Harmful Algae 8 (2009) 873–879

Contents lists available at ScienceDirect

Harmful Algae

journal homepage: www.elsevier.com/locate/hal

DSP toxin production *de novo* in cultures of *Dinophysis acuminata* (Dinophyceae) from North America

Jeremiah D. Hackett [a], Mengmeng Tong [b,c], David M. Kulis [c], Elie Fux [d], Philipp Hess [d], Ronel Bire [d], Donald M. Anderson [c,*]

[a] *Department of Ecology and Evolutionary Biology, University of Arizona, Tucson, AZ 85719, USA*
[b] *College of Environmental Science and Engineering, Nankai University, Tianjin 300071, China*
[c] *Biology Department, Woods Hole Oceanographic Institution, Woods Hole, MA 02543, USA*
[d] *Marine Institute, Rinville, Oranmore, Co. Galway, Ireland*

ARTICLE INFO

Article history:
Received 7 January 2009
Received in revised form 14 April 2009
Accepted 14 April 2009

Keywords:
Dinophysis
Diarrhetic shellfish poisioning (DSP)
Okadaic acid

ABSTRACT

For decades, many aspects of *Dinophysis* biology have remained intractable due to our inability to maintain these organisms in laboratory cultures. Recent breakthroughs in culture methods have opened the door for detailed investigations of these important algae. Here, for the first time, we demonstrate toxin production in cultures of North American *Dinophysis acuminata*, isolated from Woods Hole, MA. These findings show that, despite the rarity of *Dinophysis*-related DSP events in North America, *D. acuminata* from this area has the ability to produce DSP toxins just as it does in other parts of the world where this species is a major cause of DSP toxicity. In our cultures, *D. acuminata* cells were observed feeding on *Myrionecta rubra* using a peduncle. Culture extracts were analyzed using LC–MS/MS, providing unequivocal evidence for the toxin DTX1 in the *Dinophysis* cultures. In addition, a significant amount of an okadaic acid diol ester, OA-D8, was detected. These results suggest that this *Dinophysis* isolate stores much of its OA as a diol ester. Also, toxin PTX-2 and a hydroxylated PTX-2 with identical fragmentation mass spectrum to that of PTX-11, but with a different retention time, were detected in this *D. acuminata* culture. This demonstration of toxin production in cultured North American *Dinophysis* sets the stage for more detailed studies investigating the causes of geographic differences in toxicity. It is now clear that North American *Dinophysis* have the ability to produce DSP toxins even though they only rarely cause toxic DSP events in nature. This may reflect environmental conditions that might induce or repress toxin production, genetic differences that cause modifications in toxin gene expression, or physiological and biochemical differences in prey species.

1. Introduction

The dinoflagellate genus *Dinophysis* is interesting from both ecological and evolutionary perspectives. Some members of this genus are responsible for diarrhetic shellfish poisoning (DSP)—a syndrome associated with human consumption of shellfish that have accumulated *Dinophysis* toxins, and less frequently, toxins from *Prorocentrum lima* (Lawrence et al., 2000). DSP is a major public health and economic problem for many countries (Lee et al., 1989; Boni et al., 1993; Giacobbe et al., 2000) and is among the most important and widely distributed of the harmful algal bloom (HAB) associated poisoning syndromes (Van Dolah, 2000). The diarrhetic shellfish toxins (DTXs) are a class of polyether compounds with at least eight congeners and the parent compound, okadaic acid (OA). OA, DTX1, and DTX2 are the primary toxins involved in poisonings, with others believed to be either precursors or shellfish metabolites of the toxins. The DTXs are inhibitors of serine/threonine protein phosphatases and, when ingested by humans, cause rapid onset of gastrointestinal symptoms (vomiting and diarrhea) lasting several days.

On a global scale, DSP is widespread, documented in Western Europe, East and Southeast Asia, South America, South Africa, New Zealand and Australia. The situation in North America is particularly interesting since DSP toxins have only rarely been detected in North America despite years of testing of shellfish and plankton tows by many investigators. Two events in Canada were attributed to the benthic dinoflagellate, *P. lima*, found in association with filamentous algae growing on raft cultures of mussels (Quilliam et al., 1993; Lawrence et al., 2000). OA was detected in cultures of *P. lima* isolated from the Gulf of Maine

* Corresponding author at: Biology Department, Woods Hole Oceanographic Institution, Redfield 3-32, MS #32, Woods Hole, MA 02543, USA.
Tel.: +1 508 289 2351; fax: +1 508 457 2134.
E-mail address: danderson@whoi.edu (D.M. Anderson).

1568-9883/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.hal.2009.04.004

(Morton et al., 1999; Maranda et al., 2007), and in 2002, LC–MS analysis for OA revealed low concentrations of the toxin in plankton net tow material during an extremely dense bloom of *Dinophysis acuminata* in the Chesapeake Bay (Tango et al., 2002). Dickey et al. (1992) detected okadaic acid in oysters in the northern Gulf of Mexico in 1990. The most significant DSP event attributed to *Dinophysis* sp. in North American shellfish occurred in 2008 in Southern Texas (Texas Department of State Heath Services news release, March 7th 2008).

This very low level of DSP toxin in North America is noteworthy even though the *Dinophysis* species known to be toxic elsewhere (e.g., *D. fortii*, *D. acuminata*, *D. caudata*, *D. norvegica*, and *D. acuta*), have all been observed in high abundance in US and Canadian waters (Marshall, 1996; Rehnstam-Holm et al., 2002). The reasons for this remarkable geographic disparity in toxicity remain unresolved, but could reflect genotypic heterogeneity among strains of *Dinophysis*, or, given recent findings about feeding and culturing (Park et al., 2006), differential toxin synthesis might be due to the variability in cryptophyte and ciliate prey.

We have isolated *D. acuminata* from Eel Pond in Woods Hole, MA and established cultures grown on *Myrionecta rubra* and its cryptophyte prey to investigate toxin production in North American *Dinophysis* species. Here we report the first description of toxin production in a North American culture of *Dinophysis*.

2. Materials and methods

2.1. Culture methods

A unialgal (but not clonal) culture of *D. acuminata* was established from Eel Pond, Woods Hole, MA in September of 2006 by isolating cells from a water sample into individual wells of a 48 well tissue culture plate. These cells were cultured using a two-step feeding system described by Park et al. (2006) where the cryptophyte, *Geminigera cryophyla* (CCMP2564), is fed to the ciliate, *M. rubra* (CCMP2563), which is in turn fed to *D. acuminata* (DAEP01). All three cultures were maintained in modified f/2-Si medium (Anderson et al., 1994) at 3 °C on a 14:10 h L:D cycle (ca. 100 μmol photons m^{-2} s^{-1} irradiance provided by cool white fluorescent bulbs). Borosilicate glass culture tubes (25 mm × 150 mm) containing 25 mL medium were used. When the cell density of the cryptophyte exceeded 500,000 cells mL^{-1} it was used as food for *M. rubra* by combining 2 mL of *G. cryophyla* with 3 mL *M. rubra* in 20 mL f/2 medium. Following complete consumption of the cryptophyte cells by *M. rubra*, the ciliate was fed to *D. acuminata* by adding 3 mL *M. rubra* (~14,000 cells mL^{-1}) with 2 mL *D. acuminata* (~1800 cells mL^{-1}) in 20 mL f/2 medium. Weekly cell counts and observations on *D. acuminata* DAEP01 were conducted on samples preserved in Utermöhls solution (Utermöhl, 1958) and examined microscopically at 100× using a Sedgwick Rafter counting chamber. Additional *Myrionecta* culture was added to the *D. acuminata* cultures when the *Myrionecta*/*Dinophysis* cell ratio fell to less than 1:1. Cultures of *D. acuminata* were transferred when the cell density exceeded 1800 cells mL^{-1} and maximum cell densities greater than 7000 cells mL^{-1} could be easily achieved by these techniques.

The SSU rDNA gene was amplified by PCR from *D. acuminata* DAEP01 using primers CrN1f and BR as previously described (Hoef-Emden et al., 2002). The PCR products were sequenced on both strands using an ABI3730 sequencer and deposited in GenBank (accession FJ869120). The *D. acuminata* SSU rDNA sequence was aligned with homologues from other dinoflagellates using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The alignment was used to construct a minimum evolution tree using the Kimura 2-parameter correction with 500 bootstrap replicates in Phylip (Felsenstein, 2004, Fig. 1B).

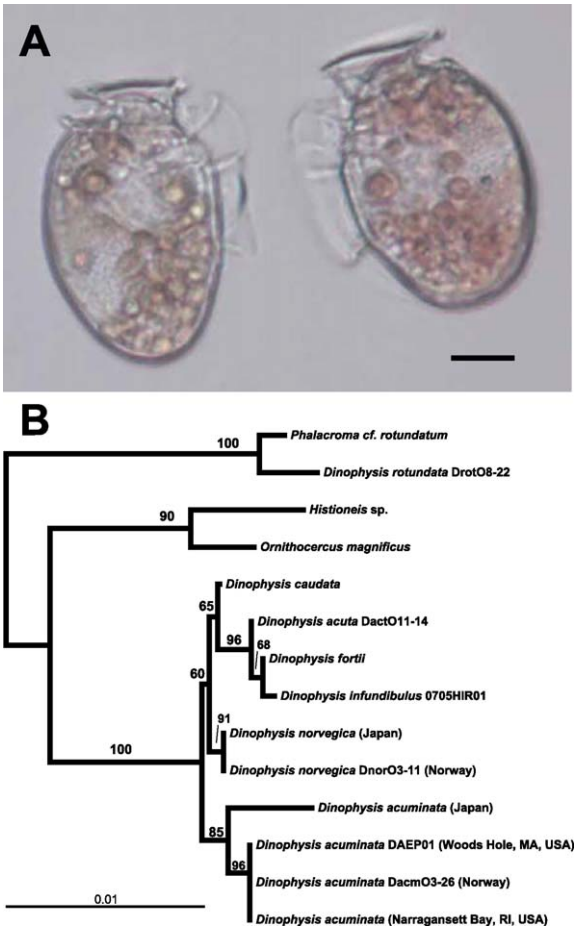

Fig. 1. (A) Light micrograph of *D. acuminata* (DAEP01) cells (scale bar = 10 μm). (B) Minimum evolution tree of *Dinophysis* sp. Numbers above the branches are the results of 500 bootstrap replicates.

The *D. acuminata* culture for toxin analysis was grown under high light (300 μmol photons m^{-2} s^{-1}) in a Fernbach flask containing 1300 mL of f/2-Si medium at 4 °C. The initial cell density of *M. rubra*, CCMP2563, and *D. acuminata*, DAEP01, was calculated to be 2000 and 100 cells mL^{-1}, respectively. A sample for toxin analysis was collected from this culture during the late exponential/early stationary phase where 120 mL of culture was passed through a 20 μm Nitex sieve to collect the *Dinophysis* cells. At this point in the growth cycle, all of the *Myrionecta* cells had been eaten, thus, none were contained in the harvested aliquot. The material caught on the sieve was backwashed into a pre-weighed 15 mL centrifuge tube and duplicate, 200 μL aliquots for cell counts were dispensed from the well-mixed sample into separate microcentrifuge tubes containing 1 mL volumes of filtered seawater and 5% formalin. The 15 mL tube was weighed again and centrifuged at 3000 × *g* for 5 min. The medium overlying the resulting cell pellet was carefully aspirated so that a minimal volume (<250 μL) was remaining above the cell pellets. The samples were flash frozen and stored in liquid nitrogen, and later shipped on dry ice for toxin analysis. There were approximately 236,000 cells in the cell pellet as determined by averaging the toxin cell counts and multiplying this value by the total volume within the 15 mL centrifuge tube. Control cultures of *M. rubra* and *G. cryophyla* were grown as described above, harvested via centrifugation (3000 × *g*, 5 min), and assayed for toxins as well. The *M. rubra* cell pellets contained 256,800 cells while 1,000,000 cells were concentrated for the *G. cryophyla* cell pellets.

2.2. Toxin extraction

At the Irish Marine Institute in Galway (MI), the cell pellet was thawed at room temperature and 200 μL of methanol was added. The sample was sonicated (Ultrasonic Bath, Branson 5510, The Netherlands) for 15 min and then centrifuged at 4200 × *g* for 5 min. The supernatant was transferred to a spin filter (0.2 μm) and centrifuged at 13,000 × *g* for 5 min using a microcentrifuge. A high recovery LC vial was used to collect the filtrate and this extraction procedure was repeated another three times to ensure complete extraction efficiency. The extracts were evaporated to dryness on a heating block at 40 °C under a stream of nitrogen and the residues were resuspended in 1 mL of methanol for LC–MS/MS analysis.

2.3. Analyses using LC–MS/MS hybrid Quadrupole:Time of Flight Mass Spectrometer

The LC–MS/MS analyses were conducted using a 2795 Waters HPLC coupled to a Micromass Ultima (Waters Micromass) quadrupole–time of flight hybrid (QTOF) equipped with a z-spray ESI source. The QTOF was used in TOF-MS/MS mode, where the molecular ion (for OA toxins) and ammonium adduct (PTXs) were isolated in the quadrupole and where after collision in the collision cell, the whole fragmentation spectrum was obtained in the TOF. OA, DTX1 and DTX2 were analyzed in negative ionization (ES−). Accurate mass data was acquired on the instrument operated in W-mode. Glu-Fib (or Leucin) solution (accurate mass: 785.8426 Da) was infused into a dual-lockspray source for in situ calibration of the instrument. Fragment ions of Glu-Fib (*m*/*z* 684.3579 and 333.1881) were used for the calibration of the entire mass range for fragment ion analysis.

This system used a binary mobile phase, with both A and B each containing 2 mM ammonium formate and 50 mM formic acid. Phase A was 100% aqueous while phase B consisted of 95% aqueous acetonitrile. A volume of 50 μL was injected using a switching valve that allowed the injection of large volumes. The chromatographic method was adapted from Hardstaff et al. (2006) and was reported in detail for the analysis of azaspiracids in shellfish extracts (Vale et al., 2008). The toxins were first concentrated on an OASIS HLB column (20 mm × 2.1 mm; 5 μm) and subsequently backwashed and separated on a BDS Hypersil C8 (150 mm × 1 mm; 3 μm) using the gradient described in Table 1. Accurate mass of DTX1 was measured on a standard (not certified) obtained from Bluebiotek (Germany).

2.4. LC–MS/MS analysis using triple quadrupole mass spectrometer: OA and DTX analysis

LC–MS/MS analyses were performed using a 2695 Waters HPLC coupled to a Quattro Ultima triple quadrupole (TQ) (Waters Micromass) with separation being achieved on a C8 Hypersil column (50 mm × 2.1 mm; 3.5 μm particle size) maintained at 25 °C. The flow rate was set at 0.25 mL/min and a volume of 5 μL was injected. Binary mobile phase was used, with phase A (100% aqueous) and phase B (95% aqueous acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid. A gradient elution was employed, starting with 30% B, rising to 90% B over 8 min, held for 0.5 min, then decreased to 30% B in 0.5 min and held for 3 min to equilibrate at initial conditions before the next run started. Quantitation was performed by LC–MS/MS using the molecular ion or the ammonium adduct as precursor ions (Table 2). The diol ester derivative of OA, OA-D8, was not quantified but a reference solution kindly provided by M. Quilliam was used to confirm the presence of the toxin. A set of OA standards (7 levels) were used for the quantification of OA and DTX1 and were prepared using the NRC OA certified reference material (CRM-OA-b). An internal laboratory reference material prepared from mussels *Mytilus edulis* and containing OA, DTX1 and DTX2 was extracted with MeOH (2 g extracted in 25 mL) and used for the comparison of the retention times.

Table 1
Gradient conditions used for the LC–MS/MS analysis of the cell extract.

Time (min)	A%	B%	Flow (mL/min)
0.00	90	10	0.200
2.00	90	10	0.200
2.01	90	10	0.020
2.05	90	10	0.075
2.50	70	30	0.075
12.50	0	100	0.075
30.50	0	100	0.075
31.00	70	30	0.075
40.00	70	30	0.075

Table 2
Transitions used for LC–MS/MS analysis.

	Precursor ion	Precursor mass	Fragment mass
OA	$[M-H]^-$	803.5	255.5
DTX1	$[M-H]^-$	817.5	255.5
OA-D8	$[M+NH_4]^+$	946.5	751.5
PTX-2	$[M+NH_4]^+$	876.5	823.5
PTX-11	$[M+NH_4]^+$	892.5	839.5
PTX-2sa	$[M+NH_4]^+$	894.5	823.5

2.5. PTX analysis

An Ultra Performance Liquid Chromatography (UPLC) method was used for the quantification of PTXs. UPLC–MS/MS analyses were performed using a Waters Acquity system coupled to a Quattro Ultima TQ (Waters Micromass). Separation was achieved on a C8 BEH Acquity column (50 mm × 2.0 mm; 1.7 μm particle size) maintained at 30 °C. The flow rate was set at 0.4 mL/min and a volume of 5 μL was injected. Binary mobile phase was used, with phase A (100% aqueous) and phase B (95% aqueous acetonitrile), both containing 2 mM ammonium formate and 50 mM formic acid. A gradient elution was employed, starting with 30% B, rising to 90% B over 10 min, held for 0.5 min, then decreased to 30% B in 0.1 min and held for 2.4 min to equilibrate at initial conditions before the next run started. Quantitation of PTX-2, PTX-11 and PTX-2sa was performed by LC–MS/MS using the ammonium adduct as precursor ions (Table 2) as described previously (Fux et al., 2007). A reference solution of PTX-11 was kindly obtained from C. Miles. PTX-11 and PTX-2sa were quantified against certified standard of PTX-2 from the NRC, Canada.

3. Results

Cells isolated from Eel Pond (DAEP01) were identified as *D. acuminata* using morphology and SSU rDNA sequence similarity (Fig. 1). The SSU rDNA sequence from our strain is identical to the SSU rDNA sequence from *D. acuminata* isolated from Norway (Genbank accession AJ506972) and had one nucleotide difference with a *D. acuminata* isolate from Narragansett Bay, RI, USA (EU130569). Our sequence was different at nine nucleotides compared to the SSU sequence of a Japanese isolate. In phylogenetic analyses, our strain was included in a highly supported clade with other *D. acuminata* isolates (Fig. 1B).

We were unable to isolate co-occurring *Myrionecta* sp. or *Geminigera* sp., so cultures isolated from the Ross Sea (Gustafson et al., 2000) were purchased from the Provasoli-Guillard Culture Collection for Marine Phytoplankton (CCMP) and used as the

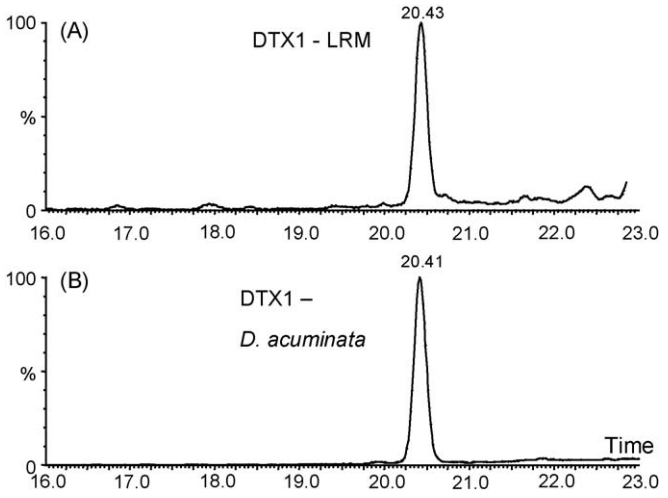

Fig. 2. Example of chromatogram obtained by large-volume injection with Hybrid Quadrupole:Time of Flight Mass Spectrometer: (A) Laboratory Reference Material Chromatogram and (B) chromatogram of cultured *Dinophysis* cells, strain, DAEP01.

Dinophysis food source. The Eel Pond *D. acuminata* cells are able to tolerate the low temperatures (3 °C) required to maintain these strains of *Myrionecta* and *Geminigera* while growing at rates of ~0.1 divisions day^{-1}. At 10 °C, *Dinophysis* achieves a growth rate of ~0.34 divisions day^{-1}, however, at this temperature, most *Myrionecta* appear to die before being consumed by *D. acuminata*. At either temperature, cell densities >7000 mL^{-1} can be routinely obtained using the feeding regimen described above. *D. acuminata* cells were observed feeding on *M. rubra* using a peduncle as described in Park et al. (2006).

Chemical analyses of the DSP toxins were initially performed using a large-volume injection system. OA and DTX1 were detected (retention times 19.03 and 20.43 min, respectively) and their QTOF fragmentation spectra matched the standards and reference compounds of DTX1 (Fig. 2) and OA (data not shown). The ion ratio in the *Dinophysis* sample was compared for parent and fragment ion, 817.8 and 255.2, respectively, and found to be within 2.5% of the ratio of the DTX1 standard. Additionally, the molecular ion masses of DTX1 in the standard and DTX1 in the sample were compared and found to be within acceptance criteria for accurate mass measurement (less than 5 ppm difference, Fig. 3). The retention time and the fragmentation spectrum of OA contained in the cells were identical to those of the OA standard. However, the low concentration of OA in the sample did not allow for accurate mass measurement to be performed.

LC–MS/MS analyses of the cell extract using the TQ showed the presence of a peak of OA (8.77 min) and DTX1 (10.27 min, Fig. 4). The concentration of OA detected in our samples was 18.0 fg cell^{-1} whereas DTX1 concentration was 203.3 fg cell^{-1}. Additionally, the presence of a diol ester of OA, OA-D8, was confirmed by comparison to a reference standard. The amount of OA-D8 was not quantified because no standards were available for this compound. However, the signal intensity revealed that a significant amount of OA-D8 was present in the cells. The polyether-lactone, PTX-2, was found at a concentration of 20.40 pg cell^{-1}. The degradation product of PTX-2, PTX-2 seco-acid (PTX-2sa) was quantified at 76.88 fg cell^{-1} and its more thermodynamically stable epimeric confirmation, 7-epi-PTX seco-acid was witnessed but not quantified. PTX-11 was not detected in the *D. acuminata* cells. However, the PTX-11 transition trace (Table 2) that was set on the UPLC–MS/MS method allowed for the detection of one compound in the sample that eluted before PTX-11 from the reference solution. Quantitation of the analog performed against PTX-2 standards indicated a concentration of 731.6 fg cell^{-1}. Analysis on the QTOF system allowed for the comparison of the fragmentation of PTX-11 and the compound detected in *D. acuminata* (data not shown). It appeared that the compound observed in the culture of *D. acuminata* is an isomer of PTX-11 likely to be a previously unreported hydroxylated PTX-2 analog. More detailed information on this will be reported separately. Cultures of *Geminigera* and *Myrionecta* were analyzed for OA and PTX compounds. No toxins were detected in the control cultures confirming that the toxin is clearly produced by *Dinophysis*.

4. Discussion

We report here the first description and unequivocal evidence of diarrhetic shellfish poisoning toxin production in cultured North American *Dinophysis*. These findings indicate that this suite of cultures can now be used to study toxin production in these

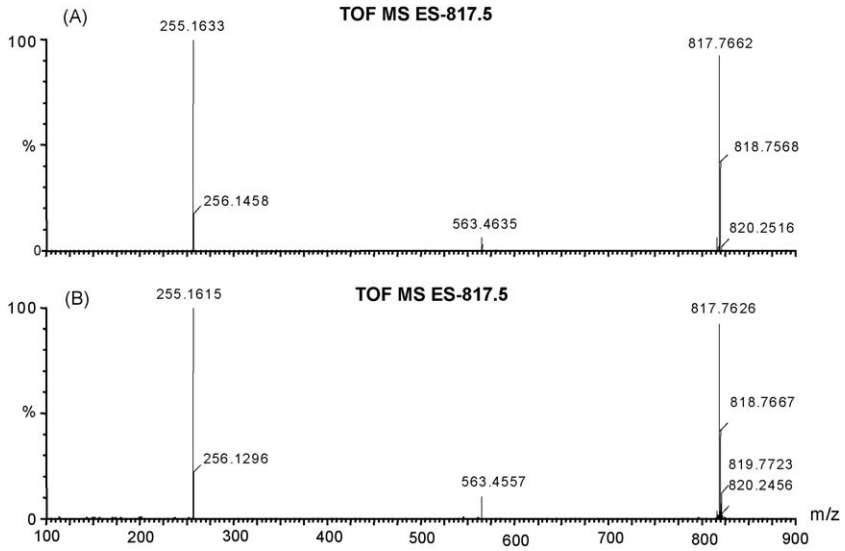

Fig. 3. High resolution fragmentation spectrum of the molecular ion of DTX1 $[M-H]^-$ (817.5) obtained by LC–MS/MS Quadrupole/Time of Flight Mass Spectrometer from (A) the DTX1 standard (BlueBiotech) and (B) the cell extract DAEP01 1-4 with a collision energy of 50 V.

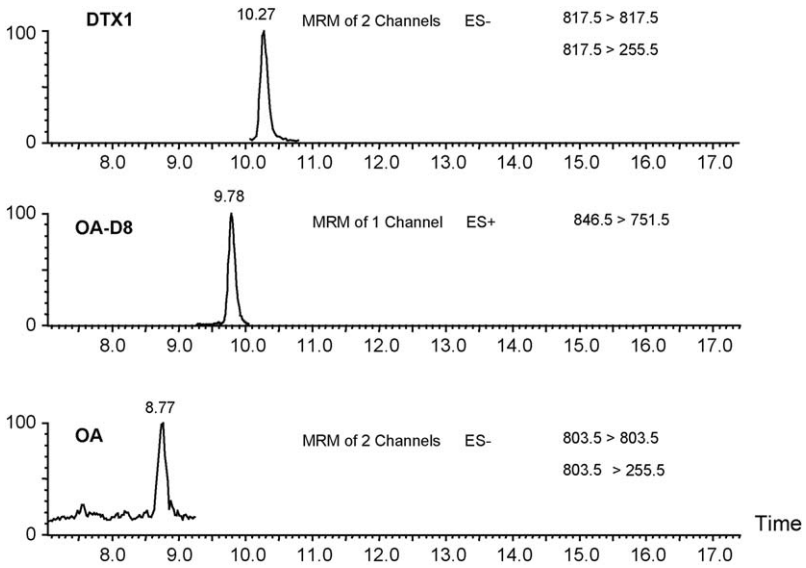

Fig. 4. Example of chromatogram obtained from DAEP01 obtained by LC–MS/MS triple quadrupole using the gradient method on a Hypersil C8 column. OA and DTX1 analyses were performed in negative ionization mode and OA-D8 in positive mode.

organisms, and in particular, the reasons behind the dramatic differences in toxicity between geographically separated strains of *Dinophysis*.

Initial trials on the chemical analysis of the cultured *Dinophysis* were carried out in order to establish if the culture is capable of producing DSP toxins. Confirmation of DTX1 and OA production in the *Dinophysis* sample is based on the comparison of the corresponding spectra with those of the OA and DTX1 standards. Additional evidences for DTX1 were obtained by LC–MS/MS using a QTOF instrument equipped with a switching valve and included: (1) retention time in 2-dimensional chromatography (<0.05 min shift); (2) mass spectral comparison through ion ratios (<2.5% difference); and (3) mass spectral comparison through mass accuracy (<5 ppm). The exact mass of OA in the *Dinophysis* sample was not measured due to its low concentration.

Quantitation of the samples was then performed by LC–MS/MS using the TQ system, which allowed for the analyses with higher sensitivity, wider linearity range and the capabilities of monitoring compounds in positive (OA-D8) and negative (OA and DTX1) in a single analytical run. The DTX1 and OA concentrations in the Woods Hole *D. acuminata* were approximately 203 and 18 fg cell^{-1}, respectively. These are at the low end of toxin content measurements for *D. acuminata*, which range from 100 fg cell^{-1} (Yasumoto, 1985) to 158 pg cell^{-1} (Marcaillou et al., 2005) for DTX1 and 0.1–2.7 pg cell^{-1} for OA (MacKenzie et al., 2005). In the only other documented account of DSP toxins in cultured *Dinophysis*, Kamiyama and Suzuki (2008) found that their *D. acuminata* isolated from Kesennuma Bay, Japan produced 2.5–4.8 pg cell^{-1} of DTX1, 14.7–14.8 pg cell^{-1} of PTX-2 and undetectable amount of OA. These cultures were grown in a manner similar to the culture described here, however, they utilized prey and *Dinophysis* species isolated from Japanese waters and the cultures were incubated at 15 °C. Suzuki et al. (2009) found similar DTX1 per cell concentrations in wild *D. acuminata* from Hokkaido Bay, Japan. Those cells also had amounts of PTX-2 comparable to those found in our culture, as mentioned below.

In addition to DTX1 and OA, the Woods Hole *Dinophysis* culture produced the diol ester of OA, OA-D8. Although no standard solution was available, pronounced signals indicated that a significant amount of the toxin was present. The relative concentration of OA and OA-D8 suggests that there is little free OA in the cell—i.e., that most of it is stored in the ester form in the Woods Hole *Dinophysis*. This is in agreement with toxin profiles for *P. lima* and *D. acuta* samples from New Zealand (Suzuki et al., 2004; Quilliam et al., 1996). The diol ester forms of OA are rapidly converted into free toxins in culture media as well as in shellfish and, therefore, have the potential to cause DSP.

Quantitation of PTXs was performed by UPLC–MS/MS with TQ detection because separation on the Hypersil C8 did not allow for the separation of the isomer of PTX-11 and PTX-2sa. Separation of these compounds was required because the isomer of PTX-11 was detected in the PTX-2sa trace due to the detection of its isotopes which have their molecular mass increased by 2 mass units (894.5 m.u. instead of 892.5 m.u.). PTX-2 was present in the cell extracts at a concentration of 20.40 pg cell^{-1} and the isomer of PTX-11 at 400 fg cell^{-1}. The concentrations of PTX-2 are consistent with some published values for *D. acuminata* (e.g., Miles et al., 2004b; MacKenzie et al., 2005; Kamiyama and Suzuki, 2008), however, they are lower than those reported by Blanco et al. (2007) who found PTX concentrations of 180 pg cell^{-1} in *D. acuminata* cells isolated during a bloom. PTX-2 can quickly degrade through hydrolysis in shellfish (Suzuki et al., 2001; Miles et al., 2004a) and by the release of algal enzymes during sample processing (MacKenzie et al., 2002) into PTX-2 seco-acid which epimerizes into its more thermodynamically stable confirmation, 7-epi-PTX seco-acid (Miles et al., 2004a). The hydroxylated PTX-2 exhibited identical fragmentation pattern as PTX-11 and eluted earlier than the latter. This suggests that oxidation of PTX-2 may have occurred at a different position than for PTX-11 and MS evidence has indicated that this new hydroxylated PTX is different to PTX-1 and to PTX-13. Further work is required to assign the structure of this compound. The role of PTX compounds in DSP is unclear and their toxic manifestations are not well understood; in mice PTX-2 and PTX-11 are toxic when injected intraperitoneally, however, these compounds along with 7-epi-PTX seco-acid are much less toxic when given orally to mice (Miles et al., 2004a; Suzuki et al., 2006). Presently PTX-1 and PTX-2 are included in shellfish safety regulations in the European Union.

This demonstration of toxin production in cultured *Dinophysis* sets the stage for more detailed studies investigating the causes of geographic differences in toxin content. It is now clear that North American *Dinophysis* have the ability to produce DSP toxins, but appear to only rarely cause toxic DSP events. Although Dickey et al. (1992) detected OA in oysters in the northern Gulf of Mexico in

1990 and Tango et al. (2002) reported trace levels of OA in shellfish in the Chesapeake Bay, the first significant episode in North America whereby shellfish became highly toxic during a *Dinophysis* bloom occurred only recently (2008) along the Texas coast, and most efforts to find DSP toxins during previous *Dinophysis* blooms have failed. There are several possible causes for the regional differences in toxin content. The first are environmental conditions such as temperature, light, nutrient availability, or other physical factors that might induce or repress toxin production. Production of toxins in other dinoflagellates is influenced by some of these conditions. For example, cellular concentrations of saxitoxin in *Alexandrium* sp. increase under phosphorus stress and decrease under nitrogen stress (Boyer et al., 1987; Anderson et al., 1990). Likewise, individual cultures of the diatom *Pseudo-nitzschia* show considerable variability in toxin content under different growth conditions (Bates et al., 1998). The second are genetic differences that cause modifications in toxin gene expression. In *Dinophysis*, these could be changes in promoters or transcription factors that regulate toxin gene expression. These alterations could cause an overall decrease in toxin production, or change how toxin production is regulated in response to environmental conditions. A third explanation invokes differences in species or strains of *M. rubra* or *G. cryophila*, the prey and plastid source of *Dinophysis*. This possibility is particularly intriguing given that DSP toxins have been localized to the plastid and PAS-bodies in *Prorocentrum* species (Zhou and Fritz, 1994; Lawrence and Cembella, 1999; Barbier et al., 1999). This distribution of DSP toxins in the cell has been suggested to be a sequestration strategy to prevent the toxin from affecting the cells own protein phosphatases (Barbier et al., 1999). *Prorocentrum* sp. have typical dinoflagellate plastids containing peridinin, not cryptophyte kleptoplasts like those found in *Dinophysis* sp. It is possible that some cryptophyte plastids are better than others for toxin production or sequestration. If North American *Dinophysis* are not able to effectively sequester toxins, they may respond by reducing toxin production, or shifting the relative proportion of the different congeners. OA and DTX1 have been shown to have an inhibitory effect on the growth of non-DSP producing algae (Windust et al., 1996; Sugg and VanDolah, 1999), indicating that poor control of toxin localization could have adverse effects on *Dinophysis* cells. It is currently unknown what role the plastid may play in toxin production, however we now have the tools to test if local *M. rubra* or *G. cryophila* strain availability influences toxin production.

Acknowledgements

This work was partially funded by a Cooperative Institute for Climate and Ocean Research postdoctoral scholarship and research funds provided by the BIO5 Institute at the University of Arizona to JDH. Analyses carried out at the Marine Institute were funded through the EU-FP7 project BIOTOX (STREP FOOD-CT-2004-514074). Support for M. Tong provided by the China Scholarship Council (CSC) 2007 and Nankai University. Research was also supported by grants (to DMA) from the Woods Hole Center for Oceans and Human Health through grants NIEHS P50 ES 012742 and NSF OCE-043072. Support to DMA also provided by NSF OCE-0402707 and OCE-0850421, and by the Ocean Life Institute at the Woods Hole Oceanographic Institution. The authors would like to acknowledge Dr. M. Quilliam and Dr. C. Miles for providing the reference solutions of OA-D8 and PTX-11.[SS]

References

Anderson, D.M., Kulis, D.M., Sullivan, J.J., Hall, S., Lee, C., 1990. Dynamics and physiology of saxitoxin production by the dinoflagellates *Alexandrium* spp. Mar. Biol. 104, 511–524.

Anderson, D.M., Kulis, D.M., Doucette, G.J., Gallager, J.C., Balech, E., 1994. Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeast United States and Canada as determined by morphology, bioluminescence, toxin composition, and mating compatibility. Mar. Biol. 120, 467–478.

Barbier, M., Amzil, Z., Mondeguer, F., Bhaud, Y., Soyer-Gobillard, M.-O., Lassus, P., 1999. Okadaic acid and PP2A cellular immunolocalization in *Prorocentrum lima* (Dinophyceae). Phycologia 38, 41–46.

Bates, S.S., Garrison, D.L., Horner, R.A., 1998. Bloom dynamics and physiology of domoic-acid-producing pseudo-nitzschia species. In: Anderson, D.M., Cembella, A.D., Hallegraeff, G.M. (Eds.), Physiological Ecology of Harmful Algal Blooms. Springer-Verlag, pp. 267–292.

Blanco, J., Álvarez, G., Uribe, E., 2007. Identification of pectenotoxins in plankton, filter feeders, and isolated cells of a *Dinophysis acuminata* with an atypical toxin profile, from Chile. Toxicon 49, 710–716.

Boni, L., Milandri, A., Poletti, R., Pompei, M., 1993. DSP cases along the coasts of Emilia-Romanga (Northwest Adriatic Sea). In: Smayda, T.J., Shimizu, Y. (Eds.), Toxic Phytoplankton Blooms in the Sea. Elsevier, Amsterdam, pp. 475–481.

Boyer, G.L., Sullivan, J.J., Andersen, R.J., Harrison, P.J., Taylor, F.J.R., 1987. Effects of nutrient limitation on toxin production and composition in the marine dinoflagellate *Protogonyaulax tamarensis*. Mar. Biol. 96, 123–128.

Dickey, R.W., Fryxell, G.A., Granade, H.R., Roelke, D., 1992. Detection of the marine toxins okadaic acid and domoic acid in shellfish and phytoplankton in the Gulf of Mexico. Toxicon 30, 355–359.

Felsenstein, J., 2004. PHYLIP (Phylogeny Inference Package) version 3.6. Distributed by the author. Department of Genome Sciences, University of Washington, Seattle, WA, USA.

Fux, E., McMillan, D., Bire, R., Hess, P., 2007. Development of an ultra-performance liquid chromatography–mass spectrometry method for the detection of lipophilic marine toxins. J. Chromatogr. A 1157, 273–280.

Giacobbe, M.G., Penna, A., Ceredi, A., Milandri, A., Poletti, R., Yang, X., 2000. Toxicity and ribosomal DNA of the dinoflagellate *Dinophysis sacculus* (Dinophyta). Phycologia 39, 177–182.

Gustafson, D.E., Stoecker, D.K., Johnson, M.D., Van Heukelem, W.F., Sneider, K., 2000. Cryptophyte algae are robbed of their organelles by the marine ciliate Mesodinium rubrum. Nature 405: 1049–1052.

Hardstaff, W.R., Lewis, N.I., Aasen, J., Quilliam, M.A., 2006. Analysis of phycotoxins in hand-picked plankton cells by micro-column liquid chromatography–tandem mass spectrometry. In: Poster Presented at the XIIth Harmful Algal Bloom Conference, Copenhagen, Denmark, 4–8 September.

Hoef-Emden, K., Marin, B., Melkonian, M., 2002. Nuclear and nucleomorph SSU rDNA phylogeny in the Cryptophyta and the evolution of cryptophyte diversity. J. Mol. Evol. 55, 161–179.

Kamiyama, T., Suzuki, T., 2008. Production of dinophysistoxin-1 and pectenotoxin-2 by a culture of *Dinophysis acuminata* (Dinophyceae). Harmful Algae 8, 312–317.

Lawrence, J.E., Grant, J., Quilliam, M.A., Bauder, A.G., Cembella, A.D., 2000. Colonization and growth of the toxic dinoflagellate *Prorocentrum lima* and associated fouling macroalgae on mussels in suspended culture. Mar. Ecol. Prog. Ser. 201, 147–154.

Lawrence, J.E., Cembella, A.D., 1999. An immunolabelling technique for the detection of diarrhetic shellfish toxins in individual dinoflagellate cells. Phycologia 38, 60–65.

Lee, J.S., Igarashi, T., Fraga, S., Dahl, E., Hovgaard, P., Yamamoto, T., 1989. Determination of diarrhetic shellfish toxins in various dinoflagellate species. J. Appl. Phycol. 1, 147–152.

MacKenzie, L., Beuzenberg, V., Holland, P., McNabb, P., Suzuki, T.S.A., 2005. Pectenotoxin and okadaic acid-based toxin profiles in *Dinophysis acuta* and *Dinophysis acuminata* from New Zealand. Harmful Algae 4, 75–85.

MacKenzie, L., Holland, P., McNabb, P., Beuzenberg, V., Selwood, A., Suzuki, T., 2002. Complex toxin profiles in phytoplankton and Greenshell mussels (*Perna canaliculus*), revealed by LC–MS/MS analysis. Toxicon 40, 1321–1330.

Maranda, L., Corwin, S., Dover, S., Morton, S.L., 2007. *Prorocentrum lima* (Dinophyceae) in northeastern USA coastal waters. II. Toxicity in the epibiota and in shellfish. Harmful Algae 6, 632–641.

Marcaillou, C., Mondeguer, F., Gentien, P., 2005. Contribution to toxicity assessment of *Dinophysis acuminata* (Dinophyceae). J. Appl. Phycol. 17, 155–160.

Marshall, H.G., 1996. Toxin producing phytoplankton in Chesapeake Bay. Vac. J. Sci. 47, 29–37.

Miles, C.O., Wilkins, A.L., Munday, R., Dines, M.H., Hawkes, A.D., Briggs, L.R., Sandvik, M., Jensen, D.J., Cooney, J.M., Holland, P.T., 2004a. Isolation of pectenotoxin-2 from *Dinophysis acuta* and its conversion to pectenotoxin-2 seco acid, and preliminary assessment of their acute toxicities. Toxicon 43, 1–9.

Miles, C.O., Wilkins, A.L., Samdal, I.A., Sandvik, M., Petersen, D., Quilliam, M.A., Naustvoll, L.J., Rundberget, T., Torgersen, T., Hovgaard, P., Jensen, D.J., Cooney, J.M., 2004b. A novel pectenotoxin, PTX-12, in *Dinophysis* spp. and shellfish from Norway. Chem. Res. Toxicol. 17, 1423–1433.

Morton, S.L., Leighfield, T.A., Hayes, B.L., Petitpain, D.L., Busman, M.A., Moeller, P.D.R., Bean, L., McGowan, J., Hurst Jr., J.W., Van Dolah, F.M., 1999. Evidence of diarrhetic shellfish poisoning along the coast of Maine. J. Shellfish Res. 18, 681–686.

Park, M.G., Kim, S., Kim, H.S., Myung, G., Kang, Y.G., Yih, W., 2006. First successful culture of the marine dinoflagellate *Dinophysis acuminata*. Aquat. Microb. Ecol. 45, 101–106.

Quilliam, M.A., Hardstaff, W.R., Ishida, N., McLachlan, J.L., Reeves, A.R., Rose, N.W., Windust, A.J., 1996. Production of diarrhetic shellfish poisoning (DSP) toxins by

Prorocentrum lima in culture and development of analytical methods. In: Yasumoto, T., Oshima, Y., Fukuyo, Y. (Eds.), Harmful and Toxic Algal Blooms. IOC of UNESCO, Senday, pp. 289–292.

Quilliam, M.A., Gilgan, M.W., Pleasance, S., Defreitas, A.S.W., Douglas, D., Fritz, L., Hu, T., Marr, J.C., Smyth, C., Wright, J.L.C., 1993. Confirmation of an incident of diarrhetic shellfish poisoning in eastern Canada. In: Smayda, T.J., Shimizu, Y. (Eds.), Toxic Phytoplankton Bloom in the Sea, Newport, RI, USA. Elsevier Sci. Publ., New York, pp. 547–552.

Rehnstam-Holm, A.-S., Godhe, A., Anderson, D.M., 2002. Molecular studies of *Dinophysis* (Dinophyceae) species from Sweden and North America. Phycologia 41, 348–457.

Sugg, L.M., VanDolah, F.M., 1999. No evidence for an allelopathic role of okadaic acid among ciguatera-associated dinoflagellates. J. Phycol. 35, 93–103.

Suzuki, T., Beuzenberg, V., Mackenzie, L., Quilliam, M.A., 2004. Discovery of okadaic acid esters in the toxic dinoflagellate *Dinophysis acuta* from New Zealand using liquid chromatography/tandem mass spectrometry. Rapid Commun. Mass Spectrom. 18, 1131–1138.

Suzuki, T., Mackenzie, L., Stirling, D., Adamson, J., 2001. Pectenotoxin-2 seco acid: a toxin converted from pectenotoxin-2 by the New Zealand Greenshell mussel. Perna canaliculus. Toxicon 39, 507–514.

Suzuki, T., Miyazono, A., Baba, K., Sugawara, R., Kamiyama, T., 2009. LC–MS/MS analysis of okadaic acid analogues and other lipophilic toxins in single-cell isolates of several Dinophysis species collected in Hokkaido. Japan. Harmful Algae. 8, 233–238.

Suzuki, T., Walter, J.A., LeBlanc, P., MacKinnon, S., Miles, C.O., Wilkins, A.L., Munday, R., Beuzenberg, V., MacKenzie, A.L., Jensen, D.J., Cooney, J.M., Quilliam, M.A., 2006. Identification of pectenotoxin-11 as 34S-hydroxypectenotoxin-2, a new pectenotoxin analogue in the toxic dinoflagellate Dinophysis acuta from New Zealand. Chem. Res. Toxicol. 19, 310–318.

Tango, P., Butler, W., Lacouture, R., Eskin, R., Goshorn, D., Michael, B., Magnien, R., Beatty, W., Brohawn, K., Wittman, R., Hall, S., 2002. An unprecedented bloom of *Dinophysis acuminata* in Chesapeake Bay. In: Steidinger, K.A., Landsberg, J.H., Tomas, C.R., Vargo, G.A. (Eds.), Xth International Conference on Harmful Algae, St. Pete Beach, FL, p. 358.

Utermöhl, H., 1958. Zur Vervollkommnung der quantitativen Phytoplankton-Methodik. Mitt. Int. Ver. Theor. Angew. Limnol. 9, 1–38.

Vale, P., Bire, R., Hess, P., 2008. Confirmation by LC–MS/MS of azaspiracids in shellfish from the Portuguese north-western coast. Toxicon 51, 1449–1456.

Van Dolah, F.M., 2000. Marine algal toxins: origins, health effects, and their increased occurrence. Environ. Health Perspect. 108, 133–141.

Windust, A.J., Wright, J.L.C., McLachlan, J.L., 1996. The effects of the diarrhetic shellfish poisoning toxins, okadaic acid and dinophysistoxin-1, on the growth of microalgae. Mar. Biol. 126, 19–25.

Yasumoto, T., 1985. Recent progress in the chemistry of dinoflagellate toxins. In: Anderson, D.M., White, A.W., Baden, D.G. (Eds.), Toxic Dinoflagellates. pp. 259–270.

Zhou, J., Fritz, L., 1994. The pas/accumulation bodies in and *Prorocentrum maculosum* (Dinophyceae) are dinoflagellate lysosomes. J. Phycol. 30, 39–44.