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 ≥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 × 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 × 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