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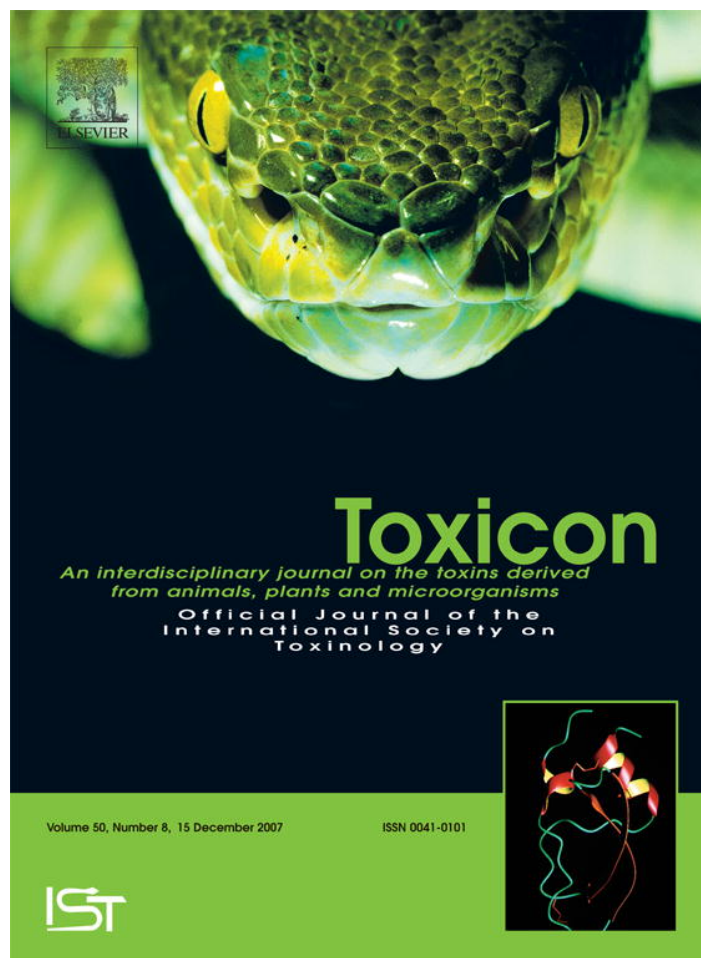

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Toxicon 50 (2007) 1175–1191

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Fate and distribution of brevetoxin (PbTx) following lysis of *Karenia brevis* by algicidal bacteria, including analysis of open A-ring derivatives

Patricia B. Roth[a,b,1], Michael J. Twiner[a,*,1], Zhihong Wang[a], Marie-Yasmine Bottein Dechraoui[a], Gregory J. Doucette[a]

[a] *Marine Biotoxins Program, Center for Coastal Environmental Health and Biomolecular Research, NOAA/National Ocean Service, 219 Fort Johnson Road, Charleston, SC 29412, USA*

[b] *Grice Marine Laboratory, College of Charleston, Charleston, SC 29412, USA*

Received 3 May 2007; received in revised form 6 August 2007; accepted 7 August 2007
Available online 14 August 2007

Abstract

Flavobacteriaceae (strain S03) and *Cytophaga* sp. (strain 41-DBG2) are algicidal bacteria active against the brevetoxin (PbTx)-producing, red tide dinoflagellate, *Karenia brevis*. Little is known about the fate of PbTx associated with *K. brevis* cells following attack by such bacteria. The fate and distribution of PbTx in *K. brevis* cultures exposed to these algicidal strains were thus examined by receptor binding assay and liquid chromatography/mass spectrometry (LC/MS) in three size fractions (>5, 0.22–5, $<0.22\,\mu$m) over a 2-week time course. In control cultures, brevetoxin concentrations in the $>5\,\mu$m particulate size fraction correlated with changes in cell density, whereas significant increases in dissolved (i.e., $<0.22\,\mu$m) toxin were observed in the later stages of culture growth. Exposure of *K. brevis* to either of the two algicidal bacteria tested caused cell lysis, coinciding with a rapid decline in the $>5\,\mu$m PbTX size fraction and a simultaneous release of dissolved toxin into the growth medium. Upon cell lysis, dissolved brevetoxin accounted for ca. 60% of total toxin and consisted of 51–82% open A-ring derivatives. Open A-ring PbTx-2 and PbTx-3 derivatives bound with lower affinity (approximately 22- and 57-fold, respectively) to voltage-gated sodium channels and were considerably less cytotoxic (86- and 142-fold, respectively) to N2A cells than their individual parent toxins (i.e., PbTx-2 and PbTx-3). These novel findings of changes in PbTx size-fractioned distribution and overall reduction in *K. brevis* toxicity following attack by algicidal bacteria improve our understanding of potential trophic transfer routes and the fate of PbTx during red tide events. Moreover, this information will be important to consider when evaluating the potential role of algicidal bacteria in harmful algal bloom (HAB) management strategies involving control of bloom populations.
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Keywords: Brevetoxin (PbTx); Algicidal bacteria; Harmful algal bloom (HAB); Open A-ring; LC/MS; Receptor binding assay; N2A cytotoxicity assay; Extracellular; Hydrolytics

1. Introduction

Karenia brevis (Davis) Hansen and Moestrup (= *Gymnodinium breve*), one of the roughly 30 toxic

*Corresponding author. Tel.: +1 843 762 8649; fax: +1 843 762 8700.
E-mail address: Mike.Twiner@noaa.gov (M.J. Twiner).

[1]These authors have equal authorship status.

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doi:10.1016/j.toxicon.2007.08.003

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microalgal species found in the Gulf of Mexico, is well documented to cause fish and marine mammal mortalities (Steidinger et al., 1998; Landsberg, 2002). This dinoflagellate produces a suite of toxins known as brevetoxins (PbTx), which are ladder-like polycyclic ether compounds that are heat stable and lipid soluble (Kirkpatrick et al., 2004). The proximate pharmacological target of brevetoxins is site 5 on the voltage-gated sodium channel (Catterall and Risk, 1981), where they bind with high affinity (K_d 1–50 nM; Poli et al., 1986). Once bound, these toxins alter the voltage sensitivity of the channel by interfering with the voltage sensor and inactivation gate, ultimately resulting in excessive depolarization of the cell. Type B brevetoxins, specifically PbTx-2 and PbTx-3, dominate *K. brevis* toxin profiles (Baden and Tomas, 1988); however, type A brevetoxins (e.g., PbTx-1) are generally more potent (Rein et al., 1994). In shellfish, the presence of oxidized forms of both type-A and type-B brevetoxin parent molecules, PbTx-1 and PbTx-2, respectively, have been reported in which the R-group on the 'tail' of the molecule is converted to a carboxylic acid from an aldehyde (Fig. 1) (Ishida et al, 2004; Wang et al., 2004; Plakas et al., 2004). Additional brevetoxin derivatives modified on the 'head' or A-ring lactone of the molecule are more polar and confirmed as hydrolyzed open A-ring products of the parent toxins. These derivatives have been isolated from *in vitro* metabolic studies (Radwan and Ramsdell, 2005), *K. brevis* cultures, natural bloom samples, and Eastern oysters exposed to *K. brevis* (Abraham et al., 2006), yet their toxic potency relative to the parent compounds remains unknown. Some of the known brevetoxin structures, including the oxidized and open A-ring hydrolyzed products, are shown in Fig. 1.

Human health effects of brevetoxins generally result from neurotoxic shellfish poisoning (NSP) and/or respiratory illness caused by inhalation of aerosolized toxin. The former can occur following consumption of contaminated shellfish that have accumulated sufficient levels of toxin while filtering *K. brevis* cells as part of their normal diet. This illness typically affects the nervous and gastrointestinal systems; however, all symptoms are reversible and to date there have been no deaths associated with NSP (McFarren et al., 1965; Van Dolah, 2000). Humans can also be exposed to brevetoxins in aerosolized form when the fragile, unarmored *K. brevis* cells burst due to wave action, releasing toxins into the air (Woodcock, 1948; Pierce et al., 2005; Backer et al., 2003), causing irritation and burning of the throat and upper respiratory tract (Asai et al., 1984; Baden et al., 1995).

In addition to *K. brevis*-produced brevetoxins acting as potent ichthyotoxins (Steidinger et al., 1972; Baden and Mende, 1982), a study by Bossart et al. (1998) revealed brevetoxin immunoreactivity in the lung, liver, and lymphoid tissues of manatees collected from a 1996 mortality event, suggesting that brevetoxins, at least in part, can be lethal to mammals via the inhalation of aerosolized toxins (Van Dolah et al., 2003). Although a comprehensive understanding of brevetoxin trophic transfer is lacking, it is clear that finfish (e.g., Woofter et al., 2005) and certain types of seagrass (i.e., *Thalassia testudinum*) can accumulate or be associated with brevetoxins and play a primary role in brevetoxin-induced unusual mortality events (UMEs) (Flewelling et al., 2005).

K. brevis was established as the primary causative agent of Florida's red tides, which have been linked to fish mortalities occurring as early as 1884 (Davis, 1948). Yet, there is still much to be learned about the population dynamics and ecology of these events, as well as the factors regulating bloom initiation, development, maintenance, and termination. Such information is essential for the development of strategies aimed at preventing, controlling, and/or mitigating harmful algal blooms (HABs) and their many adverse effects on coastal communities and ecosystems. One possible approach to controlling HABs involves biological agents such as zooplankton grazers, parasites, viruses, and bacteria that can be highly specific for their target species (reviewed by Anderson et al., 2001; Kim, 2006). The focus of many studies involving biological control agents is to better understand their role as natural regulators of bloom dynamics in order to evaluate their possible use in controlling HABs. An obvious concern associated with any type of control strategy is establishing the fate and distribution of intracellular toxins following lysis or disruption of the algal cells.

Two algicidal bacteria that target *K. brevis*, strains 41-DBG2 (*Cytophaga* sp.) and S03 (*Flavobacteriaceae*), were isolated from the west Florida shelf, USA. Strain 41-DBG2 releases an algicidal agent into the surrounding medium (Doucette et al., 1999), while strain S03 requires direct contact with target algal cells to induce mortality (Roth, 2005; Roth et al., submitted). The primary aim of this study was to examine the size-fractioned

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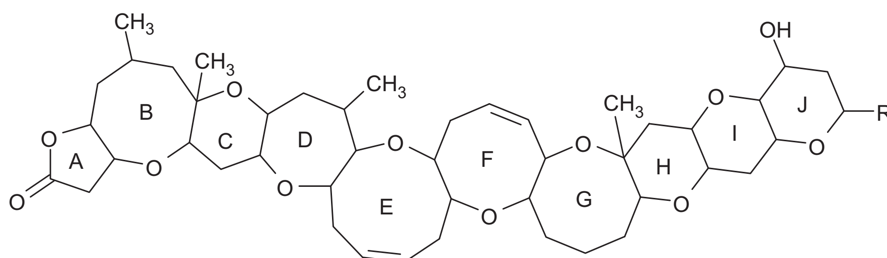
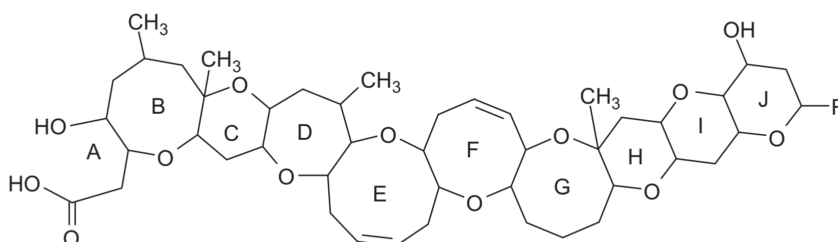
Type A brevetoxins:



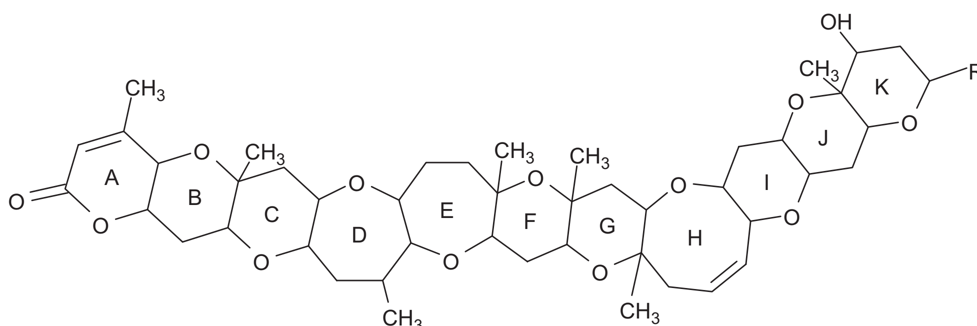
		Nominal mass of MH^+
PbTx-1	R = $CH_2C(=CH_2)CHO$	867
PbTx-7	R = $CH_2C(=CH_2)CH_2OH$	869
Oxidized PbTx-1	R = $CH_2C(=CH_2)COOH$	883

Open A-ring Forms of Type A brevetoxins:



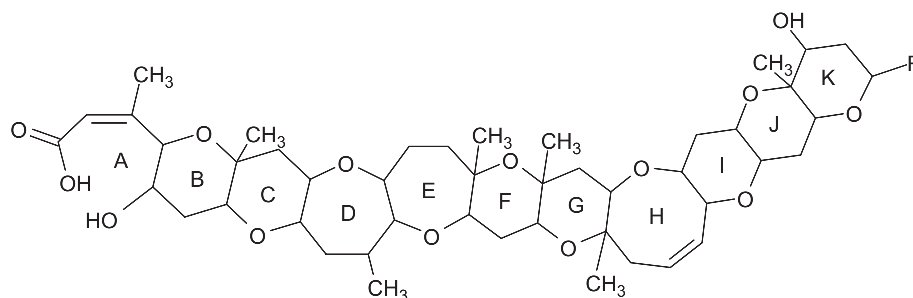
		Nominal mass of MH^+
Open A-ring PbTx-1	R = $CH_2C(=CH_2)CHO$	885
Open A-ring PbTx-7	R = $CH_2C(=CH_2)CH_2OH$	887
Open A-ring oxidized PbTx-1	R = $CH_2C(=CH_2)COOH$	901

Type B brevetoxins:



		Nominal mass of MH^+
PbTx-2	R = $CH_2C(=CH_2)CHO$	895
PbTx-3	R = $CH_2C(=CH_2)CH_2OH$	897
PbTx-9	R = $CH_2C(=CH_3)CH_2OH$	899
Oxidized PbTx-2	R = $CH_2C(=CH_2)COOH$	911
PbTx-2 adduct	putatively PbTx-2 methanol adduct	927

Open A-ring Forms of Type B brevetoxins:



		Nominal mass of MH^+
Open A-ring PbTx-2	R = $CH_2C(=CH_2)CHO$	913
Open A-ring PbTx-3	R = $CH_2C(=CH_2)CH_2OH$	915
Open A-ring oxidized PbTx-2	R = $CH_2C(=CH_2)COOH$	929

Fig. 1. Structural representation of type A and B brevetoxins and their corresponding open A-ring hydrolyzed and oxidized derivatives.

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distribution and composition of brevetoxin in *K. brevis* cultures during exposure to these algicidal bacteria. In addition, the sodium channel binding activities and cytotoxic potencies of two open A-ring brevetoxin congeners found predominantly in the dissolved (i.e., $<0.22\,\mu m$) fraction following algal cell lysis were characterized. This information on the fate and distribution of brevetoxins will aid in understanding the possible effects of natural HAB termination on toxin trophic transfer routes as well as identifying some of the potential effects on bloom toxicity following application of suggested control agents (e.g., algicidal bacteria).

2. Materials and methods

2.1. Culture conditions

2.1.1. Algal cultures

The *K. brevis* C2 clonal isolate (Charlotte Harbor, FL, USA), donated by Dr. K. Steidinger (Fish and Wildlife Research Institute, St. Petersburg, FL, USA), was maintained in 25 mL of *f*/2 medium (–Si) (Guillard, 1975) in 50 mL borosilicate glass tubes at 20 °C with a photon flux rate of $\sim 75\,\mu mol\,m^{-2}\,s^{-1}$ provided on a 16:8 light:dark regimen. Bacteria-free *K. brevis* cultures were obtained by simultaneous treatment with dihydrostreptomycin ($250\,\mu g\,mL^{-1}$), neomycin ($250\,\mu g\,mL^{-1}$), and penicillin G ($500\,\mu g\,mL^{-1}$) for 8 continuous days. Following antibiotic treatment, at least five successive transfers were performed over a $\geqslant$6-week period into autoclaved, antibiotic-free culture medium to eliminate residual antibiotics (and their effects on inoculated control and treatment bacteria) prior to experimentation. Bacterial contamination was checked periodically by DAPI (4′, 6-diamidino-2-phenylindole) staining with epifluorescence microscopy, and with PCR using eubacterial primers (341f and 907r; Lane et al., 1985; Muyzer et al., 1993) followed by cloning and sequencing (C. Mikulski, personal communication). Although the antibiotic-treated cultures displayed no morphological or growth rate effects relative to the bacteria-containing cultures, the impacts of antibiotic treatment on brevetoxin production or profile are currently unknown. It should also be noted that although antibiotics were effective in eliminating bacteria, such cultures were occasionally susceptible to fungal contamination during the late stationary phase. Exponentially growing, bacteria-free *K. brevis* C2 cultures served as the test/target organism throughout this study.

2.1.2. Bacteria cultures

Three members of the *Cytophaga–Flavobacterium–Bacteroides* complex were used in this study: strain S03 (*Flavobacteriaceae*, Genbank accession number EU021292), strain 41-DBG2 (*Cytophaga* sp., Genbank accession number AF427479), and *Aquimarina latercula* (formerly *stanierella latercula*; Nedashkovskaya et al., 2006) (Genbank accession number M58769). Strains S03 and 41-DBG2 are algicidal bacteria isolated from the Gulf of Mexico and demonstrated previously to lyse certain *K. brevis* isolates (Mayali and Doucette, 2002; Roth, 2005). *Aquimarina latercula*, a non-algicidal strain closely related to strains S03 and 41-DBG2 (91% identity match to both), was obtained through the American Type Culture Collection (ATCC # 23177; Manassas, VA, USA). All three bacteria were stored in 10% glycerol under liquid nitrogen vapor. Approximately 100 µL of the frozen material was thawed, added to 3 mL of growth medium, and incubated at 20 °C in a shaker bath for about 24 h to grow each bacterium. Strain S03 and *S. latercula* were cultivated in seawater complete medium (SWC: 2.5 g tryptone, 1.5 g yeast extract, 3 mL glycerol, 500 mL natural seawater (30 ppt)) (Haygood and Nealson, 1985), while strain 41-DBG2 was grown in DBG/5 medium (Doucette et al., 1999). Prior to initiating experiments, cells from liquid cultures were washed twice using 0.22 µm-filtered, autoclaved seawater (30 ppt) with centrifuging at 6500*g* between wash steps.

2.2. Experimental method

Triplicate mid- to late-exponentially growing cultures of *K. brevis* C2 (32 mL total volume) were inoculated with strain S03 (10^2 cell mL^{-1}), strain 41-DBG2 (10^3 cell mL^{-1}), or *S. latercula* (10^3 cell mL^{-1}). Sampling of all control and treatment cultures involved harvesting of individual, replicate culture tubes to avoid repeated sampling of a single culture. For all bacterial additions, inoculation volumes were $<0.1\%$ (v/v). Control tubes receiving no bacterial addition were amended with an equivalent volume of 0.22 µm-filtered, autoclaved seawater (30 ppt). At each time point after bacteria were introduced (Days 0, 1, 2, 3, 5, 9, and 15), 1 mL from each replicate ($n = 3$ per day per treatment) was preserved with formalin (10%) prior to DAPI

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staining (Porter and Feig, 1980) and bacterial enumeration on a Zeiss Axiovert S100 epifluorescence microscope (360 nm excitation/460 nm emission; Thornwood, NY, USA). An additional 1 mL from each replicate was preserved with Lugol's (1%) for algal cell counts performed using a Palmer-Maloney chamber on a Nikon FX-35A light microscope (Melville, NY, USA). Algal and bacterial growth rates (div day^{-1}) were determined using the equation of Guillard (1975): $\mu = \log(T_1/T_0) \times (3.322/(T_1 - T_0))$.

The $>5\,\mu$m and 0.22–5 μm size fractions were collected from the remaining volume (~30 mL) in each culture tube via sequential filtration through 5 and 0.22 μm pore-size polycarbonate filters (Osmonics, Livermore, CA, USA), respectively, using gravity or mild vacuum pressure (<5 kPa). This procedure adequately retained intact algal cells on the 5 μm filter and bacteria on the 0.22 μm filter. Both sets of filters and the $<0.22\,\mu$m filtrates were stored frozen at $-20\,^{\circ}$C until brevetoxins were extracted. Total brevetoxin levels were determined by the sum of the three size fractions.

2.3. Brevetoxin extraction

The 5 and 0.22 μm filters were extracted for brevetoxins with 5 mL of methanol overnight at $-20\,^{\circ}$C, sonicated for 10 min, and centrifuged for 4 min at 4400*g*. This procedure was repeated the following day. Supernatants were removed and filters were briefly (5 min) extracted a third time with an additional 2 mL of methanol. All supernatants (12 mL) were pooled, transferred to disposable 13 mm × 150 mm glass tubes and dried under vacuum centrifugation using a SC210A Speedvac plus (Thermo Savant, Irvine, CA, USA). Dried extracts were resuspended in 250 μL methanol and stored at $-20\,^{\circ}$C until analyzed.

Brevetoxins contained in the $<0.22\,\mu$m size fraction culture filtrates were extracted using SPEC® discs (Varian Chromatography, Lake Forest, CA, USA) as most recently described by Twiner et al. (2007).

2.4. Preparation of open A-ring PbTx-2 and PbTx-3 derivatives

Methanolic PbTx-2 or PbTx-3 was adjusted to pH above 10 with NaOH in aqueous methanol (20%) and vortexed intermittently for 10 min to yield their open A-ring hydrolysis products. A molar equivalent of aqueous HCl equal to that of the NaOH added was used to neutralize the solution, which was then treated by reversed-phase C18 solid-phase extraction (SPE) (Varian Bond Elut, 500 mg, 10 mL) to remove salts and to concentrate the brevetoxins. Briefly, the SPE cartridge was conditioned with one column volume of methanol and then water, the open A-ring PbTx solution in 20% aqueous methanol was loaded onto the SPE, followed by 5 mL of 10% aqueous methanol to wash the sample tube and the cartridge. The cartridge was further washed with 2.5 SPE column volumes of water. Brevetoxins were eluted with 5.5 mL of methanol (100%) and the eluant was evaporated under nitrogen gas using a Turbovap LV evaporator (Zymark, Hopkinton, MA, USA). The dried residue was resuspended in methanol and analyzed by liquid chromatography/mass spectrometry (LC/MS). Production of open A-ring PbTx-2 and PbTx-3 through the hydrolysis process and their recovery after SPE clean-up and evaporator drying was evaluated by LC/MS.

2.5. Receptor binding assay

The PbTx receptor binding assay (RBA) is an *in vitro* bioassay in which non-radiolabeled PbTx competes with radiolabeled PbTx ([^{3}H]-PbTx-3; Amersham, NJ, USA), for site 5 on voltage-gated sodium channels contained in a crude rat brain membrane preparation (Poli et al., 1986). The RBA was used to quantify brevetoxin-like activity in *K. brevis* extracts and to characterize the binding activity of PbTx-2 and PbTx-3 open A-ring derivatives (see Section 2.4). The assay was performed in a 96-well plate format with all samples run in triplicate as described by Van Dolah et al. (1994) and Twiner et al. (2007). Assay data generated using a four-parameter logistic curve-fitting equation (MultiCalc; Perkin-Elmer Wallac, Turku, Finland) were expressed as PbTx-3 equivalents mL^{-1}. Processing of data for open A-ring PbTx-2 and PbTx-3, available in quantities insufficient to produce complete competition curves, was conducted with Prism (ver. 4.00; GraphPad Software, San Diego, CA, USA) using sigmoidal regression curve analysis. Estimated IC_{50} values were determined by constraining the bottom curve values equal to that of the respective parent toxin.

2.6. N2A assay

Sodium channel-dependent cytotoxic activity was measured using the N2A neuroblastoma

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cytotoxicity assay developed by Manger et al. (1993) and modified recently by Bottein Dechraoui et al. (2006). Briefly, 1 μL samples (standards, extracts, or open A-ring derivative solutions) were added directly to ouabain/veratridine (0.5/0.05 mM) pre-treated N2A cells plated in 100 μL of medium (RPMI supplemented with 5% FBS and 1 mM sodium pyruvate). After 18–20 h of incubation, cell viability was assessed using the tetrazolium salt MTT. Data were processed with Prism (ver. 4.00; GraphPad Software, San Diego, CA, USA) using sigmoidal regression curve analysis. Processing of data for open A-ring PbTx-2 and PbTx-3, available in quantities insufficient to produce complete competition curves, was conducted with GraphPad Prism using sigmoidal regression curve analysis. Estimated EC_{50} values were determined by constraining the bottom curve values equal to that of the respective parent toxin.

2.7. *Liquid chromatography/mass spectrometry (LC/MS)*

LC/MS was performed on an Agilent HP1100 LC system (Agilent Technologies, Palo Alto, CA, USA) coupled to an Applied Biosystems/MDS Sciex 4000 Q TRAP hybrid triple quadrupole/linear ion trap mass spectrometer equipped with a Turbo V™ source (Applied Biosystems, Foster City, CA, USA). LC separations were performed on a Luna C8(2) column (150 × 2 mm, 5 μm, Phenomenex, Torrance, CA, USA) using a mobile phase of water (A) and acetonitrile (B), with 0.3% acetic acid as an additive. For quantitation of brevetoxin congeners in *K. brevis* culture extracts, the LC was operated under a gradient elution: 2 min at 50% B, linear gradient to 80% at 22 min, 95% B at 23 min, held at 95% B for 6 min, returned to 50% B at 30 min, and held for 7 min before the next injection. For analysis of the purity of open A-ring PbTx-2 and PbTx-3, the LC was operated under isocratic conditions with 64% B for 14 min. The mobile-phase flow rate was 0.2 mL min^{-1} and the column temperature was 40 °C. The 4000 QTRAP was operated in positive ion mode, with the turbo ion spray as the ionization source. Nitrogen was used as the nebulizer gas, turbo heater gas, curtain gas, and collision gas. The ion spray voltage was 5 kV and the declustering potential was 64 V. For determination of brevetoxin congeners in culture extracts, the mass spectrometer was operated in Q1 multiple ion scan mode (also called selected ion monitoring, SIM). Using the SIM method, injection of 0.025 ng of PbTx-3 on the LC column produced a response for the protonated molecule (MH^+: m/z 897) with a signal/noise ratio of >10 and 0.025 ng of PbTx-2 on the LC column produced a response for the protonated molecule (MH^+: m/z 895) with a signal/noise ratio of >20. Six PbTx-2 and six PbTx-3 calibration standards were used. Due to the unavailability of standards for many of the brevetoxin congeners, all data were expressed in PbTx-3 equivalents mL^{-1}. In the absence of these congener peaks, data are presented as not detected (nd). For assessing the purity of open A-ring PbTx-2 and PbTx-3 solutions after SPE clean-up and evaporator drying (Section 2.4), the mass spectrometer was operated in an enhanced MS (EMS) scan using a linear ion trap mode scanning from m/z 500 to 1500 or Q1 multiple ion scan mode, scanning masses of all the known brevetoxin congeners. For examination of the conversion of PbTx-2 or PbTx-3 to its hydrolysis product (open A-ring PbTx-2 or PbTx-3), the mass spectrometer was operated under a multiple reaction monitoring (MRM) mode. The hydrolysis solution was diluted with methanol to a calculated concentration for PbTx-2 or PbTx-3 that was above their limits of quantitation by the MRM method assuming that about 1% of PbTx had not been hydrolyzed and analyzed by LC/MS to determine the amount of un-hydrolyzed PbTx. The MRM method was also used to evaluate the recovery of open A-ring PbTx-2 and PbTx-3 (MRM transition m/z 913→877 for open A-ring PbTx-2 and m/z 915→743 for open A-ring PbTx-3) in the hydrolysis solution after SPE clean-up and evaporator drying. The hydrolysis solution was diluted with methanol in which the molar concentration of NaCl was about 5×10^{-5} M to allow for direct injection to LC/MS for quantitative comparison. The dried residue of open A-ring PbTx-2 or open A-ring PbTx-3 was dissolved in methanol and was diluted to the same extent with its hydrolysis solution for LC/MS analysis. For LC/MS analysis of the production of open A-ring PbTx-2 or open A-ring PbTx-3 through the hydrolysis process, a switching valve before the mass spectrometer ion source was used to elute the salt component to waste, thereby reducing the negative effect of salt on the mass spectrometer and maintaining signal stability.

The identities of brevetoxin congeners were confirmed by their product ion spectra in an enhanced product ion scan using a linear ion trap mode. PbTx-2 adduct (MH^+: 927) is a derivative of

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PbTx-2, which was confirmed by MS^3: the product ion spectrum of fragment m/z 895 from the precursor PbTx-927 at m/z 927 matched that of PbTx-2 (MH^+: 895) obtained through MS^3 with the linear ion trap function. The PbTx adduct is likely a methanol brevetoxin derivative produced during sample preparation, associated with the use of methanol and heat for toxin extraction. This derivative was not observed when acetonitrile solvent was used.

3. Results

3.1. Algal and bacterial growth

Bacteria-free *K. brevis* (C2) cultures in the late exponential phase inoculated with sterile seawater exhibited a growth rate (μ; mean ± SE, $n = 3$) of 0.35 ± 0.04 div day^{-1} between Days 0 and 5. Cell concentrations remained relatively constant from Days 5 through 15 (i.e., stationary phase) at an average density of 29,900 cells mL^{-1} (Fig. 2A). *K. brevis* inoculated with the non-algicidal bacterium *A. latercula* had a growth rate of 0.28 ± 0.06 div day^{-1} between Days 0 and 5, after which an average density of 21,100 cells mL^{-1} was maintained through Day 15 (Fig. 2A).

K. brevis cultures treated with the algicidal bacteria strains S03 or 41-DBG2 on Day 0 showed no adverse growth effects over 24 h (Fig. 2A). However, between Days 1 and 3, intact *K. brevis* cell counts in both treatments decreased markedly from ~14,000 to 60 cells mL^{-1}. Between Days 5 and 15, while the controls remained above 20,000 cells mL^{-1}, triplicate cell counts were often below the limit of detection (10 cells mL^{-1}) and were recorded as 0 cells mL^{-1}. Statistical analysis of algal cell counts over the time course illustrated that the growth of *K. brevis* following addition of each algicidal bacterium was different than for both of the controls ($p < 0.0001$). The *a posteriori* Tukey's HSD test revealed that the cultures treated with the non-algicidal bacterium differed significantly from those treated with the two algicidal bacteria, but not from the sterile seawater control ($p < 0.01$).

Cultures receiving the non-algicidal bacterium *S. latercula* exhibited exponential bacterial growth between Days 0 and 2 ($\mu_{max} = 4.1$ div day^{-1}) and averaged ca. 7.4×10^5 bacterial cells mL^{-1} through Day 15 (Fig. 2B). Inoculation of strains S03 or 41-DBG2 into cultures of *K. brevis* was followed by exponential bacterial growth between Days 0 and 2

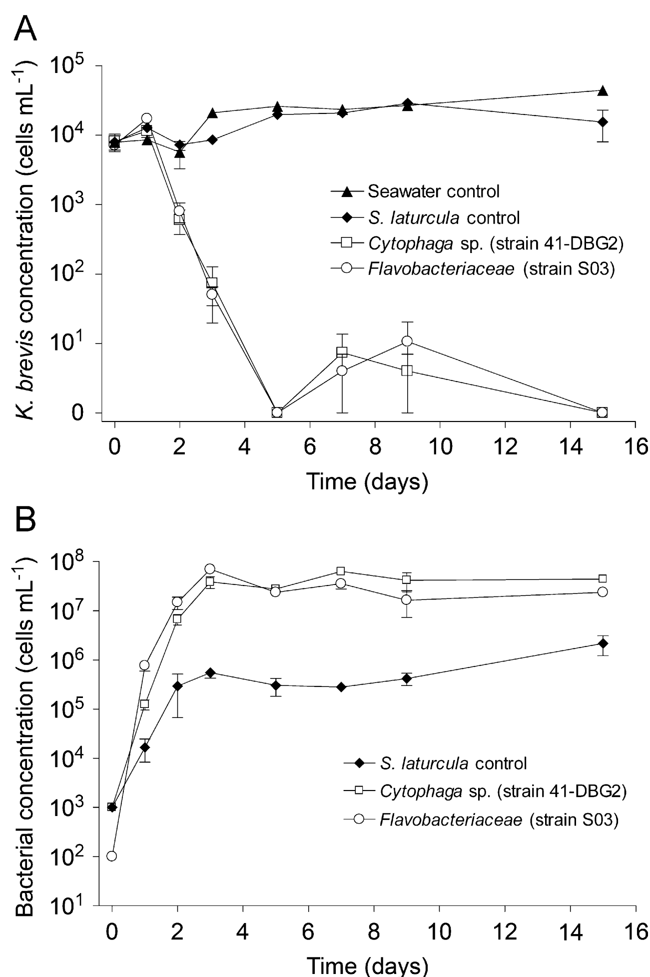

Fig. 2. Growth of *K. brevis* and bacteria in controls and algicidal bacteria treatments. Algal (A) and bacterial (B) cell counts in bacteria-free cultures of *Karenia brevis* (C2) inoculated with sterile seawater, the non-algicidal bacterium *Aquimarina latercula* (10^3 cells mL^{-1}), algicidal strain S03 (10^2 cells mL^{-1}), or algicidal strain 41-DBG2 (10^3 cells mL^{-1}). Values are mean ± SE ($n = 3$). Bacterial counts on Day 0 are based on the calculated inoculum concentrations.

with μ_{max} values of 8.6 and 6.4 div day^{-1}, respectively (Fig. 2B). Throughout the remainder of the time course, concentrations of both algicidal bacteria were relatively constant between 3.4 and 4.3×10^7 bacterial cells mL^{-1}, exceeding levels attained by *S. latercula* by about 50-fold. No detectable bacteria were observed in the seawater controls at any time point (data not shown).

3.2. Brevetoxin concentrations and profiles in the >5, 0.22–5, and <0.22 μm size fractions

Extracts from the >5, 0.22–5, and <0.22 μm size fractions were analyzed for brevetoxin concentrations on Days 0, 1, 2, 3, 5, 7, 9, and 15 by brevetoxin

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RBA (mean ± SE, $n = 3$ culture replicates with triplicate determinations per assay) for both controls (seawater and non-algicidal *S. latercula* addition) and both algicidal bacteria treatments (Fig. 3). Total brevetoxin levels in all of these treatments over the same time course are presented in Fig. 3A for comparison. Selected samples from this time series (Days 0, 2, and 9) were analyzed further for brevetoxin concentrations and identification of individual brevetoxin congeners using LC/MS (mean, $n = 2$) (Tables 1–4).

3.2.1. Brevetoxins in the >5 μm size fraction

The >5 μm size fraction represents toxins associated with intact *K. brevis* cells or large aggregates of cell debris following cell lysis. In the sterile seawater and non-algicidal bacterium-addition controls, overall increases in PbTx activity levels corresponded initially to changes in *K. brevis* cell concentration, yet continued to increase for about 4 days after cessation of cell division to maximum values of about 170 and 120 PbTx-3 equivalents mL^{-1}, respectively (Fig. 3B). Brevetoxin cell quotas ranged from 4 to 12 pg $cell^{-1}$ for both control cultures. LC/MS analysis of toxin in these cultures on Days 0, 2, and 9 displayed a similar trend, but tended to yield higher values relative to the RBA (cf. Fig. 3B and Table 1). On Day 9, the major brevetoxin congener profile for the *S. latercula*-addition control comprised PbTx-2 (51%), PbTx-3 (6%), PbTx-1 (11%), PbTx-7 (7%), and PbTx-2 adduct (23%) (Table 1). Open A-ring brevetoxin derivatives (hydrolyzed forms of PbTx) constituted <1% of the >5 μm size fraction.

K. brevis cultures inoculated with either of the algicidal bacteria exhibited a dramatic decrease in brevetoxin RBA activity for the >5 μm size fraction with the onset of bacterially induced lysis between Days 1 (~100 ng PbTx-3 equivalents mL^{-1}) and 2 (<15 ng PbTx-3 equivalents mL^{-1}) (Fig. 3B). LC/MS analyses on Days 0, 2, and 9 yielded brevetoxin concentrations similar to the RBA data (cf. Fig. 3B and Tables 3 and 4). The major brevetoxin congener profile of the two algicidal treatments on Day 9 consisted of PbTx-2 (42%), PbTx-3 (21%), PbTx-1 (5%), PbTx-7 (6%), and PbTx-2 adduct (1%). Open A-ring brevetoxin derivatives represented 25% of the >5 μm size fraction, which was mostly hydrolyzed PbTx-2 and PbTx-7.

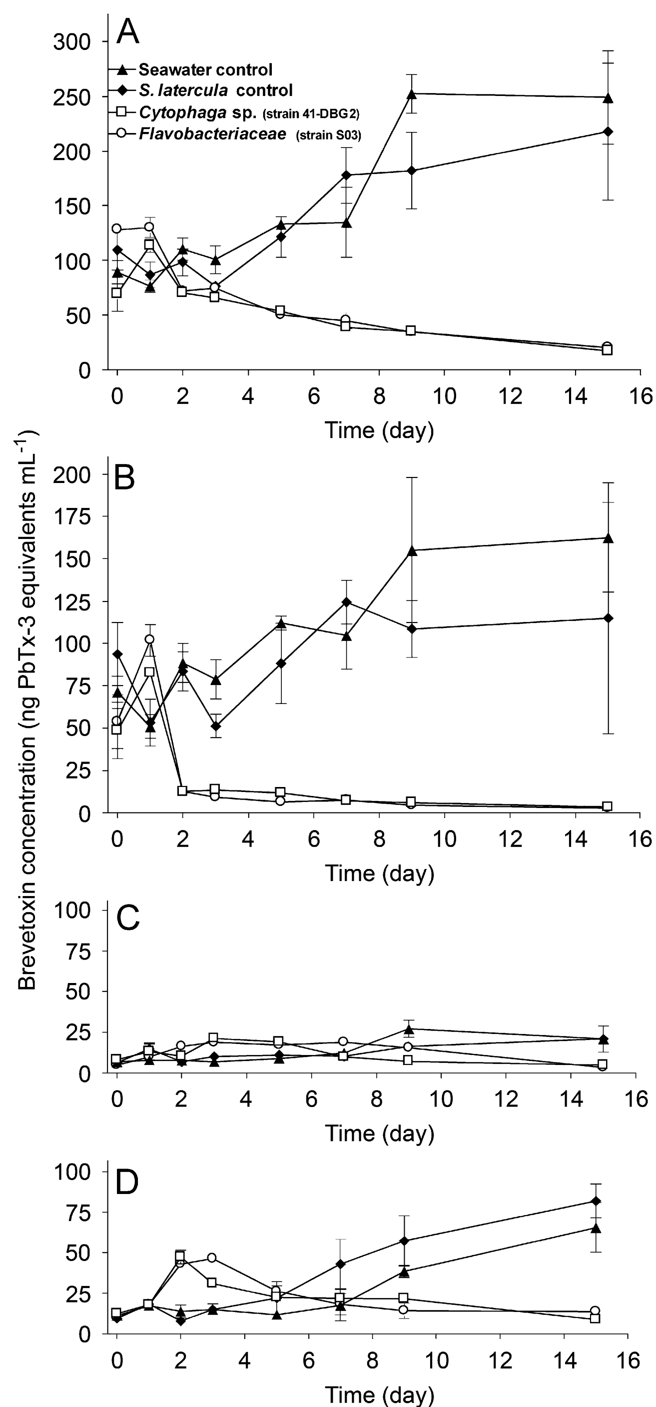

Fig. 3. Effect of algicidal bacteria on brevetoxin size partitioning in bacteria-free cultures of *Karenia* brevis (strain C2). On Day 0, cultures were inoculated with the non-algicidal bacterium *Aquimarina latercula* (10^3 cells mL^{-1}), with algicidal strain S03 (10^2 cells mL^{-1}), or with algicidal strain 41-DBG2 (10^3 cells mL^{-1}). Samples for brevetoxin extraction were collected on selected days from three size fractions and illustrated as (A) total brevetoxin, (B) >5 μm, (C) 0.22–5 μm, and (D) <0.22 μm. Brevetoxin analysis was performed by receptor binding assay (RBA). Values are mean ± SE ($n = 3$). All brevetoxin data are expressed as ng PbTx-3 equivalents mL^{-1} culture. Corresponding LC/MS data for Days 0, 2, and 9 are given in Tables 1–3. Due to a filtering error in the S03 treatment on Day 0, data from one of the replicate cultures were removed from analysis.

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Table 1
Brevetoxin congener profiles in co-cultures of *K. brevis* with non-algicidal bacterium *Aquimarina latercula*

Brevetoxin congener	>5 μm			0.22–5 μm			<0.22 μm			Total		
	Day 0	Day 2	Day 9	Day 0	Day 2	Day 9	Day 0	Day 2	Day 9	Day 0	Day 2	Day 9
PbTx-1	11.98	3.74	22.26	1.16	0.78	2.95	nd	0.06	3.56	13.15	4.57	28.78
PbTx-7	26.43	17.83	13.88	0.60	0.44	1.67	0.19	0.25	3.08	27.21	18.52	18.63
PbTx-2	45.79	18.96	102.46	6.33	6.12	16.13	3.77	4.75	94.03	55.88	29.82	212.62
PbTx-3	37.35	25.35	12.16	2.20	2.38	2.52	5.71	6.11	23.93	45.27	33.84	38.61
PbTx-9	0.28	0.31	nd	nd	nd	nd	nd	nd	0.05	0.28	0.31	0.05
Oxidized PbTx-2	0.06	0.33	1.88	nd	nd	nd	nd	nd	0.24	0.06	0.33	2.12
PbTx-2 adduct	8.31	7.89	46.66	nd	0.11	1.65	nd	0.65	11.73	8.31	8.65	60.04
Open A-ring PbTx-1	nd	nd	nd	0.04	0.11	nd	0.60	0.87	6.05	0.64	0.98	6.05
Open A-ring PbTx-7	0.48	0.36	0.51	0.49	0.77	0.45	9.94	10.71	33.61	10.91	11.83	34.57
Open A-ring oxidized PbTx-1	nd	nd	nd	nd	nd	nd	nd	nd	0.25	nd	nd	0.25
Open A-ring PbTx-2	nd	nd	nd	0.20	0.30	nd	3.87	4.61	28.99	4.07	4.91	28.99
Open A-ring PbTx-3	0.20	0.28	0.16	0.31	0.54	0.20	20.81	21.34	55.48	21.33	22.15	55.84
Open A-ring oxidized PbTx-2	nd	nd	nd	nd	nd	nd	nd	nd	0.25	nd	nd	0.25
Total	130.89	75.04	199.98	11.34	11.54	25.57	44.89	49.34	261.24	187.12	135.92	486.78
% Open A-ring derivatives	0.5	0.8	0.3	9.3	14.9	2.5	78.5	76.1	47.7	19.7	29.3	25.9

Congener concentrations (ng PbTx-3 equivalents mL^{-1} culture) are given for Days 0, 2, and 9 in the >5, 0.22–5, <0.22 μm and total size fractions. Data are means of two replicate cultures. The detection limit for PbTx-2 and PbTx-3 was ~0.03 ng mL^{-1} culture. Samples denoted with 'nd' represent not detected.

Table 2
Brevetoxin congener profiles in cultures of *Karenia brevis*

Brevetoxin congener	<0.22 μm		
	Day 0	Day 2	Day 9
PbTx-1	0.37	0.17	1.24
PbTx-7	0.36	0.43	1.17
PbTx-2	6.77	9.36	37.15
PbTx-3	6.77	8.63	14.06
PbTx-9	nd	nd	nd
Oxidized PbTx-2	nd	nd	nd
PbTx-2 adduct	1.05	1.45	4.95
Open A-ring PbTx-1	0.89	0.87	3.28
Open A-ring PbTx-7	7.84	11.51	30.40
Open A-ring oxidized PbTx-1	nd	nd	nd
Open A-ring PbTx-2	4.70	4.49	14.00
Open A-ring PbTx-3	14.53	20.96	50.11
Open A-ring oxidized PbTx-2	nd	nd	nd
Total	43.29	57.88	156.36
% Open A-ring derivatives	64.6	65.4	62.5

Congener concentrations (ng PbTx-3 equivalents mL^{-1} culture) are given for Days 0, 2, and 9 in the <0.22 μm size fraction. Data are means of two replicate cultures. The detection limit for PbTx-2 and PbTx-3 was ~0.03 ng mL^{-1} culture. Samples denoted with 'nd' represent not detected. *Note*: Data for the >5 and 0.22–5 μm fractions for the seawater controls are not available.

3.2.2. Brevetoxins in the 0.22–5 μm size fraction

The 0.22–5 μm size fraction putatively represents toxins associated with bacteria and/or debris from lysed *K. brevis* cells. Brevetoxin RBA activity in this fraction from both control cultures were ~5–15 times lower than those for the >5 μm size fraction (Fig. 3C and Table 1) and showed a slight, gradual increase from ~6 to 20 ng PbTx-3 equivalents mL^{-1} over the experiment. LC/MS values for total brevetoxin concentration on Days 0, 2, and 9 corresponded closely to results of the RBA (cf. Fig. 3C and Table 1). The major brevetoxin congeners present in the *S. latercula* control on Day 9 included PbTx-2 (63%), PbTx-3 (10%), PbTx-1 (12%), PbTx-7 (7%), and PbTx-2 adduct (7%) (Table 1). Only 3% of the 0.22–5 μm size fraction was made up of open A-ring brevetoxin derivatives.

Brevetoxin RBA activity in *K. brevis* cultures receiving either algicidal bacterium, as in the controls, changed little over the time course and reached maximum levels of about 20 ng mL^{-1} on Day 3, followed by a gradual decline to about half this amount by the end of the experiment (Fig. 3C). LC/MS analyses of material collected on Days 0, 2, and 9 showed close agreement with the RBA measurements of brevetoxin concentration. On Day 9, the average percentages of brevetoxin congeners in the two algicidal treatments were PbTx-2 (47%), PbTx-3 (25%), PbTx-1 (4%), PbTx-7 (8%), and PbTx-2 adduct (3%) (Tables 3 and 4). In this 0.22–5 μm fraction, open A-ring brevetoxin derivatives made up 12% of the total

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Table 3
Brevetoxin congener profiles in co-cultures of *Karenia brevis* with the algicidal bacterium *Flavobacteriaceae*, strain S03

Brevetoxin congener	>5 μm			0.22–5 μm			<0.22 μm			Total		
	Day 0	Day 2	Day 9	Day 0*	Day 2	Day 9	Day 0	Day 2	Day 9	Day 0	Day 2	Day 9
PbTx-1	6.55	0.90	0.38	0.38	2.30	0.66	0.41	0.65	0.09	7.34	3.84	1.14
PbTx-7	11.76	0.90	0.53	0.28	2.76	1.15	0.41	1.82	0.55	12.45	5.47	2.23
PbTx-2	25.04	6.36	3.18	4.25	10.08	8.49	11.32	23.22	13.55	40.61	39.66	25.22
PbTx-3	18.63	1.45	1.48	1.85	3.61	3.67	9.35	17.33	13.29	29.83	22.39	18.43
PbTx-9	0.10	nd	nd	nd	nd	nd	nd	nd	nd	0.10	nd	nd
Oxidized PbTx-2	nd	nd	nd	nd	nd	nd	nd	0.09	0.11	nd	0.09	0.11
PbTx-2 adduct	3.57	0.06	0.05	0.45	0.98	0.12	1.60	2.00	0.81	5.62	3.03	0.98
Open A-ring PbTx-1	nd	0.09	0.19	0.12	0.08	0.20	1.12	2.37	8.85	1.24	2.54	9.24
Open A-ring PbTx-7	0.38	0.34	0.62	0.48	0.33	0.80	11.11	11.34	23.24	11.97	12.01	24.66
Open A-ring oxidized PbTx-1	nd	nd	nd	nd	nd	nd	nd	nd	0.09	nd	nd	0.09
Open A-ring PbTx-2	nd	0.13	0.67	0.19	0.06	0.61	5.83	11.81	52.22	6.02	11.99	53.50
Open A-ring PbTx-3	0.15	0.20	0.35	0.38	0.17	0.27	23.55	22.91	37.44	24.09	23.28	38.06
Open A-ring oxidized PbTx-2	nd	nd	nd	nd	nd	nd	nd	nd	0.15	nd	nd	0.15
total	66.19	10.42	7.44	8.38	20.36	15.97	64.71	93.54	150.39	139.28	124.31	173.79
% Open A-ring derivatives	0.8	7.2	24.6	14.0	3.1	11.8	64.3	51.8	81.1	31.1	40.1	72.3

Congener concentrations (ng PbTx-3 equivalents mL^{-1} culture) are given for Days 0, 2, and 9 in the >5, 0.22–5, <0.22 μm and total size fractions. Data are means of two replicate cultures. The detection limit for PbTx-2 and PbTx-3 was ~0.03 ng mL^{-1} culture. Samples denoted with 'nd' represent not detected. Due to a filtering error as indicated by the asterisk (*), only one of the replicate cultures was used for analysis.

Table 4
Brevetoxin congener profiles in co-cultures of *Karenia brevis* with the algicidal bacterium *Cytophaga* sp., strain 41-DBG2

Brevetoxin congener	>5 μm			0.22–5 μm			<0.22 μm			Total		
	Day 0	Day 2	Day 9	Day 0	Day 2	Day 9	Day 0	Day 2	Day 9	Day 0	Day 2	Day 9
PbTx-1	4.97	1.08	0.16	1.66	1.67	0.53	0.44	0.56	0.29	7.07	3.31	0.99
PbTx-7	6.80	0.94	0.21	0.85	1.77	1.09	0.28	1.23	0.41	7.93	3.93	1.72
PbTx-2	21.63	5.28	1.86	8.64	7.32	4.47	4.41	21.07	11.36	34.68	33.66	17.69
PbTx-3	15.14	1.69	1.01	3.18	2.98	3.24	5.47	20.99	11.89	23.78	25.66	16.15
PbTx-9	0.13	nd	nd	nd	nd	nd	nd	nd	nd	0.13	nd	nd
Oxidized PbTx-2	0.03	0.09	0.05	nd	0.05	0.24	0.00	0.59	8.79	0.03	0.72	9.08
PbTx-2 adduct	2.68	0.48	0.04	0.13	0.87	0.74	0.78	2.63	1.62	3.59	3.98	2.40
Open A-ring PbTx-1	nd	0.14	0.15	0.10	0.04	0.13	0.61	2.76	9.96	0.71	2.95	10.24
Open A-ring PbTx-7	0.25	0.24	0.32	0.54	0.20	0.80	6.60	15.52	27.35	7.39	15.96	28.47
Open A-ring oxidized PbTx-1	nd	nd	nd	nd	nd	nd	nd	nd	2.83	nd	nd	2.83
Open A-ring PbTx-2	nd	0.14	0.42	0.17	0.05	0.24	4.07	12.44	61.35	4.23	12.63	62.00
Open A-ring PbTx-3	0.28	0.27	0.27	0.23	0.14	0.33	13.40	29.34	48.89	13.90	29.75	49.48
Open A-ring oxidized PbTx-2	nd	nd	nd	nd	nd	nd	nd	nd	2.48	nd	nd	2.48
Total	51.90	10.34	4.49	15.48	15.08	11.82	36.05	107.13	187.23	103.44	132.55	203.53
% Open A-ring derivatives	1.0	7.6	25.7	6.6	2.8	12.7	68.4	56.1	81.6	25.4	46.2	76.4

Congener concentrations (ng PbTx-3 equivalents mL^{-1} culture) are given for Days 0, 2, and 9 in the >5, 0.22–5, <0.22 μm and total size fractions. Data are means of two replicate cultures. The detection limit for PbTx-2 and PbTx-3 was ~0.03 ng mL^{-1} culture. Samples denoted with 'nd' represent not detected.

toxin, which occurred mostly as hydrolyzed forms of PbTx-2, PbTx-7, and PbTx-3.

3.2.3. *Brevetoxins in the <0.22 μm size fraction*

The <0.22 μm size fraction, or dissolved component, was operationally defined as that toxin passing through a 0.22 μm filter, although some of this toxin in the growth medium may be adsorbed to organic material. Dissolved brevetoxin RBA activity in both control cultures remained essentially constant over the first 3–5 days and then increased to a maximum of ca. 75 ng PbTx-3 equivalents mL^{-1} on Day 15

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(Fig. 3D), although levels were consistently lower in the sterile seawater control. While the trend of increasing PbTx concentrations was similar for LC/MS and RBA measurements on Days 0, 2, and 9, actual LC/MS values overestimated the RBA by up to 5-fold (cf. Fig. 3D and Tables 1 and 2). The major brevetoxin congeners present in the *S. latercula* controls on Day 9 were PbTx-2 (36%), PbTx-3 (9%), PbTx-1 (1%), PbTx-7 (1%), and PbTx-2 adduct (4%), and open A-ring brevetoxin derivatives accounted for 48% of the total dissolved toxin (Table 1). The major brevetoxin congeners present in the sterile seawater controls on Day 9 were PbTx-2 (24%), PbTx-3 (9%), PbTx-1 (0.8%), PbTx-7 (0.7%), and PbTx-2 adduct (3%), and open A-ring brevetoxin derivatives accounted for 63% of the total dissolved toxin (Table 2).

K. brevis cultures inoculated with either of the algicidal bacteria showed a clear pulse of dissolved brevetoxin RBA activity (ca. 45 ng PbTx-3 equivalents mL^{-1}) being released into the medium on Day 2, which declined to about 25 ng PbTx-3 equivalents mL^{-1} by Day 5 and changed little over the remaining time course (Fig. 3D). In marked contrast to the RBA data, LC/MS analyses on Days 0, 2, and 9 did not show a decline in dissolved brevetoxin concentrations following its initial release with algal cell lysis and overestimated RBA values by ~2–9-fold. On Day 9, the parent forms of type A brevetoxins (PbTx-1 and PbTx-7) each comprised $<0.5\%$ of the average brevetoxin profile for the two algicidal treatments and the remaining primary congeners (i.e., PbTx-2, PbTx-3, and PbTx-2 adduct) were all $<10\%$ (Tables 3 and 4). In both algicidal bacteria treatments the absolute amount of open A-ring PbTx-2 increased 5-fold between Days 2 and 9 following algal cell lysis and accounted for the majority of the dissolved fraction by the end of the experiment (Tables 3 and 4). Interestingly, the percentages of oxidized PbTx-2, open A-ring oxidized PbTx-2, and open A-ring oxidized PbTx-1 in the dissolved fraction of 41-DBG2 treatments exceeded those in the S03 additions by at least an order of magnitude (Tables 3 and 4). Open A-ring brevetoxin derivatives represented 51–82% of the $<0.22\,\mu m$ size fraction.

3.3. Binding activity and cytotoxicity of open A-ring brevetoxin derivatives

LC/MS was used to examine production of open A-ring PbTx-2 and PbTx-3 through the hydrolysis process. No detectable amounts of un-hydrolyzed PbTx-2 or PbTx-3 were observed in their respective hydrolysis solutions. After SPE clean-up and evaporator drying, the recovery was estimated to be 87.2% for open A-ring PbTx-2 and 98.3% for open A-ring PbTx-3. The methanolic solution of open A-ring PbTx-2 or PbTx-3 was $\geqslant 99\%$ in purity based on examination of their LC/MS chromatograms and MS spectra using MS scans as described in Section 2.7.

Open A-ring PbTx-2 and PbTx-3 were tested for binding activity and cytotoxicity using the RBA and N2A assay, respectively. In the former, concentration-dependent competition against titrated PbTx-3 was observed for both open A-ring derivatives. Due to a shortage of material, a complete competition curve was not possible. However, sigmoidal regression curve analysis was used to generate an extrapolated curve based on the available data points and a fixed lower curve value estimated based on their respective parental brevetoxins. The half-maximal inhibitory concentrations (IC_{50}) estimated for PbTx-3 and its open A-ring derivative were 11.7 and 668 ng mL^{-1}, respectively, representing an approximately 57-fold decrease in binding affinity for the open A-ring PbTx-3 derivative (Fig. 4A). Similarly, the IC_{50} estimated for PbTx-2 and its open A-ring derivative were 9.7 and 208 ng mL^{-1}, respectively, although the reduction in binding affinity for the hydrolyzed derivative was somewhat less (~22-fold).

Brevetoxin-3-induced N2A cytotoxicity resulted in a half-maximal effective concentration (EC_{50}) of 2.4 ng mL^{-1} compared to a value of 342 ng mL^{-1} for the open A-ring derivative of PbTx-3 (Fig. 4B), a 142-fold decline in potency. By comparison, the estimated potency of open A-ring PbTx-2 ($EC_{50} =$ 445 ng mL^{-1}) was 86-fold less than its parent PbTx-2 ($EC_{50} = 5.2$ ng mL^{-1}) (Fig. 4B).

4. Discussion

In this study, we examined how the size-fractioned quantity and proportion of brevetoxin congeners in *K. brevis* cultures changed in response to attack by algicidal bacteria, and characterized the binding and cytotoxic activity of two open A-ring brevetoxin derivatives that predominated in the dissolved toxin fraction. To our knowledge, this is the first report to describe phycotoxin fate and distribution following lysis of a HAB species by algicidal bacteria. The onset of bacterially mediated

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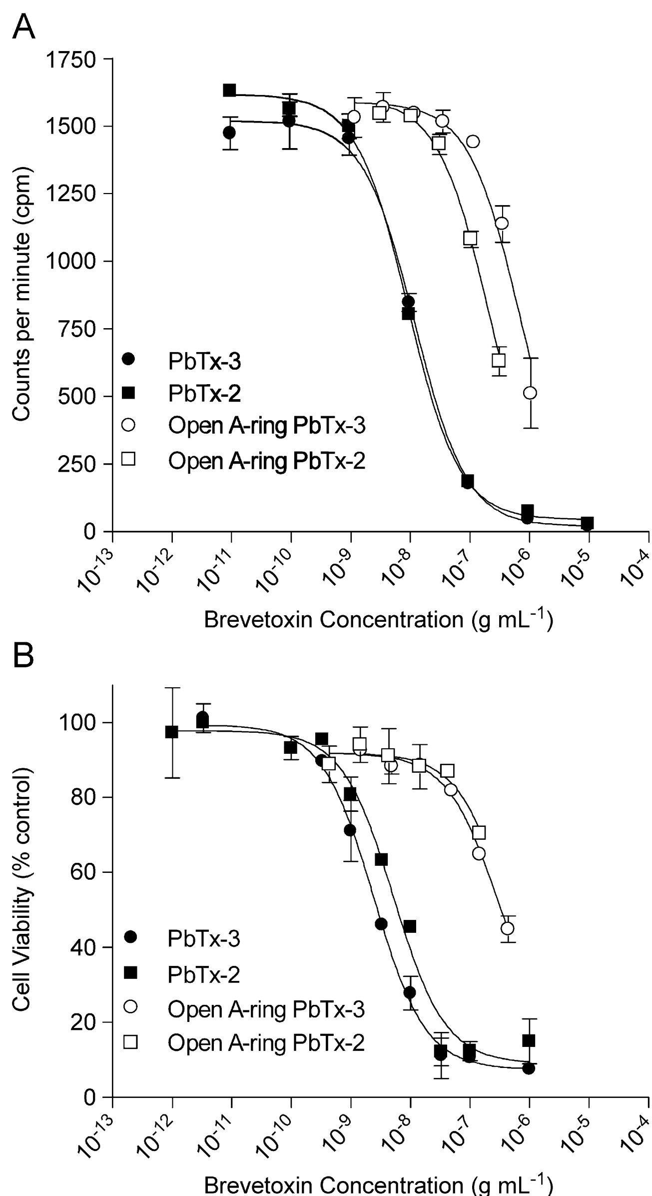

Fig. 4. Activity and potency characterization of open A-ring brevetoxin derivatives. Parent PbTx-2 and PbTx-3 (closed symbols) and hydrolyzed PbTx-2 and PbTx-3 derivatives (open symbols) were tested for (A) sodium channel binding activity on the receptor binding assay (RBA) and (B) cytotoxic potency on the N2A assay. Values are mean ± SE where $n = 3$ for RBA data and $n = 2$ for N2A data. The RBA IC_{50} values for PbTx-3, PbTx-2, open A-ring PbTx-3, and open A-ring PbTx-2 were 11.7, 9.7, 668, and 208 ng mL^{-1}, respectively. The N2A cytotoxicity EC_{50} values for PbTx-3, PbTx-2, open A-ring PbTx-3, and open A-ring PbTx-2 were 2.4, 5.2, 342, and 445 ng mL^{-1}, respectively.

disruption of *K. brevis* cells led to a rapid reduction in the total mass of the more 'active' brevetoxin congeners. Toxins released into the dissolved extracellular pool upon cell disruption were hydrolyzed from highly active/potent parent molecules into less active/potent derivatives (i.e., open A-ring hydrolysis products). These findings indicate that attack by algicidal bacteria reduces not only the growth of *K. brevis* but also its overall toxicity, although evidence supporting the importance of these processes in natural bloom populations is presently lacking. Further examination of algicidal bacteria and their effects on growth and toxicity of *K. brevis* blooms is clearly warranted, given the potential implications for managing and mitigating bloom impacts.

Both algicidal bacteria tested herein, one releasing a dissolved algicidal agent (strain 41-DBG2) and the other requiring direct contact with algal target cells (strain S03), exhibited strikingly similar activity against *K. brevis*. These bacteria began to suppress algal growth within 2 days of exposure, coincident with the initiation of cell lysis as reported previously (Roth, 2005; Roth et al., submitted). Interestingly, and similar to earlier studies, achieving algicidal bacteria concentrations of about 10^6 cells mL^{-1} was a prerequisite for algal cell lysis (Roth, 2005; Mayali and Doucette, 2002; Skerratt et al., 2002); yet, the significance of this apparent threshold (e.g., quorum sensing, effective cell concentration, etc.) remains uncertain. In contrast, the non-algicidal bacterium, *S. latercula*, closely related to strain 41-DBG2 and selected to control for effects associated with bacterial introduction alone (e.g., protein shock, nutrient cycling), showed little effect on *K. brevis* growth and brevetoxin profiles compared to seawater-addition controls receiving no bacteria. However, toxin levels in the $>5\,\mu$m size fraction of the *S. latercula*-exposed cultures were lower than the seawater controls after Day 7, possibly caused by an enhanced 'leakiness' of stationary-phase algal cells in the presence of bacteria and reflected in the higher quantities of dissolved toxin in the former.

Maximum growth rates attained by both algicidal bacteria upon exposure to *K. brevis* cells (S03 $\mu_{max} = 8.6$ day^{-1}; 41-DBG2 $\mu_{max} = 6.4$ day^{-1}) were 1.5–2-fold that of the non-algicidal bacterium, *S. latercula* ($\mu_{max} = 4.1$ day^{-1}). Moreover, maximum cell concentrations achieved by the algicidal strains (S03, 3.4×10^7 cells mL^{-1}; 41-DBG2, 4.3×10^7 cells mL^{-1}) were almost two orders of magnitude greater than for *S. latercula* (7.4×10^5 cells mL^{-1}). Such responses are consistent with the ability of algicidal bacteria to react quickly to the presence of target algal cells and to utilize effectively the organic material released upon lysis. The diversity and

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biomass of natural bacterial populations are often influenced by the development and stage of algal blooms (Fukami et al., 1991; Imai et al., 1998), also suggesting a response to organic nutrients associated with phytoplankton. We suspect that in the specific case of algicidal bacteria targeting either harmful or benign algae, these microbes can make efficient use of complex algal polymers and thereby gain a competitive advantage over other members of the bacterial community. Indeed, the algicidal strain 41-DBG2 used herein can reach maximal growth rates when supplied solely with Tween 20 (polyoxyethylene sorbitan monolaurate), a complex, high molecular weight polysorbate compound (unpublished observation). Overall, the rapid reproductive rates and high cell yields of algicidal bacteria observed herein and by others (e.g., Imai et al., 1993; Yoshinaga et al., 1995) would seem to represent an effective growth strategy in nature, although actual field measurements of the growth and abundance of these bacteria are still needed.

Total brevetoxin concentrations corresponded positively with increases in *K. brevis* cell number in all control cultures. A similar trend has been well established for other phycotoxins such as saxitoxins in *Alexandrium fundyense* (Anderson et al., 1990) and domoic acid (DA) in *Pseudo-nitzschia* spp. (Pan et al., 1996). Brevetoxin was partitioned predominantly and expectedly into the $>5\,\mu m$ size fraction throughout the control experiments, given that the majority of *K. brevis* cells remained intact. Also, it appeared that *K. brevis* cells in stationary phase continued to produce brevetoxin (i.e., Fig. 3A), although this coincided with an increase in dissolved ($<0.22\,\mu m$) brevetoxin levels comprised largely (48–78%) of open A-ring brevetoxin derivatives. Hydrolysis of dissolved brevetoxins in the seawater controls suggests that the conversion was related more to the alkalinity of the seawater (pH ~8.2) than to any bacterially mediated effect. The production of open A-ring brevetoxin derivatives under alkaline conditions (see Section 2.4) supports this idea. Studies by Abraham et al. (2006) also corroborate this finding, where the ratio of open A-ring to closed A-ring brevetoxin molecules was highest in the cell-free filtrates. Interestingly, DA is also released from *Pseudo-nitzschia* cells during the later phases of culture growth (Bates et al., 1995). It remains unclear in either case (i.e., PbTx or DA) whether this is a passive process related to the 'leaky' nature of aging cultures or an active excretion in response to some external stimulus, although release of DA by *Pseudo-nitzschia* spp. to promote iron uptake and/or reduce copper toxicity has been proposed (e.g., Wells et al., 2005).

Size-fractioned toxin analyses demonstrated that previously intracellular parent brevetoxins were released into the culture medium upon lysis of *K. brevis* cells by algicidal bacteria. Immunolocalization studies of another lipophilic marine biotoxin, okadaic acid, have revealed that this toxin and its derivatives occur in the cytoplasm (Rausch de Traubenberg et al., 1995) and the chloroplasts of the dinoflagellate *Prorocentrum lima* (Zhou and Fritz, 1994). If brevetoxins are distributed similarly in *K. brevis* cells, disruption of the outer cell membrane would be expected to result in rapid entry into the dissolved toxin pool as was observed. Over time, parent brevetoxin molecules in the intermediate (0.22–5 μm) and dissolved ($<0.22\,\mu m$) fractions appeared to be hydrolyzed into open A-ring brevetoxin derivatives and constitute ~80% of the dissolved brevetoxin pool. Type B brevetoxins (i.e., PbTx-3, PbTx-2, PbTx-2 adduct) accounted consistently for a 2–5-fold greater proportion of total toxin than did type A brevetoxins for all size fractions. This relationship is similar to other *in vitro* experiments and *in situ* observations where type A brevetoxins (i.e., PbTx-1, PbTx-7) have typically been present at low relative concentrations (Wang et al., 2004; Plakas et al., 2004; Abraham et al., 2006) and likely reflects a biosynthetic bias toward the former toxin group.

A dramatic difference between the brevetoxin levels detected via the activity-based RBA versus the analytical LC/MS method was noted in this study, particularly for samples representing the dissolved size fraction. Although the type B brevetoxins dominated the dissolved toxin profile, the majority of these congeners occurred as open A-ring brevetoxin derivatives of their parent toxins. As the major parent, type B brevetoxins (i.e., PbTx-2 and -3) have similar receptor binding affinities (Rein et al., 1994), this discrepancy was likely due to a combination of reduced binding activity (PbTx RBA) of the open A-ring brevetoxins that would, by comparison, not have caused a reduction in total brevetoxin detected by LC/MS, and the expression of the LC/MS data in PbTx-3 equivalents. Without available standards for the various brevetoxin congeners, it is impossible to accurately determine individual congener concentrations. This inherent difficulty may also have contributed to the apparent production of

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brevetoxin in algicidal treatments after many of the *K. brevis* cells were lysed (see Tables 3 and 4 between Days 2 and 9). Although the few remaining *K. brevis* cells can account for some of the increase in total brevetoxin, much of the apparent production according to the LC/MS data may result from the high predominance of the open A-ring brevetoxin congeners and their expression as PbTx-3 equivalents.

Binding activity and cytotoxicity studies of open A-ring PbTx-2 and PbTx-3 derivatives suggested that the open A-ring brevetoxins have at least 25-fold less affinity than the parent toxins and 86-fold less cytotoxic activity than their respective parent toxins. However, it is worth noting that the open A-ring brevetoxin solutions obtained through hydrolysis of their parent toxins contained ca. 1% parent toxin contamination. Therefore, the contribution of the parent PbTx-2 and PbTx-3 to the observed binding and cytotoxicity experiments are not negligible, suggesting that fully purified open A-ring brevetoxin derivatives would be less toxic than determined. Structure–activity-based studies would support this finding, where it has been shown that an open A-ring PbTx-3 compound binds with ~92-fold less affinity than a parental type B PbTx (Michelliza et al., 2004). Similarly, 2,3-dihydro PbTx-3 A-ring diol, another open A-ring brevetoxin, has been shown to have an IC_{50} binding affinity value and a LC_{50} ichthyotoxicity value of >25 and $>2\,\mu M$ (Rein et al., 1994; Jeglitsch et al., 1998; Gawley et al., 1995), representing reductions in binding and toxicity of 6250- and 127-fold relative to the parent PbTx-3, respectively. Similarly, the A-ring lactone has been shown to be required for binding and activity (Baden et al., 1994).

The results of this study provide important insights into the size-fractioned distribution of brevetoxin produced by *K. brevis* under control conditions and following cell lysis by algicidal bacteria. During normal growth in batch culture, dissolved brevetoxin levels increased several fold during the late exponential phase and into the stationary phase while total toxin concentration also continued to rise. These findings are consistent with the laboratory and field investigations of Pierce et al. (2001), who reported that the concentration of extracellular brevetoxins increased relative to intracellular levels with the progression of *K. brevis* cultures or blooms. Nonetheless, the pulse of dissolved ($<0.22\,\mu m$) brevetoxin released into the medium following attack by algicidal bacteria observed herein was somewhat unexpected. Given their lipophilic nature, it was thought initially that adsorption of dissolved toxin onto particles such as membrane fragments and organic debris in the intermediate 0.22–5 μm size fraction would predominate, yet the toxin appeared to not only remain in the <0.22 size fraction but also become hydrolyzed to minimally toxic derivatives.

Certainly, the fate and distribution of the toxins produced and released by an algal species must be considered in the context of evaluating control strategies for the management and mitigation of HABs and their effects. Since toxin content and partitioning between intra- and extracellular forms vary with physiological status and bloom stage, the effects of algicidal bacteria on the fate and distribution of brevetoxins will vary accordingly over *K. brevis* bloom events and require further investigation to evaluate critically. Indeed, as bloom termination begins through physical, chemical, and/or biological means, our experimental data suggest that most of the intracellular toxins released into the surrounding water would be hydrolyzed rapidly into open A-ring brevetoxin derivatives of lesser activity and potency. While such a process is advantageous from ecological and human health perspectives, as well as potential management strategies that may disrupt *K. brevis* cells, careful examination of dissolved brevetoxin hydrolysis is still required to confirm its occurrence in the field and potential role in modulating toxin accumulation by various trophic compartments, including shellfish, fish, and marine mammals.

Acknowledgments

The authors wish to thank Dr. K. Steidinger for generously providing the *Karenia brevis* isolate used in this study. Support for this research was provided in part by the US ECOHAB Program sponsored by NOAA, the US EPA, NSF, NASA, and ONR. This is contribution number 249 from the US ECOHAB Program.

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