Harmful Algae 12 (2011) 119–124

Contents lists available at SciVerse ScienceDirect

Harmful Algae

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

Tracking losses of brevetoxins on exposure to phytoplankton competitors: Ecological impacts

Clare H. Redshaw [a,b,1], Denise A. Sutter [a], Krista Lim-Hing [a], Melanie L. Heckman [a], Stina L. Jakobsson [a], Jerome Naar [c], Julia Kubanek [a,b,*]

[a] School of Biology, Georgia Institute of Technology, 310 Ferst Drive, Atlanta, GA 30332-0230, USA
[b] School of Chemistry and Biochemistry, Georgia Institute of Technology, 901 Atlantic Drive, Atlanta, GA 30332-0400, USA
[c] Center for Marine Science, University of North Carolina at Wilmington, 5600 Marvin K. Moss Lane, Wilmington, NC 28409, USA

ARTICLE INFO

Article history:
Received 14 April 2011
Received in revised form 13 September 2011
Accepted 20 September 2011
Available online 29 September 2011

Keywords:
Aiptasia
Artemia
Biological control
Brevetoxin
Karenia brevis
Toxicology
Skeletonema

ABSTRACT

The frequent occurrence of devastating blooms of the harmful dinoflagellate *Karenia brevis* in the Gulf of Mexico has motivated research into bloom dynamics and potential mitigation strategies. The use of competing phytoplankton to lower waterborne concentrations of the most abundant and toxic brevetoxins produced during these blooms has been proposed. However the ecological impacts of using such biocontrol agents have not been addressed. This study investigated the impact on marine invertebrates of lowered brevetoxin concentrations due to the presence of competing phytoplankton. Even at low brevetoxin concentrations, the presence of the common diatom *Skeletonema grethae* ameliorated harmful toxic effects of brevetoxins upon the brine shrimp, *Artemia salina*, and reduced the incidence of negative physiological and morphological responses of the sea anemone *Aiptasia pallida*. In addition, brevetoxin biotransformation products formed by competing phytoplankton appear to be non-toxic or do not trigger the same physiological responses as brevetoxins in the model organisms used. These findings may impact the interpretation of ecotoxicological data gathered during bloom events, since the presence of phytoplankton competitors in *Karenia* blooms is likely to reduce the harmful effects seen on many marine invertebrates.

1. Introduction

A suite of neurotoxic cyclic polyether compounds, brevetoxins, periodically cause large scale epizootic events in the Gulf of Mexico, resulting in severe environmental and economic impacts (Anderson et al., 2000; Van Dolah, 2000). These events, known as red tides, occur during blooms of the dinoflagellate *Karenia brevis*, which produces brevetoxins. Brevetoxin analogs are classified as type A (e.g. PbTx-1, -7, -10) and type B (e.g. PbTx-2, -3, -5, -6, -9, -11, -12) and are often found alongside structurally related non-toxic compounds (e.g. brevenal, tamulamides A and B) (Nakanishi, 1985; Van Dolah, 2000; Bourdelais et al., 2005; Satake et al., 2008; Truxal et al., 2010).

The deleterious effects of brevetoxins can be seen on a wide range of organisms including fish, turtles, and seabirds (Bossart et al., 1998; Fire et al., 2007; Gannon et al., 2009), and brevetoxicosis is well documented for marine mammals such as manatees and dolphins (Trainer and Baden, 1999; Flewelling et al., 2005; Landsberg et al., 2009). Organisms from the lower trophic levels, such as small bivalves, epiphytes, copepods, amphipods and urchins generally survive red tides with some acting as toxin vectors to benthic and pelagic food webs (Tester et al., 2000; Flewelling et al., 2005; Landsberg et al., 2009; Sotka et al., 2009). However, invertebrate mortality events occasionally occur, such as in 2005 when for more than a month benthic mortality extended over 2000 km off the West Florida shelf due to the effects of a red tide, exacerbated by other environmental factors (Landsberg et al., 2009).

Despite extensive environmental monitoring which utilizes coastal *K. brevis* cell concentrations and mouse toxicity assays of shellfish extracts, predictions of bloom toxicity are difficult (Heil, 2009). Meteorological and oceanographic factors as well as concentrations of brevetoxin antagonists such as brevenal, *K. brevis* cell concentration, biochemical or strain differences, osmotic stress, and the presence of competitor phytoplankton species have all been proposed as reasons for the variable toxic potency seen

* Corresponding author at: School of Biology, Georgia Institute of Technology, 310 Ferst Drive, Atlanta, GA 30332-0230, USA. Tel.: +1 404 894 8424; fax: +1 404 385 4440.
E-mail addresses: clare.redshaw@pcmd.ac.uk (C.H. Redshaw), julia.kubanek@biology.gatech.edu (J. Kubanek).
[1] Present address: European Centre for Environment and Human Health, Peninsula College of Medicine and Dentistry, Royal Cornwall Hospital, Truro, Cornwall, TR1 3HD, UK; School of Geography, Earth and Environmental Science, University of Plymouth, Drake Circus, Plymouth, Devon, PL4 8AA, UK.

1568-9883/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.hal.2011.09.007

during blooms (Bourdelais et al., 2004; Van Dolah et al., 2009; Errera and Campbell, 2011).

Previous work by Myers et al. (2008) and Redshaw et al. (2010) showed that a wide range of competitor phytoplankton species are able to decrease waterborne concentrations of brevetoxins, specifically PbTx-1 and PbTx-2, which are the most toxic and most abundant brevetoxins in the field, respectively (Roszell et al., 1989). It may be possible to eventually use competing phytoplankton or proteins derived from phytoplankton as biocontrol agents to lower waterborne brevetoxin concentrations and potentially reduce ecosystem-wide impacts. However, before any biocontrol strategies are applied, impacts upon marine organisms must be assessed and the potential toxicity of biotransformation products such as protein–brevetoxin complexes (Redshaw et al., 2010; Wang and Ramsdell, 2011), should be considered.

2. Materials and methods

2.1. Culturing and maintenance of organisms

Phytoplankton strains were obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP; USA). Stock cultures of *Skeletonema grethae* (strain 775) and *K. brevis* (strain 2228) were maintained in 5 L Fernbach flasks at 22 °C under a 12:12 h light:dark cycle (Percival incubator with Philips F32T8/TL741 Universal/Hi-Vision vertically mounted fluorescent bulbs, irradiance 100–145 μmol m^{-2} s^{-1}). Growth media consisted of sterile filtered natural seawater from Maine (36 ppt; CCMP) modified with L1 media with silicate (CCMP; Guillard and Hargraves, 1993). When required, phytoplankton was gradually acclimatized to different conditions over a period of 4–16 days (Δ temperature 1 °C day^{-1}; Δ salinity 2 ppt every 4 days). Growth status was monitored using *in vivo* chlorophyll *a* fluorescence (Turner Designs TD-700, chlorophyll *a* calibrated) and cell counts (acid Lugols preserved samples; 125 μL Palmer settling chamber; Olympus IX50 microscope).

Artemia salina cysts were hatched in an aerated glass 1 L separatory funnel containing 500 mL of 28 ppt natural seawater, pH $\geq$8.5 (1 g certified *Artemia* cysts, ARGENT Chemical Laboratories, WA, USA; 800 mL). Cysts were incubated at 26 °C with a 12:12 h light:dark cycle. First naupliar stage, a non-feeding molt stage, was achieved at 24 h and second naupliar stage, a feeding stage, at 48–60 h.

Sea anemones (*Aiptasia pallida*) ranging from 0.6 to 3.8 cm in length were obtained from WARD's Natural Science, Rochester, NY, USA and were maintained in 10 L aquaria at 23 °C under fluorescent light with 12:12 h light:dark cycle in 6 L of 38 ppt artificial seawater (Instant Ocean salts in deionized water).

2.2. Brevetoxin source

PbTx-2 used in experiments was extracted and purified from *K. brevis* cultures. In brief, *K. brevis* cultures in late exponential growth phase ($[2.6 \pm 0.1] \times 10^4$ cells mL^{-1}) were subjected to exhaustive extraction with ethyl acetate, followed by partitioning of dried extracts between 90% aqueous methanol and petroleum ether. Reversed phase solid phase extraction (SPE) was used to isolate brevetoxins from the aqueous methanol fraction (SPE; 10 g ENVI-18, Sigma, 85% aqueous acetonitrile eluent). Final purification of PbTx-2 was achieved by reversed phase high performance liquid chromatography with UV detection (HPLC-UV; Altima C_{18}, 5 μm, 250 mm $\times$ 10 mm column; 70–100% aqueous methanol; 3 mL min^{-1}). HPLC electrospray-ionization mass-spectrometry (HPLC-ESI-MS) in positive ionization mode was used to quantify PbTx-2, which was stored dry at −20 °C. HPLC-ESI-MS was performed with a Waters-Micromass single quadrupole ZQ2000 mass spectrometer coupled to a SM4 HPLC Separations module (Waters, Milford, MA), using reversed phase chromatography with gradient elution (Phenomenex Luna C_{18}, 3 μm, 150 mm $\times$ 3 mm i.d. column; 80–100% aqueous Fisher Optima acetonitrile modified with 0.1% acetic acid over 7 min; 0.25 mL min^{-1}). Ions characteristic of PbTx-2 were acquired from extracted ion chromatograms of selective ion reaction analyses: $[M-H_2O+H]^+$, $[M+H]^+$ and $[M+Na]^+$; *m/z* 876.6, 895.7 and 917.6. The summed integration areas of these ions were used to quantify PbTx-2, using linear regression against an external PbTx-2 calibration series (0.05–2 μg mL^{-1}; Redshaw et al., 2010).

2.3. Experiment 1: Impact of competitor phytoplankton upon brine shrimp physiology

A. salina, although not an environmentally relevant species, is sensitive to a wide range of toxins and environmental contaminants, hence their common use as a model species in ecotoxicological studies (Kanwar, 2007). For these reasons, *A. salina* was selected as a target organism to measure differential toxicological effects of exposure to PbTx-2 in the presence and absence of competing phytoplankton.

Twenty-four hour prior to the commencement of the exposure assay, treatment solutions were established in glass culture tubes with sterile caps, with 28 ppt natural seawater: solvent control (2% DMSO v/v); *S. grethae* control (1.4×10^5 cells mL^{-1} + 2% DMSO v/v); PbTx-2 treatment (2 μg mL^{-1} PbTx-2 in 2% DMSO v/v) and *S. grethae* + PbTx-2 treatment (1.4×10^5 cells mL^{-1} + 2 μg mL^{-1} PbTx-2 in 2% DMSO v/v). These treatment solutions were not replicated. DMSO was selected as carrier solvent based on its lack of toxicity to both *A. salina* and *S. grethae* (data not shown). Establishing treatment solutions 24 h before the assay start allowed *S. grethae* to lower waterborne PbTx-2 concentrations and form any potential biotransformation products. Aliquots of the treatment solutions taken post incubation, analysed by ELISA, were used to measure the extent of brevetoxin losses caused by *S. grethae*.

After incubation, treatment solutions were placed in the top rows of 96-well polycarbonate plates (n = 18 per treatment) and serially diluted 3-fold down the plate (2.0–0.30 μg mL^{-1} PbTx-2 for the PbTx-2 only treatment; sterile 28 ppt natural seawater used for dilution). The bottom row of each plate was used as a negative control; i.e., no treatment solution was added. *A. salina* were light aggregated and aliquots of the culture were transferred to each well (8 μL; ~15 *A. salina* individuals). Following 24 h exposure, the number of dead and impaired (twitching, limited motility, difficulty moving) individuals in each well was counted under a dissecting microscope (Olympus SZ61, 61x, Japan). Acid Lugols solution was then added to each well to preserve *A. salina*, and a count of the total number of individuals in each well was recorded.

The percentage of *A. salina* deleteriously affected was calculated by summing the counts for dead and impaired individuals in each well, dividing by the total number of individuals in the well and multiplying by 100. Statistically significant differences between the two treatments at each concentration were detected by 2-way ANOVA with repeated measures, followed by a Bonferroni post-test (GraphPad Prism V4.0); statistical significance taken at $P < 0.05$ (Fig. 1). To assess differences in the percentage of *A. salina* deleteriously affected in each treatment relative to controls (*S. grethae* and solvent control) a 1-way ANOVA was performed upon treatment and control data from each exposure concentration.

2.4. Experiment 2: Effect of competitor phytoplankton upon sea anemone physiology

The sea anemone *A. pallida* which is native to U.S. Gulf of Mexico waters and southern Atlantic coasts (OBIS, 2010), was selected as

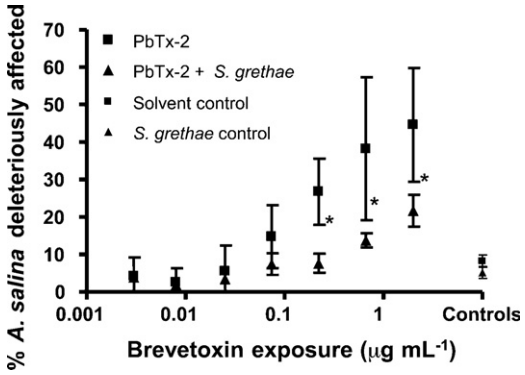

Fig. 1. Competitor phytoplankton ameliorate the deleterious effects of PbTx-2 upon brine shrimp. Toxic effects of PbTx-2, either in the presence or absence of *S. grethae*, upon brine shimp (*Artemia salina*), reported as a percentage of brine shrimp deleteriously affected (dead and impaired individuals; Experiment 1). Brevetoxin concentrations were measured by ELISA, therefore brevetoxin exposure is as PbTx-3 equivalents. Statistical differences detected by a 2-way ANOVA with repeated measures (for treatment and concentration $P < 0.0001$), followed by a Bonferroni post-test ($P < 0.05$; significant differences between PbTx-2 and PbTx-2 + *S. grethae* treatments are represented by *).

an ecologically relevant invertebrate model for testing the negative behavioural and physical consequences of exposure to PbTx-2, in the presence and absence of competitor phytoplankton.

Treatment solutions were established 24 h prior to exposure and incubated (12:12 h light:dark cycle; 22 °C): *S. grethae* control (7.1×10^5 cells mL^{-1} + 1.5% ethanol v/v); PbTx-2 treatment (38 ppt L1 + silicate natural seawater + PbTx-2 in 1.5% ethanol v/v); *S. grethae* + PbTx-2 (7.1×10^5 cells mL^{-1} + PbTx-2 in 1.5% ethanol v/v; $n = 1$ for each treatment). This allowed time for *S. grethae* to reduce PbTx-2 concentrations and for biotransformation products to form. Aliquots of the 10 ng mL^{-1} assay treatment solutions were taken before and after the 24 h incubation and subjected to SPE, followed by HPLC-ESI-MS to measure the decrease in PbTx-2 concentrations as described below.

A. pallida were acclimatized in individual crystallizing dishes with 100 mL, 38 ppt artificial seawater for 24 h, then incubated treatment solutions were added (10 ng mL^{-1} PbTx-2 for PbTx-2 only treatment; $n = 13$). Individuals were continuously monitored for physiological or behavioural responses for 3 h, and again after 24 h. At the end of the assay the seawater was subjected to SPE and brevetoxin concentrations measured by HPLC-ESI-MS. This experiment was later repeated with an initial PbTx-2 exposure concentration of 0.28 μg mL^{-1} ($n = 5$).

Brevetoxin concentrations in the exposure medium at 24 and 48 h were converted into a percentage of the concentration at the previous time point, i.e., 24 h concentration expressed as % of 0 h concentration. To ascertain whether the proportion of healthy anemones (uncoiled symmetrical body shape, tentacles displayed, no discolouration, see Fig. 2) at the termination of the exposure was significantly different from controls, 2-tailed Fishers Exact tests were performed on percentage healthy data. Results were considered statistically significant when $P < 0.05$ (Experiment 2).

2.5. Experiment 3: Effect of competitor phytoplankton cell density upon waterborne PbTx-2 concentrations

As the use of competitor phytoplankton has been proposed as a red tide bioremediation mechanism, the influence of competitor cell concentrations upon brevetoxin losses was assessed. *S. grethae* at mid-exponential growth phase was diluted with L1 + silicate media in 30 culture tubes (10 mL; high 8.4×10^5; medium 4.2×10^5; low 8.5×10^4 cells mL^{-1}; $n = 10$ each). Abiotic control tubes were also prepared (10 mL L1 + silicate media; $n = 20$). PbTx-2 was added to all treatments and controls (2 μg PbTx-2 in 5 μL dimethyl sulfoxide (DMSO) and 95 μL sterile seawater tube^{-1}).

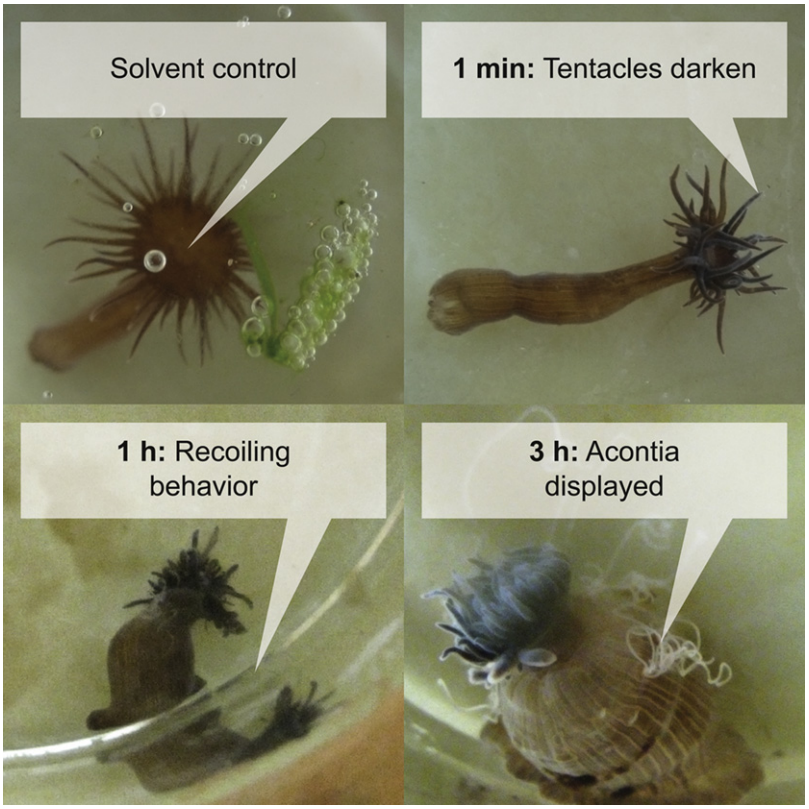

Fig. 2. Sea anemones exhibit stress symptoms from brevetoxin exposure. Negative morphological and physiological responses of sea anemones, *A. pallida*, upon exposure to PbTx-2 (0.28 μg mL^{-1} assay; Experiment 2). A healthy individual is represented by the solvent control.

Ten abiotic controls were immediately subjected to SPE to obtain start concentrations of brevetoxins. All remaining treatments and controls were incubated for 24 h, then subjected to SPE and brevetoxins were quantified by HPLC-ESI-MS as described below.

Percentage recovery of PbTx-2 was calculated from HPLC-ESI-MS data by dividing the concentration of brevetoxin in each sample by the mean concentration of the initial non-incubated abiotic control, and multiplying by 100. This value was then subtracted from 100 to give percentage lost. These data were then subjected to statistical analysis using GraphPad Prism V4.0 at the 95% confidence interval (1-way ANOVA with Tukey post hoc test, Fig. 3).

2.6. Sample extraction and analysis

Interfering components were removed from the sample matrix of aqueous samples prior to analysis by HPLC-ESI-MS (Experiment 3; Experiment 2 treatment solutions and exposure medium). Samples were passed through a conditioned and equilibrated SPE cartridge, and compounds eluted with acetone and dried (1 g ENVI-18 SPE cartridges, Sigma). Extracts were then dissolved in aqueous methanol and filtered through a 0.2 μm nylon syringe filter to remove particulates. HPLC-ESI-MS was performed as described above. Ions characteristic of brevetoxins were acquired from extracted ion chromatograms of selective ion reaction analyses: $[M-H_2O+H]^+$, $[M+H]^+$ and $[M+Na]^+$; *m/z* 876.6, 895.7 and 917.6 for PbTx-2; *m/z* 878.6, 897.7 and 919.6 for PbTx-3; *m/z* 918.6, 937.7 and 959.6 for PbTx-5; *m/z* 892.6, 911.7 and 933.6 for PbTx-6; *m/z* 880.6, 899.7 and 921.6 for PbTx-9; *m/z* 848.6, 867.7 and 889.6 for PbTx-11; *m/z* 906.6, 925.6 and 947.6 for PbTx-12; $[M+Na]^+$ and $[M+2Na+H]^+$ *m/z* 933.3 and 955.1 for BTX-B5. The summed integration areas of these ions were used to quantify brevetoxins, using linear regression against an external PbTx-2 standard calibration series (0.05–2 μg mL^{-1}).

A competitive enzyme-linked immuno-sorbent assay (ELISA) was used to quantify type B brevetoxins (i.e., brevetoxins with the same backbone structure, including PbTx-2, -3, -5, -6, -9, -11, -12, BTX-B5) in treatments and assay medium (Experiment 1). This ELISA, with a 2-step signal amplification procedure, utilized a PbTx-3 bovine serum albumin conjugate, an anti-PbTx goat polyclonal primary antibody and a rabbit anti-goat horseradish peroxidase secondary antibody, with 3,3′,5,5′-tetramethylbenzidine and H_2SO_4 to visualize at 450 nm, as described by Naar et al. (2002). Inhibition curves generated from a PbTx-3 calibration series were used for type B brevetoxin quantification. Brevetoxin detection is specific to type B brevetoxins and their metabolites as the primary antibody recognises the H–K ring structure of the type B brevetoxin backbone. PbTx-3 is the reduced form of PbTx-2, in which the side chain displays an alcohol rather than an aldehyde. ELISA data are expressed as PbTx-3 equivalents (equiv.) since PbTx-3 is used for the generation of inhibition curves and as the competitive component (PbTx-3 and PbTx-2 possess the same reactivity towards the PbTx antibody). Direct analysis of aqueous samples (Experiment 1) without sample extraction was used, limiting possible losses.

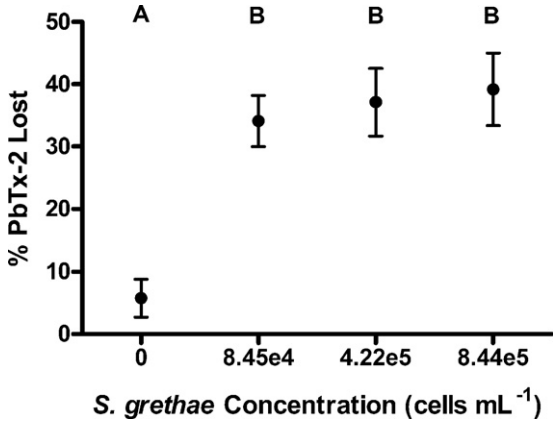

Fig. 3. Competitor phytoplankton cell concentration has minimal influence upon brevetoxin losses. Losses of waterborne PbTx-2 after 24 h incubation with *S. grethae* at various cell concentrations ($n = 10$; Experiment 3). Statistical differences detected by 1-way ANOVA and Tukey post-hoc test ($P < 0.05$) are represented by different capitalized letters. Error bars represent +1 (or ±1) standard deviation in all figures.

3. Results and discussion

3.1. Competitor phytoplankton ameliorate the harmful effects of PbTx-2 upon brine shrimp

S. grethae reduced overall toxicity of PbTx-2 to brine shimp (*A. salina*), as indicated by the different proportions of individuals deleteriously affected in the 2 treatments, and also ameliorated all toxic effects at some concentrations (vs. solvent controls; Fig. 1). During the 24 h pre-assay incubation, the presence of *S. grethae* caused a 43% decline in brevetoxin concentrations (from 2.0 to 1.1 μg mL^{-1} PbTx-3 equiv. vs. <1% loss in absence of *S. grethae*), which resulted in a 49% decrease in the number of brine shrimp deleteriously affected at the highest exposure concentration. Statistical analysis (ANOVA) of the exposure assay data, at each exposure concentration tested, indicated that the presence of *S. grethae* had partially ameliorated the toxic effect of PbTx-2 upon *A. salina* at 2.0 μg mL^{-1} PbTx-2 ($P < 0.001$). At toxin concentrations below this (≤0.67 μg mL^{-1}) the presence of *S. grethae* completely ameliorated all negative physiological effects of PbTx-2 (vs. controls $P > 0.05$).

Another statistical analysis, a 2-way ANOVA with repeated measures and a Bonferroni post-test, supported this finding (significant differences between treatments at toxin concentrations ≥0.22 μg mL^{-1}; $P < 0.0001$; Fig. 1). This analysis also revealed that the different PbTx-2 test concentrations caused significantly different effects upon the brine shrimp ($P < 0.0001$), which indicates that brine shrimp were sensitive to small changes in toxin concentration. These findings suggest that using a competitor phytoplankton species as a biocontrol agent would result in toxin reductions substantial enough to significantly reduce detrimental impacts upon this model organism, while also implying that biotransformation products formed in the presence of *S. grethae* are not toxic to brine shrimp.

3.2. Negative morphological and physiological responses of sea anemones to PbTx-2 are reduced by competitor phytoplankton

The sea anemone, *A. pallida*, is susceptible to the toxic effects of PbTx-2, exhibiting immediate severe morphological and physiological symptoms upon exposure (0.28 μg mL^{-1}; Fig. 2). In the presence of *S. grethae* the extent of these morphological responses and the incidence of distressed behaviour decreased, with the majority of anemones returning to a healthy state after 24 h exposure.

Negative responses of *A. pallida* to PbTx-2 were most apparent at the relatively high toxin concentration of 0.28 μg mL^{-1} PbTx-2. Anemones in both treatments responded immediately upon the addition of toxin: initially tentacles darkened in color, followed by recoiling behaviour (Fig. 2). After 3 h exposure to PbTx-2 100% of anemones began to show a distressed response (acontia exuded), however only 40% of *A. pallida* initiated the same response in the presence of *S. grethae* and PbTx-2. Displaying acontia is generally considered to be a defensive response, although the presence of

nematocysts (stinging cells) within the acontia also provides anemones with the ability to stun and digest prey by injecting toxins or digestive enzymes (Anderluh and Macek, 2002; Schlesinge et al., 2009). Chemical stimuli have been associated with exudation of acontia (La Spada et al., 2002). At the termination of the assay 80% of anemones exposed to *S. grethae* + PbTx-2 had returned to a healthy state, whereas only 50% of those exposed to 0.28 $\mu g\ mL^{-1}$ PbTx-2 in the absence of *S. grethae* were healthy. At lower exposure concentrations (10 $ng\ mL^{-1}$ PbTx-2) these responses were not observed until the full 24 h exposure time was achieved. Nevertheless, the presence of *S. grethae* provided protection, with 92% anemones remaining healthy (vs. only 42% remaining healthy when exposed to PbTx-2 without *S. grethae*; $P = 0.030$). This indicates that *S. grethae* is able to reduce the negative physiological and morphological responses of a marine invertebrate, *A. pallida*, on exposure to PbTx-2 even at low concentrations. *K. brevis* cell densities during blooms can range from 5 to 1000 cells mL^{-1} (Heil, 2009) and brevetoxin concentrations are typically 10–17 pg $cell^{-1}$ in Florida waters (Hua et al., 1995; Pierce et al., 2003), but have been reported as high as 126 pg $cell^{-1}$ (Greene et al., 2000); this results in wide ranging exposure concentrations of 50 pg mL^{-1} to 126 ng mL^{-1}, which are comparable to this experimental design. Our design mimicked exposure of marine organisms to waterborne PbTx-2, i.e., extracellular toxins released upon cellular lysis of *K. brevis* which occurs at bloom termination. *S. grethae* is not only able to reduce the detrimental impact of PbTx-2 on anemones, but also any PbTx-2 biotransformation products formed by *S. grethae* are clearly not toxic to *A. pallida*, or at least do not result in the same morphological and physiological distress symptoms, during the 24 h exposure time period tested.

Consistent with previous studies, in the presence of competitor phytoplankton waterborne concentrations of PbTx-2 declined during the pre-assay incubation by 85% (Myers et al., 2008; Redshaw et al., 2010). During the subsequent exposure assay with *A. pallida*, PbTx-2 concentrations declined further still; $47 \pm 20\%$ lost in PbTx-2 only treatment and >97% (<LOD; 0.04 $\mu g\ mL^{-1}$) for *S. grethae* + PbTx-2 treatment. Given the observed losses in the PbTx-2 only treatment, biotransformation of PbTx-2 by *A. pallida* or by endosymbiotic zooxanthellae (dinoflagellates found within *A. pallida*) is possible. It is feasible that the *A. pallida* zooxanthellae are able to remove waterborne PbTx-2, as has been shown in numerous species of phytoplankton (Myers et al., 2008; Redshaw et al., 2010), thereby providing the anemone with protection against the toxic effects of PbTx-2. However, it is also known that endosymbiotic zooxanthellae and their host cnidarians share resources including photosynthate and organic and inorganic metabolites (Muscatine and Hand, 1958; Cook, 1971; Trench, 1979; Muscatine, 1980; Carroll and Blanquet, 1982; Clayton and Laker, 1984;). Therefore the further losses of PbTx-2 could be due to the results of a symbiotic relationship, as opposed to the dinoflagellates alone.

3.3. Competitor phytoplankton cell concentration has minimal influence upon brevetoxin losses

All cell concentrations of *S. grethae* in the range of 8.5×10^4 to 8.4×10^5 cells mL^{-1} significantly decreased waterborne PbTx-2 concentrations ($P < 0.001$ for all). A very modest but non-statistically significant trend was observed, with marginally increasing losses of PbTx-2 at higher cell concentrations (Fig. 3). This implies that within the narrow range tested, the cell concentration of *S. grethae* does not have a substantial impact upon of the ability of this diatom to remove PbTx-2, as a 10 fold increase in cell density only resulted in 5% additional loss. In two subsequent experiments testing *S. grethae* concentrations down to 1 cell mL^{-1}, we found brevetoxin removal to be insignificant near or below 10^4 cells mL^{-1}, although these experiments employed high starting concentrations of PbTx-2 (200 $ng\ mL^{-1}$; data not shown). If the interaction between *S. grethae* and PbTx-2 is stoichiometric as we predict, then at typical bloom concentrations of waterborne brevetoxins, diatoms and other phytoplankton in the range of 10^3 to 10^4 cells mL^{-1} may effectively modulate bloom toxicity. This could be important in the design and application of a biocontrol strategy, as it allows the quantities of competitor cells added to be optimized for the required reduction in PbTx-2 concentrations. The lack of literature data on field concentrations of *S. grethae* and the added complexity of *S. grethae* reclassification (formerly *S. costatum*; Kooistra et al., 2008) make it difficult to assess the environmental relevance of cell concentrations used in this experiment. However, the selection of *S. grethae* as a model competitor phytoplankter is relevant, as *Skeletonema* spp. are commonly found in coastal marine and estuarine waters and often form dense blooms (Kooistra et al., 2008). Additionally, *Skeletonema* is dominant in coastal waters (e.g., Tampa Bay, Florida), is present year round, and has been found during *K. brevis blooms* (Turner and Hopkins, 1974; Badylak et al., 2007; Prince et al., 2008). Myers et al. (2008) demonstrated that *S. grethae* rapidly outcompetes *K. brevis* in co-cultures; therefore the possibility of adding small quantities of *S. grethae* as a biocontrol agent, to allow the required threshold density for brevetoxin removal to be reached within a given time period, should be further investigated.

4. Concluding remarks

Competing phytoplankton are able to significantly decrease waterborne PbTx-2 concentrations due to the release of proteins that complex brevetoxins (Fig. 3; Myers et al., 2008; Redshaw et al., 2010). Wang and Ramsdell (2011) showed that PbTx-2 undergoes conjugation with human serum albumin resulting in both covalent and non-covalent interactions with cysteine, histidine and lysine amino acid residues. This is likely the same mechanism as occurs in the presence of competing phytoplankton, in which waterborne proteins associated with phytoplankton are responsible for brevetoxin removal (Redshaw et al., 2010). It has been shown that bloom toxicity does not correlate well with *K. brevis* cell concentrations; the evidence presented herein highlights just one of the factors that may disrupt this relationship.

The current study suggests that use of *S. grethae* as a biocontrol would significantly decrease the harmful impacts upon some marine invertebrates, even if only a modest decrease in brevetoxin concentration was achieved, as these organisms are sensitive to small changes in brevetoxin concentration (Figs. 1 and 2). Additionally, biotransformation products formed in the presence of *S. grethae* appear to be either non-toxic or do not trigger the same physiological responses in the model organisms studied. However, it should be noted that exposure times used during these assays were relatively short, and the potential for future release of brevetoxins, or trophic transfer of protein–brevetoxin adducts stored in marine organisms should be considered in future studies.

Studies on biotoxin control and resulting ecological impacts represent a largely neglected area of research. Due to the complex nature of ecological interactions involving brevetoxins and *K. brevis*, it would be pertinent to explore more of these interactions in the presence of competing phytoplankton, towards the potential longer-term application of phytoplankton as biocontrol agents. While it might not be practical to seed large patches of coastal ocean with lab-grown phytoplankton, addition of a limiting nutrient (e.g., silicate) could facilitate diatom growth in anticipation of a *K. brevis* bloom moving onshore. This might partially protect coastal invertebrates from deleterious effects of brevetoxins. Alternatively, protein exudates from mass-cultured phytoplankton or other environmentally benign and commercially

available marine-derived proteins may be effective at reducing waterborne concentrations of brevetoxins in localized areas, particularly near vulnerable populations of invertebrates. Each of these possibilities is the subject of ongoing investigation.

Acknowledgements

This research was supported by a grant from the Florida Fish and Wildlife Conservation Commission (FWC-07179) and by a National Science Foundation grant (OCE-0726689) awarded to J. Kubanek. ELISA analyses were funded by NOAA-OHHI # NA05NOS478 and NOAA-MERHAB # NA05NOS393 awarded to J. Naar. Georgia Tech Presidential Undergraduate Research Awards awarded to D. A. Sutter supported this student's project. The study sponsors played no role in study design, experimental work, data interpretation, report writing, or in the decision to submit this paper for publication.[SS]

References

Anderluh, G., Macek, P., 2002. Cytolytic peptide and protein toxins from sea anemones (Anthozoa: *Actiniaria*). Toxicon 40 (2), 111–124.

Anderson, D.M., Hoagland, P., Kaoru, Y., White, A.W., 2000. Estimated Annual Economic Impacts from Harmful Algal Blooms (HABs) in the United States. Woods Hole Oceanographic Institution, p. 97.

Badylak, S., Philips, E.J., Baker, P., Fajans, J., Boler, R., 2007. Distributions of phytoplankton in Tampa Bay Estuary, U.S.A. 2002–2003. Bull. Mar. Sci. 80 (2), 295–317.

Bossart, G.D., Baden, D.G., Ewing, R.Y., Roberts, B., Wright, S.D., 1998. Brevetoxicosis in manatees (*Trichechus manatus latirostris*) from the 1996 Epizootic: gross, histologic, and immunohistochemical features. Toxicol. Pathol. 26 (2), 276–282.

Bourdelais, A.J., Campbell, S., Jacocks, H., Naar, J., Wright, J.L.C., Carsi, J., Baden, D.G., 2004. Brevenal is a natural inhibitor of brevetoxin action in sodium channel receptor binding assays. Cell. Mol. Neurobiol. 24 (4), 553–563.

Bourdelais, A.J., Jacocks, H.M., Wright, J.L.C., Bigwarfe, P.M.J., Baden, D.G., 2005. A new polyether ladder compound produced by the dinoflagellate *Karenia brevis*. J. Nat. Prod. 68, 2–6.

Carroll, S., Blanquet, R.S., 1982. Transport of amino acids from the cnidarian *Cassiopea* to its endosymbiotic zooxanthellae. Am. Zool. 22, 872.

Clayton, W.S., Laker, H.R., 1984. Host feeding regime and zooxanthellal photosynthesis in the anemone, *Aiptasia pallida* (Verrill). Biol. Bull. 167, 590–600.

Cook, C.B., 1971. Transfer of ^{35}S-labelled material from food ingested by *Aiptasia* sp. to its endosymbiotic zooxanthellae. In: Lenhoff, H.M., Muscatine, L., Davids, L.V. (Eds.), Experimental Coelenterate Biology. University of Hawaii Press, Honolulu, pp. 218–224.

Errera, R.M., Campbell, L., 2011. Osmotic stress triggers toxin production by the dinoflagellate *Karenia brevis*. Proc. Natl. Acad. Sci. 108 (26), 10591–10601.

Fire, S.E., Fauquier, D., Flewelling, L.J., Henry, M., Naar, J., Pierce, R., Wells, R.S., 2007. Brevetoxin exposure in bottlenose dolphins (*Tursiops truncatus*) associated with *Karenia brevis* blooms in Sarasota Bay, Florida. Mar. Biol. 152, 827–834.

Flewelling, L.J., Naar, J.P., Abbott, J.P., Baden, D.G., Barros, N.B., Bossart, G.D., Bottein, M.-Y.D., Hammond, D.G., Haubold, E.M., Heil, C.A., Henry, M.S., Jacocks, H.M., Leighfield, T.A., Pierce, R.H., Pitchford, T.D., Rommel, S.A., Scott, P.S., Steidinger, K.A., Truby, E.W., Van Dolah, F.M., Landsberg, J.H., 2005. Red tides and marine mammal mortalities. Nature 435, 755–756.

Gannon, D.P., McCabe, E.J.B., Camilleri, S.A., Gannon, J.G., Brueggen, M.K., Barleycorn, A.A., Palubok, V.I., Kirkpatrick, G.J., Wells, R.S., 2009. Effects of *Karenia brevis* harmful algal blooms on nearshore fish communities in southwest Florida. Mar. Ecol. Prog. Ser. 378, 171–186.

Greene, R.M., Walker, C.C., Murrell, M.C., Kurtz, J.C., Stanley, R.S., Genthner, F.G., 2000. High brevetoxin concentrations in *Gymnodinium breve* blooms along the northwest Florida coast during 1999. J. Phycol. 36, 25–26.

Guillard, R.R.L., Hargraves, P.E., 1993. Stichochrysis immobilis is a diatom, not a chrysophyte. Phycologia 32, 234–236.

Heil, D.C., 2009. *Karenia brevis* monitoring, management, and mitigation for Florida molluscan shellfish harvesting areas. Harmful Algae 8, 608–610.

Hua, Y., Lu, W., Henry, M.S., Pierce, R.H., Cole, R.B., 1995. On-line high-performance liquid chromatography-electrospray ionization mass spectrometry for the determination of brevetoxins in Red Tide algae. Anal. Chem. 67, 1815–1823.

Kanwar, A.S., 2007. Brine shrimp (*Artemia salina*) – a marine animal for simple and rapid biological assays. J. Chin. Clin. Med. 2 (4), 236–240.

Kooistra, W.H.C.F., Sarno, D., Balzano, S., Gu, H., Andersen, R.A., Zingone, A., 2008. Global diversity and biogeography of *Skeletonema* species (Bacillariophyta). Protist 159, 177–193.

La Spada, G., Sorrenti, G., Soffli, A., Montaleone, B., Marino, A., Musci, G., 2002. Thiol-induced discharge of acontial nematocytes. Comp. Biochem. Physiol. B: Biochem. Mol. Biol. 132 (2), 367–373.

Landsberg, J.H., Flewelling, L.J., Naar, J., 2009. *Karenia brevis* red tides, brevetoxins in the food web, and impacts on natural resources: decadal advancements. Harmful Algae 8, 598–607.

Muscatine, L., 1980. Productivity of zooxanthellae. In: Falkowski, P.G. (Ed.), Primary Productivity in the Sea. Plenum Press, New York.

Muscatine, L., Hand, C., 1958. Direct evidence for the transfer of materials from symbiotic algae to the tissues of a coelenterate. Proc. Natl. Acad. Sci. 44, 1259–1263.

Myers, T.L., Prince, E.K., Naar, J., Kubanek, J., 2008. Loss of waterborne brevetoxins from exposure to phytoplankton competitors. Harmful Algae 7 (6), 762–771.

Naar, J., Bourdelais, A., Tomas, C., Kubanek, J., Whitney, P.L., Flewelling, L., Steidinger, K., Lancaster, J., Baden, D.G., 2002. A competitive ELISA to detect brevetoxins from *Karenia brevis* (formerly *Gymnodinium breve*) in seawater, shellfish, and mammalian body fluid. Environ. Health Perspect. 110 (2), 179–185.

Nakanishi, K., 1985. The chemistry of brevetoxins: a review. Toxicon 23 (3), 473–479.

OBIS, 2010. *Aiptasia pallida*: Data Extent Map (from CMAR c-Squares Mapper). Ocean Biogeographic Information System.

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

Prince, E.K., Myers, T.L., Kubanek, J., 2008. Effects of harmful algal blooms on competitors: allelopathic mechanisms of the red tide dinoflagellate *Karenia brevis*. Limnol. Oceanogr. 53 (2), 531–541.

Redshaw, C.H., Sutter, D., Myers, T.L., Naar, J., Kubanek, J., 2010. Tracking losses of brevetoxins on exposure to phytoplankton competitors: mechanistic insights. Aquat. Toxicol. 100, 365–372.

Roszell, L.E., Schulman, L.S., Baden, D.G., 1989. Toxin profiles are dependent on growth stages in cultured *Ptychodiscus brevis*. In: Granelli, E., Sundstrom, B., Edler, L., Anderson, D.M. (Eds.), Toxic Marine Phytoplankton. Elsevier Science, New York, pp. 403–440.

Satake, M., Bourdelais, A.J., Van Wagoner, R.M., Baden, D.G., Wright, J.L.C., 2008. Brevisamide: an unprecedented monocyclic ether alkaloid from the dinoflagellate *Karenia brevis* that provides a potential model for ladder-frame initiation. Org. Lett. 10 (16), 3465–3468.

Schlesinge, A., Zlotkin, E., Kramarsky-Winter, E., Loya, Y., 2009. Cnidarian internal stinging mechanism. Proc. R. Soc. B 276 (1659), 1063–1067.

Sotka, E.E., McCarty, A., Monroe, E.A., Oakman, N., Van Dolah, F.M., 2009. Benthic herbivores are not deterred by brevetoxins produced by the Red Tide dinoflagellate *Karenia brevis*. J. Chem. Ecol. 35, 851–859.

Tester, P.A., Turner, J.T., Shea, D., 2000. Vectorial transport of toxins from the dinoflagellate *Gymnodinium breve* through copepods to fish. J. Plankton Res. 22 (1), 47–61.

Trainer, V.L., Baden, D.G., 1999. High affinity binding of red tide neurotoxins to marine mammal brain. Aquat. Toxicol. 46, 139–148.

Trench, R.K., 1979. The cell biology of plant–animal symbiosis. Annu. Rev. Plant Physiol. 30, 485–531.

Truxal, L.T., Bourdelais, A.J., Jacocks, H., Abraham, W.M., Baden, D.G., 2010. Characterization of Tamulamides A and B, polyethers isolated from the marine dinoflagellate *Karenia brevis*. J. Nat. Prod. 73, 536–540.

Turner, J.T., Hopkins, T.L., 1974. Phytoplankton of the Tampa Bay system, Florida. Bull. Mar. Sci. 24, 101–124.

Van Dolah, F.M., 2000. Marine algal toxins: origins, health effects, and their increased occurrence. Environ. Health Perspect. 108 (1), 133–141.

Van Dolah, F.M., Lidie, K.B., Monroe, E.A., Bhattacharya, D., Campbell, L., Doucette, G.J., Kamykowski, D., 2009. The Florida red tide dinoflagellate *Karenia brevis*: new insights into cellular and molecular processes underlying bloom dynamics. Harmful Algae 8, 562–572.

Wang, Z., Ramsdell, J.S., 2011. Analysis of interactions of brevetoxin-B and human serum albumin by liquid chromatography/mass spectrometry. Chem. Res. Toxicol. 24, 54–64.