Available online at www.sciencedirect.com
ScienceDirect
Analytical Biochemistry 369 (2007) 128–135
ANALYTICAL BIOCHEMISTRY
www.elsevier.com/locate/yabio

Extraction and analysis of lipophilic brevetoxins from the red tide dinoflagellate *Karenia brevis*

Michael J. Twiner [a,*], Marie-Yasmine Bottein Dechraoui [a], Zhihong Wang [a], Christina M. Mikulski [a], Michael S. Henry [b], Richard H. Pierce [b], Gregory J. Doucette [a]

[a] *Marine Biotoxins Program, Center for Coastal Environmental Health and Biomolecular Research, NOAA/National Ocean Service, Charleston, SC 29412, USA*
[b] *Mote Marine Laboratory, Sarasota, FL 34236, USA*

Received 5 April 2007
Available online 22 June 2007

Abstract

Efficient extraction and accurate analysis of lipophilic brevetoxins (PbTxs), produced by the harmful algal bloom (HAB) species *Karenia brevis,* are essential when assessing the toxicological potential of this dinoflagellate. One of the most commonly used brevetoxin extraction methodologies employs C18 solid-phase extraction (SPE). In this study, C18 SPEC discs were tested for extraction of spiked PbTx-3 in seawater and naturally produced brevetoxins from *K. brevis.* Quantification of brevetoxin in the extracts was determined using four independent methods: receptor binding assay (RBA), radioimmunoassay (RIA), neuroblastoma (N2A) cytotoxicity assay, and liquid chromatography/mass spectrometry (LC/MS). In addition to quantification of the brevetoxin concentration, LC/MS analysis provided identification of individual congeners and each of their hydrolyzed products. SPEC disc extractions prepared from sonicated cultures of non-brevetoxin-producing *Karenia mikimotoi* cultures spiked with PbTx-3 yielded extraction efficiencies of 108, 99, and 125% as determined by the RBA, RIA, and N2A cytotoxicity assay, respectively. In SPEC disc extracts of brevetoxin-producing *K. brevis* (isolate SP3) cultures, LC/MS analysis yielded the highest total concentrations, possibly due to the concurrent detection of hydrolytic brevetoxin congeners that accounted for up to 20% of the congener profile. Relative to the brevetoxin concentration as determined by LC/MS, the RBA, RIA, and N2A cytotoxicity assay detected 73, 83, and 51% of the total brevetoxin concentration. Stability experiments demonstrated that brevetoxins remain stable on the SPEC discs for at least 30 days, making this extraction method suitable for shipboard collections.

Keywords: Brevetoxin; Extraction; Harmful algal bloom; LC/MS; N2A cytotoxicity assay; Radioimmunoassay; Receptor binding assay

Harmful algal blooms of the toxic dinoflagellate *Karenia brevis* (formerly *Gymnodinium breve* and *Ptychodiscus brevis*) are prominent in the U.S. Gulf of Mexico along the west Florida shelf [1] and along the coast of New Zealand [2]. Economic losses in the United States attributed to this toxic algal species exceed U.S. $38 million annually, due primarily to contamination and mortality of fishery resources as well as to lost tourism revenues [3]. The harmful effects of *K. brevis* result from production of potent neurotoxins called brevetoxins (PbTxs)[1] that bind with high affinity to site 5 of voltage-gated sodium channels (VGSCs) [4]. On binding, brevetoxins cause a shift in the threshold potential for channel activation concurrent with inhibition

* Corresponding author. Fax: +1 843 762 8700.
E-mail address: Mike.twiner@noaa.gov (M.J. Twiner).

[1] *Abbreviations used:* PbTx, brevetoxin; VGSC, voltage-gated sodium channel; NSP, neurotoxic shellfish poisoning; SPE, solid-phase extraction; RBA, receptor binding assay; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; N2A, neuroblastoma cytotoxicity assay; LC/MS, liquid chromatography/mass spectrometry; BSA, bovine serum albumin; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid; SIM, selected ion monitoring; ANOVA, analysis of variance; amu, atomic mass units.

0003-2697/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ab.2007.06.031

of the inactivation, resulting in enhanced Na^+ ion influx and excitotoxicity [4,5].

Brevetoxins associated with blooms of *K. brevis* can elicit various effects on marine organisms and humans. Cell densities as low as 10^3 cells/L have been documented to cause acute respiratory irritation from wave action that aerosolizes the brevetoxins from lysed *K. brevis* cells, whereas slightly greater concentrations of more than 5×10^3 cells/L can induce neurotoxic shellfish poisoning (NSP) in humans ingesting contaminated shellfish [6,7]. Cell densities of more than 10^5 cells/L can result in massive fish kills, and cell densities of more than 10^6 cells/L also cause water discoloration [6].

K. brevis samples collected for brevetoxin analysis often require processing for concentrating these lipophilic toxins and eliminating interfering matrices. The most commonly used methods for extracting brevetoxins from seawater are solid-phase extraction (SPE) techniques such as those that employ C18 columns or SPEC discs [8]. Multiple methods can be used for quantifying either individual brevetoxin congeners or composite brevetoxin-like activity such as a receptor binding assay (RBA) [9], a radioimmunoassay (RIA) [10–12], an enzyme-linked immunosorbent assay (ELISA) [13], a neuroblastoma (N2A) cytotoxicity assay [14], and liquid chromatography/mass spectrometry (LC/MS) [15].

In this article, we characterize the SPEC disc methodology for extracting brevetoxin from brevetoxin-producing cultures of *K. brevis* and PbTx-3 from spiked nontoxic cultures as detected using four analytical methods: RBA, RIA, N2A cytotoxicity assay, and LC/MS. We demonstrate that this SPE method can extract multiple brevetoxin congeners, including the recently identified brevetoxin hydrolytic congeners, and is readily applicable for field sampling.

Materials and methods

Cultures

All experiments employed batch cultures of either *Karenia mikimotoi* (strain NOAA-2, isolated by S. Morton, Marine Biotoxins Program, Charleston, SC, USA), originating from the east coast of Florida, or *K. brevis* (strain SP3, isolated by T. Villareal, Marine Science Institute, University of Texas at Austin, TX, USA), originating from the Texas Gulf coast. Cultures were grown in *f*/2 seawater medium with added selenium [16] under 70 to 100 μE/m^2/s on a 16:8-h light/dark cycle at 20 °C. Samples were removed from exponentially growing cultures for experimental analysis, and cell concentrations were determined by light microscopy using a Palmer–Maloney counting chamber.

To assess the efficiency of the brevetoxin extraction method, cultures of the non-brevetoxin-producing *K. mikimotoi* were used as a matrix control. Whole cultures were disrupted on ice with a probe sonicator (Branson Sonifier, model 450, Branson Ultrasonics, Danbury, CT, USA) by pulsing at 40% output for 3×1-min intervals. Complete cell lysis was confirmed microscopically. One-half of a sonicated culture was spiked with a final concentration of 350 ng/ml PbTx-3 standard reference material (Calbiochem, San Diego, CA, USA). The remaining half of this culture served as an unspiked control, and an equivalent amount of the methanol vehicle (0.035%, v/v) was added. Subsamples of both the brevetoxin-spiked and vehicle control cultures were stored frozen at −20 °C for direct (i.e., no extraction) analysis of brevetoxin in the seawater.

To evaluate the extraction and measurement methods using naturally produced brevetoxin, exponentially growing cultures of *K. brevis* (strain SP3) were sonicated as described above and processed accordingly. Additional aliquots of sonicated samples were stored at −20 °C for direct analysis of brevetoxin in the seawater.

Standard solid-phase SPEC extraction of brevetoxins

SPE of brevetoxins from sonicated cultures of toxic *K. brevis* and PbTx-3-spiked cultures of *K. mikimotoi* were performed using C18 SPEC discs (Varian Chromatography, Lake Forest, CA, USA). SPEC discs are 47 mm C18AR SPE discs consisting of two glass fiber filters encasing C18 resin. Discs were supported using an Empore six-station vacuum manifold (Varian Chromatography) and preconditioned with 20 ml of methanol (100%) followed by 20 ml of high-purity Milli-Q water. Sonicated culture samples (10 ml of spiked or nonspiked *K. mikimotoi* or 40 ml of brevetoxin-producing *K. brevis*) were applied to the discs and filtered at a flow rate not exceeding 50 ml per minute, allowing adsorption of brevetoxin onto the C18 resin (maximum vacuum pressure = 35 kPa). Salts and debris were removed with 60 ml of Milli-Q water, and the discs were dried for 5 min. Using the standard extraction protocol, brevetoxins were eluted with 20 ml of methanol (100%) and then dried by centrifugation using an SC210A SpeedVac Plus (Thermo Savant, Milford, MA, USA). Dried extracts were resuspended in 1 ml of methanol and stored at −20 °C until analyzed for brevetoxin by the methods described below. It should be noted that extensive studies of elution solvents and drying techniques have shown methanol to be a superior elution solvent, although some loss of brevetoxin can occur during the drying process. However, toxin profiles remained relatively constant during these procedures (data not shown).

Modified solid-phase SPEC extraction of brevetoxins

For examination of toxin stability on the SPEC discs for long-term storage (i.e., 1 month) following shipboard collections, a modified elution protocol (i.e., modified extraction) was used. After filtration of the sample, discs were rinsed and dried under vacuum as described above for the standard extraction protocol; however, brevetoxins

were not eluted immediately with methanol but instead were stored frozen at −20 °C. Following a specified time period (0, 7, 15, or 30 days), triplicate discs were extracted in 50-ml centrifuge tubes by the addition of 20 ml methanol and incubated on a shaker (60 rpm, Titramax 100, Heidolph Instruments, Cinnaminson, NJ, USA) for 20 min at room temperature. The methanol extract was removed and dried as noted above. Day 0 SPEC discs extracted using the modified shaking SPEC extraction protocol were frozen at −20 °C for 1 h.

Brevetoxin measurement by receptor binding assay

The brevetoxin RBA is a functional bioassay in which an unknown quantity of nonradiolabeled brevetoxin (standards or samples) competes with radiolabeled brevetoxin ([^{3}H]PbTx-3, Amersham Biosciences, Piscataway, NJ, USA) for the site 5 receptor of the VGSC. Crude membranes prepared from rat brain provide the biological receptor [9,17]. The assay was performed in a 96-well plate format, and all samples were run in triplicate. Here 5 µl of sample extracts or PbTx-3 standards was diluted in 30 µl of ice-cold brevetoxin binding buffer (50 mM Hepes buffer [pH 7.4], 130 mM choline chloride, 5.5 mM glucose, 5.4 mM potassium chloride, 0.8 mM magnesium sulfate, 1 mg/ml bovine serum albumin [BSA], and 0.01% Emulophor-EL 620) just prior to the addition of 35 µl of [^{3}H]PbTx-3 (1.51 nM final) and 140 µl of the rat brain membrane preparation (1 mg protein/ml). For each assay, competition standard curves for assay calibration were generated using PbTx-3 (Calbiochem) concentrations ranging from 10^{-5} to 10^{-11} M (final). After incubating for 1 h at 4 °C, the contents of each well were transferred to a 96-well glass fiber filter mat (PerkinElmer, Boston, MA, USA), filtered, and washed twice with brevetoxin binding buffer (4 °C). The filter mat was dried on a slide warmer, sealed in a plastic bag with solid wax scintillant (MeltiLex, PerkinElmer), and heated at approximately 60 °C for 1 min or until the wax scintillant was completely melted. Once cooled, the mat was counted directly in a microplate scintillation counter (Microbeta 1450, PerkinElmer). Sample extract concentrations were determined using MultiCalc software (PerkinElmer, Finland) based on the PbTx-3 competition standard curve run in parallel. Quality control of each assay was evaluated by testing a known concentration of PbTx-3 (10.5 nM final). Data are expressed as PbTx-3 equivalents per milliliter.

Brevetoxin measurement by RIA

RIAs were performed using a sheep antiserum prepared against a PbTx-2–fetuin conjugate [18] as described previously by Baden and coworkers [11] and Poli and coworkers [10]. RIAs were run in triplicate tubes (final assay volume of 500 µl) containing PbTx-3 standard or the sample with anti-PbTx-2 antiserum (1:4000) and [^{3}H]PbTx-3 (0.4 nM) in phosphate-buffered saline (PBS) supplemented with 0.01% Emulphor-EL 620 (GAF, New York, NY, USA). The tubes were placed on a shaker (Titramax 100) and incubated for 1 h, after which Sac-Cel (Alpco Diagnostics, Windham, NH, USA) was added to achieve separation of bound and unbound brevetoxin. Antibodies were filtered onto 25-mm glass fiber filters, and each assay tube was rinsed with PBS (3 × 2 ml) using a 48-sample Semi-Auto Harvester (Brandel, Gaithersburg, MD, USA). The filters were placed in 5.0 ml of ScintiVerse (Fisher Scientific, Suwanee, GA, USA), and the radioactivity was determined on a Tri-Carb 3100TR Liquid Scintillation Counter (Packard–PerkinElmer, Wellesley, MA, USA). Data were analyzed with GraphPad Prism software (version 4.01, GraphPad Software, San Diego, CA, USA) by a sigmoidal concentration–response (variable slope) curve and expressed as PbTx-3 equivalents per milliliter.

Brevetoxin measurement by N2A cytotoxicity assay

The N2A cytotoxicity assay developed by Manger and coworkers [14] was used, with some modification [19], to quantify the toxic activity in extracts from *K. brevis* and PbTx-3-spiked *K. mikimotoi* cultures. This assay is based on the combined cytotoxic activity of ouabain (0.5 mM), veratridine (0.05 mM), and sodium channel activator toxins such as brevetoxin. N2A cells were harvested by application of trypsin–ethylenediaminetetraacetic acid (EDTA) solution and plated into a 96-well plate (Costar, Cambridge, MA, USA) 24 h prior to treatment at a density of 30,000 cells/well in 100 µl of RPMI growth medium supplemented with 5% fetal bovine serum and 1 mM sodium pyruvate. After 20 h of treatment with known PbTx-3 concentrations, vehicle controls (methanol), or triplicate samples (1 µl in methanol), cell viability was measured using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) CellTiter 96 nonradioactive cell proliferation assay (Promega, Madison, WI, USA). Data were analyzed with GraphPad Prism software by a sigmoidal concentration–response (variable slope) curve and expressed as PbTx-3 equivalents per milliliter.

Brevetoxin measurement by LC/MS

LC separations were performed on a Luna C8(2) column (150 × 2 mm, 5 µm, Phenomenex, Torrance, CA, USA) using an Agilent Technologies 1100 LC system (Palo Alto, CA, USA). Mobile phase was water (A) and acetonitrile (B) with 0.1% acetic acid additive. The LC gradient was as follows: 2 min 50% B with a linear gradient to 80% at 22 min, 95% B at 23 min and held for 6 min, returned to 50% B at 30 min and held for 7 min prior to the next injection. The mobile phase flow rate was 0.2 ml/min. The eluant from the LC was analyzed by an Applied Biosystems/MDS Sciex 4000 QTRAP triple quadrupole/linear ion trap mass spectrometer equipped with a TurboIonSpray interface (Foster City, CA, USA). For quantification of toxin congeners, LC/MS was operated in positive ion mode using selected ion

monitoring (SIM). For identification of the toxin congeners, LC/MS was operated either in positive or negative ion using full scan mode or in positive ion using enhanced product ion scan mode. Because standards for many toxin congeners are not available, the data are expressed in PbTx-3 equivalents per milliliter for quantification purposes. Ions monitored (with corresponding mass/charge ratios) included PbTx-1 (m/z 867), PbTx-7 (m/z 869), hydrolytic PbTx-1 (m/z 885), hydrolytic PbTx-7 (m/z 887), PbTx-2 (m/z 895), PbTx-3 (m/z 897), PbTx-9 (m/z 899), hydrolytic oxidized PbTx-1 (m/z 901), oxidized PbTx-2 (m/z 911), hydrolytic PbTx-2 (m/z 913), hydrolytic PbTx-3 (m/z 915), PbTx-927 (m/z 927; PbTx-2 adduct), and hydrolytic oxidized PbTx-2 (m/z 929).

Statistical analysis

Sample calibration for the RBA, RIA, and N2A cytotoxicity assay was performed by sigmoidal concentration–response analysis using GraphPad Prism software and expressed as PbTx-3 equivalents. RBA, RIA, and N2A cytotoxicity assay data are presented as means ± standard deviations. Significant differences between means were assessed by an analysis of variance (ANOVA) followed by a Tukey multiple t test with $P < 0.01$ being considered significant.

Results

Extraction efficiency

The normalized extraction efficiencies of the C18 SPEC disc method from spiked samples as determined using the RBA, RIA, and N2A cytotoxicity assay are shown in Fig. 1. Triplicate extractions of the PbTx-3 spiked into *K. mikimotoi* cultures ($1.5–4.3 \times 10^7$ cells/L) using this method consistently showed near quantitative recovery, 108 ± 11, 99 ± 16, and $125 \pm 31\%$ for RBA, RIA, and N2A cytotoxicity assay, respectively. No significant differences were observed among the RBA, RIA, and N2A cytotoxicity assay detection methods for the spiked PbTx-3. To assess potential algal matrix issues, matching nonspiked *K. mikimotoi* control samples were extracted in parallel but revealed no brevetoxin-like signal for any of the extracts at the dilutions used in the brevetoxin spike experiments (data not shown). Spiked samples that were applied directly to the RBA (i.e., without SPEC disc extraction) yielded brevetoxin concentrations representing only $9.5 \pm 6\%$ of the spiked amount. This finding suggests that seawater/algal matrices must be removed or reduced sufficiently for quantitative recovery and analysis by the RBA.

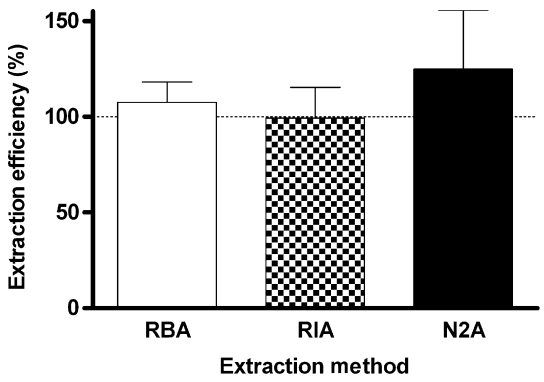

Fig. 1. Brevetoxin (PbTx-3) extraction efficiency using the SPEC disc method. PbTx-3 was spiked (350 ng/ml final) into sonicated cultures of *K. mikimotoi,* a non-brevetoxin-producing dinoflagellate. Extracts were analyzed by RBA, RIA, and N2A cytotoxicity assay. Data (means ± SD) are normalized to the amount of spiked brevetoxin.

Extraction and measurement of brevetoxins from K. brevis cultures

Relative to LC/MS, the RBA, RIA, and N2A cytotoxicity assay detected 73, 83, and 51% of the total brevetoxin concentration (Fig. 2). LC/MS analyses revealed that PbTx-2 was the major congener (~56% of the total), whereas PbTx-3, PbTx-1, and PbTx-7 represented approximately 9, 4, and 1% of the total brevetoxin, respectively (Table 1). In addition, hydrolytic products of PbTx-3 (~9%), PbTx-2 (~5%), PbTx-7 (~2%), and PbTx-1 (~2%) were also detected by LC/MS and represented approximately 20% of the brevetoxin in the SPEC disc extracts (Table 1). Due to the lack of corresponding brevetoxin congener standards, all data are expressed as PbTx-3 equivalents. Extraction of the separate culture replicates generally yielded brevetoxin quantities that were directly proportional to cell densities. One exception where the brevetoxin quantity was not proportional to cell density was with the third replicate culture; this lower density culture produced a midrange brevetoxin response by RIA. The brevetoxin profile may be influenced by cell density such that this particular culture contained an unknown brevetoxin congener(s) not currently detected by LC/MS.

Stability of brevetoxin on SPEC discs

Exponentially growing *K. brevis* SP3 cultures (40 ml at a density of 2.9×10^7 cells/L) were sonicated and filtered through SPEC discs. At time 0, one set of SPEC discs (day 0, standard extraction) were extracted according to the standard method (see Materials and methods) and a total of approximately 5.3 μg brevetoxin was detected by RBA (Fig. 3). A modified extraction protocol was developed to allow elution of previously frozen SPEC discs. Frozen SPEC discs stored at −20 °C for time periods of up to 30 days revealed no significant differences in brevetoxin recovery compared with the day 0 values.

Confirmation of brevetoxin hydrolytics

For brevetoxin hydrolytic products, the lactone in the A ring is opened [20,21]. Their structures were confirmed by LC/MS on the basis of the following. First, the molecular

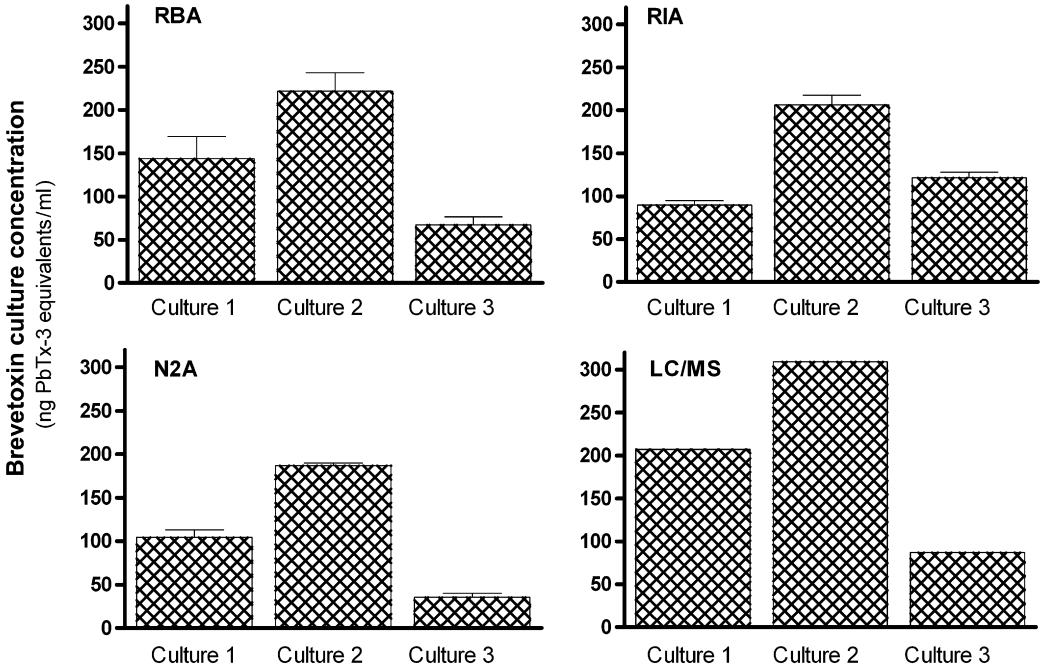

Fig. 2. Total brevetoxin culture concentrations as determined by extraction of *K. brevis* (strain SP3) cultures using the SPEC disc extraction method followed by analysis with RBA, RIA, N2A cytotoxicity assay, and LC/MS. Cultures of different cell densities were used: culture $1 = 2.2 \times 10^7$ cells/L; culture $2 = 2.6 \times 10^7$ cells/L; culture $3 = 6.2 \times 10^6$ cells/L. RBA, RIA, and N2A cytotoxicity assay data are means ± standard deviations. All data are expressed as nanogram PbTx-3 equivalents per milliliter of culture (including both intracellular and extracellular toxins).

Table 1
Total culture concentrations of various brevetoxin congeners as determined by extraction of *K. brevis* (strain SP3) cultures using the SPEC disc extraction method followed by analysis with LC/MS

Brevetoxin congener	*m/z*	Culture concentration (ng/ml)		
		Culture 1	Culture 2	Culture 3
PbTx-1	867	8.43	15.63	3.50
PbTx-2	895	116.48	178.63	47.58
PbTx-3	897	17.10	21.58	10.55
PbTx-7	869	2.35	2.55	1.08
PbTx-9	899	0.30	0.50	0.10
PbTx-927	927	13.40	28.23	4.98
Hydrolytic PbTx-1	885	2.45	4.03	2.08
Hydrolytic PbTx-2	913	7.85	12.18	5.90
Hydrolytic PbTx-3	915	25.55	29.75	5.15
Hydrolytic PbTx-7	887	5.60	6.50	2.28
Hydrolytic oxidized PbTx-1	901	1.48	2.00	0.88
Hydrolytic oxidized PbTx-2	926	4.88	5.45	1.33
Oxidized PbTx-2	911	1.68	2.45	1.43
Total concentration		207.53	309.45	86.80
Percentage hydrolytics		23.03	19.36	20.28

Note. All data are expressed as nanogram PbTx-3 equivalents per milliliter of culture and include both intracellular and extracellular toxins. Cultures of different cell densities were used: culture $1 = 2.2 \times 10^7$ cells/L; culture $2 = 2.6 \times 10^7$ cells/L; culture $3 = 6.2 \times 10^6$ cells/L. Detection limits for each congener in the concentrated extracts were approximately 1 ng/ml, the equivalent of 0.025 ng/ml culture.

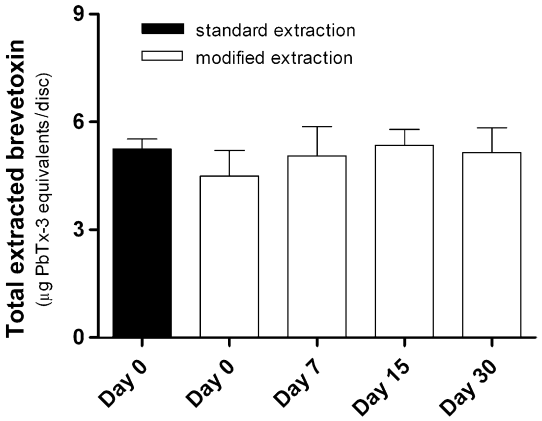

Fig. 3. Stability of brevetoxins on SPEC discs. Sonicated cultures of *K. brevis* (strain SP3) were filtered through SPEC discs and adsorbed brevetoxins were extracted using the standard SPEC extraction technique (day 0) or by a modified SPEC extraction technique (days 0, 7, 15, and 30) following storage of the discs at –20 °C. RBA data (means ± SD) are presented as the total mass of brevetoxins per disc in PbTx-3 equivalents.

weight of each hydrolytic compound increased by 18 atomic mass units (amu) compared with its parent compound. Second, the negative ion full scan showed the ions' negative ion character that was consistent with the presence of the carboxylic group. Third, the fragment ion $[M - H_2O + H]^+$ produced via collision-induced dissociation at the LC/MS interface from each hydrolytic compound $[M + H]^+$ was subjected to collision-activated dissociation at the second quadrupole and yielded a product ion spectrum consistent with that of its corresponding nonhydrolytic compound. Fourth, the mass spectra of PbTx-3 and PbTx-2 hydrolytic products matched those produced by chemical derivatization of PbTx-3 and PbTx-2 by raising the pH above 10.

Discussion

K. brevis sample collection and subsequent brevetoxin quantification often are hindered by relatively low toxin concentrations in natural material and/or the associated matrix effects (e.g., seawater, organic matter). Tests using brevetoxin spiked into the matrix represented by sonicated cultures of *K. mikimotoi,* closely related to *K. brevis* but not producing brevetoxins [6], revealed that the RBA method detected only approximately 10% of the introduced toxin without prior extraction. Adsorption and sequestering of toxin by cell particles or lipids likely interfered with brevetoxin detection. The efficient extraction of lipophilic brevetoxins from seawater and algal matrices, thus, would appear to be essential for assessing the toxicological potential of *K. brevis.* In this study, C18 SPEC discs were characterized for their ability to extract PbTx-3 from spiked, nontoxic *K. mikimotoi* cultures and natural brevetoxins from toxic *K. brevis* cultures using four independent detection methods.

The extraction efficiency of the SPEC disc method was approximately 100% for PbTx-3 spiked into sonicated *K. mikimotoi* cultures as detected by the RBA, RIA, and N2A cytotoxicity assay. Although spike recovery experiments in the current study employed only PbTx-3, our findings are consistent with those of Pierce and coworkers [22,8], who reported SPEC disc extraction efficiencies (means $\pm$ SD based on HPLC detection) for PbTx-3 and PbTx-2 of 100 ± 14 and $105 \pm 5\%$, respectively. Analyses of spiked sample extracts herein using the RBA, RIA, and N2A cytotoxicity assay suggest that each assay can detect the spiked PbTx-3 quantitatively. Because the RBA and N2A cytotoxicity assay methods are at least 10-fold less sensitive to the hydrolytic forms of PbTx-2 and PbTx-3 [23], these data suggest that hydrolysis of the introduced PbTx-3 into less active/potent derivatives was negligible during the less than 5-min period prior to extraction. This is consistent with previous experiments indicating that hydrolysis of parent brevetoxins in seawater appears to occur over hours to days [23]. When naturally produced brevetoxins from *K. brevis* cultures were stored frozen on SPEC discs for up to 1 month, there seemed to be little loss of toxin or conversion into less active brevetoxin hydrolytics given that RBA activities remained constant during this time.

In the experiments using cultures of *K. brevis,* it is clear that the four independent detection methods are not equally capable of measuring naturally produced brevetoxins. Brevetoxin detection by LC/MS, which does not rely on toxic activity or antibody cross-reactivity but rather relies on LC retention times and ion mass/charge ratios, consistently yielded the highest total brevetoxin quantities. However, a common impediment for all brevetoxin detection methods is the absence of specific congener reference standards. Therefore, quantification relied on expressing brevetoxin concentrations as PbTx-2 or PbTx-3 equivalents that, depending on the congener profile, may have over- or underestimated the real concentration.

Relative to LC/MS values, the RBA, RIA, and N2A cytotoxicity assay detected 73, 83, and 51%, respectively, of the total brevetoxin extracted from *K. brevis* cultures. Although the RIA is based on binding of a PbTx-2 antibody that recognizes multiple brevetoxin congeners, antibody binding does not appear to recognize all forms detected by LC/MS to the same degree. As noted previously by Poli and coworkers [10], this discrepancy may be due in part to the presence of the hydrolyzed and oxidized brevetoxin congeners with variable antibody cross-reactivity. Similarly, the RBA, which involves competition between unlabeled and radiolabeled brevetoxins for VGSCs, also yielded lower concentrations relative to LC/MS, probably due to variable affinities of brevetoxin congeners for VGSCs [24,25]. In fact, our recent investigation of semisynthesized PbTx-2 and PbTx-3 hydrolytics demonstrated 25- and 59-fold reductions in binding affinity, respectively [23]. The same study showed that the N2A cytotoxicity assay was even less effective at detecting these two hydrolytics given that the response declined by approximately 900- and 150-fold, respectively, and this would help to explain the approximately 50% lower brevetoxin concentration compared with LC/MS quantification herein. These data demonstrate that, unlike the PbTx-3 spiking experiments where all detection methods showed equivalent quantitative recoveries, mixed brevetoxin congener profiles produced by *K. brevis* cultures yield different results depending on the method used. Specifically, values generated for the same *K. brevis* culture extract ranged as follows: N2A cytotoxicity assay $<$ RBA $<$ RIA $<$ LC/MS. Therefore, researchers should be cautious in selecting a detection method and the interpretation of data generated.

Interestingly, a study of brevetoxin detection in shellfish extracts involving a multilaboratory comparison of the mouse bioassay regulatory method with the RBA, N2A cytotoxicity assay, LC/MS, and ELISA was published recently [26]. Not unlike the current spike recovery experiments, recoveries of PbTx-3 introduced into shellfish extracts as detected by RBA, ELISA, and LC/MS were 136, 87, and 78%, respectively. For naturally incurred brevetoxin in shellfish, the RBA and ELISA methods performed well, whereas the N2A cytotoxicity assay was highly variable and LC/MS, although offering unambiguous structural confirmation, was hindered by lack of reference standards. In general, these observations on toxin detection techniques in a shellfish matrix compared similarly with our study, which is the first to evaluate multiple methods for measuring brevetoxin concentrations in *K. brevis* cultures and seawater.

In the current study, the LC/MS method yielded profiles of many of the known brevetoxin congeners (total of 13), and this may explain why its toxin concentration values exceeded those of the other three techniques. Although expressed as PbTx-3 equivalents, the brevetoxin profiles of *K. brevis* cultures based on SPEC disc samples contained PbTx-2 ($\sim$56%), PbTx-3 ($\sim$9%), PbTx-1 ($\sim$4%), PbTx-2 hydrolytics ($\sim$5%), PbTx-3 hydrolytics ($\sim$9%), PbTx-1

hydrolytics (2%), and PbTx-*m*/*z* 927 (7%). Although these findings are generally consistent with other reports [27], it should be noted that the current study not only included data for brevetoxin hydrolytics (representing ~20% of total brevetoxin), but also incorporated both intracellular and extracellular fractions. We have shown that most hydrolytics are formed on release from the cells into the surrounding alkaline seawater (~pH 8.2) [23]. Similarly, Abraham and coworkers [20] found that the majority of the open-ring, polar brevetoxin derivatives (i.e., hydrolytics) were present in the bloom water filtrates, whereas PbTx-2 and PbTx-1 dominated the cell-rich fractions. It is likely that measurements of only the intracellular brevetoxins during the current study would have yielded closer agreement among the various methods tested.

In conclusion, we have demonstrated that the C18 SPEC disc method is an excellent one for extracting multiple brevetoxin congeners, including many hydrolytic forms, from water samples. The SPEC disc method can be employed to extract brevetoxins from up to six samples simultaneously (~15 min/sample depending on volume filtered) and requires only a minimal volume (20 ml) of methanol for preconditioning. Brevetoxins extracted from *K. brevis* cultures were stable on the SPEC discs when frozen at −20 °C for at least 1 month. Collectively, these data show that the SPEC disc protocol is a suitable method for the in situ collection and subsequent storage of brevetoxin field samples prior to laboratory analysis. In addition, we compared four independent detection methods representing a range of toxin recognition strategies (i.e., receptor interactions, antibody recognition, MS) for measuring brevetoxin in *K. brevis* and seawater. Each assay can accurately measure spiked amounts of PbTx-3 in SPEC disc sample extracts and can also be confidently applied to the analysis of intracellular brevetoxins. Alternatively, caution must be used in the analysis and data interpretation when these methods are used for quantification of samples that may contain a mixed profile of brevetoxin congeners, particularly the hydrolytic forms that are observed in high concentrations in extracellular extracts. The absence of suitable reference standards for individual brevetoxin congeners is a severe hindrance for both quantification purposes and detection method comparisons where response to individual congeners may differ significantly among the detection methods. Although the LC/MS method appears to be the best method for identification and quantification of brevetoxin congeners, it cannot be used to infer biological activity and/or toxicity.

Acknowledgments

This work was funded by the National Oceanic and Atmospheric Administration/National Ocean Service (NOAA/NOS). M.J.T. was partially supported by a National Research Council Associateship Award through NOAA/NOS/National Centers for Coastal Ocean Science (NCCOS)/Center for Coastal Environmental Health and Biomolecular Research (CCEHBR). This publication does not constitute an endorsement of any commercial product or intend to be an opinion beyond scientific or other results obtained by NOAA. No reference shall be made to NOAA, or this publication furnished by NOAA, to any advertising or sales promotion that would indicate or imply that NOAA recommends or endorses any proprietary product mentioned herein or that has as its purpose an interest to cause the advertised product to be used or purchased because of this publication.

References

[1] P. Tester, K. Steidinger, *Gymnodinium breve* red tide blooms: initiation, transport, and consequences of surface circulation, Limnol. Oceanogr. 42 (1997) 1039–1051.

[2] H. Ishida, A. Nozawa, K. Totoribe, N. Muramatsu, H. Nukaya, K. Tsuji, K. Yamaguchi, T. Yasumoto, H. Kaspar, N. Berkett, T. Kosuge, Brevetoxin B1, a new polyether marine toxin from the New Zealand shellfish, *Austrovenus stutchburyi*, Tetrahedron Lett. 36 (1995) 725–728.

[3] D.M. Anderson, P. Hoagland, Y. Kaoru, A.W. White, Woods Hole Oceanographic Institution Technical Report (WHOI-2000-11), Woods Hole, MA, 2000.

[4] D.G. Baden, Brevetoxins: unique polyether dinoflagellate toxins, FASEB J. 3 (1989) 1807–1817.

[5] F.W. Berman, T.F. Murray, Brevetoxins cause acute excitotoxicity in primary cultures of rat cerebellar granule neurons, J. Pharmacol. Exp. Ther. 290 (1999) 439–444.

[6] P. Gentien, Bloom dynamics and physiology of the *Gymnodinium mikimotoi* species complex, in: D.M. Anderson, A.D. Cembella, G.M. Hallegraeff (Eds.), Physiological Ecology of Harmful Algal Blooms, NATO ASI Series, 1998, pp. 155–173.

[7] L.C. Backer, L.E. Fleming, A. Rowan, Y.-S. Cheng, J. Benson, R.H. Pierce, J. Zaias, J. Bean, G.D. Bossart, D. Johnson, Recreational exposure to aerosolized brevetoxins during Florida red tide events, Harmful Algae 2 (2003) 19–28.

[8] R.H. Pierce, M.S. Henry, P.C. Blum, J. Lyons, Y.S. Cheng, D. Yazzie, Y. Zhou, Brevetoxin concentrations in marine aerosol: human exposure levels during a *Karenia brevis* harmful algal bloom, Bull. Environ. Contam. Toxicol. 70 (2003) 161–165.

[9] F.M. Van Dolah, E.L. Finley, B.L. Haynes, G.J. Doucette, P.D. Moeller, J.S. Ramsdell, Development of rapid and sensitive high throughput pharmacologic assays for marine phycotoxins, Nat. Toxins 2 (1994) 189–196.

[10] M.A. Poli, K.S. Rein, D.G. Baden, Radioimmunoassay for PbTx-2-type brevetoxins: Epitope specificity of two anti-PbTx sera, J. AOAC Intl. 78 (1995) 538–542.

[11] D.G. Baden, T.J. Mende, J. Walling, D.R. Schultz, Specific antibodies directed against toxins of *Ptychodiscus brevis* (Florida's red tide dinoflagellate), Toxicon 22 (1984) 783–789.

[12] R.T. Woofter, M.-Y. Bottein Dechraoui, I. Garthwaite, N.R. Towers, C.J. Gordon, J. Cordova, J.S. Ramsdell, Measurement of brevetoxin levels by radioimmunoassay of blood collection cards after acute, long-term, and low-dose exposure in mice, Environ. Health Perspect. 111 (2003) 1595–1600.

[13] J. Naar, A. Bourdelais, C. Tomas, J. Kubanek, P.L. Whitney, L. Flewelling, K. Steidinger, J. Lancaster, D.G. Baden, A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid, Environ. Health Perspect. 110 (2002) 179–185.

[14] R.L. Manger, L.S. Leja, S.Y. Lee, J.M. Hungerford, M.M. Wekell, Tetrazolium-based cell bioassay for neurotoxins active on voltage-sensitive sodium channels: semiautomated assay for saxitoxins, brevetoxins, and ciguatoxins, Anal. Biochem. 214 (1993) 190–194.

[15] S.M. Plakas, Z. Wang, K.R. El Said, E.L.E. Jester, H.R. Granade, L. Flewelling, P. Scott, R.W. Dickey, Brevetoxin metabolism and elimination in the Eastern oyster (*Crassostrea virginica*) after controlled exposures to *Karenia brevis*, Toxicon 44 (2004) 677–685.

[16] R.R.L. Guillard, Culture of phytoplankton for feeding marine invertebrates, in: W.L. Smith, M.H. Chanley (Eds.), Culture of Marine Invertebrate Animals, Plenum, New York, 1975, pp. 29–60.

[17] G.J. Doucette, M.M. Logan, J.S. Ramsdell, F.M. Van Dolah, Development and preliminary validation of a microtiter plate-based receptor binding assay for paralytic shellfish poisoning toxins, Toxicon 35 (1997) 625–636.

[18] I. Garthwaite, K. Ross, C. Miles, L.R. Briggs, N.R. Towers, T. Borrell, P. Busby, Integrated enzyme-linked immunosorbent assay screening system for amnesic, neurotoxic, diarrhetic, and paralytic shellfish poisoning toxins found in New Zealand, J. AOAC Intl. 84 (2001) 1643–1648.

[19] M.-Y. Bottein Dechraoui, J.S. Ramsdell, Type B brevetoxins show tissue selectivity for voltage-gated sodium channels: comparison of brain, skeletal muscle, and cardiac sodium channels, Toxicon 41 (2003) 919–927.

[20] A. Abraham, S.M. Plakas, Z. Wang, E. Jester, K.R. El Said, H. Granade, M. Henry, P. Blum, R. Pierce, R.W. Dickey, Characterization of polar brevetoxin derivatives isolated from *Karenia brevis* cultures and natural blooms, Toxicon 48 (2006) 104–115.

[21] A. Abraham, S.M. Plakas, Z. Wang, E.L. Jester, K.R. Said, H.R. Granade, R.W. Dickey, R.H. Pierce, M.S. Henry, Characterization of brevetoxin derivatives from *Karenia brevis* natural blooms and laboratory cultures, in: Toxin Symposia and Task Force Meeting at AOAC Annual Meeting, Orlando, FL, 2005.

[22] R.H. Pierce, M.S. Henry, L.S. Proffitt, A.J. deRosset, Evaluation of solid sorbents for the recovery of polyether toxins (brevetoxins) in seawater, Bull. Environ. Contam. Toxicol. 49 (1992) 479–484.

[23] P.B. Roth, M.J. Twiner, Z. Wang, M.-Y. Bottein Dechraoui, G.J. Doucette, Fate and distribution of brevetoxin (PbTx) following lysis of *Karenia brevis* by algicidal bacteria, including analysis of open A-ring derivatives, Toxicon, in press.

[24] K.S. Rein, B. Lynn, R.E. Gawley, D.G. Baden, Brevetoxin B: chemical modifications, synaptosome binding, toxicity, and an unexpected conformational effect, J. Org. Chem. 59 (1994) 2107–2113.

[25] M.-Y. Dechraoui, J. Naar, S. Pauillac, A.-M. Legrand, Ciguatoxins and brevetoxins, neurotoxic polyether compounds active on sodium channels, Toxicon 37 (1999) 125–143.

[26] R. Dickey, S. Plakas, E. Jester, E.S.K. Rein, J. Johannessen, L. Flewelling, P. Scott, D. Hammond, F. Van Dolah, T. Leighfield, M.-Y. Bottein Dechraoui, J. Ramsdell, R. Pierce, M. Henry, M. Poli, K. Walker, J. Kurtz, J. Naar, D. Baden, S. Musser, K. White, P. Truman, T. Hawryluk, M. Wekell, D. Stirling, M. Quilliam, J. Lee, Multi-laboratory study of five methods for the determination of brevetoxins in shellfish tissue extracts, in: K. Steidinger, J. Landsberg, C.R. Tomas, G.A. Vargo (Eds.), Harmful Algae 2002, Florida Fish and Wildlife Conservation Commission, Florida Institute of Oceanography, and Intergovernmental Oceanographic Commission of UNESCO, St. Petersburg, FL, 2004, pp. 300–302.

[27] L.E. Roszell, L.S. Schulman, D.G. Baden, Toxin profiles are dependent on growth stages in cultured *Ptychodiscus brevis*, in: E. Graneli (Ed.), Toxic Marine Phytoplankton, Elsevier Science, New York, 1990, pp. 403–406.