and Moestrup (syn. *Gymnodinium* F. Stein, in part), including producers of brevetoxin (BTX), gymnodimine and other as yet uncharacterized bioactive compounds, have been identified in New Zealand's coastal waters (Haywood *et al.,* 1996; Haywood, 2001). BTXs can cause neurotoxic shellfish poisoning (NSP) in consumers of contaminated shellfish, although detection of BTX in New Zealand shellfish has been rare (Hay *et al.,* 2000). *Karenia mikimotoi* Miyake et Kominami ex Oda, the most toxic BTX producer identified in New Zealand (Todd, 2002), can be differentiated from other dorso-ventrally flattened *Karenia* species, and from the closely related genera *Gymnodinium* F. Stein and *Karlodinium* J. Larsen, using routine light microscope techniques. However, cell distortions can occur following fixation with Lugol's iodine, and the delicate live cells in untreated samples can lyse during transport. DNA probes provide a rapid and reliable option for species confirmation and have been offered as a commercial service to the shellfish industry and public health regulators in New Zealand since 1997 for the amnesic shellfish poisoning genus, *Pseudo-nitzschia* (H. Peragallo) (Rhodes *et al.,* 2002). This has led to a refinement of biotoxin risk assessments for harvesters and regulators and an improvement in the information available on which to base management decisions. *Karenia* probes will further strengthen the current phytoplankton monitoring program in New Zealand, and it is proposed that in due course they will be used with phytoplankton data to trigger bioassays for BTXs in New Zealand's regulatory programs for shellfish safety. For example, in approved areas with no NSP history, shellfish meat testing may be reduced from weekly to monthly, bioassays being triggered in the intervening weeks when *K.* cf. *mikimotoi* cell counts reach ≥5000 cells L^{-1}. DNA probes have been designed to differentiate between *K. brevisulcata* (Chang) G. Hansen and

Table 1 Microalgae tested for species-specific rRNA binding of DNA probes. Code: Cawthron Culture Collection designation.

Species	Code	Species	Code
Akashiwo sanguinea (Hirasaka) G. Hansen and Moestrup	CAWD01	*K. brevis* (Davis) G. Hansen and Moestrup	CAWD04
Alexandrium tamarense (Lebour) Balech	CAWD20	*K. brevisulcata* (Chang) G. Hansen and Moestrup	CAWD82
Amphidinium operculatum Claparède and Lachmann	CAWD42	*K. longicanalis* Yang, Hodgkiss and G. Hansen	CAWD65
Chattonella antiqua (Hada) Ono	CAWR18	*K. mikimotoi* (Miyake and Kominami ex Oda) G. Hansen and Moestrup	CAWD63
Chrysochromulina ericina Parke and Manton	CAWP01	*K. mikimotoi*	CAWD123
Coolia monotis Meunier	CAWD77	*K. papilionacea* Haywood and Steidinger	CAWD91
Gymnodinium aureolum (Hulburt) G. Hansen	CAWD87	*K. selliformis* Haywood, Steidinger and MacKenzie	CAWD79
G. catenatum HW Graham	CAWD113	*Karlodinium micrum* (Leadbeater and Dodge) J. Larsen	CAWD66
G. chlorophorum Elbrachter and Schnepf	CAWD62	*Prorocentrum balticum* (Lohmann) Loeblich III	CAWD38
G. impudicum Fraga and Bravò	CAWD03	*P. compressum* (Bailey) Abé ex Dodge	CAWD31
cf. *Gymnodinium* sp	CAWD71,80,81,85,86,90,93,115	*P. gracile* Schütt	CAWD112
Gyrodinium instriatum Freudenthal and Lee	CAWD64	*P. micans* Ehrenberg	CAWD34
Heterocapsa triquetra (Ehrenberg) Stein	CAWD36	*Protoceratium reticulatum* (Claparède and Lachmann) Bütschli	CAWD40
Heterosigma akashiwo (Hada) Hada	CWAR05		
Karenia bidigitata Haywood and Steidinger	CAWD92	*Pseudo-nitzschia pungens* (Grunow ex Cleve) Hasle	CAWB49

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Moestrup, *K. mikimotoi, K. papilionacea,* and *K. selliformis* (Haywood *et al.,* in press) and validated for use in whole cell assay format based on fluorescent *in situ* hybridization (FISH).

Materials and Methods

Microalgae for assessing specificity of the DNA probes were maintained in the Cawthron Microalgae Culture Collection (Table 1) at 90 μMol m^{-2} s^{-1} photon flux (12:12 h light:dark); 18°C ± 1°C.

DNA probes for differentiation of the *Karenia* species were designed on the basis of previously determined DNA sequence data (refer Haywood 2001), and DNA sequence alignments were generated using Pileup. GenBank accession numbers were U92249, U92250 and U92252 for *K. mikimotoi, K. selliformis,* and *K. papilionacea,* respectively. Oligomers that specifically bound to the target species rRNA were selected for further trials. Whole cell (*in situ*) hybridization of the selected large subunit rRNA-targeted species-specific oligonucleotide probes (Oligos Etc, USA) was optimized against *Karenia* cultures for temperature and salinity using the filtration method (Scholin *et al.,* 1997; Miller and Scholin, 1998); microalgae were tested for cross-reactivity (Table 1) as described by Scholin *et al.* (1996). Exponentially growing cultures (≈2 mL per filter stack) were filtered (3 μm, Nucleopore) in the custom filter manifold and cells fixed for 1 h, then hybridized for 1 h at 50°C using 1× SET buffer. Probe response was observed using epifluorescence microscopy (ex., 490 nm; em., 520 nm). Field samples (≈10 mL per filter stack) from routine phytoplankton monitoring programs were assayed using the optimized conditions. Lugol's iodine-treated samples had decolorizer added (3% sodium thiosulphate, 20 μL per 10 mL; Thronsden, 1978). To prevent cell lysis, untreated samples containing live cells were never filtered to dryness.

Results and Discussion

In New Zealand, from December 1992 to January 1993, *Karenia mikimotoi* (identified at the time as *Gymnodinium* cf. *aureolum*; Rhodes *et al.,* 1993) was implicated as the causative agent of 180 cases of illness fitting the case definition for NSP, including respiratory irritation syndrome (RIS) in beach visitors (Jasperse, 1993; MacKenzie *et al.,* 1995). The incidents occurred along the northeast coast from Northland to the Bay of Plenty, including the Hauraki Gulf, and resulted in the closure of the entire New Zealand coastline for shellfish harvesting until a comprehensive biotoxin monitoring program was operational. BTXs were detected in shellfish from the area (Todd, 2002). One year later, a bloom of the gymnodimine producer *K. selliformis* was responsible for shellfish deaths along the south and eastern coastline of the South Island (MacKenzie *et al.,* 1996). In 1998, *K. brevisulcata* was implicated in the massive deaths of marine fauna and flora in Wellington Harbor with associated respiratory irritation in humans (Chang, 1999). The dorso-ventrally flattened and toxic *Karenia* complex posed some difficulties for light microscope identification,

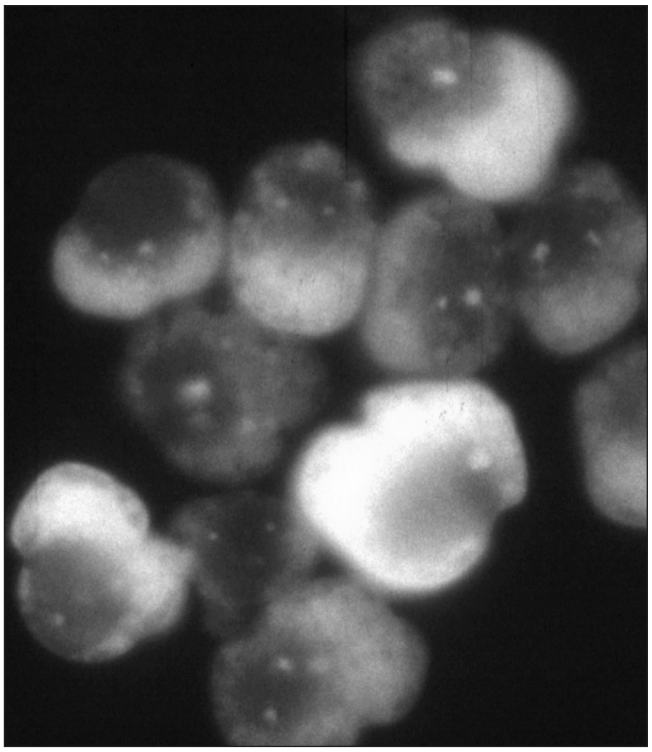

Figure 1 Positive binding of *Karenia mikimotoi* to a species-specific DNA probe.

and DNA probes now offer a rapid alternative. The ability to differentiate between the species will allow better correlations to be made between species presence and toxins in shellfish and/or effects on marine life.

The DNA probes designed to target the rRNA of *K. mikimotoi, K. brevisulcata, K. papilionacea,* and *K. selliformis* proved species-specific, with no cross-reactivity against the 36 microalgae tested (Table 1). Only the *K. mikimotoi* probe showed some binding to *K. selliformis* at low salinities and the assay was adjusted to eliminate this binding. There was no cross-reactivity between the *K. selliformis* probe and *K. mikimotoi* cultures, which gave further confidence in the assay. The fluorescence following probe binding tended to be patchy, particularly in *K. papilionacea* cells, but was unmistakable. Double FITC fluors improved the response but would add to the cost for monitoring purposes.

The latest *Karenia* bloom occurred from late September through October 2002 in the Hauraki Gulf, with reports of mass deaths of fish and eels. Seawater samples were collected throughout that time and assayed with the species-specific DNA probes. The bloom proved to be predominantly *K. mikimotoi* (Fig. 1) with low numbers (≈5% total *Karenia* species) of *K. brevisulcata* toward the end of the bloom. No BTX was detected in shellfish samples collected from the area, and three *K. mikimotoi* isolates have been cultured to help determine the causative agent of the fish deaths. Lysis of cells in untreated samples during transport of samples led to the use of Lugol's iodine (LI)-treated samples with addition of decolorizer prior to DNA probe assaying. In

Table 2 Comparative *Karenia mikimotoi* DNA probe assays of untreated (U) and Lugol's iodine-treated plus decolorizer (L+D) Hauraki Gulf bloom samples. FITC fluorescence: +++ very bright; ++ moderate; + pale; - neg; nd. no cells detected.

Site	Date 2002 (D/M)	Probes UNIC U	UNIC L+D	UNIR U	UNIR L+D	*K. mikimotoi* U	*K. mikimotoi* L+D	Phytoplankton monitoring results (cells L^{-1})
Whangaparoa	1/10	+	+	-	-	+	++	8,650
Whangaparoa	4/10	++	+	-	-	+	++	3,600
Whangaparoa	9/10	nd	+	-	-	nd	++	1,400
Port Fitzroy	26/9	++	++	-	-	++	++	154,000
Te Kapa	9/10	++	++	-	-	+	++	32,000
Te Kapa	12/10	+++	++	-	-	+++	++	59,000
Te Kapa	30/9	nd	++	-	-	nd	++	58,000

some untreated samples, all cells appeared to have lysed either in transit or during the filtration process, as evidenced by the comparative probe results and phytoplankton monitoring data (Table 2). A yellow background was observed following assaying in LI-treated samples, which made species identification difficult. However, with the added decolorizer, a clear green fluorescence was observed with positive probe binding, the fluorescence being enhanced in comparison with untreated control samples. LI-treated samples were also stored for one month following the original assay and retested. However, the resulting fluorescence was pale and the background autofluorescence was bright, suggesting that treated field samples should be processed as soon as possible after collection.

The eventual commercial application of *Karenia* DNA probes will allow greater specificity and confidence in the use of phytoplankton to trigger NSP testing in areas approved for reduced shellfish testing. The Cawthron Phytoplankton Laboratory analysts have International Accreditation NZ for this assay (recognized under ISO 17025).

Acknowledgements

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Utility of the Algal Photopigment Gyroxanthin-diester in Studies Pertaining to the Red Tide Dinoflagellate *Karenia brevis* (Davis) G. Hansen and Moestrup

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Abstract

Discrimination of the algal photo-pigment gyroxanthin-diester (through High Performance Liquid Chromatography) can be applied to studies of *Karenia brevis* (Davis) G. Hansen and Moestrup as a biomarker and an indicator of both phytoplankton taxonomy and photophysiology. Four years of HPLC data, collected in nine independent field studies of the ECOHAB Florida project, were used to demonstrate the utility of gyroxanthin-diester in studies involving *K. brevis.* A weak linear relationship existed between the abundance of *K. brevis* and the concentration of gyroxanthin-diester; however, the presence and relative abundance of *K. brevis* was still determined using HPLC data. When gyroxanthin-diester was present, its ratio to chlorophyll *a* was found to be constant. A comparison of the ratios of gyroxanthin-diester: chlorophyll *a* and gyroxanthin-diester: diadinoxanthin also provided information concerning photo-physiological state. Taxonomic composition, including the contribution of *K brevis* to total community composition, was determined using gyroxanthin-diester and the software package ChemTax®.

Introduction

The abundance of the red tide dinoflagellate *Karenia brevis* and concentrations of the algal pigment gyroxanthin-diester have been shown to have a linear relationship (Millie *et al.,* 1995), suggesting that gyroxanthin-diester might serve as a biomarker for the presence and abundance of *K. brevis.* However, other species, as well as some other phytoplankton, contain gyroxanthin-diester. Field studies in the eastern Gulf of Mexico have shown that in *K. brevis* blooms, the gyroxanthin-diester to chlorophyll *a* ratio is constant at 0.040, suggesting that gyroxanthin-diester can be incorporated into chemotaxonomic evaluations. The ratios of gyroxanthin-diester/chlorophyll *a* and diadinoxanthin/gyroxanthin-diester should provide information pertaining to bloom light history, and four years of ECOHAB data demonstrated the utility of gyroxanthin-diester to studies of *K. brevis.*

Materials and Methods

Data for this study were compiled from four years of field surveys conducted on the west Florida continental shelf. Nine independent studies were used; a time-series transect (1999–2002) consisting of seven stations between the 10 and 30 meter isobaths offshore of Sarasota, FL, USA, and eight separate legs of yearly ECOHAB Florida cruises conducted on the west Florida continental shelf between 1998 and 2000.

Hydrographic variables were measured with Sea Bird SBE 19, Sea Bird SBE 25 or Sea Bird SBE 9/11 plus CTD profiling systems. Discrete samples were taken at depth for HPLC algal pigment analyses and microscopic enumeration of *K. brevis.* Pigment data were analyzed using a Shimadzu SPD-10A HPLC system following the methods of Wright *et al.,* 1991. *K. brevis* cells were enumerated using an inverted microscope after preservation with Utermöhl's solution and concentrating 1 mL of each sample into counting wells.

Absolute and relative contributions of *K. brevis* to total chlorophyll *a* biomass were derived from chemotaxonomic photopigments using ChemTax®, a software package incorporating factor analysis and a steepest descent algorithm (Wright *et al.,* 1996). ChemTax® optimizes chlorophyll and carotenoid pigment ratios based on published chlorophyll *a*: diagnostic pigment ratios within the representative phylogenetic groups (for critical appraisals of ChemTax® applications, see Wright *et al.,* 1996; Mackey *et al.,* 1998). Because pigment ratios within phytoplankton would be expected to vary depending upon physiological state (Descy *et al.,* 2000; Schlüter *et al.,* 2000), the pigment data set was divided into subsets by sampling transects and periods.

Results

Pigment data from three years of cruises indicated that the gyroxanthin-diester to chlorophyll *a* ratio was constant (Fig. 1). Although the r^2 value was 0.6, a linear relationship

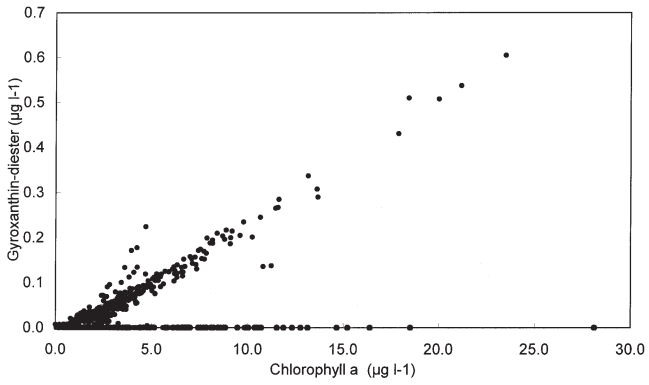

Figure 1 Concentration of gyroxanthin-diester versus chlorophyll *a* from three years of cruises during the ECOHAB Florida project (1998–2000). Many instances occurred where chlorophyll *a* values were high yet no gyroxanthin-diester was present, indicating the absence of *K. brevis* in the sample.

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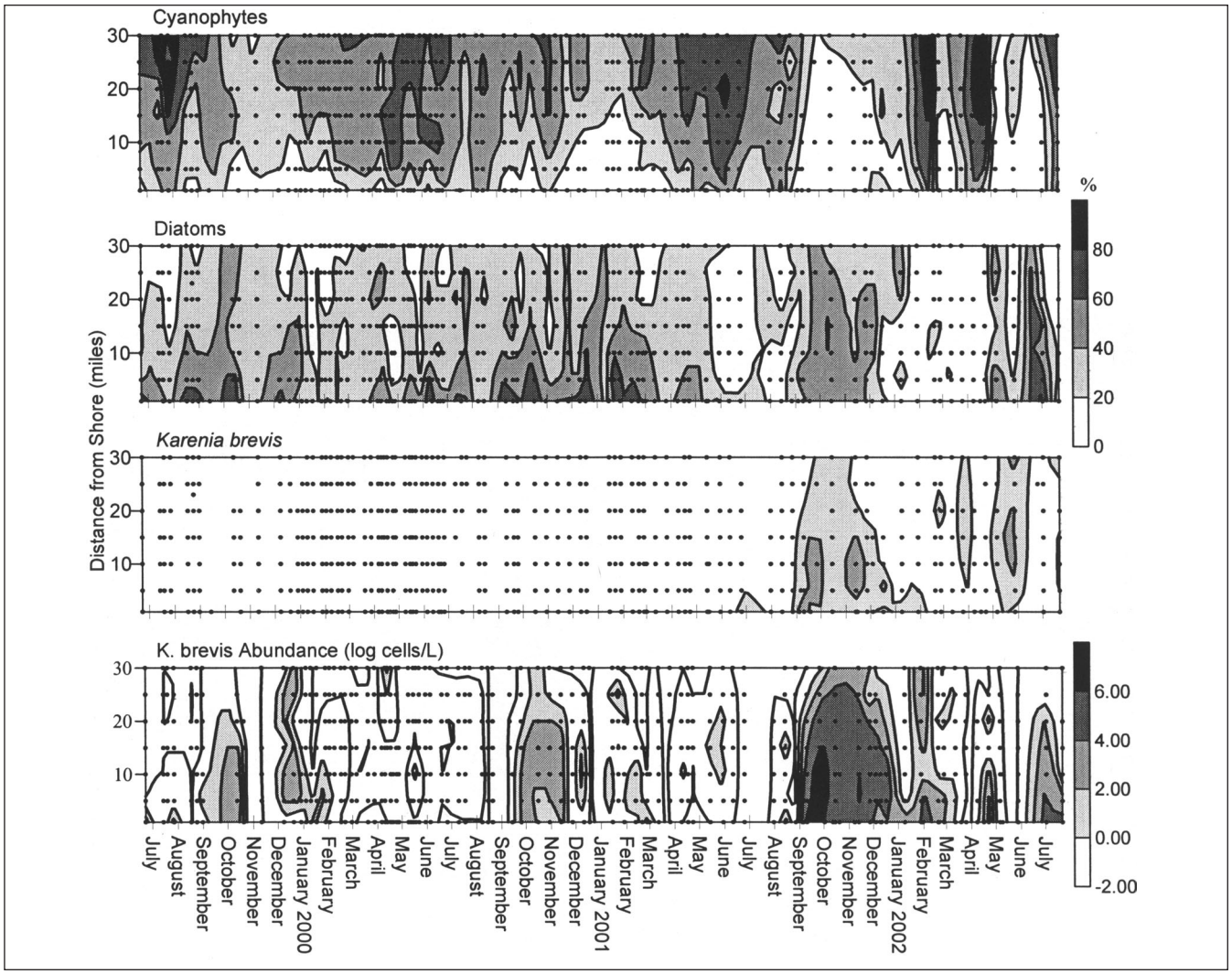

Figure 2 Contoured results from ChemTax® using surface photopigment data collected on the Sarasota transect between June 11, 1999, and July 22, 2002. Station marks apply to each series. The top three contours represent the contribution (%) of each algal group to total chlorophyll *a*. The log of *K. brevis* abundance values (determined microscopically) was used to create the contours in the bottom panel.

was also observed between gyroxanthin-diester and *K. brevis* abundance (data not shown). More pertinent to this study was the fact that when gyroxanthin-diester was present, its concentration occurred in a linear and constant relationship with chlorophyll *a*. The constant ratio allows gyroxanthin-diester to be used as a proxy in the eastern Gulf of Mexico for *K. brevis* chlorophyll biomass. This provides a means to evaluate community composition even though the gyroxanthin-diester and chlorophyll *a* contents per cell vary due to physiological changes such as light history. The ratio of other carotenoid pigments, such as fucoxanthin and diadinoxanthin, were generally linear during each cruise, but the relationship did not hold up after the data had been compiled (data not shown). The variability in these ratios between cruises may be indicative of differences in light history.

The gyroxanthin-diester to chlorophyll *a* data from the Sarasota time series transect were incorporated into the software package ChemTax® and the relative contributions of 7 different taxonomic groups were determined (Fig. 2). Contours showed that cyanophytes (offshore) and diatoms (inshore) typically dominate the region unless a bloom of *K. brevis* is present. The contribution of *K. brevis* to total chlorophyll *a* followed the pattern seen for *K. brevis* abundance (determined microscopically) except when abundance values were low.

Discussion

Over 5 years and 8 separate cruises, the gyroxanthin-diester to chlorophyll *a* ratio for *K. brevis* has remained constant. The fact that gyroxanthin-diester and chlorophyll *a* covary when *K. brevis* is present becomes a useful tool in studies pertaining to *K. brevis.* Studies involving light history, *K.brevis* distribution and algal taxonomy can all benefit from HPLC pigment analyses.

Gyroxanthin-diester proved to be a reliable indicater of

the presence of *K. brevis* in the Gulf of Mexico. While the ratio of other pigments to chlorophyll *a* varied, presumably due to light history, gyroxanthin-diester:chlorophyll *a* remained constant, allowing gyroxanthin-diester to be utilized in studies pertaining to both photophysiology and taxonomic identification. Due to the constant ratio to chlorophyll *a*, gyroxanthin-diester also makes a good marker pigment for taxonomic evaluations using HPLC and ChemTax®.

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Vertical Migration of a *Karenia brevis* Bloom: Implications for Remote Sensing of Harmful Algal Blooms

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Abstract

A bio-optical instrument package was used to vertically profile a *K. brevis* bloom on the West Florida Shelf in October 2001. The inherent optical properties of the population were used to quantify and discuss the impacts on remote sensing of algal blooms. Vertically dependent changes in attenuation coefficients on the order of 4–5 fold were measured as the population migrated to the surface and back to the bottom over the diel period. The changes in IOPs affected the spectral distribution of the remote sensing reflectance as the population migrated to the surface. This shift reflected the vertical migration and the cell cycle biology.

Introduction

Blooms of the toxic red tide dinoflagellate, *Karenia brevis* (= *Gymnodium breve* Davis), frequently occur off western Florida in the Gulf of Mexico and pose a serious public health concern (Steidinger *et al.,* 1973; Riley *et al.,* 1989; Pierce *et al.,* 1990; Shumway *et al.,* 1990). Given the recurrent nature of *K. brevis* blooms, much effort has been focused on developing non-intrusive mapping technologies using bio-optical approaches (cf. Schofield *et al.,* 1999). Pattern recognition methods have been developed that can provide maps of *K. brevis* from *in situ* measurements (Kirkpatrick *et al.,* 2000; Millie *et al.,* 2002). Remote sensing techniques, however, are more difficult as the spectral resolution of current satellite systems is limited to only a few wavelengths, satellites detect only the upper portion of the euphotic zone, and the overall reflectance signal reflects the optics characteristics of all phytoplankton present in the surface waters (Kirk 1995).

Despite these hurdles, satellite delineation of *K. brevis* blooms, compared to other harmful algae, may be uniquely promising due to its unique physiological ecology. *K. brevis* is positively phototactic (Steidinger 1975) which often results in cells concentrating (as great as 1×10^8 cells L^{-1}) at the air-sea interface. This migration leads to increasing mono-specific *K. brevis* concentrations in the surface during the day. The ocean color SeaWiFs satellite passes over the Gulf of Mexico in the early afternoon (~1300 LDT), when cells are potentially accumulating in the surface waters. Additionally, as *K. brevis* migrates into the surface waters, cells must often acclimate to the high irradiances. This results in changes in pigment concentration, pigment composition (Kiefer 1973, Harris 1980) and cellular optical cross-sections (Prezelin *et al.,* 1991). *In situ* data on the radiometric conditions in natural *K. brevis* populations is lacking. Therefore, in an effort to understand these impacts on remote sensing, we quantified the impact of vertically migrating natural populations of *K. brevis* on *in situ* optical properties which allowed us to quantify the corresponding impact on remote sensing reflectance during the October 2001 ECOHAB process cruise. This manuscript outlines those results.

Materials and Methods

During the October 2001 ECOHAB cruise on the West Florida Shelf, a bloom of *Karenia brevis* was located, mapped and tracked for several days using Langragian drifters (Fig. 1). During that cruise, the *K. brevis* bloom was sampled hourly during a 24-hour diel cycle using a vertically profiling bio-optical instrument cage. The profiles were measured with an integrated bio-optical package consisting of a Wetlabs absorption/attenuation meter (ac-9), a Seabird CTD, Satlantic spectroradiometers, and a HOBI-Labs HS-6 backscatter sensor. The measurements of the inherent optical properties (IOPs) used in this study were collected using the standard nine-wavelengths of the ac-9. The instruments were factory-calibrated prior to the cruise and clean water measurements were made throughout the cruise. Manufacturer recommended protocols (http://www.wetlabs.com) were used to track instrument

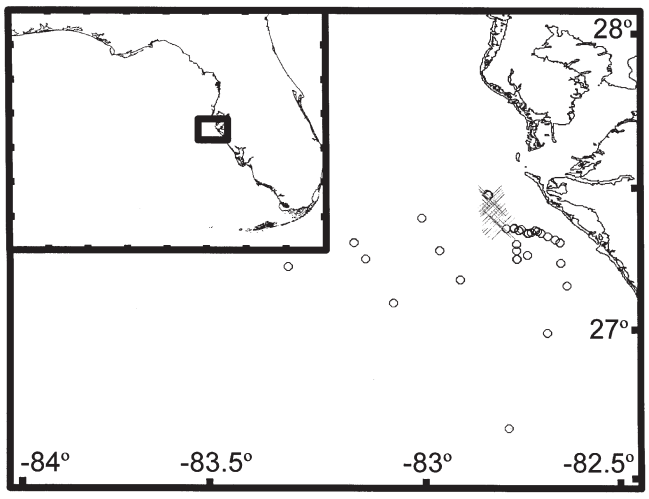

Figure 1 October 2001 ECOHAB Study site. All stations (○) and diel stations (×) are shown.

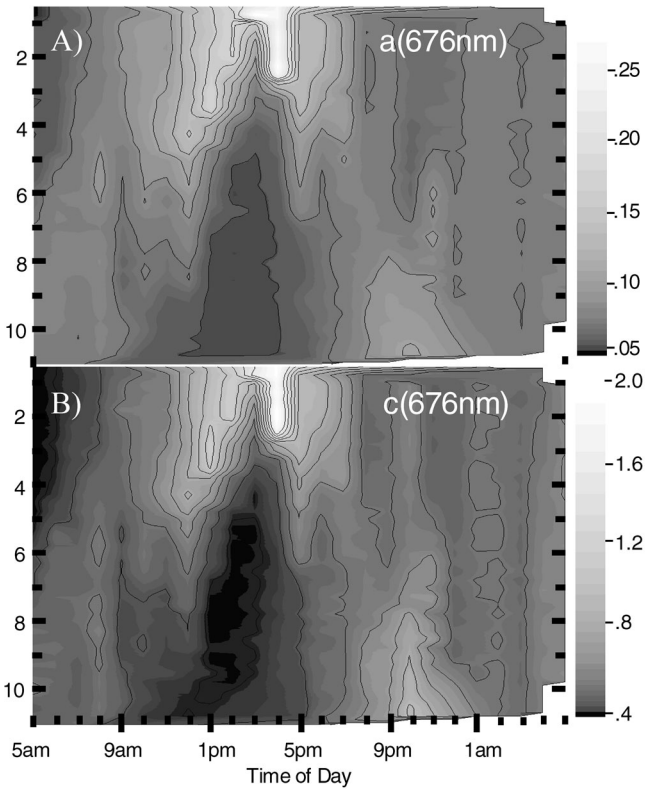

Figure 2 Diel time series of the vertical changes in **A**) a(676 nm) (m^{-1}) and **B**) c(676 nm).

drift, and clean water, temperature, and salinity corrections were performed. To estimate the reflectances encountered for all the different cell distributions over the entire diel migration (note this allows for reflectances to be calculated during the night stations), the measured IOPs were used as inputs to a radiative transfer model to calculate reflectances at local noon. In brief, absorption, attenuation and backscatter data were averaged into 0.25 m bins and were used as inputs to Hydrolight (v4.2), to model the spectral scalar irradiances and associated apparent optical properties from 400–700 nm. The runs used the pure water absorption values of Pope and Fry (1997). Hydrolight computed a new spectral scattering phase function when the backscatter to total scatter ratio changed by more than 0.001. Default atmospheric parameters were used for all runs and all calculations were set to provide estimates of the apparent optical properties with the sun directly overhead.

Results and Discussion

Over the diel cycle there were no significant changes observed in the hydrography and the ship maintained close proximity to drifters; therefore it was likely that the same population was sampled at each station. Over the course of the day the absorption, a(λ), and attenuation, c(λ), signals exhibited a dramatic increase in the surface waters (0–2 meters) with highest values observed by 16:00 LST (Fig. 2). During the evening this situation reversed itself as the cells migrated downwards. The a(λ) and c(λ) signals increased fivefold during the daylight hours. The increases in these optical signals were mirrored by an increase in *K. brevis* cell counts in the surface waters. The SeaWiFs and MODIS satellites pass over the Western coast of Florida at approximately 13:00 LDT. Although this is 3 hours before the maximum a(λ) and c(λ) values were observed, these values are still a factor of 4 higher than when the population is near the bottom. Therefore, vertical migration of *K. brevis* disproportionately increases the potential remote

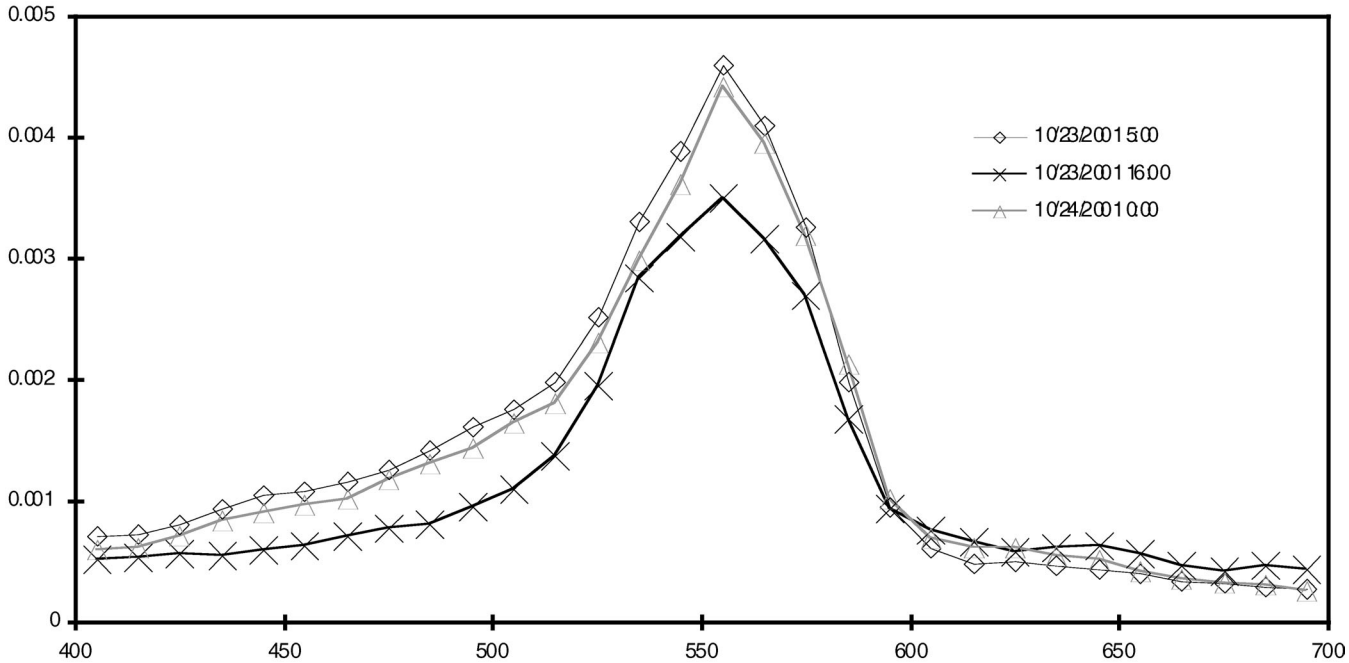

Figure 3 Modeled $R_{rs}(\lambda)$ using measured optics and Hydrolight v4.2. The peak abundance of *K. brevis* was encountered at 16:00 LDT.

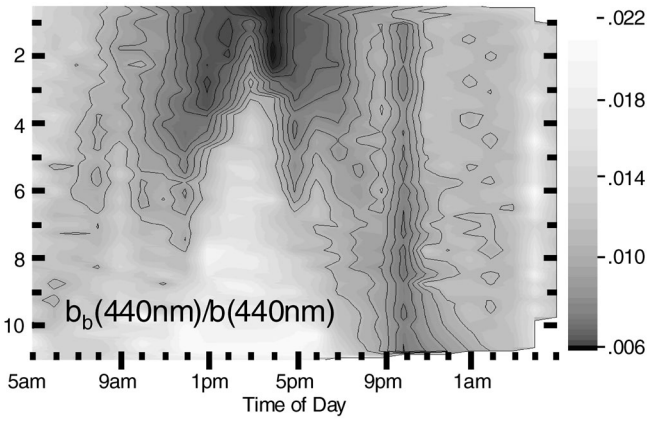

Figure 4 The diel cycle of the backscatter to scatter ratio during the vertical migration of *K. brevis*.

sensing signal of this harmful algae compared to the other algae present in the water column.

The presence of a near-surface population dramatically altered the remote sensing reflectance, $R_{rs}(\lambda)$, over the course of the day.

The surface populations of *K. brevis* altered both the magnitude and spectral shape of the remote sensing reflectance, R_{rs} (Fig. 3). $R_{rs}(\lambda)$ is related to both the absorption of light and the amount of backscattered light. Both of these optical parameters were impacted by the increases in *K. brevis*. The increase in surface *K. brevis* resulted in an overall decrease in remote sensing reflectance due to the enhanced absorption. The decreases in reflectance are spectrally-dependent. For example, in the blue and green wavelengths of light, there was a decrease in reflectance associated with the *K. brevis* photosynthetic pigmentation. In contrast, there was an increase in red light reflectance associated to the increased backscatter of light associated with *K. brevis*. We hypothesize that the increase in backscattered light associated with a near-surface layer would lower the average downward red photon path-length in the ocean and this would increase the red light scattered out of the ocean by decreasing the probability of red light absorption. This increase in backscattered light with the surface layer may contribute to why red tides are red. The reddish color is not directly related to any unique phytoplankton pigmentation (McLeroy-Etheridge and Roesler 1998). This light-scattering factor is magnified by red light from solar stimulated fluorescence. In *K. brevis,* however, the population is not as red as might be expected given the high cell numbers in the surface waters. Total backscatter is to first order a function of cell concentration, but the amount of backscattered light per cell is modulated by the cell cycle biology in *K. brevis*. Larger cells have proportionally more forward-scattered light then smaller cells (Van De Hulst 1957), and over the course of the day as cells grew in size, the ratio of backscattered to scattered light (b_b/b) decreased. This minimized the amount of red light reflected out of the ocean. Given this, it was not surprising that the b_b/b ratio increased later in the evening as *K. brevis* underwent cellular division after cells had migrated to depth (Leighfield, pers. comm., 2001).

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A Novel Optical Classification Technique for Detection of Red Tides in the Gulf of Mexico

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Abstract

Optical field data were collected on the West Florida Shelf (WFS) as part of the Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) program in October 2000. Stations were assigned to one of four phytoplankton taxonomic groups based on cell count and High Performance Liquid Chromatography (HPLC) pigment data. Relationships between chlorophyll *a* concentration and absorption and backscattering coefficients were evaluated to discriminate blooms of the toxic dinoflagellate, *Karenia brevis,* from populations of diatoms, nitrogen-fixing cyanophytes (*Trichodesmium* spp.), and prochlorophytes. A novel technique for classifying these four phytoplankton groups using remote-sensing reflectance (R_{rs}(l)) data is introduced based on the relationship between [chl *a*] and particulate backscattering. The classification technique was applied to Sea-viewing Wide Field-of-view Sensor (SeaWiFS) data to determine phytoplankton composition on the WFS.

Introduction

Harmful algal blooms of the toxic dinoflagellate, *Karenia brevis,* occur regularly on the West Florida Shelf (WFS), routinely killing marine mammals, posing a threat to human health, and resulting in millions of dollars of economic loss. Monitoring the origin and subsequent transport of these blooms, therefore, is a pressing need, hampered by inadequate spatial and temporal data. Since *K. brevis* blooms discolor oceanic surface waters, satellite ocean color sensors which measure the fraction of light entering the ocean that is reflected or the remote-sensing reflectance ($R_{rs}(\lambda)$) may provide a means for detecting and monitoring blooms from space.

$R_{rs}(\lambda)$ is dominated by the ratio of light backscattered by particles (phytoplankton and detritus) ($b_{bp}(\lambda)$) and water ($b_{bw}(\lambda)$) to light absorbed by phytoplankton ($a_{ph}(\lambda)$), detritus ($a_d(\lambda)$), colored dissolved organic matter ($a_{CDOM}(\lambda)$), and water ($a_w(\lambda)$). That is

$$R_{rs}(\lambda) \propto \frac{b_{bp}(\lambda) + b_{bw}(\lambda)}{a_{ph}(\lambda) + a_d(\lambda) + a_{CDOM}(\lambda) + a_w(\lambda)} \qquad (1)$$

where $b_{bw}(\lambda)$ and $a_w(\lambda)$ are constant and known. While [chl *a*] can successfully be derived from ratios of $R_{rs}(\lambda)$ [Gordon *et al.,* 1983; Carder *et al.,* 1999], algal taxonomy cannot be determined from [chl *a*] since all phytoplankton contain chlorophyll *a*. Classifying various algal groups from $R_{rs}(\lambda)$, however, may be possible if unique absorption or backscattering properties exist.

Materials and Methods

Optical data were collected in surface waters on the WFS from 4–6 October 2000 during an Ecology and Oceanography of Harmful Algal Blooms (ECOHAB) cruise. *K. brevis* cell concentrations were determined on live seawater samples using a dissecting microscope within thirty minutes of collection. Phytoplankton pigment concentrations were measured using High Performance Liquid Chromatography (HPLC). Methodological details and ref-

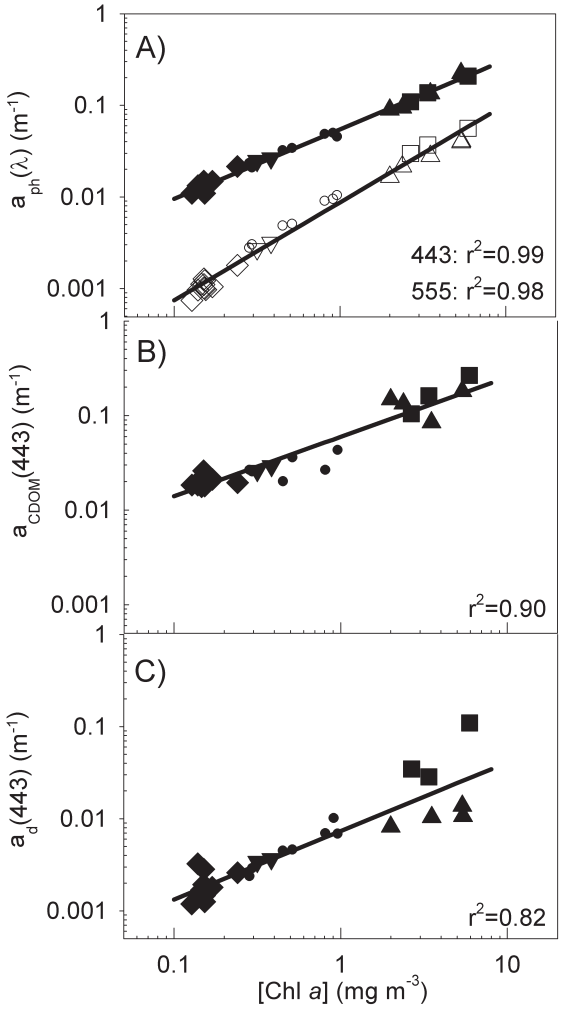

Figure 1 Relationships between [chl *a*] and **A** phytoplankton absorption at 443 nm (closed symbols) and 555 nm (opened symbols), **B** CDOM absorption at 443 nm, and **C** detrital absorption at 443 nm. Symbols represent phytoplankton taxonomic composition: diatoms (■), *K. brevis* (▲), *Trichodesmium* (▼), and prochlorophytes (♦). Mixed assemblages were considered transitional (●). Linear regression lines (solid) were generated on log-transformed data (n = 26).

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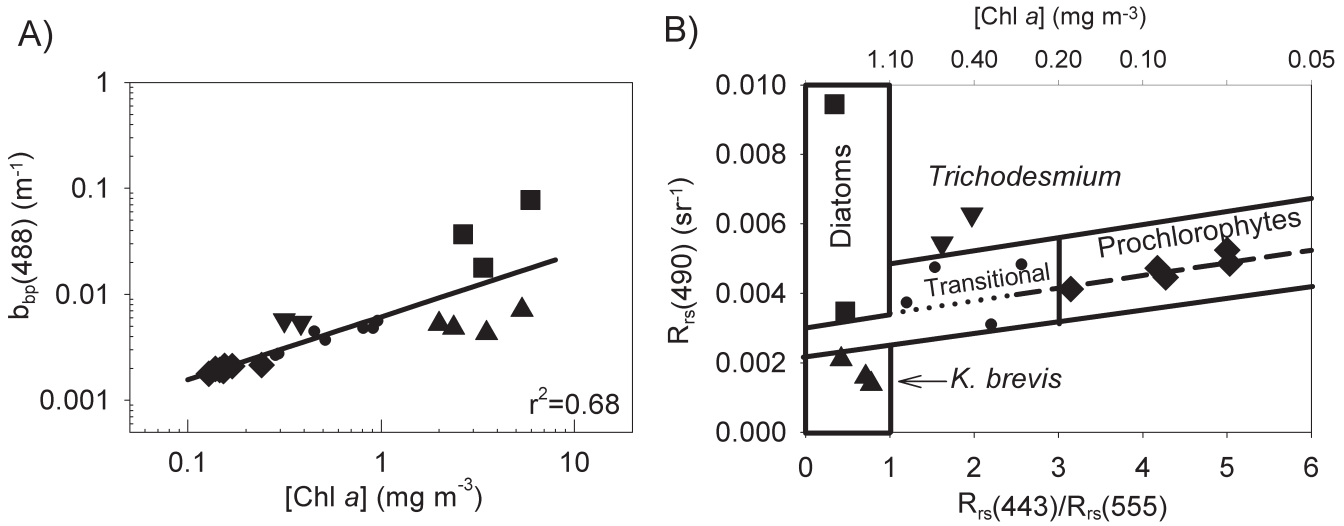

Figure 2 Relationships between **A** [chl *a*] and particulate backscattering at 488 nm with a linear regression line (solid) generated on log-transformed data (n = 26) and **B** $R_{rs}(443)/R_{rs}(555)$ and $R_{rs}(490)$ with an "oligotrophic" line (dashed) (extrapolated to zero (dotted) for reference purposes) generated from optically deep Florida Current and Bahamian data (not shown). $R_{rs}(490) = 3.65\ 10^{-4}$ $[R_{rs}(443)/R_{rs}(555)]+3.05\ 10^{-3}$ ($r^2 = 0.94$, n = 22). Symbols same as in Fig. 1.

erences for collecting and processing discrete seawater samples for [chl *a*] and [phaeopigment] fluorometrically, $a_{ph}(\lambda)$ and $a_d(\lambda)$ using the quantitative filter technique, and aCDOM(λ) spectrophotometrically along with measuring above-water $R_{rs}(\lambda)$ are provided in Carder *et al.* (1999). Underway $b_{bp}(488)$ was measured in a flow-through system with a Hydroscat-2 (HOBI Labs). Sea-viewing Wide Field-of-view Sensor (SeaWiFS) data were processed using SeaDAS (version 4.3) and analyzed using ENVI (version 3.4).

Results and Discussion

Relationships between [chl *a*] and absorption and backscattering coefficients (Figs. 1 and 2a) were examined for shipboard data assigned to one of four phytoplankton taxonomic groups (diatoms, *K. brevis, Trichodesmium,* and prochlorophytes). Cell count and HPLC diagnostic pigment criteria for identifying these groups are included below. Stations containing mixed algal assemblages were considered "transitional."

Chlorophyll *a* concentration is highly correlated (r^2 >0.90) with absorption due to both phytoplankton (Fig. 1a) and CDOM (Fig. 1b). High-chlorophyll waters (>1.0 mg m^{-3}) containing high concentrations of *K. brevis* (>10^4 cells L^{-1}), however, exhibit low detrital absorption (Fig. 1c) and low particulate backscattering (Fig. 2a) relative to high-chlorophyll, diatom-dominated estuarine waters (<10^4 cells L^{-1} of *K. brevis* and [fucoxanthin]/[chl *a*] > 0.5).

Since particulate backscattering is predominantly due to submicron particles, these results indicate that *K. brevis* blooms may exhibit a paucity of detritus compared to diatom-dominated estuarine waters. This may be due to 1) relatively low estuarine supplies of high-backscattering suspended sediment associated with *K. brevis* blooms and/or 2) reduced grazing pressure caused by cellular toxicity as indicated by a fourfold reduction in [phaeopigment]/[chl *a*] observed in *K. brevis* blooms.

Trichodesmium and prochlorophyte were the dominant phytoplankton in low-chlorophyll waters (<1.0 mg m^{-3}). *Trichodesmium* populations ([zeaxanthin]/[chl *a*] > 0.4 and [chl a_2]/[chl *a*] < 0.1) exhibit relatively high particulate backscattering (Fig. 2a) due to gas vacuoles used for buoyancy regulation. Prochlorophytes ([zeaxanthin]/[chl *a*] > 0.4 and [chl a_2]/[chl *a*] > 0.3) exhibit low absorption, low backscattering coefficients, and [chl *a*]'s less than ~0.2 mg m^{-3} (Figs. 1 and 2a).

The greatest separation between the four taxonomic groups (*i.e.,* the weakest correlation (r^2 =0.68)) is displayed in the relationship between [chl *a*] and $b_{bp}(488)$ (Fig. 2a). Modeling studies indicate that these changes in chlorophyll-specific particulate backscattering significantly influence $R_{rs}(\lambda)$. Therefore, space-based surrogates for these parameters were sought since they cannot be determined directly from space. Assuming that 1) the ratio $R_{rs}(443)/R_{rs}(555)$ is inversely related to [chl *a*] [Gordon *et al.,* 1983] and 2) $R_{rs}(490)$ provides an estimate of $b_b(488)/a(488)$ at a wavelength where absorption influences are relatively weak, the relationship between these two surrogates was chosen to classify populations of diatoms, *K. brevis*, *Trichodesmium,* and prochlorophytes from space using satellite-based ocean color data from SeaWiFS.

Indeed, shipboard $R_{rs}(l)$ data sorted into the same four taxonomic groups as in Fig. 1 (Fig. 2b) exhibit similar patterns as observed in the [chl *a*] versus $b_{bp}(488)$ relationship (Fig. 2a). Prochlorophyte-dominated waters are located on or about an "oligotrophic" line generated from optically deep Florida Current and Bahamian data with [chl *a*] < ~0.2. *Trichodesmium* are located in waters with [chl *a*]<1.0 mg m^{-3}

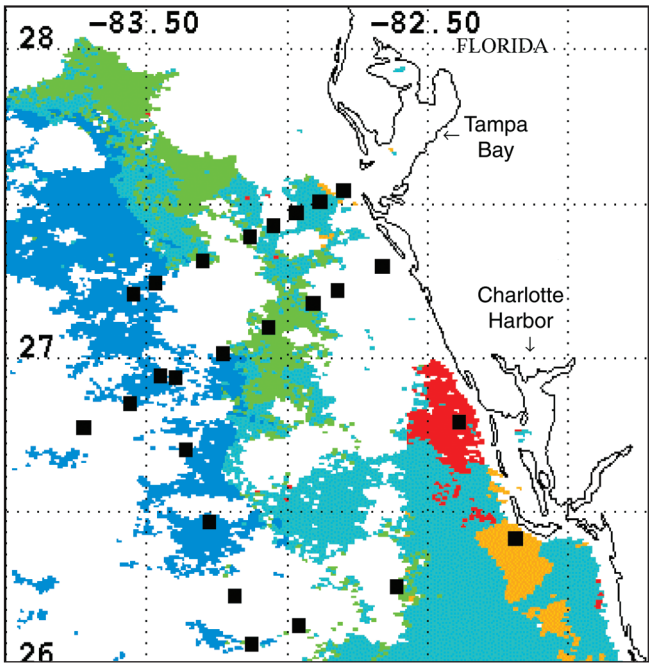

Figure 3 SeaWiFS image (6 October 2000) of the WFS classified using the criteria illustrated in Fig. 2b. Colors represent diatoms (orange), *K. brevis* (red), *Trichodesmium* (green), prochlorophytes(blue), and transitional (light blue) regions and land/clouds (white). ECOHAB station locations are denoted with filled squares.

and relatively high $R_{rs}(490)$ (*e.g.,* high backscattering). Diatom-dominated estuarine waters with [chl *a*] > 1.0 mg m^{-3} also exhibit high reflectivity. *K. brevis* blooms exhibit [chl *a*] > 1.0 mg m^{-3} and low $R_{rs}(490)$ values (*e.g.,* low backscattering).

Application of the classification criteria illustrated in Fig. 2b to SeaWiFS data collected on 6 October 2000 (Fig. 3) indicates that the four phytoplankton taxonomic groups were located as follows: diatoms in low salinity waters associated with Charlotte Harbor, *K. brevis* north of this estuarine plume, *Trichodesmium* offshore of the *K. brevis* population, and prochlorophytes west of the 50 m isobath.

Using this technique, note that high-chlorophyll *K. brevis* blooms are successfully discriminated from high-chlorophyll diatom blooms. Also, the juxtaposition of *Trichodesmium* and *K. brevis* populations on the WFS supports the theory proposed by Walsh and Steidinger [2001] that nitrogen fixed by *Trichodesmium* may provide an important nutrient source for *K. brevis* blooms.

Acknowledgements

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Detection of *Alexandrium fundyense* Bloom Initiation and Transport in the Western Gulf of Maine, USA, Using Mussels (*Mytilus edulis*) on Offshore Hydrographic Moorings

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Abstract

Shellfish toxins that cause Paralytic Shellfish Poisoning (PSP) are seasonally detected in the blue mussel (*Mytilus edulis*) along the western Gulf of Maine (GOM) shoreline, most notably in Casco Bay, Maine. As part of the ECOHAB-GOM field program, mussels were placed in nylon mesh bags and attached to hydrographic moorings deployed both inshore and offshore of Casco Bay. In 1998, toxicity was first detected in the mussels at the most offshore mooring when *Alexandrium fundyense* concentrations reached ca. 200 cells L^{-1}, two weeks prior to detection at the inshore monitoring sites. In contrast, the first toxin detection of year 2000 occurred at an inshore monitoring site in Casco Bay, suggesting local initiation. In both years, however, high levels of toxicity (above quarantine) within Casco Bay were likely the result of delivery and accumulation of *A. fundyense* populations within the Bay from the offshore waters due to both alongshore and onshore transport associated with downwelling-favorable winds. Therefore, monitoring mussels attached to offshore buoys or at offshore islands can complement inshore monitoring programs in regions where large-scale hydrographic processes are responsible for harmful algal blooms (HABs).

Introduction

In the Gulf of Maine (GOM), toxic blooms of *Alexandrium fundyense* occur nearly every year during the spring and summer months, affecting shellfish resources along large stretches of the New England coastline (Anderson, 1997). Several studies have demonstrated that populations of *A. fundyense* are associated with coastal currents that are part of the general circulation of the GOM. Franks and Anderson (1992) observed cells in low-salinity coastal waters and suggested that blooms in the western GOM may originate near the mouth of the Kennebec River. Indeed, higher abundances of *A. fundyense* have been observed early in the bloom season near the frontal boundary of the Kennebec plume as it enters the coastal ocean (Anderson and Keafer, 1993; Anderson *et al.,* submitted). More recently, Townsend *et al.* (2001) demonstrated that offshore *A. fundyense* blooms were associated with the cold, nutrient-rich, tidally well-mixed waters of the Eastern Maine Coastal Current (EMCC) which often deflects offshore of Penobscot Bay, but sometimes can also branch alongshore to join the Western Maine Coastal Current (WMCC; see Fig. 1).

The first detection of shellfish toxicity along the Maine coastline during the spring commonly occurs in Casco Bay, near the Kennebec River mouth. Toxicity usually reaches higher levels and often persists longer than in other areas along the western GOM coast. These observations suggest that Casco Bay is a site for initiation of local blooms or at least an area favorable for accumulation and growth of advected populations, but very little shellfish toxicity data is available from the adjacent offshore waters where *A. fundyense* is known to occur. The objective of this study was to use mussels (*Mytilus edulis*) as biosensors at offshore hydrographic mooring sites to determine if the timing and magnitude of toxicity at the offshore sites could provide evidence of offshore bloom initiation and alongshore and cross-shore transport processes in the Casco Bay region.

Materials and Methods

Hydrographic moorings were deployed both within and offshore of Casco Bay as part of the ECOHAB-Gulf of Maine field program and provided the surface buoys from which to hang mussels (Fig. 1). These moorings provided continuous measurement of temperature (T), salinity (S) and current velocity/direction at 5 m. Three moorings were deployed near Casco Bay in 1998 (M1, M2, and M3). In year

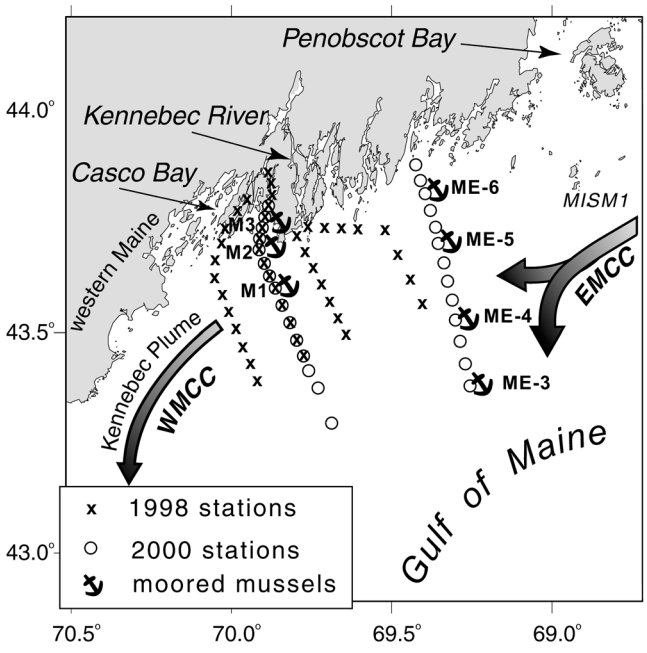

Figure 1 Map of the study area showing the major coastal currents, the EMCC and the WMCC, influencing the western GOM coastline. Hydrographic stations and mussel locations at hydrographic moorings for each study year are indicated.

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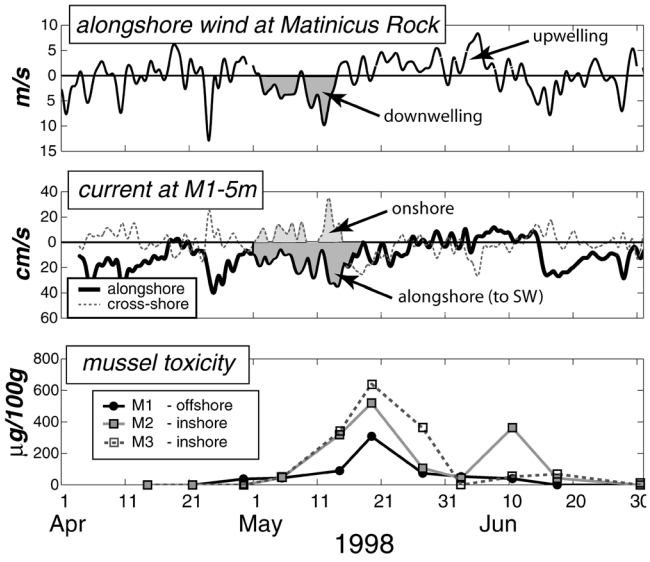

Figure 2 Time series of alongshore wind, alongshore and cross-shore current at M1 mooring, and mussel toxicity (STX eq.) at selected stations in year 1998. The initial outbreak was associated with downwelling-favorable conditions and current flow along and onto the coast.

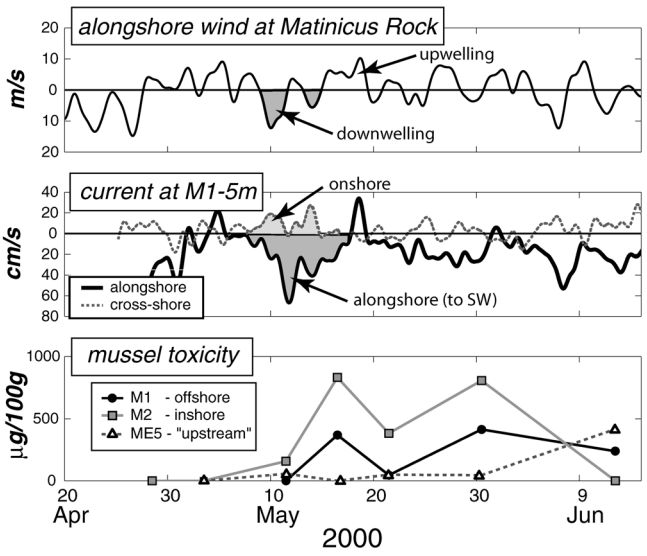

Figure 3 Time series of alongshore wind, alongshore and cross-shore current at M1 mooring, and mussel toxicity (STX eq.) at selected stations in year 2000. As in 1998, toxicity increased with downwelling winds. It persisted with the alongshore and onshore current flow.

2000, additional mussel moorings were established "upstream," out of the influence of the Kennebec River plume (ME-3, ME-4, ME-5, and ME6). Continuous winds were recorded at Matinicus Rock (NOAA # MISM1).

Prior to the bloom season, wild mussels free of PSP toxins were placed in nylon-mesh bait bags and deployed on each buoy at a depth of about 1 m. Each week, snorkelers harvested a mussel bag and attached a replacement (free from toxin) bag. In addition, a bag that was never deployed in the survey region was used as a control. All mussels were assayed for PSP toxins using the standard mouse bioassay (AOAC, 1984).

During the weekly surveys, a CTD/rosette profiler provided T, S measurements and water samples to determine *A. fundyense* cell abundance. Water samples were concentrated with 20 µm sieves and preserved with 5% formalin. *A. fundyense* cells were counted using an immunofluorescent protocol.

Results and Discussion

In 1998, shellfish toxicity was first detected during late April at the most offshore site, M1, followed 1 week later by detection at two moorings, M2 and M3, within Casco Bay. Toxicity at the intertidal monitoring sites in Casco Bay and along the western Maine coast was not detected until two weeks after the offshore levels increased. Although toxic cells were observed within Casco Bay prior to toxin detection in mussels, the highest cell abundance was observed in the offshore waters near the frontal boundary of the Kennebec River plume. The progression of toxicity from offshore to onshore was associated with downwelling-favorable wind conditions that transported the offshore *A. fundyense* population (ca. 200–500 cells L^{-1}) into Casco Bay as indicated by the alongshore and onshore components of the current velocity at M1 (Fig. 2). The results are consistent with a possible offshore source for blooms within Casco Bay.

In contrast, in 2000, shellfish toxicity was first detected at the intertidal monitoring sites within Casco Bay even with the expansion of offshore mussel sites. This observation indicated that a local source of *A. fundyense* cells within Casco Bay might also exist. However, the evidence suggests that the contribution from the offshore waters was more important. First, a denser offshore population (300–500 cells L^{-1}; two- to threefold greater than inshore) existed near the outer Kennebec plume front, similar to 1998, but that population was seaward of the M1 mooring site and therefore was not detected early by the offshore mussels at that mooring. Second, an "upstream" population was present early near mussel sites ME4 and ME5, but it was near the lower limit of cell concentrations that can be detected by the mussels (ca. 200 cells L^{-1}). As toxicity rose rapidly inshore within Casco Bay, toxicity was detected at the same time at two upstream mussel sites (ME4 and ME5), suggestive of an offshore and upstream source. Finally, the rise in toxicity within Casco Bay was associated with downwelling-favorable conditions and transport of surface waters onshore and alongshore at the M1 mooring, comparable to 1998 (Fig. 3). Either the local Bay population grew very rapidly or the offshore population was advected and accumulated within Casco Bay. The rapid rise in both toxicity (from 40 µg 100 g^{-1} to >2000 µg 100 g^{-1}) and cell abundance (from ca 200 cells L^{-1} to >2000 cells L^{-1}) in less than a week, and the association of this rise with downwelling-favorable conditions in both 1998 and 2000, favors the latter inter-

pretation. Transport and accumulation likely dominated local growth processes over these short time scales. Thus, despite the detection of an early season inshore population by the mussel bags in year 2000, significant populations existed <10–20 km offshore. These populations could easily be pulsed into the inshore embayments with wind events and replenished with the flow from the upstream waters. When the flow reversed and moved offshore during strong upwelling, the source population was presumably cut-off from the inshore areas and shellfish toxicity generally declined (*e.g.,* Fig. 2, saxitoxin equivalents).

The westernmost extension of an upstream population of *A. fundyense* associated with the EMCC is thus the most likely source population for PSP outbreaks along the western Maine coastline. This current provides a nutrient-rich environment for growth of *Alexandrium* sp. and other phytoplankton in the GOM (Townsend *et al.,* 2001). The growing population is transported into the western GOM in the spring, facilitated by downwelling-favorable conditions, but toxicity is not detected until the population reaches about 200 cells L^{-1}, which may occur first either offshore (*e.g.,* 1998) or inshore (*e.g.,* 2000). The confluence of the western branch of the EMCC and the river plumes that contribute to the WMCC create an environment where cells can accumulate at frontal boundaries and/or be entrained in the buoyant plume waters. Once in the plume, toxic cells can be delivered into Casco Bay and other embayments along the western GOM coastline with transport strongly influenced by the wind, causing shellfish closures as far south as Massachusetts Bay (Anderson *et al.,* submitted).

This study demonstrated that offshore-moored mussels can serve as useful indicators of impending PSP outbreaks from adjacent offshore waters in the GOM. To be most beneficial, mussels must be deployed at both inshore and offshore sites located based on an understanding of the local and regional circulation patterns. The results must be interpreted with caution, however, due to plankton patchiness and the fact that offshore populations can rapidly develop into inshore blooms during downwelling-favorable conditions. Therefore, wind events and their influence on the behavior of the EMCC and WMCC are critical for prediction of PSP events along the western GOM coastline. These mussel bag data will eventually be used to develop a shellfish toxicity submodel that will be incorporated into bio-physical models currently under development for the prediction of PSP in the GOM (*e.g.,* McGillicuddy *et al.,* 2003). Meanwhile, the state of Maine has expanded their monitoring program to include offshore islands and the deployment of mussels in the offshore waters adjacent to the well-established inshore monitoring sites to improve seafood safety in the region.

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Validation of an LC-MS Method to Detect ASP and DSP Toxins in Shellfish

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Abstract

A method for the routine detection and quantitation of domoic acid, gymnodimine, okadaic acid, pectenotoxin-2 and yessotoxin has been developed and an intra-laboratory validation completed. In addition, the method is semi-quantitative for seven other toxins or classes of toxin. Extractability estimates were made using naturally toxic shellfish and completing exhaustive extractions. Recovery was calculated where pure reference material was available.

Introduction

The use of animal-based test methods is becoming increasingly unpopular (Coghlan, 2002) and so alternative test methods are being used in New Zealand to protect shellfish consumers from the effects of harmful algal toxins (Holland *et al.,* in press). Traditional mouse bioassay methods have often been introduced to fill immediate needs or in response to emergency situations. Frequently these methods are not validated and little or no performance data is available (Hannah *et al.,* 1995). The introduction of test methods based on LCMS (MacKenzie *et al.,* 2002) has required extensive validation (Holland, 2002). Some of the validation findings are presented here.

Materials and Methods

Sample Preparation A blended shellfish homogenate (2.0 g) was extracted with methanol/water 9+1v/v (18 mL). Following centrifugation (3000 g, 10 min), a 2 mL aliquot of supernatant was washed with 5 mL of hexane. The methanol/hexane mixture was centrifuged (3000g, 10 min) and the methanol extract transferred to an autosampler vial for LC-MS/MS analysis. The proportions of esterified forms of DSP toxins ('DTX3') were determined by analysis of OA and DTX1 concentrations in sample extracts before and after alkaline hydrolysis (Mountfort *et al.,* 2001).

LC-MS/MS Analysis A Waters 2790 LC system and Quattro Ultima triple quadrupole mass spectrometer system (Micromass Ltd., UK) was used. Chromatographic separation was performed using a Phenomonex Luna C18 column (150 × 2 mm) with 5 µm packing. A gradient from 13% acetonitrile to 77% acetonitrile containing a constant concentration of buffer (4 mM ammonium hydroxide and 50 mM formic acid) was run between 2 and 10 minutes and held at 77% until 25 minutes. The electrospray ionisation interface (ESI) was operated in both positive and negative modes (source temperature 80°C, cone gas flow 50 L N_2/hr, desolvation temperature 350°C and gas flow 500 L N_2/hr). The mass spectrometer was operated in MS-MS modes with collision cell gas pressure (argon) set at 1.2×10^{-3} T. For multiple reaction monitoring (MRM) the mass channels and optimal cone voltages and collision energies for each biotoxin were established from daughter ion studies with standards or extracts of contaminated shellfish. The MRM channels were monitored in windows that covered the elution of the compounds of interest (parent > daughter): ESI positive, capillary 3.5 kV; Domoic acid (DA) 312.2 > 161.1 and 312.2 > 266.15, gymnodimine (GYM) 508.4 > 392.3 and 508.4 > 490.3, pectenotoxin-2 (PTX2) 876.7 > 823.5, pectenotoxin-1 (PTX11) 892.7 > 839.5, pectenotoxin seco acid (PTX2 SA) 894.7 > 805.4, azaspiracid-1 (AZA1) 842.6 > 672.6. ESI negative, capillary 3.0 kV: Okadaic acid (OA) and dinophysis toxin-2 (DTX2) 803.5 > 255.0, dinophysis toxins-1 (DTX1) 817.5 > 255.0, 45 hydroxy yessotoxin (45OH-YTX) 1157.5 > 1077.5, yessotoxin (YTX) 1141.5 > 1061.5.

Materials Whole tissues of Greenshell™ mussel (*Perna canaliculus*), pacific oyster (*Crassostrea gigas*) and cockle (*Austrovenus stutchburyi*), and roe of scallop (*Pecten novaezelandiae*) were studied. Certified reference material (CRM) containing ASP toxins (mussel, MUS-1B, NRC, Canada) and DSP toxins (mussel, MUS-2, NRC Canada) was diluted into blank Greenshell™ mussel homogenate to provide suitable levels for accuracy/bias studies. Shellfish samples naturally contaminated by ASP, DSP, PTX and YTX toxins were used for extractability studies.

Authentic material was used to calibrate the LCMS response for DA, GYM, PTX2, OA and YTX, the Quan toxins. PTX11, PTX2 SA, DTX1, DTX2, 45 OH-YTX and AZA1 were quantified using the response factor of the most closely related Quan toxin. In the case of AZA1 the GYM response was used as only small amounts of contaminated shellfish were available (courtesy of the Marine Institute, Ireland) and no local samples have been found to contain this toxin.

Results and Discussion

Validation Scope and Parameters The method detailed above was developed to replace mouse bioassay testing in the comprehensive New Zealand Marine Biotoxin Monitoring Programme. The toxins detected and the limits of detection required were considered prior to beginning to develop the method so that once fully validated this LCMS method could be used for regular monitoring in New Zealand. This approach has reduced the number of mouse bioassays for DSP completed in New Zealand by over half. Validation of the LC-MS/MS method followed guidelines

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Table 1 Summary of key validation parameters.

	Recovery	CRM	R_r (RSD)	R_R (RSD)	LOD (mg/kg)	Horrat
DA	99%	82%	4.6%	5.1%	0.020	1.05
GYM	94%	NA	9.6%	10.2%	0.0006	0.46
OA	99%	93%	14.8%	16.9%	0.016	0.75
PTX-2	101%	NA	16.0%	16.4%	0.010	0.73
YTX	71%	NA	14.2%	15.4%	0.016	0.68
Other	79–160%	NA	7–17%—Concentration from Rf		0.01–0.02	NA

for new methods published by the New Zealand regulatory authority (Burrow and Seamer, 2001). The following method parameters were studied during this validation study: Linearity, recover, repeatability, reproducibility, extractability, comparison to other methods, robustness, standard stability and uncertainty. A result summary is presented in Table 1.

Certified Reference Material (CRM) The CRMs available from NRC Canada were extremely valuable for establishing accuracy and the excellent results obtained for DA, OA and DTX-1 give confidence of the method validity. The calculated amount of DA was 83% of the CRM value due to the use of 90% methanol as an extractant. In addition, this and other data from fortification experiments have shown the method precision to be very high. Currently those samples with levels greater than 10 mg/kg are further tested using a DA-only method that uses 50% methanol for extraction. OA and DTX-1 gave amounts of 92% and 104% of expected, within the experimental error. The DTX-1 result confirms the use of response factors (Rf's) for calibration as valid. The CRM's were tested after dilution with the most common local species, Greenshell™ mussel, so that any effects due to matrix could be observed.

Contaminated Shellfish Greenshell™ mussel samples from recent bloom events (MacKenzie *et al.,* 2002) which contained DSP, PTX and YTX toxins were used to test a number of parameters. Following dilution of homogenate or contaminated extract into blank sample matrix the linearity of response was tested for all compounds. A plot of the percentage of contaminated sample against the LCMS response gave highly linear responses except YTX at the highest levels studied (>200 ng/mL).

Recoveries for the PTX seco acids were enhanced by up to 160%. Repeat analyses showed the non Quan toxins to have similar precision parameters to the related Quan toxin and previous fortification experiments had shown that repeatability of LCMS response was a large contributor to recovery and precision so this type of experiment incorporates most sources of variation.

Extractability Contaminated shellfish was extracted three times to assess the extractability for each toxin. The levels of PTX11, PTX2 and OA were too low to be useful. However the CRM data already collected showed that a single extraction gave acceptable results for OA. The recovery of PTX seco acids and GYM was greater than 90% using a single extraction. YTX and 45 OH-YTX were 75% extracted in a single extraction and the second and third extraction contained significant levels of both. The combination of LCMS suppression effects, which are likely to be less in subsequent extractions and tissue binding most likely explain this result. Levels of YTX and/or 45 OH-YTX above 0.5 mg/kg require a second extraction in routine sample processing. Samples containing DA, including the CRM, were extracted once with 90% methanol then the pellet was re-extracted with 50% methanol. The 90% methanol extraction contained 82% of the DA while the combined extracts contained 97%.

Conclusions

By completing a comprehensive validation study and assessing the parameters listed we have shown that LCMS is a suitable technique to apply to the routine testing of shellfish for ASP and DSP toxins. Furthermore by using the data from the validation study method uncertainty can be calculated. For example based on Eurachem the authors calculate that a domoic acid level of 1.8mg/kg would be between 1.3mg/kg and 2.3mg/kg, with a 95% degree of confidence. The validation study allowed the collection of data that were used to develop quality control criteria such as minimum LCMS response to achieve LODs and acceptable spike recoveries. In the case of YTX and DA the validation showed that additional laboratory procedures needed to be developed to ensure accurate levels are reported.

Calibrations were all highly linear in the range 5–200 ng/mL ($R^2 > 0.98$). Repeatability (R_r—same day same operator) and Reproducibility (R_R—different day, different operator) was measured at 0.05–0.20 mg/kg. Limit of detection (LOD) three times the peak to peak noise in blank samples, with comparison to low level spikes. The DA and YTX recovery is the average for all matrices although enhancement of DA with Cockle and suppression of YTX with Scallop Roe were observed.

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Competitive ELISA: An Accurate, Quick and Effective Tool to Monitor Brevetoxins in Environmental and Biological Sample

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Abstract

A competitive Enzyme-Linked Immuno-Sorbent Assay (competitive ELISA) has been developed for analyzing brevetoxins (PbTxs). Antibodies to brevetoxins were used in combination with a multi-step signal amplification procedure for the detection of toxins. This procedure minimizes non-specific signals and background noise often observed in complex matrices. Therefore, analysis can be performed with various samples (seawater, air filter, mammalian body fluids, shellfish, etc.) without the need for extensive extraction and/or purification steps. Brevetoxin analysis in liquid samples like seawater, urine and serum can be performed without pretreatment, dilution or purification. The limit of quantification of PbTxs is 2 ng mL^{-1} in any of the liquid sample matrices tested. For shellfish monitoring, analyses are performed after homogenization of shellfish meat (5 g) with brevetoxin-ELISA buffer (200 mL) and can be performed on tissue from a single mollusk as well as on a pool of shellfish meat. Comparative quantification of PbTxs achieved in buffer, seawater, mammalian body fluid and shellfish homogenate spiked with equal amounts of toxin (10 ng mL^{-1} sample) varied by no more than 5%. These data suggest that the matrix composition of the sample does not affect the performance of the assay. Because this assay is not affected by matrix composition and can be performed in shellfish homogenate, this procedure can be used to prevent or diagnose human exposure to PbTxs and has the potential to replace the currently used mouse bioassay for monitoring PbTxs in shellfish.

Introduction

Almost every year, and sometimes several times in the same year, blooms of the dinoflagellate *Karenia brevis* are observed in the Gulf of Mexico. These blooms, also called red tides, usually affect the west coast of Florida, but on several occasions have had an impact on every state that borders the Gulf of Mexico. Red tides appear to have had a long history in Florida, with the earliest recorded event dating to the 1800s. Red tides almost always result in fish kills and temporary closures of local shellfish harvesting areas. Local tourism activities such as boating, recreational fishing and beach-related activities are also negatively affected. Red tides are a threat to both human and environmental health. *K. brevis* produces brevetoxins, potent neurotoxins that are found in the organisms at a concentration of approximately 10 pg $cell^{-1}$ as well as in the seawater supporting blooms. In addition to the health effects associated with neurotoxic shellfish poisoning, there have been multiple anecdotal reports of respiratory irritation and possibly immunologic effects associated with the inhalation of aerosolized seawater during Florida red tides. Recent die-offs of the endangered Florida manatee have also been associated with brevetoxins. Research in sheep and other laboratory animals has confirmed the ability of aerosolized red tide toxins to cause reversible bronchospasm. Brevetoxins induce toxicity at very low concentrations. Therefore, effective monitoring requires an analytical procedure having sufficient sensitivity and specificity to detect toxin at sub-symptomatic levels. Since the matrices in which the toxins are found (algae, shellfish, body fluid and seawater) are diverse and complex, current methods of quantitative analysis are laborious and imprecise, requiring many steps of extraction and purification. The difficulties in quantifying brevetoxins in biological samples have led us to pursue an alternate analytical approach. This study reviews the use of the sensitive and accurate ELISA method developed in recent research on shellfish management and monitoring, and human and animal exposure to aerosolized brevetoxins (Naar *et al.*, 2002).

Materials and Methods

Competitive ELISA Development of this method is fully described in Naar *et al.* (2002).

A: Liquid samples (seawater, urine, serum, etc.) Samples were serially diluted (dilution factor = 2) with buffer into the sensitized ELISA plate. Anti-brevetoxin antibodies were added into all the wells and incubated for 1 hour. After incubation, the plate is washed, and the revelation of immobilized antibodies is obtained by successive addition of the secondary antibody, the horseradish peroxidase conjugate, and the enzyme substrate. The reaction is terminated by addition of sulfuric acid. Absorbance is recorded at 492 nm to quantify the amount of brevetoxin present in the sample.

B: Shellfish samples Shellfish meat was homogenized in buffer using a commercial blender. The homogenate can be analyzed directly using the protocol describe above. Alternatively, brevetoxins can be extracted from shellfish with organic solvents (ethyl ether or acetone). After extraction, the residue is dried and dissolved in buffer for analyses as performed above.

Results and Discussion

Exposure to aerosolized brevetoxins The Gulf Coast is a region highly dependent on tourism and other coastal industries. Faced with intermittent aerosol exposures resulting in possible acute and chronic respiratory effects, a new public health and epidemiologic investigation has been instituted in Florida. To investigate the human health effects of environmental exposure to red tide toxins, an interdis-

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ciplinary team of scientists has been formed (Fleming *et al.,* 2003). When a red tide moves near shore where people might be exposed, this team rapidly assembles at the site to collect environmental samples and epidemiologic data.

With the development of the competitive ELISA methodology for brevetoxin analysis, levels of contamination by brevetoxins in water and in sea sprays (Chung *et al.,* 2003) were precisely measured on the day samples were collected. During moderate and high exposure periods in Jacksonville, FL, brevetoxin concentrations of 36 to 80 ng/m^3 were measured in the air (Pierce *et al.,* 2003). An average adult breathes in 25 L min^{-1} of air during light exercise. People visiting the beaches during these periods were inhaling up to 54–120 ng of brevetoxin hr^{-1}, or an inhaled dose of 0.77–1.71 ng kg^{-1} hr^{-1} (Backer *et al.,* 2003). During the same period, using the ELISA methodology, analysis of air filters from a personal air sampler revealed a direct correlation between presence on the beach and exposure to brevetoxins (unpublished data).

During the fall of 2001, an extensive Florida red tide was studied off the coast of Sarasota, Florida. The data obtained included *K. brevis* counts in the water, the amount of toxin in the water, toxin in aerosols transported on shore, subsequent exposure of humans through respiration, and both throat swab and epidemiologic data on occupationally exposed individuals (lifeguards). These data were supported by meteorological measurements. ELISA was used to quantify total toxin on site. ELISA and LC-coupled mass spectrometry were used subsequently in the lab (Baden *et al.,* 2003). During the 5-day study, toxin concentrations in the water were measured at six different locations and ranged from 20 ng mL^{-1} to 400 ng mL^{-1}. *K. brevis* cell counts ranged from 1,000 to 15 million cells L^{-1}. The amount of toxin on impact air sampler filters was 80–467 ng cm^{-2}. Symptoms of sneezing, eye irritation, and coughing were experienced by the lifeguard subjects and scientists. Toxin levels and symptoms were inversely correlated with distance from the shoreline (Baden *et al.,* 2003).

During calm weather, offshore seawater samples show a strong correlation between cell concentration and toxin amount in the water. However, the cell counts from inshore water samples (at the beach) are not a good predictor of the toxin concentration (unpublished data). It is suspected that wind and wave action, combined with low water depth, induce cell lysis and subsequent accumulation of brevetoxins in the water. Brevetoxin concentrations can be high even as the cell densities remain low. Thus, we believe that measuring the cell abundance to monitor water quality and predict respiratory irritation and possible deleterious health consequences at the beach should be replaced by toxin analysis.

Management and Monitoring

Prevention of Neurotoxic Shellfish Poisoning in the United States relies upon environmental monitoring for *K. brevis* and timely closure of affected shellfish resources. The reopening of resources is contingent upon results from mouse bioassay of extracts from exposed shellfish. Recent research has shown that brevetoxins are rapidly accumulated and metabolized by shellfish and that the mouse bioassay method does not account for potential toxicity of the metabolites. With the goal of replacing the mouse bioassay, a multi-laboratory comparative study was undertaken to test the accuracy and precision of four alternative methods for the determination of brevetoxins in shellfish. These include the N2a neuroblastoma cytotoxicity assay, two variations of the sodium channel receptor binding assay, the competitive ELISA, and LC/MS (Dickey *et al.,* 2003). Results of this study indicate that the ELISA methodology is the best candidate to replace the mouse bioassay. Because of its sensitivity, ELISA analysis can be performed on a single or even part of a single bivalve mollusk. Individual analyses of shellfish harvested at the same time from the same bed have shown a low variability in toxin concentration (Weidner *et al.,* 2003), indicating monitoring of shellfish by ELISA can be very precise while reducing the time and cost of analysis. However, depending on the protocol used (homogenization or organic solvent extraction), parent brevetoxins and brevetoxin metabolites can be measured together or individually in shellfish (Naar *et al.,* 2003). We believe that the regulatory agencies should seriously consider evaluating the different types of toxins that need to be monitored to prevent human intoxication, whether they are parent toxins, toxin metabolites or both.

Since the discovery of other brevetoxin-producing algae in US coastal waters (Bourdelais *et al.,* 2002), brevetoxin production by algae from the genera *Chattonella* and *Fibrocapsa* has been described (Bridgers *et al.,* 2003). Again, the ELISA methodology was used to rapidly and precisely measure brevetoxin production by theses organisms.

The competitive ELISA assay is reliable, inexpensive, quantitative, and not limited to a single application (*i.e.,* no matrix effects are observed). Since its development in 2002, this assay has already been used by several laboratories and regulatory agencies. Current applications include diagnosis of exposure in birds, marine mammals and humans as well as monitoring of shellfish, seawater and sea aerosols.

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NSP (*Karenia brevis*) Toxins and Metabolites in Oysters, Clams, and Whelks

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Abstract

Three species of shellfish (clams, oysters and whelks) were collected during and following an intensive *Karenia brevis* bloom that occurred from August to December 2001, in Sarasota Bay, Florida, USA. The purpose was to monitor the accumulation of brevetoxins and production of metabolites (toxin-conjugates) in clams and oysters exposed to the same natural harmful algal bloom and to observe trophic transfer of neurotoxic shellfish poisoning (NSP) components to whelks feeding on contaminated clams. The ultimate goal is to identify the toxins and toxin-conjugates responsible for NSP. Two previously reported toxin-metabolites were observed in clams and oysters (*m*/*z*1018 and 1034), with little or no parent brevetoxins detected, and no trophic transfer was observed in whelks.

Introduction

The harmful alga *Karenia brevis* (formerly *Gymnodinium breve* Davis) (Daugbjerg *et al.,* 2001) is a dinoflagellate that produces a suite of polyether neurotoxins responsible for neurotoxic shellfish poisoning (NSP) (Poli *et al.,* 1986; Shimizu *et al.,* 1990; Baden *et al.,* 1995). Filter-feeding bivalve mollusks, such as clams, oysters, and mussels, accumulate high concentrations of these toxins as they ingest the harmful algal cells from the water (Steidinger, 2002). Identification and quantitation of toxins responsible for NSP in the shellfish is complicated by shellfish metabolism of the parent toxins to toxin-conjugates, the toxicity of which is not known (Poli *et al.,* 2000; Plakas *et al.,* 2002). Evidence for shellfish metabolism of brevetoxins appeared following a 1992–1993 outbreak of NSP in New Zealand. Ishida *et al.* (1995) isolated a C-42 N-taurine conjugate of PbTx-2 (named BTX-B1) in the cockle *Austrovenus stutchburyi.* Murata *et al.* (1998) found an oxidized (sulfoxide) S-cysteine conjugate (named BTX-B2) in the greenshell mussel *Perna canaliculus* and proposed biosynthetic routes from either PbTx-2 or -3. Morohashi *et al.* (1995) described fatty acid esters of PbTx-2 in *P. canaliculus* formed by cleavage of ring D, esterification with palmitic or myristic acids, and oxidation of the terminal aldehyde, which they named BTX-B3. Further evidence for toxin metabolites was obtained during a 1996 Gulf of Mexico *K. brevis* bloom when metabolic conjugates of PbTx, with mass to charge (*m*/*z*) ratios of 1018 and 1034 for the cystine and cystine sulfate conjugates, respectively, along with PbTx-3, were isolated from oysters, *Crassostrea virginica,* collected from affected areas (Dickey *et al.,* 1999; Poli *et al.,* 2000). Trophic transfer of neurotoxins persisting in shellfish also has resulted in human intoxication from NSP (Poli *et al.,* 2000). We investigated brevetoxins and metabolites in clams (*Mercenaria mercenaria*), oysters (*Crassostrea virginica*) and the whelk (*Busycon* sp.), all exposed to the same *K. brevis* bloom in Sarasota Bay during August to December 2001. Two of the previously reported metabolites were observed in clams and oysters, but none were observed in the whelks.

Materials and Methods

Shellfish were collected routinely from a common site in Sarasota Bay, Florida, during and following an intensive *K. brevis* bloom that lasted from August 2001 into December 2002. Shellfish were placed on ice and shipped overnight to the FDA Lab for processing. A minimum of 18 clams and 24 oysters were shucked under clean conditions, the liquid drained off and the tissue homogenized as one composite sample for each species. Only one or two whelks were collected each time to avoid depletion of the local mudflat population. The homogenate was separated into a 100 g aliquot for mouse bioassay and into 1 g aliquots for analyses by liquid chromatography-mass spectrometry (LC-MS). Brevetoxins and metabolites were extracted from shellfish tissue in acetone with additional separation and clean up by solid-phase extraction (Plakas *et al.,* 2002). Toxicity of the shellfish tissue was determined with the standard mouse bioassay utilizing 100 g shellfish tissue from which toxins were extracted into diethyl ether. The residue was dissolved in cottonseed oil, 1 mL was injected (ip) into mice and the time to death observed (APHA, 1970).

Toxins and toxin-conjugates were analyzed by LC-MS, using Agilent Technologies (Palo Alto, CA) Model 1100 LC system and Thermo Finnigan (San Jose, CA) Navigator AQA MS detector with electrospray ion source, on a YMC J'Sphere ODS-L80 S-4, 2.0 × 250 mm column (YMC Inc., Wilmington, NC) under gradient conditions: mobile phase reservoirs were (A) 0.1% glacial acetic acid in LC grade water; (B) 0.1% glacial acetic acid in acetonitrile. Gradient conditions were: 35% B for 2 min., linear gradient to 70% B at 45 min., ramp to 100% B at 50 min., hold for 15 min. (to wash column), return to initial conditions (in 5 min.), and condition column for 25 min. before next injection. Flow rate was 0.2 mL/min. and column temperature, 40°C. Sample injection volume was 12.5:L. MS data were acquired by full scan mode in the range *m*/*z* 700–1200 or by selected ion monitoring (SIM). Standards of pure PbTx-2 and -3 were provided by Dr. Baden at UNC Wilmington. Although standards were not available for the metabolites, probable metabolite structures were previously identified by

Steidinger, K. A., J. H. Landsberg, C. R. Tomas, and G. A. Vargo (Eds.). 2004. Harmful Algae 2002. Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO.

Table 1 Toxicity by mouse bioassay and concentrations by LC-MS (in PbTx-3 equivalents) of brevetoxin and metabolites in oysters collected from 10/08/01 through 3/20/02 in Sarasota Bay, Florida, with *K. brevis* cell counts (*m/z* 1018 = cystine conjugate, *m/z* 1034 = cystine sulfate conjugate).

Date of Collection	Species	Mouse Bioassay MU/100 g	LC-MS PbTx-3	µg/g PbTx-3 Equivalent *m/z* 1018	*m/z* 1034	Total	*K. brevis* Cells/mL
10/08/01	oyster	34	0.0	6.9	3.1	10.0	
11/13/01	oyster	108	0.4	12.2	3.9	16.5	660
12/04/01	oyster	146	0.0	8.2	2.7	10.9	310
12/17/01	oyster	58	0.0	3.9	1.0	4.9	0
01/04/02	oyster	29	0.0	1.8	0.0	1.8	0
01/18/02	oyster	31	0.0	1.8	0.7	2.5	0
02/13/02	oyster	34	0.0	6.5	1.1	7.6	306
03/20/02	oyster	31	0.0	2.3	0.6	2.9	0

LC/MS/MS using a Micromass Q-Tof II hybrid quadrupole/orthogonal time-of-flight mass spectrometer (Micromass, Manchester, UK), (Plakas *et al.,* 2002).

Results

Results of the mouse bioassay toxicity and LC-MS analyses to identify the brevetoxins and metabolites present in oysters collected from 10/08/01 through 3/20/02 are given in Table 1 along with *K. brevis* cell counts in the water. These results show that no PbTx-2 and only a trace of PbTx-3 was observed in oyster tissue, while two of the previously reported toxin-metabolites were readily abundant (*m/z* 1018 and 1034). The *K. brevis* cell counts indicate that the oysters were exposed to high concentrations throughout October and November 2001, and diminished to background levels in December. Although the toxin-metabolite concentration diminished along with the cell counts, oysters retained mouse toxicity and levels of the metabolites remained above background for more than 6 weeks, until a small bloom was again observed in the area in mid February.

A comparison of brevetoxins and toxin-metabolites observed in the three species of shellfish is given in Table 2. These results show no accumulation of PbTx-2 or -3 in the clams, consistent with the above results for oysters. One of the whelk samples indicated the presence of PbTx-3, but no metabolites were observed in the whelk tissue. Both clams and oysters exhibited accumulation of the metabolites. All three species exhibited mouse toxicity.

Discussion

Both clams and oysters were found to accumulate the brevetoxin conjugates (*m/z* 1018 and 1034), but not the parent toxins. The toxin-conjugates were observed in higher concentrations in the oysters relative to the clams during exposure to the bloom; however, both species retained approximately the same level of conjugates after the bloom subsided. An important observation is that the mouse toxicity and toxin-conjugate contamination persisted for six (6) weeks following the bloom, indicating a persistent public health risk. That clams and oysters exhibited mouse toxicity, yet contained no detectable amounts of parent toxins, would suggest that the toxin-conjugates represented the NSP toxic components. This conclusion is complicated by the fact that, although less toxic than the clam, the whelk still exhibited some mouse toxicity. However, the two primary conjugates (*m/z* 1018 and 1034) were not observed in the whelk tissue, contrary to the findings of Poli *et al.* (2000). This may be due to the presence of other toxic conjugates not recognized with the LC-MS method, or to difficulty in extraction and recovery of toxins and toxin-conjugates from the whelk. Although parent toxin and

Table 2 Mouse toxicity, brevetoxins and metabolites in oysters, clams, and whelks collected from Sarasota Bay from 12/04/01 through 1/22/01.

Date	Species	MU/100 g	PbTx-3	*m/z* 1018	*m/z* 1034	LC-MS Total
12/04/01	oyster	146	0.0	8.2	2.7	10.9
12/04/01	clam	69	0.0	1.8	0.9	2.7
12/04/01	whelk	22	0.0	0.0	0.0	0.0
01/08/02	oyster	29	0.0	1.8	0.7	2.5
01/08/02	clam	27	0.0	1.6	0.6	2.2
01/08/02	whelk	16	0.3	0.0	0.0	0.3
01/22/02	oyster	31	0.0	1.8	0.7	2.5
01/22/02	clam	24	0.0	1.3	0.5	1.8
01/22/02	whelk	13	0.0	0.0	0.0	0.0

metabolite recovery have been verified from spiked clam and oyster tissue, further validation of methods for whelk tissue is required before conclusions can be drawn regarding trophic transfer of toxins and metabolites from clams to whelks.

Acknowledgements

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Recent Developments in the Analysis of Algal Toxins

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Abstract

The development of novel analytical strategies for detecting low, yet toxic, concentrations of algal toxins is an important concern for field scientists. Improvements in sensitivity, selectivity and rapid analysis are clearly needed. The complexity of the sample matrix also plays an important role in such developments. For this reason, special emphasis has been placed on the improvement of sample preparation strategies for faster and more efficient sample extraction and clean-up. We report the results of techniques that improve both sample pre-treatment and analysis using modern analytical approaches such as Accelerated Solvent Extraction (ASE) and Microwave Assisted Processes (MAP). These strategies have been applied to the extraction of Paralytic Shellfish Poisoning (PSP) toxins, with applications of High Performance Liquid Chromatography (HPLC), Capillary Electrophoresis (CE) and Capillary Electrochromatography (CEC) for Amnesic Shellfish Poisoning (ASP) toxins and Yessotoxins (YTX). The results demonstrate these approaches enhance sensitivity, selectivity and short analysis times for HPLC and CE. Evaluation of the CEC approach, as a hybrid technique between CE and HPLC, led us to conclude that this is a promising technique, although further research in the development of alternative stationary phases is still required.

Introduction

Considerable effort has been devoted over the last few years to the development of analytical methodologies for algal toxins. Mass spectrometry has clearly contributed to advancements in this field due to its potential for confirmation and identification of new toxins.

The complex matrix containing these toxins, as well as the low concentrations at which they occur, make sample preparation a tedious and time-consuming step and a source of large errors. Supporting the need for improvements in sample preparation protocols, analytical goals for sensitivity, selectivity, efficiency, reliability and accuracy are not always accomplished. We have focused on improved methods for sample extraction and clean-up, applying new strategies that have been reported as efficient alternatives for a range of organic contaminants.

Regarding extraction, Accelerated Solvent Extraction (ASE) uses conventional liquid solvents at elevated temperatures to increase extraction efficiency (Richter *et al.,* 1996). Microwave Assisted Process (MAP) extraction is based upon the fact that different chemical substances absorb microwave energy at different levels, resulting in selective and rapid extraction of algal toxins (Jassie *et al.,* 1997).

Additional approaches for sample extraction and clean-up include SPME and Immunoaffinity (IAC) extraction (Theodoridis *et al.,* 2000; Van Emon, 2001), based on the specificity of antigen-antibody interactions (Van Regenmorte *et al.,* 1997). Finally, we used CE to analyse YTX and CEC to analyse ASP toxins.

CE applications have increased over the last few years, resulting in a simple and fast alternative that can be efficiently applied for a wide range of analytes. Its versatility improved with the development of a number of CE modes, such as Micellar Electrokinetic Chromatography (MEKC) that allow the analysis of neutral molecules. Lack of sensitivity with CE has been overcome by using isotacophoresis or Field-Amplified Sample Stacking (FASS) that concentrates the analyte. CE was used to analyse YTXs following the conditions proposed by Prof. Yasumoto (Yasumoto, pers. comm.).

Results and Discussion

Accelerated Solvent Extraction (ASE) An ASE 200 automated extractor, Dionex, was used for the extraction of mussel tissue contaminated with gonyautoxin (GTX) 2/3. The optimal extraction conditions briefly follow. The toxin is extracted on a Hydromatrix Solid sorbent support (Varian, Harbor City, CA) using a solvent of 5% acetic acid at 1500 psi with three, 5 min cycles of 100% solvent flushes at 100ºC. The ASE extracts were then derivitized with periodate or peroxide and analysed by HPLC with fluorescent detection (HPLC-FLD), following Lawrence *et al.* (1991). This extraction procedure was compared with the conventional Association of Analytical Communities (AOAC) procedure, except substituting 5% (v/v) acetic acid for hydrochloric acid. ASE efficiency had been slightly greater for most toxins except GTX 1/4; nevertheless, poor reproducibility was found and attributed to matrix effects.

MAP Extraction Different parameters affecting the microwave-assisted process, such as irradiation time, irradiation power, type of solvent and solvent volume, were tested for the extraction of PSP toxins. The optimal conditions included 3 mL of 0.25 M acetic acid, an irradiation time of 15 seconds with an irradiation power of 270 W. The efficiency of the extraction was improved, increasing sensitivity. This technique was compared with AOAC conventional extraction and with ASE extraction in this case as an example of application to GTX 2/3. Both ASE and MAP were found to efficiently extract PSP toxins rapidly and with minimal sample degradation. An example of the applica-

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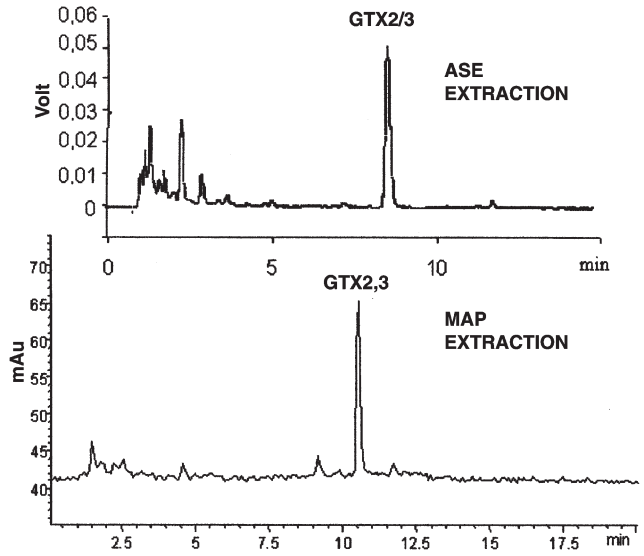

Figure 1 Comparison M.A.P. versus A.S.E. extraction.

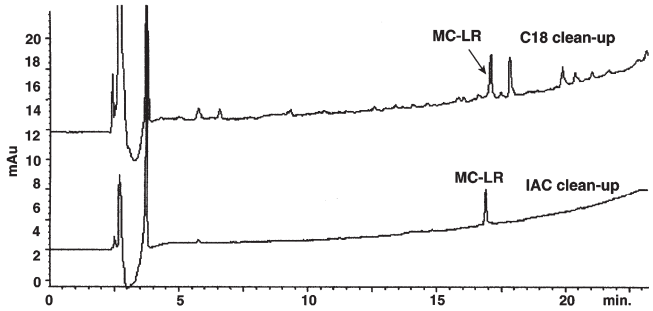

Figure 2 Comparison of different clean-up procedures for water sample naturally contaminated with Microcystin-LR.

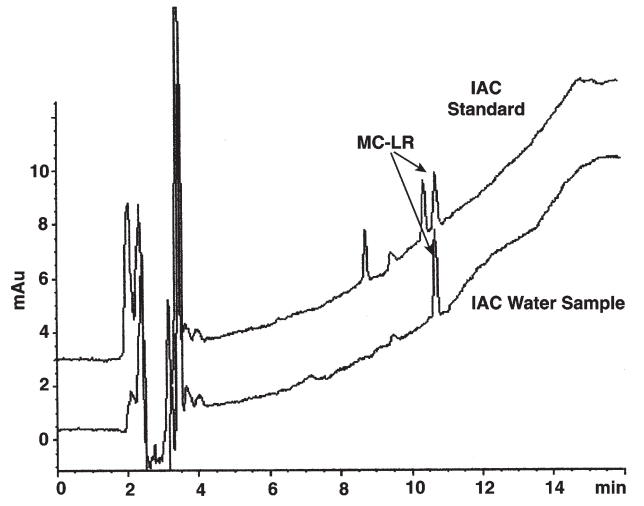

Figure 3 HPLC-UV/DAD analysis of contaminated water sample using SPME pretreatment.

tion of both ASE and MAP for the precolumn HPLC/FLD analysis of PSP toxins in "naturally" contaminated samples is shown in Fig. 1.

IAC Extraction A solid phase extraction (SPE) procedure for microcystins was tested using IAC cartridges (Abkem Iberia, Spain). Specificity of antigen-antibody interactions offered the best potential for increased selectivity and sensitivity. The sample was diluted with Phosphate Buffer Solution (PBS) to obtain a methanol concentration less than 15%. Cartridges were conditioned with 3 mL of water and 3 mL of PBS prior to loading the sample. The cartridge was rinsed with 3 mL of PBS, followed by 3 mL of water and 3 mL of methanol:water (25:75). The microcystins were eluted with 6 mL of methanol:water (80:20) containing 4% (v/v) acetic acid. The effluent was collected in a 50-mL round bottom flask and evaporated to dryness at 35°C, then dissolved in 0.1–0.5 mL of acetonitrile:water (20:80) for injection into the HPLC system.

When compared with the conventional silica SPE, this clean-up procedure more efficiently removed specific interferences and achieved high recovery, contributing to sensitivity increase, which is especially important for trace-level contamination samples. An example of the results obtained after SPE and IAC- HPLC/ultraviolet (UV) analysis of microcystins is shown in Fig. 2.

SPME SPME has been used as a modern alternative for the extraction of microcystins in contaminated waters. We have tested the optimal conditions (Gago-Martínez *et al.,* unpublished results). Briefly, the methods are: a polydimethyl- siloxane divinylbenzene (PDMS/DVB) 60 mm fiber was used to extract 3 mL of sample over 60 min. at pH 2.0 using 20% NaCl at 55ºC with an agitation of 1000 rpm, followed by HPLC/UV analysis. An example of the results is shown in Fig. 3. We conclude that SPME is a fast and efficient way of extracting microcystins in water. This method avoids further clean-up, simplifies the sample preparation protocols, and considerably reduces organic solvent use.

CE Analysis of YTXs Analysis of YTXs was performed using CE with UV-diode array detection. The initial analysis conditions were proposed by Yasumoto (pers. comm.), but have been modified to include a FASS mode and a solution of MeOH:Buffer (7:3) to overcome a lack of sensitivity with low levels of YTXs. The results, using a standard and naturally contaminated samples of YTXs, are shown in Fig. 4. From the results we concluded that CE is a simple, rapid alternative that does not require any further sample pre-treatment (*e.g.,* derivatization), and provides adequate YTX sensitivity.

CEC Analysis of ASP Toxins Preliminary work was performed by Leão *et al.* (2002) showing promising applications for CEC as an alternative to HPLC and CE in domoic acid analysis. Samples naturally contaminated with ASP were analysed by CEC. Briefly, 5 mM PBS (pH 2.5) is

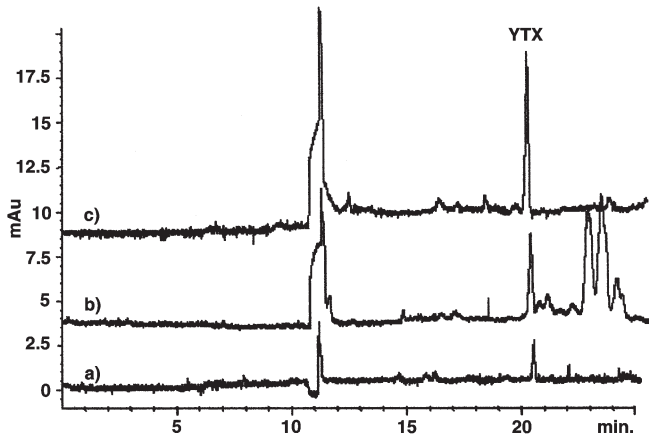

Figure 4 Electropherograms obtained for YTX analysis: **a**) standard of YTX 5 µ/mL, **b**) naturally contaminated mussel hepatopancreas sample from *P. canaliculus,* **c**) phytoplankton sample from culture *P. reticulatum.* Background buffer: 70 mM phosphate-MeOH (60:40) at pH 8.5. Injection 50 mbar for 20 s. Run voltage: 30 kV.

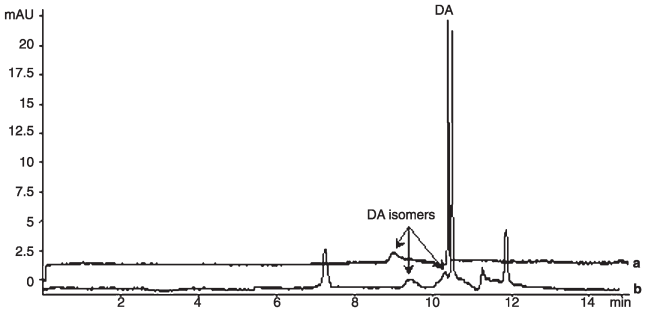

Figure 5 CEC-UV/DAD analysis of **a**) Domoic acid standard solution and **b**) Mussel Tissue Reference Material. Mobile phase: 5 mM phosphate buffer pH 2.5-MeCN (40:60). Conditions: 12 kV, 10 bar pressure, 25°C. Detection: UV at 242 nm. Injection: 10 kV for 10 s.

added to MeCN to a ratio of 40:60. The sample is then run at 12kV, 10 bar pressure, 25°C through a UV detector. Results of analyses of domoic acid and some of its isomers are shown in Fig. 5. We conclude CEC is a promising alternative for determination of ASP toxins with adequate efficiency, sensitivity and selectivity. Nevertheless, further improvements are still required, particularly regarding the lack of robustness with this approach, and limited commercial availability of stationary CEC phases. The main advantage of using CEC is greater selectivity and sensitivity compared to CE, as well as greater efficiency when compared to HPLC.

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Multi-Laboratory Study of Five Methods for the Determination of Brevetoxins in Shellfish Tissue Extracts

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Abstract

A thirteen-laboratory comparative study tested the performance of four methods as alternatives to mouse bioassay for the determination of brevetoxins in shellfish. The methods were N2a neuroblastoma cell assay, two variations of the sodium channel receptor binding assay, competitive ELISA, and LC/MS. Three to five laboratories independently performed each method using centrally prepared spiked and naturally incurred test samples. Competitive ELISA and receptor binding (96-well format) compared most favorably with mouse bioassay. Between-laboratory relative standard deviations (RSDR) ranged from 10 to 20% for ELISA and 14 to 31% for receptor binding. Within-laboratory (RSDr) ranged from 6 to 15% for ELISA, and 5 to 31% for receptor binding. Cell assay was extremely sensitive but data variation rendered it unsuitable for statistical treatment. LC/MS performed as well as ELISA on spiked test samples but was inordinately affected by lack of toxin-metabolite standards, uniform instrumental parameters, or both, on incurred test samples. The ELISA and receptor binding assay are good alternatives to mouse bioassay for the determination of brevetoxins in shellfish.

Introduction

Neurotoxic shellfish poisoning (NSP) is a disease caused by consumption of shellfish contaminated by toxins from the marine dinoflagellate *Karenia brevis* (formerly *Gymnodinium breve*). Prevention of NSP in the United States relies upon environmental monitoring, and closure of shellfish resources, when *K. brevis* densities exceed 5000 cells per liter seawater. The re-opening of resources is contingent upon results from mouse bioassay of diethyl ether extracts from affected shellfish. Results from mouse bioassay have been the sole criterion for NSP regulation for 35 years. Although this method has protected the public from NSP since inception, ethical as well as scientific issues surrounding the use of this method suggest that an alternative is needed. Toward this end, a thirteen-laboratory comparative study was undertaken to test four alternative methods for the determination of NSP toxins (brevetoxins and brevetoxin-metabolites) in shellfish.

Materials and Methods

Shellfish Extraction Extracts were prepared using tissue homogenates of shellfish collected from Mobile Bay, Alabama (non-toxic oysters), and from Appalachicola Bay, Florida during a 2001 *K. brevis* red tide (toxic oysters). Homogenates (100 g) for mouse bioassays were acidified with 1 mL 1N HCl, heated to boiling for 5 min, and extracted in diethyl ether using the American Public Health Association protocol specified for regulatory acceptability (Subcommittee on Laboratory Methods for the Examination of Shellfish, 1970). Diethyl ether was removed from the extracts by rotary evaporation, and the residues were suspended in saline containing 1% Tween-60 to achieve 10 mL total volume for mouse bioassays. Homogenates for the alternative methods were extracted in acetone (2:1 v/w). Solvent was removed from the extracts by rotary evaporation and the dried residues were re-solubilized in 80% methanol. Methanolic solutions were defatted with n-hexanes (1:1 v/v), evaporated, and resolubilized in 25% methanol. These methanolic solutions were cleaned-up by C18 solid-phase extraction (SPE) by washing with 25% methanol and eluting with 100% methanol.

Test Sample Preparation Test samples included brevetoxin-3 standard, non-toxic shellfish extracts, brevetoxin-3 spiked shellfish extracts, and extracts from shellfish where toxicity was naturally incurred. Four spiked and 4 naturally incurred toxicity levels, including non-toxic controls, were prepared by serial dilution of toxic extract solutions with non-toxic extract solution. Triplicate test samples (0.5 mL) of each treatment were dispensed into 2 mL crimp-top vials, and delivered to each participating laboratory.

Methods Mouse bioassays were performed in accordance with the APHA protocol with the exception of substitut-

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Table 1 Receptor Binding Assay (96-well format) Performance Estimates

Parameter	Sample C	Sample A	Sample E	Sample B	Sample F	Sample D
N	9	9	9	6	6	6
Assay Mean (ppm)	0.54	1.08	2.21	0.27	0.78	1.00
Spike Level (ppm)	0.4	0.8	1.6	–	–	–
Incurred Toxin (ppm)	–	–	–	0.29	0.58	1.16
Percent of Spiked Value	135	135	138			
Percent of Incurred Value				92	134	86
Repeatability SD (S_r)	0.17	0.17	0.35	0.04	0.04	0.13
Repeatability RSD_r	0.31	0.16	0.16	0.17	0.05	0.13
Repeatability Value, r ($2.8 \times S_r$)	0.47	0.48	0.98	0.12	0.11	0.38
Reproducibility SD (S_R)	0.17	0.17	0.79	0.06	0.13	0.13
Reproducibility RSD_R	0.31	0.16	0.36	0.24	0.17	0.13
Reproducibility Value, R ($2.8 \times S_R$)	0.47	0.48	2.2	0.18	0.36	0.38
HORRAT ($RSD_R\%/PRSD_R\%$)	1.78	1.00	2.51	1.25	1.00	0.84

$PRSD_R\% = 2C^{-0.1505}$ where C = (estimated mean in ppm $\times 10^{-6}$)

ing 1% Tween-60 in saline for cottonseed oil as the delivery vehicle. The alternative methods included the N2a neuroblastoma cell culture assay (Dickey *et al.,* 1999), two variations of the sodium channel receptor binding assay (*i.e.,* test-tube format: Trainer and Poli, 2000 and 96-well plate format: Van Dolah *et al.,* 1994), a competitive enzyme-linked immunosorbent assay (ELISA: Naar *et al.,* 2002), and liquid chromatography–mass spectrometry (LC/MS: Plakas *et al.,* 2002). Multiple laboratories were enlisted to independently perform each of the alternative methods using the centrally prepared test samples: 3 laboratories each for mouse bioassay, the receptor binding assays, and the ELISA; 4 laboratories for cell assay; and 5 laboratories for LC/MS.

Statistical Analysis Data were analyzed using the 1-way analysis of variance (ANOVA) for each test sample. In each analysis, the Cochran's test for homogeneity of variances was applied at the 2.5% level of significance. The repeatability and reproducibility values (r and R) indicate that the absolute difference of two test results from a single laboratory or from two laboratories (*i.e.,* one result from each laboratory) is expected to be below r or R in 95% of the cases. Horwitz ratio (HORRAT) values less than 2 indicate that the method is acceptable according to the relative reproducibility standard deviation of historical data for AOAC International validated methods. It should be noted, however, that the number of laboratories performing each of the alternative methods in this study is too small for AOAC International full collaborative validation status, but does meet the reduced format criteria for peer verification of methods.

Results and Discussion

Incurred toxin (ppm) values for test samples B, F, and D were derived from mouse bioassay and converted from mouse unit (MU) to a quantitative measure by adopting an equivalence of 4 mg brevetoxin per MU (Baden, 1982).

The homogeneity of variances for mouse bioassay passed the Cochran's test at the 2.5% level of significance. Within-laboratory variation (RSD_r) was 11% and between-laboratory variation (RSD_R) was 15%. The HORRAT was 0.96. Statistical summaries for the two best performing alternative methods, receptor binding assay (96-well format) and competitive ELISA, are shown in Tables 1 and 2, respectively. Not shown are the test-tube format receptor binding assay which showed greater variability than the 96-well format; LC/MS which performed very well on spiked test samples but was inordinately affected by lack of toxin-metabolite standards, uniform control of instrumental parameters, or both in incurred test samples; and the N2a cell culture assay where extreme data variance rendered it unsuitable for statistical analysis. In data derived from receptor binding ELISA and LC/MS methods, the homogeneity of variances for spiked test samples C, A, and E passed Cochran's test at the 2.5% level of significance, and no statistical outlying laboratories were detected. For the naturally incurred test samples B, F, and D only LC/MS passed Cochran's test for all 3 test samples. One outlying laboratory was identified for ELISA and binding assay methods.

Recoveries of brevetoxin-3 from spiked test samples averaged 97% (binding test-tube format), 136% (binding 96-well format), 87% (ELISA), and 78% (LC/MS). The lower limit of detection was not tested for any of the methods but in practice all methods easily measured spiked test

Table 2 ELISA Performance Estimates Parameter Sample

Parameter	Sample C	Sample A	Sample E	Sample B	Sample F	Sample D
N	9	9	9	9	9	9
Assay Mean (ppm)	0.30	0.71	1.53	0.47	0.86	1.91
Spike Level (ppm)	0.4	0.8	1.6	–	–	–
Incurred Toxin (ppm)	–	–	–	0.29	0.58	1.16
Percent of Spiked Value	76	89	96	–	–	–
Percent of Incurred Value	–	–	–	162	148	165
Repeatability SD (S_r)	0.03	0.10	0.22	0.03	0.08	0.20
Repeatability RSD_r	0.11	0.14	0.15	0.06	0.09	0.10
Repeatability Value, r ($2.8 \times S_r$)	0.09	0.28	0.62	0.08	.022	0.55
Reproducibility SD (S_R)	0.06	0.11	0.28	0.05	0.15	0.28
Reproducibility RSD_R	0.20	0.15	0.18	0.10	0.17	0.15
Reproducibility Value, R ($2.8 \times S_R$)	0.17	0.30	0.78	0.13	0.41	0.78
HORRAT ($RSD_R\%/PRSD_R\%$)	1.04	0.89	1.21	0.57	1.05	1.01

$PRSD_R\% = 2C^{-0.1505}$ where C = (estimated mean in ppm $\times 10^{-6}$)

samples one order of magnitude below the regulatory guidance level (*i.e.,* 0.2 MU/g or 0.8 ppm). After excluding data from outlying laboratories, within-laboratory variation for all test samples averaged 27% (binding test-tube format), 16% (binding 96-well format), 10% (ELISA), and 14% (LC/MS). Between-laboratory variation for all test samples averaged 39% (binding test-tube format), 23% (binding 96-well format), 16% (ELISA), and 44% (LC/MS). HORRAT values averaged 2.40 (binding test-tube format), 1.40 (binding 96-well format), 0.99 (ELISA), and 2.96 (LC/MS).

Conclusions

This study of methods performance shows statistically acceptable correlation of mouse bioassay with competitive ELISA and receptor binding assay for the determination of NSP toxins in shellfish. Either of the *in vitro* methods are suitable replacements for mouse bioassay. The N2a cell assay showed the greatest variability of the methods tested. Futher investigation will be needed to identify the source of this variation. LC/MS performed as well as ELISA on spiked test samples but was less consistent between-laboratories for incurred test samples. Metabolite standards, uniform control of instrumental parameters, or both may resolve this discrepancy. Nevertheless, LC/MS provides unambiguous structural confirmation of brevetoxins and metabolites in shellfish tissues.

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Application of an Ocean Color Algal Taxa Detection Model to Red Tides in the Southern Benguela

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Abstract

A forward reflectance model is used to demonstrate the sensitivity of hyperspectral ocean color observations to phytoplankton biomass, species composition and cell size. An inverse ocean color model is developed which has explicit terms for 5 taxonomic groups of phytoplankton. The model expresses the contributions of each group in terms of the magnitude of the absorption coefficient, which is proportional to biomass. The model is applied to a time series of reflectance data from an expansive red tide off the west coast of South Africa. The results are significantly correlated with those derived from microscopic cell counts. This demonstrates the utility of *in situ* ocean color detection of the composition and concentration of potentially harmful algae.

Introduction

Toxic and harmful algal blooms occur globally and are often associated with discolored water, giving rise to the misnomers "red tides" and "brown tides," among others. Until recently, it was thought that the algae responsible for such discolorations contained causative unique pigmentation. However, the taxa responsible for toxic and harmful blooms cannot be distinguished from their non-toxic and non-harmful counterparts based upon their optical properties (Etheridge and Roesler, 1998) or their pigment composition, with one minor exception (Millie *et al.,* 1997). This does not, however, negate the wealth of information that can be gleaned from the quantitative analysis of ocean color as a tool for the detection of algal species composition (Roesler and Etheridge, 1998).

In this paper, we demonstrate the sensitivity of the hyperspectral ocean color signal to variations in algal concentration, composition, and size distribution. We present an ocean color inversion model (Roesler and Perry, 1995; Roesler and Boss, 2003) modified to quantify the relative contributions of 5 groups of phytoplankton. This model is applied to hyperspectral ocean color spectra collected in the southern Benguela Upwelling System during the onset and development of a red tide event. The derived taxa composition is compared with that obtained from microscopic counts.

Materials and Methods

Expansive red tides occur annually along the western coast of South Africa during the latter half of the upwelling season (January–April). While red water is a predictable phenomenon, significant year-to-year variations are observed in bloom dynamics, spatial extent and, in particular, species composition (Pitcher *et al.,* 1998). We sampled the waters off Lamberts Bay, north of Cape Columbine, in March of 2001 during the initiation and development of an expansive red tide that was characterized by significant temporal variations in algal species composition.

A station located 2 nautical miles offshore (~60 m depth) was occupied daily for nearly three weeks. Hyperspectral downward irradiance and upward radiance were measured with a Satlantic HTSRB radiometer buoy between 10h00 and 14h00. Values were corrected for the dark signal at *in situ* temperatures, and averages were computed over periods of >100 observations where the coefficient of variation was <1%. Upward radiance, measured at a depth of 0.63 m, was corrected to the surface using spectral attenuation coefficients derived from upward radiance profiles in the top meter. Spectral reflectance was calculated from the ratio of upward radiance to downward irradiance.

Water samples were collected from the upper meter. Samples were preserved for microscopic enumeration of the phytoplankton. Spectral absorption coefficients were measured spectrophotometrically on the dissolved (<0.7 μm) and particulate fractions of each sample. The particulate fraction was further separated by parallel filtration through 5 and 20 μm Supor filters and the total and each size fraction collected on glass fiber filters. Spectral absorption coefficients were determined using the methods and corrections as in Roesler (1998). Phytoplankton absorption was determined by extraction (Kishino *et al.,* 1985).

Taxon-specific absorption spectra were statistically identified based upon co-occurence of distinct pigment absorption features and taxon dominance within a size fraction (0.7–5, 5–20 and >20 μm). The separable taxonomic groups were: diatoms, dinoflagellates, cryptophytes (which were symbiotically associated with *Mesodinium rubrum*), and chlorophytes. *Dinophysis* was further separable from the dinoflagellate group due to statistically significant deviations due to pigment packaging within its size fractionation (Fig. 1A).

The premise of the ocean color inversion model is that the spectral reflectance, R, can be theoretically expressed as a function of the absorption, a, and backscattering, b_b, coefficients (*e.g.,* Gordon *et al.,* 1983):

$$R \approx \frac{b_{bw} + b_{bp}}{a_w + a_\varphi + a_{nap} + a_{CDOM} + b_{bw} + b_{bp}}$$

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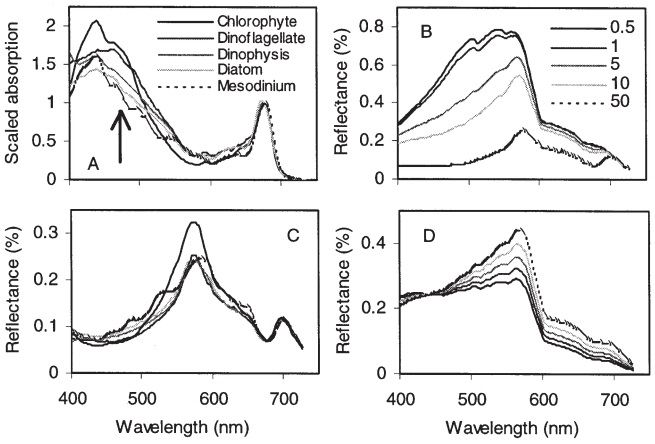

Figure 1 **A** Scaled taxon-specific absorption spectra for 5 separable groups based upon spectrophotometric analysis of size-fractionated samples and corresponding microscopic species counts (in decending order at arrow). Reflectance simulations as a function of **B** algal biomass (μg chl L^{-1}), **C** algal composition at 50 μg chl L^{-1} concentrations, symbols as in part A, **D** particle size distribution, where long wavelength reflectance increases as particle size increases.

where the subscripts w, p, φ, CPOM, and CDOM indicate water, particles, phytoplankton, and colored particulate and dissolved matter, respectively, and particulate backscattering includes the influence of both algal and non-algal particles. This model has been recently modified to express the particle backscattering as a function of parameters that have explicit dependence on the particle size spectrum (Roesler and Boss 2003). We use this expression to generate forward simulations of reflectance as a function of phytoplankton biomass, composition, and size distribution. Inversion of this equation is based upon the approach of assuming spectral shapes for each component and solving for the magnitude of each (Roesler and Perry 1995). In this paper the phytoplankton absorption component is further separated into the 5 algal groups represented by the 5 non-dimensional absorption spectra in Fig. 1A so that the contribution by each can be quantified upon inversion.

Results

Forward Ocean Color Simulations Forward simulations of ocean color reflectance demonstrate that as algal cell concentrations increase, the magnitude and the spectral shape of the reflectance changes dramatically, darkening (lower magnitude) and shifting in color from blue to red wavelengths (Fig. 1B). The resulting red color is due to selective absorption of the shorter wavelengths and enhanced scattering of the red wavelengths. Phytoplankton are weak absorbers in the red, aside from the chlorophyll *a* peak, but at high concentrations can effectively scatter red photons before they can be absorbed by water. Thus, as the concentration of phytoplankton increases, the ocean color signal arises from increasingly shallower depths.

Phytoplankton communities, owing to different pigment compositions, yield variable features in reflectance spectra (Fig. 1C). This is seen by eye most dramatically during high algal biomass conditions (~50 μg chl L^{-1}), but can be detected radiometrically or computationally at lower biomass conditions. Perhaps most dramatic are the features associated with cyano-bacterial or cryptomonad phycobilipigments and chlorophytic chl *b*. Potentially, the most difficult to distinguish would be diatoms and dinoflagellates due to the spectral overlap between their respective dominant carotenoids, fucoxanthin and peridinin. However, differences are detectable and therefore should be retrievable by inversion.

Phytoplankton size distributions play an important role in determining ocean color. For example, the pelagophyte *Aureococcus* sp., responsible for brown tides, is a small cell (~2 μm), while many dinoflagellate species responsible for red tides can exceed 25 μm in diameter. The difference in color that can be accounted for solely by changes in size distribution are dramatic (Fig. 1D) and are due to the fact that small particles scatter predominantly in the blue wavelengths, while large particles scatter all wavelengths equally or, under very distinct monospecific situations, more in the red wavelengths.

Inverse Ocean Color Model Results Absorption is a biomass-dependent parameter, a function of cell size as well as concentration. Thus, the microscopic counts were converted to biomass to account for cell size differences. Once that was done, the ocean color inversion model yielded estimates of the contribution (to absorption) by each of the taxonomic groups that compared well with those derived from microscopic counts (Fig. 2) over the time series. While some differences are expected owing to *in situ* versus discrete sampling in a highly patchy environment, in all five cases, the differences were not significantly different from zero ($\alpha = 0.05$).

Discussion

By far the most significant impact on spectral reflectance is the concentration of absorbing constituents, particularly algae, with brightness being determined by the magnitude of scattering relative to absorption. The spectral slope of backscattering, in response to variations in particle size distributions, also impacts the shape of reflectance, albeit to a lesser degree than does biomass concentration. These processes both lead to the dramatic changes in ocean color observed during blooms. Subtle variations in the color are primarily driven by pigment composition and it is this process that allows for pigment-based taxonomic identifications via ocean color observations.

The capability to deconvolve these different sources of variation using reflectance inversion techniques provides us with the capability for determining not only the algal biomass but additionally the composition and size distribution of the population. While this is still a far distance from species composition, it does allow us to monitor transitions between the major algal groups and community size struc-

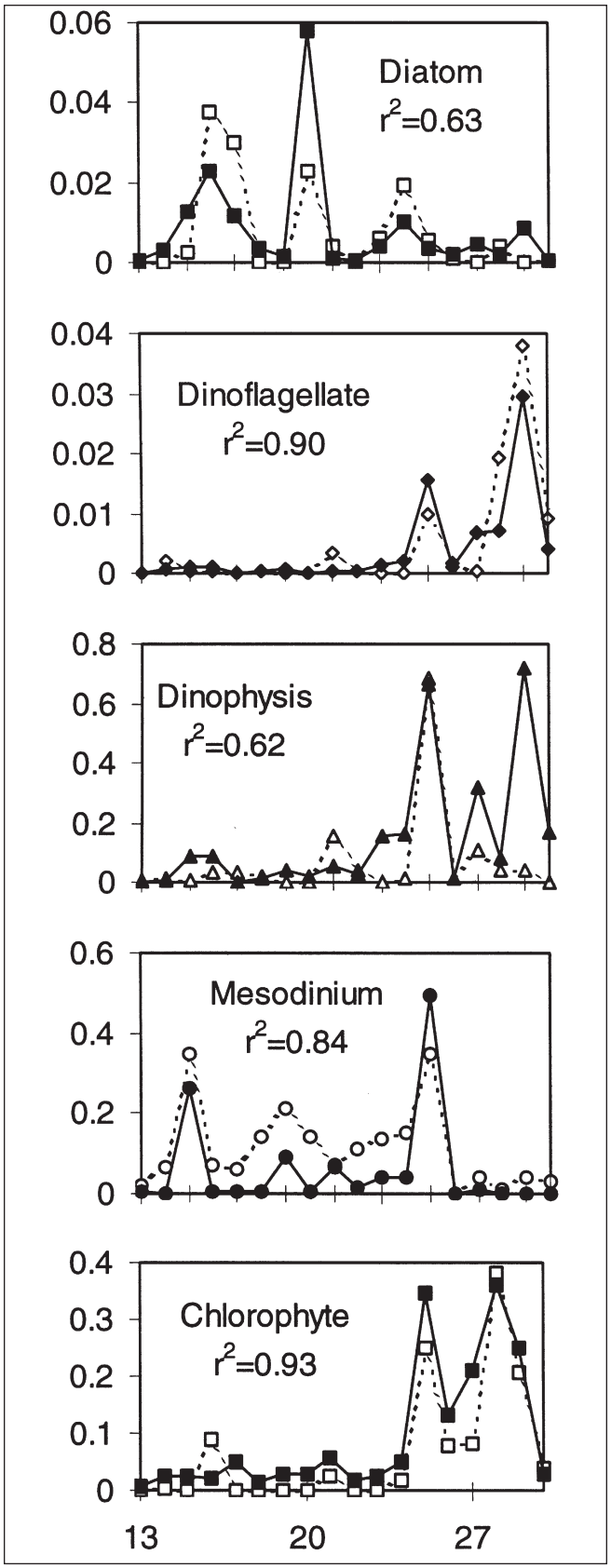

Figure 2 Absorption at 676 nm for taxonomic groups during the time series: inverse ocean color modeled values (filled) and those based upon microscopic cell counts, cell size and cellular absorption efficiency (open). X-axis is date in March 2001, correlation coefficients indicated.

ture. This is a significant improvement over simply monitoring biomass. The advantage to this approach lies in the potential for remote monitoring of ocean color in sensitive areas to detect changes in the phytoplankton community that are indicative of harmful algal species and/or harmful concentrations.

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Detection of Pectenotoxin in Norwegian Blue Mussels (*Mytilus edulis*)

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Abstract

Relatively high concentrations of pectenotoxin (PTX-1) were detected in blue mussels from the Trondheimsfjord area, mid-Norway, during the winter month of February 2002. The presence of toxin was indicated by the standard DSP mouse bioassay. Analysis by LC-MS indicated a peak level of PTX-1 of 486 µg/kg shellfish meat, declining to non-detectable level within six weeks. The levels of okadaic acid (OA) and dinophysistoxins (DTXs) as well as yessotoxins (YTXs) were low. This is the first report of toxic levels of PTX-1 in European mussels. Prior to the outbreak, potential pectenotoxin-producing dinoflagellates (*Dinophysis* spp.) were observed in trace concentrations (<40 cells/L) in water samples but were relatively abundant in net hauls. *Dinophysis norvegica* made up to 10–50% of the total phytoplankton available for the mussels prior to and after the pectenotoxin outbreak. An unexpected occurrence of *Dinophysis acuta* was observed corresponding with the peak pectenotoxin concentration in mussels. During the PTX-1 maximum, the cell numbers of *D. norvegica* and *D. acuta* were 80 cells/L and 40 cells/L, respectively, which is far below the known critical limits for the accumulation of toxins in mussels. It seems probable that *D. acuta* was the pectenotoxin-producing species, as seen from the timing of its occurrence and the level of toxins in the mussels. *Dinophysis acuta* has been associated with pectenotoxins in Irish waters and the Adriatic Sea. In Norway, *D. acuta* is known as the major cause of DSP (OA and DTXs) in mussels. The unexpected peak of pectenotoxin at low algal concentrations raises questions related to the basic metabolic ability of *D. acuta* and its relation to the environmental growth conditions, which in this case included a low light and a low temperature regime.

Introduction

Pectenotoxins PTX-1 and PTX-, were first isolated from shellfish by Professor Yasumoto and colleagues (1984). Pectenotoxins are cyclic polyether macrolides. Five PTXs have been chemically identified so far; PTX-1, -2, -3, -4, and -6. The structure of PTX-5 is not yet fully known. In addition, two acidic analogues of PTX-2, PTX-2 seco acid and 7-*epi*-PTX-2 seco acid, have been isolated from dinoflagellates and greenshell mussels (Daiguji *et al.,* 1998). PTX-2 has been associated with *Dinophysis fortii* in Japan and Italy, while the seco acids have been found in *D. acuta* in Ireland (Draisci *et al.,* 2000). Only PTX-2 was found in phytoplankton (*D. fortii*), whereas several PTXs were found in scallops, indicating transformation to other analogues within the digestive gland of shellfish (Lee *et al.,* 1988; Suzuki *et al.,* 1998). PTX-2 seco acids have recently been found in toxic mussels (*Mytilus galloprovincialis*) from the North Adriatic Sea (James *et al.,* 1999). There are very few data on oral toxicity of the PTXs, but results from mice may indicate that acute oral toxicity of PTX-1 is comparable to its intraperitoneal (i.p.) toxicity (Professor Yasumoto, pers. comm.). The liver seems to be the primary target of PTX-1 toxicity, while the toxin is not diarrhetic upon i.p. injections (Hamano *et al.,* 1985; Terao *et al.,* 1986). The mechanism of toxicity of the PTXs is still not fully known, but PTX-2 has been shown to affect actin organization in cells (Zhou *et al.,* 1994). Furthermore, PTX-1 induces apoptosis in freshly prepared rat and salmon hepatocytes. The apoptotic phenotype induced by PTX-1 gave more extreme nuclear and cellular shrinkage than OA and DTX-1 (Fladmark *et al.,* 1998). PTX-2 exhibits selective *in vitro* cytotoxicity towards the following human tumor cell lines: ovarian, renal, lung, colon, central nervous system (CNS), melanoma, and breast (Jung *et al.,* 1995). PTX-2 does not inhibit protein phosphatase 2A (Ogino, 1997).

There are no reports of human intoxications due to PTXs, while PTX-2 seco acids have been suspected of contributing to intoxications associated with the consumption of pipis (*Donax delatoides*) in Australia (Burgess and Shaw, 2001).

According to recent EU regulation (EU Commission, 2002), PTX toxins are regarded as a group of toxins separate from the DSP toxins. However, until analytical methods of detection are internationally accepted, the PTXs are included in the mouse bioassay for the DSP toxins (OA and DTXs) at a level of 160 µg/kg shellfish meat as OA equivalents. The EU Expert Working Group (2001) suggested a separate tolerance level for PTXs at 150 µg/kg shellfish meat. In Norway, samples of mussels are analysed by both the traditional mouse bioassay and by HPLC-MS prior to marketing.

Materials and Methods

Sample Site Blue mussels (*Mytilus edulis*) were collected weekly from bottom cultures in a shallow, 2.5 km long, 1–2 m deep channel (named Strommen), between the Trondheimsfjord (600 m deep) and a fjord basin (named Botn), 6 km long, 50 m deep. Due to net out-flux from Botn, mussels at Strommen are mainly exposed to phytoplankton from Botn, in addition to mixed fjord and basin phytoplankton from tidal inflow to the basin.

Phytoplankton Sampling Phytoplankton was collected at Stommen and in the main fjord close by (location named Kvithyll). Net haul samples were collected using 20 µm mesh size, at 0–1 m depth at Strommen, and 0–10 m depth

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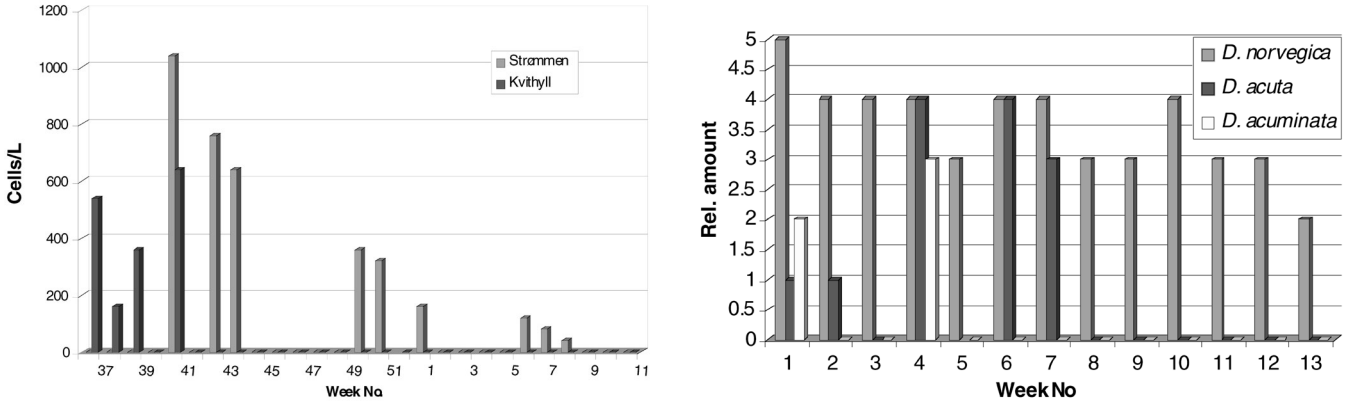

Figure 1 Population size of *Dinophysis* spp. at Strommen (mussel site) and Kvithyll (main fjord) in autumn 2001 and winter 2002 (left), and relative amount of *Dinophysis* species in net hauls from Strommen in early 2002 (right).

at Kvithyll. Water samples were collected at 0–1 m depth at Strommen and 0–3 m depth at Kvithyll.

Semiquantitative assessment of relative abundance in net samples was according to the following scale: 0 = not detected; 1 = present; 2 = several cells; 3 = 1–10% of biomass; 4 = 10–50% of biomass; 5 = 50–100% of biomass.

Chemical analysis PTX-1 and YTX were generous gifts from Professor Yasumoto, OA and DTX-1 were obtained from NRC, Canada. From 1 kg blue mussels, the digestive glands (hepatopancreas) were collected and homogenised. One-g portions were extracted with 9 mL (8+2 v/v) methanol/water, centrifuged and filtered through 0.22 µm filters, and analyzed by HPLC-MS. Isocratic chromatography was performed using methanol/water (8+2 v/v) containing 10 mM ammonium acetate, at a flow rate of 300 µL/min, and an Omnispher 5 C18 (Varian Chrompack), 150 × 2 mm analytical column operated at 35°C. Mass spectrometric analysis (Goto *et al.,* 2001) was carried out with an API-2000 triple quadrupole instrument (Sciex) using a TurboIonspray source, and data were acquired in positive ionisation mode. The presence of PTX-1 was confirmed by HPLC-MS/MS, while quantification was performed by SIM on the $[M+NH_4]^+$ ion.

Results and Discussion

Prior to the PTX outbreak, the phytoplankton biomass, which was dominated by diatoms in the early autumn, had declined to a minimum in January–February. At the mussel site Strommen, *Dinophysis* (mainly *D. norvegica*) was still present in significant amounts in December (Fig. 1), although *Dinophysis* usually is less common in the Trondheimsfjord in the winter period (Tangen and Sakshaug 2000). In January, *D. acuta* unexpectedly occurred in the net samples from Strommen, amounting to 10–50% of the phytoplankton, with a peak concentration of 40 cells L^{-1} in early February in the quantitative samples (Fig. 2), accompanied by *D. norvegica* (10–50%) and *D. acuminata* (<10 %) in net hauls (Fig. 1). These populations seem to have developed in cold water masses (0°–2°C) and low light conditions in the Botn basin from late December to February, whereas *Dinophysis* disappeared from the water samples and net hauls in the main fjord in October.

In Table 1, the results are given from the mouse bioas-

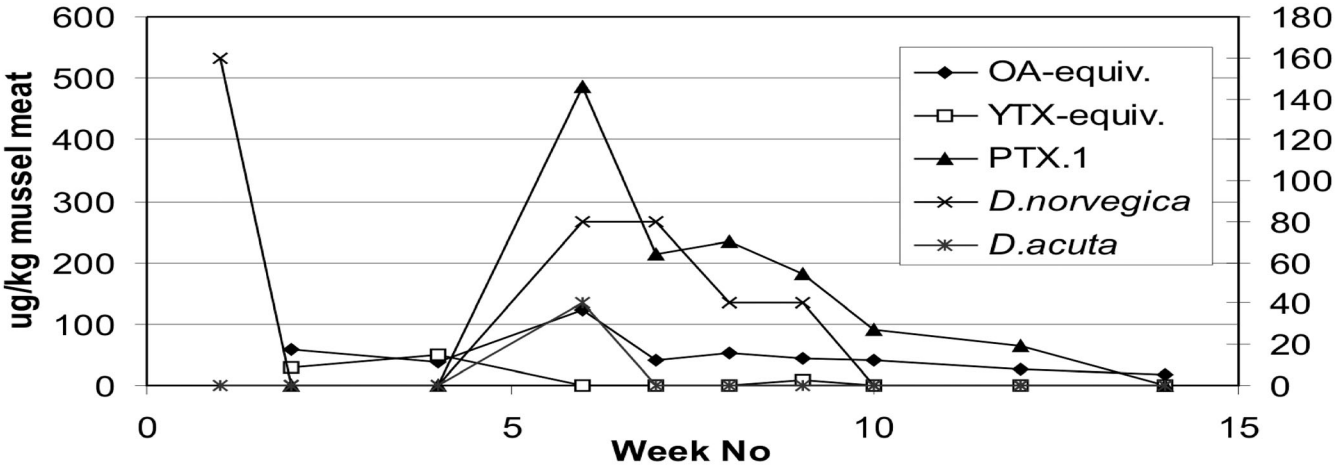

Figure 2 Toxin concentrations in mussels (left scale) and cell concentrations (cells/L) at Strommen of *D. norvegica* and *D. acuta* during first weeks of 2002 (right scale).

Table 1 Mouse bioassay and chemical analysis of blue mussels from Strommen, winter 2002. ND = Not detected.

Week No.	Mouse bioassay Survival time, hrs	OA-equivalents µg/kg mussel meat	PTX-1 µg/kg mussel meat	YTX-equivalents µg/kg mussel meat
2	ND	59	ND	29
4	ND	38	ND	51
6	1–2	122	486	ND
7		40	213	ND
8	2–4	54	235	ND
9	2–4	44	182	9
10		42	90	ND
12	ND	26	64	ND
14	ND	18	ND	ND

say and chemical analysis of toxin extracts from the blue mussels. The levels of OA and DTXs and YTXs never exceeded the lethal i.p. dose level, while the peak levels of PTX-1 corresponds well with its acute i.p. toxicity (Yasumoto *et al.,* 1989).

In Norway, OA and DTX-1 are commonly found in blue mussels in late summer and fall and may remain until early spring. *Dinophysis acuta* is the dominating toxin producer in Norwegian waters. The unsuspected mouse lethality of mussel samples from Strommen in early February 2002, when levels of OA and DTXs were low, corresponded with a peak of PTX-1 in the mussels. Dinophysis species were observed in moderate concentrations in September–October 2001 in Botn and in the main fjord. The bloom terminated in October in the main fjord, but remained until March 2002 in the Botn basin. The mussels at Strommen feed on rapidly changing diets. Typically, the toxicity in mussels is associated with toxic phytoplankton originating in the basin. The algal bloom was dominated by *D. norvegica,* but in January–February 2002, *D. acuta* made up a major part of the net samples. These populations developed in cold-water masses (0–2°C) during a short ice-free period in January. Even though the cell counts were low, *D. acuta* constituted a high relative amount of the algal biomass, corresponding with the PTX-1 peak in mussels. It is suggested that PTX-1 in blue mussels at Strommen originated from the unusual winter peak of *D. acuta.*

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Rapid Monitoring of Toxic Phytoplankton and Zooplankton with a Lateral-Flow Immunochromatographic Assay for ASP and PSP Toxins

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Abstract

A study was conducted to determine the feasibility of applying the MIST Alert™ test kit to concentrated phytoplankton net tows and zooplankton samples in the field to monitor for the presence of PSP (paralytic shellfish poisoning) and ASP (amnesic shellfish poisoning) toxins. The lateral flow immunochromatographic test was originally developed to detect these primary phycotoxins in shellfish tissue. After a simple preparation procedure, the test strip detected ASP toxins in six phytoplankton net tows containing *Pseudo-nitzschia* spp. collected during summer blooms in Ship Harbour, Nova Scotia. In addition, the MIST Alert™ for PSP yielded a positive response for PSP toxins in three phytoplankton net tows collected on board a research vessel in the Gulf of Maine when *Alexandrium* cells were present. The level of PSP toxins in zooplankton samples collected simultaneously during the cruise was below the detection limit of the test, but these toxins were readily detected in copepods fed toxic *Alexandrium* cells in the laboratory.

Introduction

The MIST Alert™ is a rapid and easily applied diagnostic test kit that provides a visual test (+/–) for the presence of ASP (domoic acid, DA) or PSP toxins, including the major toxin analogues in the saxitoxin family (Laycock *et al.,* 2000), in about 10 minutes. For shellfish samples, the MIST Alert™ test strip has been tested in parallel trials with the mouse bioassay for PSP toxin; in over 2100 regulatory samples, the test detected PSP toxin in 100% of the extracts declared to be "toxic" according to the mouse assay (Jellett *et al.,* 2002). The PSP toxin test is designed to have an average detection limit of 400 µg STXeq [saxitoxin equivalents] kg^{-1} shellfish (half the regulatory limit), and the ASP version has a detection limit of 8–10 mg DA kg^{-1} mussel tissue (well below the 20 mg kg^{-1} regulatory limit) (Cembella *et al.,* 2003). Strips consist of an absorption pad, a membrane striped with immobilized toxin (the T line) and antibody detection reagent (the C line), a sample pad and a conjugate pad containing the antibodies on colloidal gold. A positive test for PSP or ASP toxins is indicated by the absence of the toxin "T" line after 10 minutes. The presence of the "C" line indicates that the test has performed properly (Laycock *et al.,* 2000).

Both *Alexandrium ostenfeldii,* a known producer of toxic spirolides (and sometimes PSP toxins), and members of the *A. tamarense/fundyense* species complex, which produce PSP toxins, are commonly found in coastal waters of Nova Scotia and the northern Gulf of Maine. Two species of *Pseudo-nitzschia,* determined to be *P. delicatissima* and *P. seriata* by scanning electron microscopy, were found in Ship Harbour, Nova Scotia, during the summer of 2001. Certain strains of *Pseudo-nitzschia* species produce domoic acid, which is responsible for ASP. In this study, we describe the successful application of the MIST Alert™ kit for ASP toxins to net tow samples from Ship Harbour, and the use of the PSP toxin version on laboratory cultures and on-board a research vessel in the northern Gulf of Maine to screen phytoplankton and zooplankton samples for PSP toxins.

Materials and Methods

The Mist Alert™ for ASP was applied to detect toxin in live net tow (20 µm mesh) plankton collected weekly during the summer of 2001 at five stations in Ship Harbour, Nova Scotia. The following year, both zooplankton and phytoplankton samples were tested using the MIST Alert™ for PSP on-board the RV *Endeavor* in the northern Gulf of Maine during July 2002. On the oceanographic cruise, the test strips were applied to extracts of concentrated copepods to screen for PSP toxins accumulated through the food chain via consumption of toxic phytoplankton as well as to concentrated phytoplankton net tow samples.

For toxin extraction from phytoplankton samples, Nytex sieves (10 µm) were used to concentrate 250 mL of live net tow material to ~10 mL. The concentrated slurry was centrifuged (5,000 × *g*; 4°C) for 10 min. Toxins were extracted from the pellet in 0.1 M acetic acid (1:10 v:w) by crushing and resuspending with a glass stir-rod and then recentrifuging the suspension. Toxin extract (50 µL) from the supernatant was added to 200 µL Jellett Biotek phytoplankton buffer and then applied to the test strip.

The protocol for toxin extraction from zooplankton concentrates was applied to pooled individuals picked from plankton samples that had tested positive for PSP toxin by MIST Alert™. Three groups of copepods (10, 101 and 205 individuals) from the Gulf of Maine were tested with the MIST Alert™ test strip. Laboratory samples of copepods (*Eurytemora* and *Acartia*) fed toxic *Alexandrium* cells were also tested (data not reported here). Copepods were mashed up with a glass rod to liberate gut contents before centrifugation. Samples were then subjected to the same protocol as for the phytoplankton except that the buffer was replaced by zooplankton buffer.

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Table 1 Putative toxic dinoflagellates from laboratory cultures and field populations assayed by MIST Alert™ and analyzed by HPLC-FD for PSP toxins.

Species	Isolate/Sample	Origin	PSP toxin (μmol L^{-1})	Toxin quota (fmol $cell^{-1}$)	MistAlert™
		Cultured Isolates			
Alexandrium tamarense	CCMP115-1	Tamar estuary, Plymouth, UK	0	0	–
	IP-9	Indian Point, NS, Canada	7.8	71	++++
	IP-22		2.3	255	++++
	IP-37		8.8	28	++++
	ALGS26	Graves Shoal, NS, Canada	22	352	++++
	GS2-2-95		22	22	++++
	GS2-6-95		0	0	–
	GS2-8-95		18	33	++++
	GS2-10-95		6.6	4.2	++++
	GS2-23-95		20	11	++++
	GS2-26-95		9.9	21.4	++++
	GS2-26-95		21	11	++++
	GS2-36-95		57	87	++++
	GS2-42-95		4.6	2.4	++++
	GS3-96-1A		0.09	0.07	+++
	GS3-96-1Y		0	0	–
	GS4-96-4A		12	2.1	++++
Alexandrium tamarense	AI18b-1	Rimouski, Quebec, Canada	263	541	++++
cf *excavatum*	AL103		1200	608	++++
Alexandrium ostenfeldii	AOSH1	Ship Harbour, NS, Canada	0	0	–
Gymnodinium catenatum	Singl	Singapore Harbour, Singapore	1.2	24	++++
	Sing2		0.9	17	++++
Lingulodinium polyedrum	CCMP407	La Jolla, CA, USA	1.4	0.1	++++
Protoceratium reticulatum	GS2-16-95	Graves Shoal, NS, Canada	0	0	–
Pyrodinium bahamense	Pyro A	Manila Bay, Philippines	24	98	++++
var. *compressum*	Pyro B		12	50	++++
Scrippsiella trochoidea	GS2-22-95	Graves Shoal, NS, Canada	0	0	–
	GS2-13-95		0	0	–
		Field Samples			
Alexandrium monilatum	Trin-1	Trinidad	0	0	–
	Trin-2		0	0	–
	Trin-3		0	0	–

– = negative, + = <25% positive, ++ = 25–50% positive, +++ = 50–75% positive. ++++ = fully positive (100%)

Results

The data shown in Table 1, comparing the immunodiagnostic test to analysis by high-performance liquid chromatography with fluorescence detection (HPLC-FD) (Luckas *et al.,* 2003), indicate that the MISTAlert™ successfully detected PSP toxins from complex toxin profiles in various dinoflagellate matrices.

In Fig. 1, the cell concentrations of key putatively toxic species in concentrated extracts are shown in relation to the toxin detected by Mist Alert™. The level of PSP toxins was below the detection limit of the test (ca. 10 ng STX) for all of the field samples tested from Ship Harbour. Nevertheless, the ASP test detected toxin during the appearance of the *Pseudo-nitzschia* bloom. A summary of the combined results of the toxin testing with the Mist Alert™ in Ship Har-

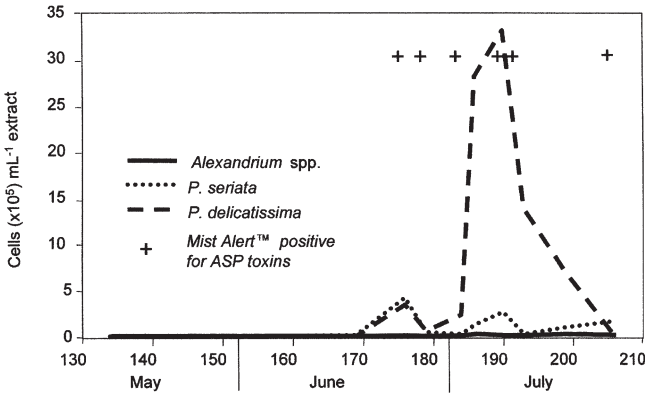

Figure 1 Cell concentration of *Alexandrium* spp., *Pseudo-nitzschia seriata* and *P. delicatissima* identified in Ship Harbour, NS, in the spring and summer of 2001. X-axis values are in Julian days.

Table 2 Presence of PSP and ASP toxins in phytoplankton and zooplankton samples from Ship Harbour, NS, and the Gulf of Maine determined by MIST Alert™.

Sample Type	Species	Date	IV/CV(mL)*	MIST Alert™
Ship Harbour				
Phytoplankton net tow	*Pseudo-nitzschia seriata* / *P. delicatissima*	June 2001	250/10	++++ASP
		June 2001	250/10	++++ASP
		July 2001	250/10	++++ASP
		July 2001	250/10	++++ASP
		July 2001	250/10	++++ASP
		Aug 2001	250/10	++++ASP
Gulf of Maine				
Phytoplankton net tow	*Alexandrium tamarense/fundyense*	July 2002	250/10	++++PSP
		July 2002	250/10	++++PSP
		July 2002	250/10	++++PSP
Pooled zooplankton	*Calanus finmarchicus*	July 2002	0 copepods	–PSP
		July 2002	101 copepods	–PSP
		July 2002	205 copepods	–PSP

*IV = Initial Volume; CV = Concentrated Volume.

bour, NS and the northern Gulf of Maine is presented in Table 2.

Discussion

The MIST Alert™ has proven to be a simple, rapid and cost-effective method of detection of both PSP and ASP toxins in phytoplankton and zooplankton samples. The negative results obtained from the MIST Alert™ test on zooplankton samples in the Gulf of Maine may be related to the low rate of feeding on *Alexandrium* cells at the particular location, although *Alexandrium fundyense* was a dominant phytoplanktonic species in the water column at the time. Based on the concurrent taxonomic identification by light microcopy, and subsequently by molecular probes, this dinoflagellate species was the likely source of the PSP toxins present in the plankton samples. In any case, laboratory studies (A. Cembella, unpublished observations) have shown no difficulties or matrix effects in the detection of PSP toxins from *Alexandrium* cells ingested by copepods. The MIST Alert™ test was specifically designed for the detection of toxins in shellfish matrices at levels consistent with regulatory limits. For critical field application in plankton matrices where toxic cell numbers may be low, the sensitivity can be readily increased by a relatively minor adjustment in the configuration of the test.

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Detection of *Kryptoperidinium foliaceum* in South Carolina Estuaries Using a Real-Time PCR Assay

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Abstract

Following observations of *Kryptoperidinium foliaceum* blooms in South Carolina estuarine waters, and the potential for adverse impacts to shellfish and finfish health, a real-time PCR assay was developed for the rapid, specific detection of *K. foliaceum* from environmental samples. With recent evidence suggesting a multi-species complex within the *Kryptoperidinium* clade, the need for specific assays for the detection of *Kryptoperidinium* isolates is critical to sort out these bloom events. A species-specific, real-time PCR assay was developed to target *K. foliaceum* sequence identity. The integration of a *K. foliaceum*-specific PCR assay into existing monitoring efforts revealed that at least one additional species is responsible for the observed blooms. Comparisons between light microscopy and real-time PCR assay demonstrate the utility of the assay in detecting *K. foliaceum.*

Introduction

Since their discovery in the spring of 1998, blooms of *Kryptoperidinium foliaceum* have been observed with increased frequency in South Carolina (SC) estuarine waters, with cell densities at times $>10^5$ cells mL^{-1} (Lewitus *et al.,* 2001; Lewitus and Holland, 2003). Laboratory studies and field evidence suggest that high cell densities of *Kryptoperidinium* may cause physiological stress to shellfish (Lewitus *et al.,* 2003). Lysosomes, typically involved in cellular defense, tissue repair, and nutrition, can become destabilized through exposure to a variety of stressors (*e.g., Kryptoperidinium* blooms). Lysosomal destabilization rates that were well above the normal range of healthy oysters have been documented from oyster deployments, laboratory exposures to bloom material, and collection of native oysters from areas where blooms commonly occur (Lewitus *et al.,* 2003; Keppler *et al.,* unpub. data).

Results from morphological and molecular analyses used to confirm the identity of these blooms suggest that *Kryptoperidinium* is not a monospecific genus (Kempton *et al.,* 2002). Differences in thecal plate tabulation and SSU and ITS rDNA in culture isolates and field populations suggest that the genus *Kryptoperidinium* is made up of multiple species. An expanded thecal plate tabulation has been described for *K. foliaceum* by Wolny *et al.* (this Proceedings), however no additional *Kryptoperidinium* species have been formally characterized to date.

Real-time PCR-based technologies have recently been adapted for the detection of HAB species. Bowers *et al.* (2000) developed species-specific, real-time PCR assays to target *Pfiesteria piscicida* and *P. shumwayae* from environmental samples. A *Karlodinium micrum* assay targeting chloroplast DNA was developed by Tengs *et al.* (2001). Real-time PCR offers many advantages over conventional PCR. Amplification and detection are combined into one system. The rapid, precise heating and cooling of reaction mixtures allows for decreased assay times and increased productivity. A dual-labeled, internal fluorescent oligonucleotide probe enhances assay specificity over conventional PCR methods. Fluorescence detection is used to measure amplification products as they are generated in real-time. These assays also hold the potential to provide quantitative data (Bowers *et al.,* 2000) and to be adapted for handheld use in environmental sampling. In an effort to sort out bloom events in SC, a real-time PCR assay was developed for the detection of *K. foliaceum.* The effectiveness of this assay was evaluated.

Materials and Methods

Kryptoperidinium foliaceum culture UTEX LB 1688 was used as the positive control for the development of the real-time PCR assay, based on morphological and molecular confirmation of its identity as *K. foliaceum* (Kempton *et al.,* 2002; Wolny *et al.,* this Proceedings). Cultures CCAP 1116/3 (*Glenodinium foliaceum*) and CS-291 (*K. foliaceum*) were purchased from the Culture Collection of Algae and Protozoa (CCAP) and the Australian Commonwealth Scientific and Industrial Research Organization (CSIRO), respectively. Both CCAP 1116/3 and CS-291 cultures are genetically distinct from the UTEX LB 1688 culture, though morphologically similar at the light microscopic level (Kempton *et al.,* 2002). These cultures, as well as non-*Kryptoperidinium* dinoflagellates, were used as negative controls.

All environmental samples were collected as surface water samples in 1-L acid washed sample bottles. Samples were collected either as part of the South Carolina Harmful Algal Bloom Program (SCHABP), or as part of the South Carolina Estuarine and Coastal Assessment Program (SCECAP). Samples collected from the SCHABP included 22 routine monitoring stations. SCECAP samples were taken from 62 stations selected using a probability-based, random tessellation, stratified sampling design (Stevens, 1997; Stevens and Olsen, 1999). Both sample sets

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include tidal creeks and larger tidal rivers, whereas SCHABP samples also included brackish stormwater detention ponds (Lewitus *et al.,* 2003). All of the SCECAP samples were analyzed by the *K. foliaceum* PCR assay (see below), but SCHABP samples were selected for the PCR assay based on confirmation of the presence of *K. foliaceum* and/or "Species B" by microscopic identification.

DNA extractions were carried out using either a rapid CTAB (cetyltrimethylammonium bromide) buffer DNA isolation technique (Schaefer 1997) or a Puregene® DNA Isolation Kit. The Puregene® DNA Isolation Kit was used for extracting DNA from Lugols-preserved samples following a protocol by Gentra Systems, Inc. for isolating DNA from *Chlamydomonas.* Some Lugols-preserved samples needed destaining by drop-wise addition of 10× sodium thiosulfate. Primers and probe were selected based on alignments of dinoflagellate SSU rDNA sequences. Sequences were downloaded from GenBank and aligned using DNASIS v.3.6. Two *K. foliaceum* sequences were used in the alignment, GenBank accession no. AF274268 (Saldarriaga *et al.,* 2001) and GenBank accession no. AF231804 (Inagaki *et al.,* 2000), both derived from the UTEX LB 1688 strain. Sequence data from the SC *K. foliaceum* isolate were identical to the AF274268 sequence data, but differed by 4 base pairs to the AF231804 sequence data (Kempton *et al.,* 2002). Based on the alignments, two *K. foliaceum*-specific primers (forward K.foli 38 primer: TCTAGGTATAAGCTTCTGTATAG and reverse K.foli 261 primer: ACTATCACTCACCAAAGCATCA) and an internal probe (K.foli 165 probe: ACACGCATAACCGCCCAACTTC) were designed. The internal probe K.foli 165 was labeled on the 5′ ends with 5-carboxyfluorescein, and on the 3′ ends, labeled with 5-carboxytetramethylrhodamine (QIAGEN Operon, Alameda, CA). The following reagents were added: primers and internal probe at a final concentration of 0.2 μM; PCR buffer at a final concentration of 1× (Promega); $MgCl_2$ at a final concentration of 2.5 mM; a dNTP mixture at a final concentration of 0.2 mM (Stratagene); bovine serum albumin at a final concentration of 10 μM; and *Taq* polymerase at a final concentration of 0.2 Units μL^{-1} (Promega). The final reaction volume was 25 μL. All reactions for the *K. foliaceum*-specific real-time PCR assay were performed using the SmartCycler® System (Cepheid, Sunnyvale, CA) using the following reaction protocol: Stage 1, 96°C for 75 sec; Stage 2, 50 cycles at 96°C for 5 sec and 60°C for 40 sec.

Results and Discussion

During the 2002 sampling season, 164 samples were designated presumptive positives for *K. foliaceum* or the morphologically similar "Species B" based on microscopic screening of live samples. To assess the distribution of *K. foliaceum* in SC waters and correlate light microscopic observations with PCR results, 123 samples were tested with the *K. foliaceum*-specific, real-time PCR assay. These included 61 samples collected through the routine monitoring efforts of the SCHABP, and an additional 62 random samples collected as part of SCECAP. The 61 samples assayed from the SCHABP were all presumptive positive for *K. foliaceum* based on light microscopy. Out of the 62 SCECAP samples collected, only four samples were presumptive positive for *K. foliaceum* and five for "Species B".

Evaluations of the *K. foliaceum*-specific PCR assay suggest that the assay is useful for discriminating *K. foliaceum* target sequence from that of "Species B." Out of 27 samples identified microscopically as "Species B," only one PCR positive for *K. foliaceum* was noted. This sample was either misidentified during initial screening via light microscopy, or more likely, *K. foliaceum* represented a small component of a mixed assemblage that included "Species B." In contrast, 19 PCR positives for *K. foliaceum* were noted from 65 samples microscopically identified to have *K. foliaceum* (SCHABP and SCECAP samples combined). One explanation for the low percentage of PCR-confirmed samples (19 of 65) is that multiple species within the *Kryptoperidinium* clade preclude distinction at the light microscopic level (Kempton *et al.,* 2002). Alternatively, samples that were presumptive positive for *K. foliaceum,* but not confirmed with PCR, may suggest false negatives based on the sensitivity of the assay when extracting from Lugols-preserved samples. Bowers *et al.* (2000) noted a decrease in sensitivity by 1 log when assaying Lugols-preserved cultures.

Results from microscopic evaluations and the *K. foliaceum* real-time PCR assay indicated a widespread distribution of *K. foliaceum* in SC estuarine waters (data not shown). It should be noted that the SCECAP samples were collected during summer (June to August), whereas *K. foliaceum* occurrence in past years has been more prevalent from March to May (Lewitus and Holland 2003; Lewitus *et al.,* 2003). Therefore, the low occurrence of *K. foliaceum* identifications (light microscopy) and PCR-confirmed positives from SCECAP sampling may be attributed to seasonality.

This real-time PCR assay exhibits specificity for *K. foliaceum* based on these evaluations. Future efforts will focus on determining the sensitivity of the assay, and test the effects of storage time on sample stability. Efforts will also be made to make this assay quantitative. In addition, the identity of the "Species B" and additional *Kryptoperidinium* species will be addressed.

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