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Effects of *Karenia brevis* on clearance rates and bioaccumulation of brevetoxins in benthic suspension feeding invertebrates

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ABSTRACT

Blooms of the toxic alga *Karenia brevis* occur along coastlines where sessile suspension feeding invertebrates are common components of benthic communities. We studied the effects of *K. brevis* on four benthic suspension feeding invertebrates common to the coast of the SE United States: the sponge *Haliclona tubifera*, the bryozoan *Bugula neritina*, the bivalve *Mercenaria mercenaria,* and the tunicate *Styela plicata.* In controlled laboratory experiments, we determined the rate at which *K. brevis* was cleared from the seawater by these invertebrates, the effect of *K. brevis* on clearance rates of a non-toxic phytoplankton species, *Rhodomonas* sp., and the extent to which brevetoxins bioaccumulated in tissues of invertebrates using an enzyme-linked immunosorbent assay (ELISA). All four invertebrate species cleared significant quantities of *K. brevis* from seawater, with mean clearance rates ranging from 2.27 to 6.71 L g h^{-1} for *H. tubifera* and *S. plicata*, respectively. In the presence of *K. brevis*, clearance rates of *Rhodomonas* sp. by *B. neritina* and *S. plicata* were depressed by 75% and 69%, respectively, while clearance rates by *H. tubifera* and *M. mercenaria* were unaffected. Negative effects of *K. brevis* were impermanent; after a recovery period of 13 h, *B. neritina* and *S. plicata* regained normal clearance rates. All four invertebrates accumulated high concentrations of brevetoxin after a 4 h exposure to *K. brevis*, but when animals were transferred to filtered seawater for 15 h after exposure, brevetoxin concentrations in the tissues of *H. tubifera* and *B. neritina* decreased by ~80%, while there was no change in toxin concentration in the tissues of *S. plicata* and *M. mercenaria*. High cell concentrations of *K. brevis* may cause a suppression of clearance rates in benthic suspension feeding invertebrates, resulting in a positive feedback for bloom formation. Also, high concentrations of toxin may accumulate in the tissues of benthic suspension feeding invertebrates that may be transferred to higher-level consumers.

1. Introduction

Blooms of the toxic dinoflagellate *Karenia brevis* occur with close to yearly frequency off the southwest coast of Florida (Steidinger et al., 1998). These blooms originate 18–74 km offshore on the mid-west Florida shelf and travel shoreward (Steidinger, 1975a). In a comprehensive survey of the benthic habitats on the southwest Florida shelf, Phillips et al. (1990) found that hard-bottom habitat was the most common feature over most of the depth ranges surveyed. Suspension feeding sponges, tunicates, and bryozoans were all common components of the communities that occupied hard-bottom habitat. These invertebrates clear large volumes of phytoplankton from the water column, and may control both the intensity and frequency of algal blooms (Petersen et al., 2006). Benthic suspension feeding invertebrates also have the potential to bioaccumulate high concentrations of algal toxins and may act as vectors of toxin to higher trophic levels (Ishida et al., 2004; reviewed by Landsberg et al., 2009; Naar et al., 2007; Plakas et al., 2008). Mindful of this potential benthic-pelagic interaction, we performed a series of laboratory investigations to determine the effects of *K. brevis* on suspension feeding invertebrates from four taxa that are common to the coast of the SW United States, including Florida.

The brevetoxins (PbTx-1 through -12) are a suite of lipid soluble polycyclic ethers produced by *K. brevis* that may be classified as Type A (PbTx-1, -7, and -10) or Type B (PbTx-2, -3, -5, -6, -9, -11, and -12) depending on differences in their structural backbones (Baden, 1989; Baden et al., 2005). Brevetoxins are potent neurotoxins that cause mass mortalities of fish, birds, and marine mammals (Flewelling et al., 2005; Forrester et al., 1977; Gannon et al., 2009; reviewed by Landsberg, 2002). These toxins also bioaccumulate in the tissue of animals that come into contact with either

Abbreviations: AFDM, ash free dry mass; ASW, artificial seawater; BWM, blotted wet mass; CF, concentration factor; CMS, Center for Marine Science; CR, clearance rate; DM, dry mass; DR, deposition rate; ELISA, enzyme-linked immunosorbent assay; ESD, equivalent spherical diameter; FSNSW, natural seawater passed through glass fiber and charcoal filters and UV-sterilized; GF/F, glass fiber filter; NSW, natural seawater; PbTx, brevetoxin.

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whole cells of *K. brevis* or dissolved toxins. Significant concentrations of brevetoxins were found in bivalves (Ishida et al., 1996, 2004; Plakas et al., 2002, 2008; Roberts et al., 1979), fish (Flewelling et al., 2005; Landsberg et al., 2009; Naar et al., 2007), birds (Forrester et al., 1977), manatees (Bossart et al., 1998; Flewelling et al., 2005), and dolphins (Flewelling et al., 2005) exposed *to K. brevis* in the wild. Brevetoxins may also accumulate in shellfish tissues under laboratory conditions after exposure to purified toxins, lysate of *K. brevis*, and whole cells (Ishida et al., 2004; Plakas et al., 2002). It is not known whether other common members of the filter-feeding community accumulate high concentrations of brevetoxin in their tissues.

Increasing evidence suggests that mortality in higher trophic levels during blooms of *K. brevis* is caused by ingestion of organisms that have bioaccumulated toxins (reviewed by Landsberg et al., 2009). Brevetoxins accumulated in the tissues of Atlantic Croaker (*Micropogonius angulatus*) and Pinfish (*Lagodon rhomboides*) after being fed toxic bivalve tissue (Naar et al., 2007). Mass mortalities of birds and dolphins were attributed to the consumption of toxic shellfish and fish, respectively (Flewelling et al., 2005; Forrester et al., 1977), and incidental consumption of toxin-containing tunicates was implicated as the cause of a die-off of endangered Florida manatees (*Trichechus manatus latirostris*) although no tunicates were found in manatee guts (O'Shea et al., 1990). Quantifying the amounts of toxins that accumulate in the tissues of benthic suspension feeders may allow predictions about the effects of blooms of *K. brevis* on higher level consumers.

Benthic suspension feeding invertebrates may play an important role in regulating blooms of *K. brevis*. However, *K. brevis* may have negative effects on clearance rates of benthic suspension feeders creating a positive feedback for blooms of *K. brevis*. Shellfish readily clear *K. brevis* from seawater but exposure to cell concentrations of 1000 cells mL^{-1} caused decreases in clearance rates ranging from 38% to 79% in four species of juvenile bivalve molluscs (Leverone et al., 2007). In other systems, mass mortalities of benthic fauna may facilitate the formation of harmful algal blooms. In Florida Bay, a 90% decrease in sponge biomass was documented following blooms of cyanobacteria in the early 1990s (Steveley and Sweat, 1995). These die-offs increased regional turnover rates of the water column by 12 days in some areas (Petersen et al., 2006). Mass mortalities of benthic fauna during blooms of *K. brevis* are not well documented due to lack of sampling. In two cases, mass mortalities of benthic fauna related to blooms of *K. brevis* were documented between July–August 1971 and August 2005 in which fish, sponges, corals, molluscs, polychaetes, and other taxa were severely affected (Fish and Wildlife Research Institute, 2005; Smith, 1975). It is unclear whether mortality in these events was caused by secondary effects such as anoxia and hydrogen sulfide production or the direct effects of toxins from *K. brevis*.

Although blooms of *K. brevis* occur with frequency off the coast of Florida, there was one recorded bloom of *K. brevis* off the coast of North Carolina that had devastating effects on the commercially important bay scallop (*Argopecten irradians*). Populations of *A. irradians* experienced 21% mortality as a result of the bloom (Summerson and Peterson, 1990). Recruitment the following year did not return bay scallops to their pre-red tide densities indicating that the red tide may have had significant multiple-year effects on population levels (Summerson and Peterson, 1990). Despite their potential role in regulating blooms, the effects of *K. brevis* on clearance rates of non-shellfish benthic suspension feeding invertebrates and the rate at which they clear *K. brevis* from seawater have not been investigated.

In the present study, we investigated the ability of benthic suspension feeding invertebrates to clear *K. brevis* from seawater, the effects of *K. brevis* on clearance rates of a non-toxic phytoplankton species in the presence of *K. brevis*, and the bioaccumulation of brevetoxins in the tissues of invertebrates exposed to *K. brevis*. We used four species of benthic suspension feeders that are found along the coastline of Florida and are also common in the North Carolina fouling community: the demosponge *Haliclona tubifera*, the bryozoan *Bugula neritina*, the bivalve *Mercenaria mercenaria*, and the tunicate *Styela plicata* (Dragovich and Kelly, 1964; Estevez and Bruzek, 1986; Keough, 1986; Maldonado and Young, 1996). These species were chosen because they are commonly available and perform well under laboratory conditions. We measured filtration of the nontoxic cryptomonad, *Rhodomonas* sp. in the presence and absence of *K. brevis* to determine if *K. brevis* has a negative effect on normal feeding activity in the four species. To determine if potential negative effects of *K. brevis* were persistent, clearance rates were measured again after a recovery period. Bioaccumulation and persistence of toxins in tissue were determined by measuring brevetoxin concentrations in tissue before and after recovery periods using an enzyme-linked immunosorbent assay (ELISA).

2. Materials and methods

2.1. Collection and maintenance of animals

All collections of suspension feeding invertebrates were performed during spring and summer 2010. Care was taken to ensure that animals of the same species were approximately the same size. Three of four suspension feeders, *H. tubifera* (sponge), *S. plicata* (tunicate), and *B. neritina* (bryozoan) were collected from floating docks at Seapath Marina (34° 12′ 44.30″ N/77° 48′ 19.53″ W), Wrightsville Beach, North Carolina, USA, 24–48 h prior to experiments. Animals were maintained together in aquaria at the Center for Marine Science (CMS), Wilmington, North Carolina, at room temperature. Aquaria contained 35 ppt natural seawater (NSW) and were maintained at room temperature (20–24 °C) with recirculating seawater that passed through a mechanical filter. Animals were not fed during the 24 h prior to experiments.

The bivalve mollusc *M. mercenaria* was collected at low tide from the intertidal zone off the Intracoastal Waterway (34° 10′ 41.22″ N/77° 50′ 39.68″ W), Wilmington, N.C. Clams were maintained in hanging cages ~1 m deep off floating docks at CMS. Clams opened irregularly during initial experiments so additional steps were necessary to acclimate them to experimental conditions. Three days prior to bioaccumulation and clearance rate assays, individual clams were transferred to separate 4 L jars. Each jar contained 3 L of 34 ppt NSW (natural seawater) that was passed through a mechanical and charcoal filter, UV-sterilized, and kept at 21–23 °C using water baths. Individuals were fed 20,000 cells mL^{-1} *Rhodomonas* sp. 48 h prior to experiments and 1.5 L of seawater in jars was replaced every day before experiments.

2.2. Determination of body size conversion metrics

The relationships between blotted wet mass (BWM), dry mass (DM), and ash free dry mass (AFDM) were determined for the relevant tissues from each of the four study species so that clearance rate measurements from clearance rate and bioaccumulation experiments could be standardized to AFDM. Clearance rate measurements for benthic suspension feeders were variably reported in terms of BWM, DM, and AFDM in previous studies. Conversion factors were used to compare clearance rate measurements from the current study with previous studies.

Invertebrates were collected and maintained in recirculating aquaria with 35 ppt NSW at room temperature (20–24° C) for 24 h prior to measurements allowing animals to eliminate gut contents. Blotted wet mass measurements were taken of the whole tissue mass of sponges, the stolon-free tissue of bryozoans, the foot

muscle and visceral tissue of clams, and the tunic and visceral tissue of tunicates. Tissue was transferred to an 80 °C drying oven for 5 days. Dry mass (DM) was measured and tissue was transferred to a muffle furnace and combusted for 12 h at 450 °C and AFDM measurements were taken. Linear regressions were performed using XLSTAT version 2010.4.01 (Addinsoft, New York, NY), to determine the relationship between BWM and AFDM measurements.

2.3. Maintenance of algal cultures

Cultures of *Rhodomonas* sp. were grown in F/2 media (Guillard, 1975) prepared from 35 ppt NSW from the UNCW Aquaculture facility that was passed through glass fiber (GF/F) and charcoal filters and UV-sterilized (FSNSW). Cultures were maintained at 22 °C on a 12:12 light:dark cycle at an irradiance of 70–85 μmol photons m^{-2} s PAR cool white fluorescent light. Algal cultures used in clearance rate experiments were 5–12 days old. Cultures of *K. brevis* (=*Gymnodium breve*; Wilson clone) were grown in Wilson's NH15 media prepared from 33 ppt artificial seawater (ASW) (Gates and Wilson, 1960). Cultures were maintained at 21–24 °C, on a 24 h light cycle at an irradiance of 57–78 μmol photons m^{-2} s PAR cool white fluorescent light. All cultures of *K. brevis* used in clearance rate experiments were 28–35 days old in stationary growth phase. All materials that came into contact with cultures of *K. brevis* were sterilized and detoxified with bleach prior to disposal.

2.4. Clearance rate and bioaccumulation assay with no recovery step

Individuals of *H. tubifera* ($n = 5$), *B. neritina* ($n = 5$), *M. mercenaria* ($n = 8$), and *S. plicata* ($n = 5$) were each transferred to 4 L jars that contained 3 L of 34 ppt FSNSW. Seawater in the jars was gently mixed by the release of air bubbles (~1 s^{-1}) from the tip of a graduated pipette at the bottom of each jar. Temperature was maintained at 21–23 °C using water baths. After a 1 h acclimation period, culture of *Rhodomonas* sp. was added to each treatment (~20,000 cells mL^{-1}) and control (~20,700 cells mL^{-1}) jar and *K. brevis* was added to each treatment jar at a concentration of ~700 cells mL^{-1} corresponding to an intermediate-level bloom of *K. brevis* (Heil and Steidinger, 2009). Immediately after the addition of algal cultures, and every h thereafter for 4 h, a 10 mL seawater sample was withdrawn from each jar. In addition to the constant bubbling, the seawater in each jar was gently stirred with the pipette prior to each sampling period. Cell concentrations in each seawater sample were determined using a Coulter Counter® Multisizer II E eletronic particle counter fitted with a 140 μm aperture. By measuring the decrease in algal cells during the course of the experiment, the rate at which suspension feeding invertebrates cleared *Rhodomonas* sp. and *K. brevis* from the seawater was determined. At the onset of each experiment, a 5 mL sample of the culture of *K. brevis* was frozen at −5 °C for later toxin analysis. At the end of the time-course, invertebrates were removed from test jars. Shells of clams, stolons from bryozoans, and tunics from tunicates were removed and discarded. Blotted wet mass (BWM) measurements were taken of the whole tissue mass of sponges, the stolon-free tissue of bryozoans, the foot muscle and visceral tissue of clams, and the tunic and visceral tissue of tunicates. Tissue samples were frozen at −5 °C for toxin analysis.

2.5. Clearance rate and bioaccumulation assay with recovery step

Experiments were conducted using the same methods described above, but rather than being sacrificed at the end of the initial time-course, individuals of *H. tubifera* ($n = 6$), *B. neritina* ($n = 6$), *M. mercenaria* ($n = 16$), and *S. plicata* ($n = 7$) were transferred to separate jars that contained 3 L of 34 ppt FSNSW. Seawater in the jars was mixed as before and the temperature kept constant by placing jars in 21–23 °C water baths. After 12 h, seawater was stirred with a pipette for 10 s and a 10 mL seawater samples was withdrawn for toxin analysis from each jar. Next, invertebrates were again transferred to separate 4 L jars that each contained 3 L of 34 ppt FSNSW. After a 1 h acclimation period, cultured *Rhodomonas* sp. was added to both treatment and control jars to a concentration of ~20,700 cells mL^{-1}. From each jar, a 10 mL sample was withdrawn immediately after the addition of algae, and every h thereafter for 2 h. Seawater was stirred with a pipette for 10 s prior to sampling. Cell concentrations in each sample were determined as before. After the 2 h time-course, invertebrates were removed from jars and tissue samples processed for toxin analysis as described previously.

2.6. Analysis of clearance rate data

Exponential AFDM-specific clearance rates of *Rhodomonas* sp. and *K. brevis* were determined by the equation $CR = V/tm \times (\mathrm{Ln}C_0 - \mathrm{Ln}C_t)$ (Coughlan, 1969) where CR, clearance rate, V, volume of suspension (L), t, time interval (h), m, AFDM of organism (g), C_0, concentration of cell suspension (cells mL^{-1}) at time 0, and C_t, concentration of cell suspension (cells mL^{-1}) after 2 h. Clearance rates were normalized to tissue mass by dividing the clearance rate for each invertebrate by the AFDM (ash free dry mass) of the tissue. For AFDM-specific clearance rate calculations the mass of whole tissue from stolon-free tissue from bryozoans, whole tissue from sponges, visceral and foot tissue from clams, and visceral tissue from tunicates was used. This procedure assumes a proportional linear relationship between mass and clearance rates. Previous studies determined exponential relationships between clearance rate and mass in tunicates and bivalves (Randlov and Riisgard, 1979; Riisgard, 1988), while linear equations were found to best describe relationships between clearance rate and mass in sponges (Lisbjerg and Petersen, 2000; Riisgard et al., 1993a). In the present study, no relationship was found between mass and clearance rates (unpublished data), but because the primary goal of this study was to determine the relative effect of *K. brevis* on different invertebrate taxa rather than the absolute clearance rates for each species, and because mass differences between individuals in experiments were small, we assumed proportional linear relationships between mass and clearance rates for all species.

Clearance rate measurements may be positively skewed due to deposition of algal cells (Hegaret et al., 2007; Summerel, 2009). Loss of algal cells from the suspension due to the effects of deposition was determined by calculating clearance rates in jars where clams were observed to remain closed during the course of experiments. Deposition rates of *Rhodomonas* sp. were calculated for control and treatment, pre- and post-recovery groups of clams. The deposition rate of *K. brevis* was calculated for the treatment pre-recovery group of clams. Deposition rates were calculated according to the equation $DR = V \times (\mathrm{Ln}C_0 - \mathrm{Ln}C_t)$ where DR, deposition rate, V, volume of suspension, C_0, concentration of cells (cells mL^{-1}) at time 0, and C_t, concentration of cells (cells mL^{-1}) after 2 h (Coughlan, 1969; Summerel, 2009).

Statistical analyses of CR data were performed using XLSTAT version 2010.4.01 (Addinsoft, New York, NY), unless otherwise noted. Bartlett's and Feltz-Miller tests were performed on CR data to test for homogeneity of variance and coefficients of variation, respectively (Zar, 1996). As clearance rate data exhibited heterogeneity of variance (Bartlett's test; $p < 0.05$) but not heterogeneity of coefficients of variation (Feltz-Miller test; $p > 0.05$) all CR data were log transformed prior to statistical analyses (Zar, 1996).

Pre-recovery clearance rate data from all experiments were combined for statistical analyses of clearance rates of *Rhodomonas*

sp. and *K. brevis*. Only clams and tunicates that were observed to be open during both experiments were included in clearance rate calculations. A 2-way ANOVA (species × treatment [with and without *K. brevis*]) was performed on initial data for clearance rates of *Rhodomonas* sp. Bonferroni corrected *T*-tests were performed to test for differences in clearance rates within invertebrate species between treatments.

For experiments with a recovery step, a repeated measures ANOVA (species × treatment × recovery step) was performed on pre- and post-recovery period clearance rates of *Rhodomonas* sp. (SAS 9.1.3, SAS Institute Inc., Cary, NC). Bonferroni corrected *T*-tests were performed to test for differences in clearance rates within invertebrate species between controls and treatments for pre- and post-recovery periods. A 1-way ANOVA was performed to test for differences in clearance rates of *K. brevis* among the four invertebrate species. Multiple comparisons were performed using Tukey's HSD test. Lillefors test was performed on residuals to test for normality following all ANOVAs (Lilliefors, 1967).

2.7. Toxin extraction and analysis

Whole individuals of *H. tubifera*, stolon-free tissue of *B. neritina*, and visceral tissue of *M. mercenaria* and *S. plicata* were analyzed for brevetoxin content. Only the tissue of clams and tunicates that were observed to be open in the first feeding assay and during the recovery step of the second feeding assay were included in analyses. Tissue was homogenized in 1:5 (BWM [g]:solvent volume [mL]) 80% MeOH and then centrifuged at 3000 rpm for 5 min at 4 °C. Supernatant was collected and the pellet was dispersed in 1:5 (BWM [g]:solvent volume [mL]) 80% MeOH by vortexing and shaking. Supernatant from the first and second extractions were combined and filtered through a 0.2 μm filter. Filtrate was analyzed for the presence of brevetoxin by enzyme-linked immunosorbent assay (ELISA). Culture samples of *K. brevis* from each experiment were vortexed and analyzed directly by ELISA.

The ELISA for brevetoxins is highly sensitive, able to detect 0.2 ng PbTx g^{-1}. The assay only detects brevetoxin congeners with type B (PbTx type B) backbone structures (PbTx-2, PbTx-3, and PbTx-9) (Naar et al., 2002). Brevetoxin congeners with type B backbones are much more common in *K. brevis* cells and in tissue of animals that bioaccumulated brevetoxins (Ishida et al., 1996, 2004; Lekan and Tomas, 2010), and therefore are a good indicator of the total toxicity level of animals (Naar et al., 2007; Plakas et al., 2008). However, measurements of tissue toxicity made by ELISA most likely underestimate the total toxicity of animal tissue due to the presence of undetectable Type A backbone brevetoxins. Cysteine conjugate metabolites of brevetoxin, with variable levels of toxicity, are commonly found in shellfish along with parent toxins (Dechraoui et al., 2007; Ishida et al., 2004; Pierce et al., 2004; Wang et al., 2004). The ELISA may overestimate or underestimate the amount of biologically active brevetoxin derivatives within samples. The affinity of the ELISA for metabolites of brevetoxins is lower than for the parent compound (Naar, personal communication) but the ELISA also detects fragments of brevetoxin epitopes that may not be biologically active (Tomas, personal communication). However, metabolism of brevetoxins by shellfish has little effect on the H-K ring regions of brevetoxins targeted by antibodies used in the ELISA assay so that conjugates of brevetoxin type B are detected (Plakas and Dickey, 2010).

Data for toxicity of animal tissues were standardized in the following way: concentration factors (CF) were calculated by using the formula $CF = T_{tx}/S_{tx}$, where T_{tx}, toxin concentration in tissue (ng g BWM^{-1}) and S_{tx}, toxin concentration in test jar seawater (ng g H_20^{-1}) at $t = 0$. Toxin concentrations were based on the mass of stolon-free tissue from bryozoans, whole tissue from sponges, and visceral tissue from clams and tunicates. All statistical analyses were conducted on concentration factor values.

Bartlett's and Feltz-Miller tests were performed on CF data to test for homogeneity of variance and coefficients of variation, respectively (Zar, 1996). As concentration factor data exhibited heterogeneity of variance (Bartlett's test; $p < 0.05$) but not heterogeneity of coefficients of variation (Feltz-Miller test; $p > 0.05$) all CF data was log transformed prior to statistical analyses (Zar, 1996). A 2-way ANOVA was performed to test for significant differences in PbTx type B concentration factor between species, and treatments with and without recovery steps. Multiple comparisons were performed using Tukey's HSD test (XLSTAT, Addinsoft, New York, NY). Lillefors test was performed on residuals to test for normality following the ANOVA (Lilliefors, 1967).

3. Results

3.1. Body size measurements

There were strong linear relationships between ash free dry mass (AFDM) and blotted wet mass (BWM) measurements for *H. tubifera* ($p < 0.01$), *B. neritina* ($p < 0.001$), *M. mercenaria* ($p < 0.001$), and *S. plicata* ($p < 0.0001$) (Fig. 1), and all regressions relating BWM to AFDM had R^2 values ≥ 0.97 (Fig. 1). There was low variation in the size of animals within the same species used in feeding assay experiments (Table 1). Ash free dry masses of individual animals ranged from 0.17 to 0.67 g in *H. tubifera*, 0.23 to 0.56 g in *B. neritina*, 1.02 to 1.66 g in *M. mercenaria*, and 0.16 to 0.46 g in *S. plicata*.

3.2. Cell concentration, size, and toxicity

The size ranges of cells of *Rhodomonas* sp. and *K. brevis* changed slightly from experiment to experiment. The range of cell sizes of *Rhodomonas* sp. was 4.64–11.84 μm equivalent spherical diameter (ESD) and the range of cell sizes of *K. brevis* particles was 15.10–27.27 μm (ESD).

Relatively consistent cell concentrations were achieved across all experiments. Mean ± SE concentrations of *Rhodomonas* sp. in pre-recovery step treatment and control jars were 19,974 ± 294 cells mL^{-1} and 19,841 ± 194 cells mL^{-1}, respectively. Mean concentrations in post-recovery step treatment and control jars were 18,740 ± 476 cells mL^{-1} and 18,744 ± 451 cells mL^{-1}, respectively. The mean cell concentration of *K. brevis* for all experiments was 701 ± 28 cells mL^{-1}. The mean toxin concentration of seawater in test jars was 28.36 ± 4.2 ng type B brevetoxin (PbTx) mL^{-1}.

3.3. Effect of K. brevis on clearance rates of Rhodomonas sp. by filter-feeding invertebrates

In the absence of *K. brevis*, *B. neritina* had the highest clearance rate of cells of *Rhodomonas* sp. at 13.58 ± 1.87 g AFDM L h^{-1} and *M. mercenaria* had the lowest clearance rate at 1.52 ± 0.36 g AFDM L h^{-1}. For all species, concentrations of *Rhodomonas* sp. decreased more rapidly when *K. brevis* was absent, indicating that *K. brevis* consistently had an inhibitory effect on the consumption of *Rhodomonas* sp. (Fig. 2). A 2-way ANOVA yielded highly significant ($p < 0.0001$) effects of treatment, species, and interactive effects on clearance rates of *Rhodomonas* sp. The presence of *K. brevis* had significant (*T*-test; $p < 0.05$) negative effects on the rates at which *B. neritina* and *S. plicata* cleared *Rhodomonas* sp., with a reduction of 75% and 69%, respectively (Fig. 3). There was no significant effect of *K. brevis* on the rate at which *H. tubifera* or *M. mercenaria* cleared *Rhodomonas* sp.

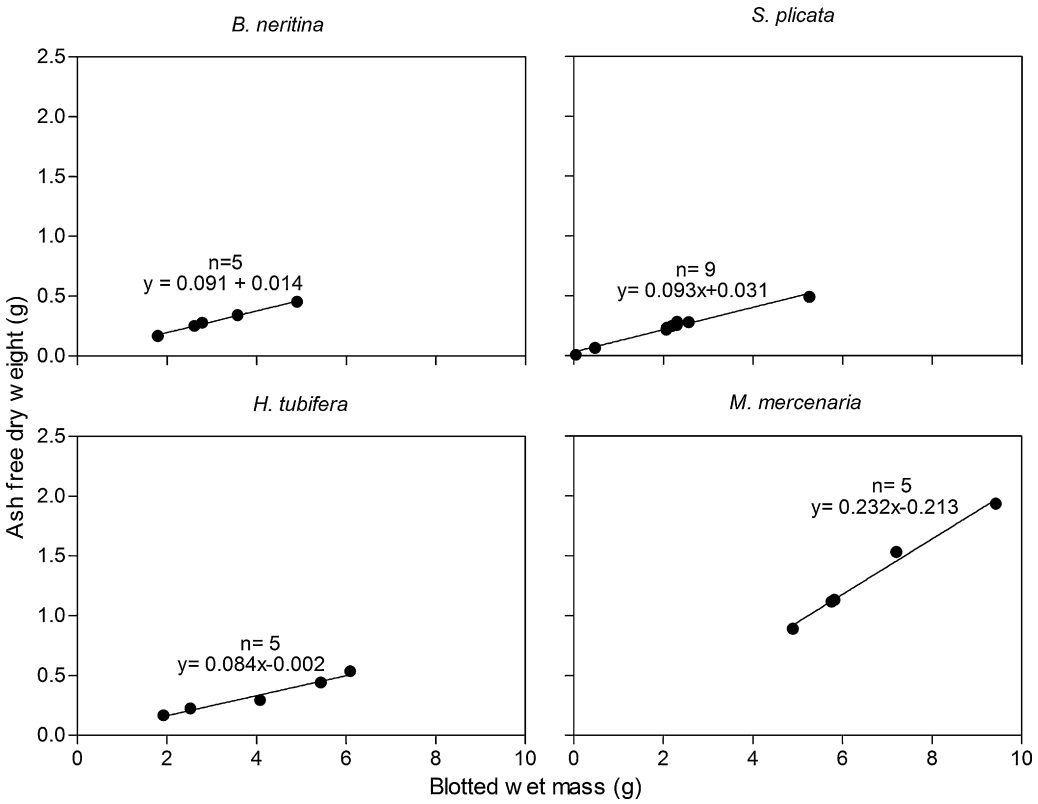

Fig. 1. Relationships between blotted wet mass of invertebrate tissue (BWM) and ash free dry mass (AFDM) for interconversions between body size measurements. Regression lines and equations describing the lines which were used to convert BWM to AFDM are shown.

3.4. Clearance rates of K. brevis

All 4 species of filter-feeding invertebrates rapidly filtered *K. brevis* from seawater (Fig. 2). Mean rates at which suspension feeders cleared *K. brevis* from seawater ranged from 2.27 ± 0.33 L g AFDM L^{-1} h^{-1} for *H. tubifera* to 6.71 ± 0.83 L g AFDM h^{-1} for *S. plicata*. There was a highly significant difference in clearance rates of *K. brevis* among species (ANOVA; $p < 0.0001$). Tukey's HSD test revealed that *B. neritina* and *S. plicata* cleared *K. brevis* from the seawater at a faster rate than *H. tubifera* and *M. mercenaria* ($p < 0.05$) (Fig. 4).

3.5. Recovery of normal clearance rates of Rhodomonas sp.

Data from treatments using *M. mercenaria* were excluded from post recovery step comparisons of clearance rates because the clams did not open during the post-recovery period. Decreases in concentrations of *Rhodomonas* sp. were similar between treatment and control groups during the post-recovery step of the other 3 species (Fig. 2). A repeated measures ANOVA revealed no difference in pre- and post-recovery clearance rates of *Rhodomonas* sp. There were significant differences ($p < 0.05$) in clearance rates of *Rhodomonas* sp. between controls (−*K. brevis*) and treatments (+*K. brevis*) (repeated measures ANOVA). Additionally there were significant ($p < 0.05$) interactive effects between species, treatment, and recovery step on rates at which suspension feeding invertebrates cleared *Rhodomonas* sp. from seawater (repeated measures ANOVA). There was a significant effect ($p < 0.05$) of *K. brevis* on the rates at which *S. plicata* and *B. neritina* cleared *Rhodomonas* sp. during the pre-recovery step, but no effect ($p > 0.05$) on *H. tubifera* (*T*-test) (Fig. 5). Both *S. plicata* and *B. neritina* recovered normal clearance rates of *Rhodomonas* sp. after the recovery step (*T*-test) (Fig. 5). The mean rate at which *B. neritina* exposed to *K. brevis* cleared *Rhodomonas* sp. rose from 2.75 ± 0.38 L g AFDM^{-1} h^{-1} to 9.58 ± 1.94 L g AFDM^{-1} h^{-1} between pre- and post-recovery steps. The clearance rate of *S. plicata* rose from 5.37 ± 0.74 to 11.88 ± 1.62 between pre- and post-recovery steps. No significant non-normality of residuals was found following statistical tests of all clearance rate data (Lilliefors test).

Table 1
Blotted wet mass (BWM) and ash-free dry mass (AFDM) measurements.

Species	Mean body size measurements (g) (SE)			
	Control		Treatment	
	BWM	AFDM	BWM	AFDM
H. tubifera	3.94 (0.38)	0.33 (0.03)	4.01 (0.50)	0.33 (0.04)
B. neritina	3.98 (0.07)	0.37 (0.02)	3.82 (0.35)	0.36 (0.03)
M. mercenaria	6.99 (0.37)	1.41 (0.09)	5.95 (0.18)	1.17 (0.04)
S. plicata	2.28 (0.19)	0.24 (0.02)	2.97 (0.24)	0.31 (0.02)

3.6. Deposition of algal cells

Loss of algal cells from suspension due to the effect of deposition represented a small proportion of the algal cells cleared from suspension as a result of the filtering activity of the suspension feeding invertebrates. Mean deposition rates of *Rhodomonas* sp. were lowest in pre-recovery period control jars (105 ± 71.5 cells mL^{-1} h^{-1}) and highest in post-recovery period control jars (206 ± 81.2 cells mL^{-1} h^{-1}). The mean deposition rate of *K. brevis* was 216 ± 337 cells mL^{-1} h^{-1}. For all experimental groups deposition rates were less than 10% of clearance rates.

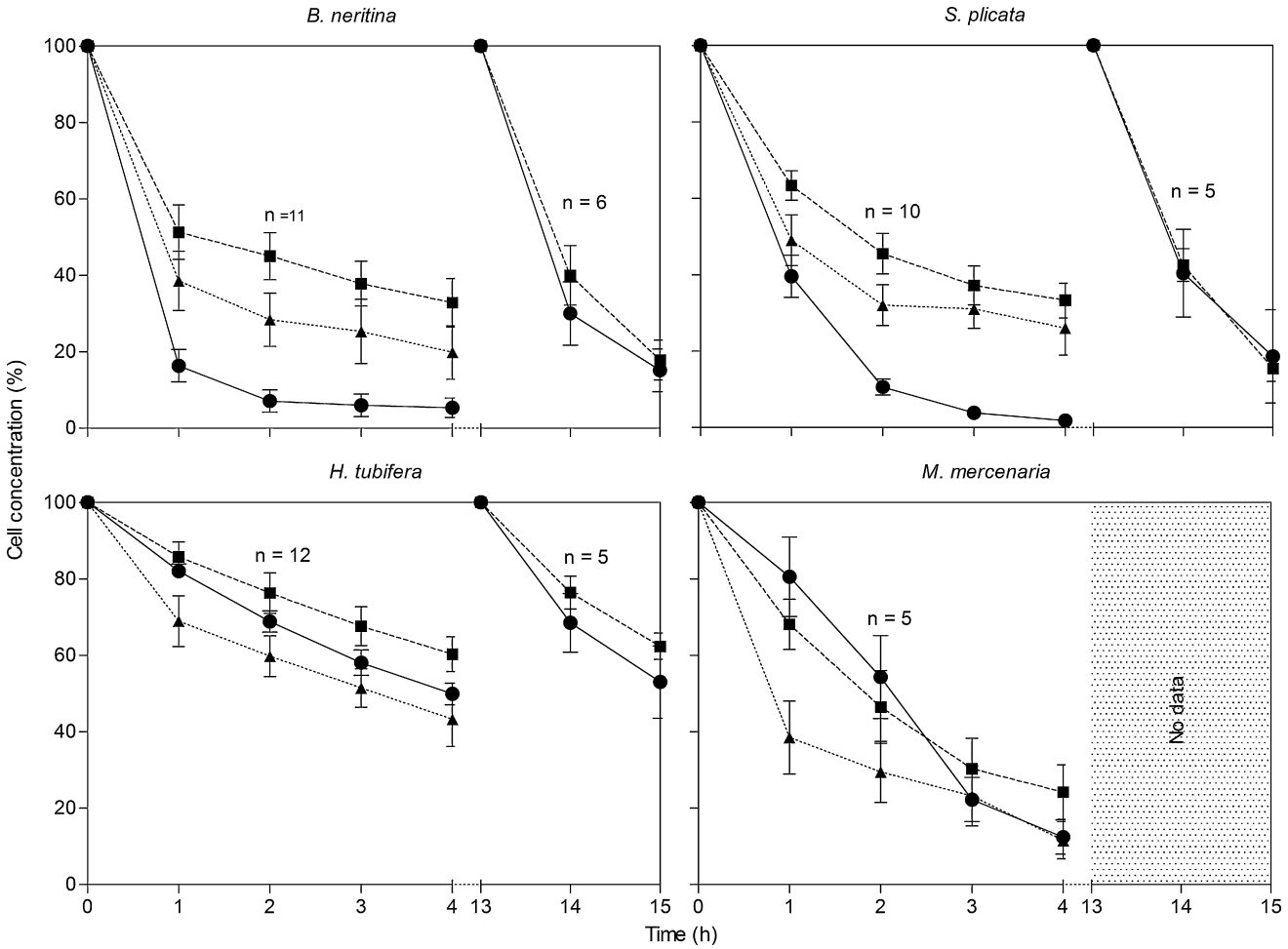

Fig. 2. Mean (±SE) change in concentrations of suspended cells of *Rhodomonas* sp. and *K. brevis* in clearance rate and bioaccumulation assays with and without recovery steps. (■), *Rhodomonas* sp. treatment (+*K. brevis*); (●), *Rhodomonas* sp. control (−*K. brevis*); (▲), *K. brevis*.

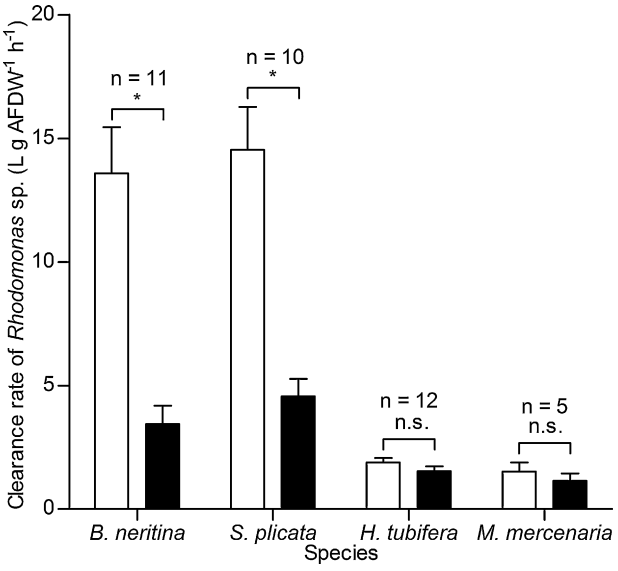

Fig. 3. Mean (±SE) clearance rates of cells of *Rhodomonas* sp. by suspension feeding invertebrates immediately after exposure to *K. brevis* (pre-recovery step). (□), Control (−*K. brevis*); (■), Treatment (+*K. brevis*). Treatments in which there was a significant difference ($p < 0.05$) between treatment and control groups are indicated by * (2-way ANOVA, Bonferroni corrected *T*-test).

3.7. Bioaccumulation and retention of brevetoxins in tissue

All four invertebrate species bioaccumulated significant concentrations of brevetoxin in their tissues after exposure to *K.*

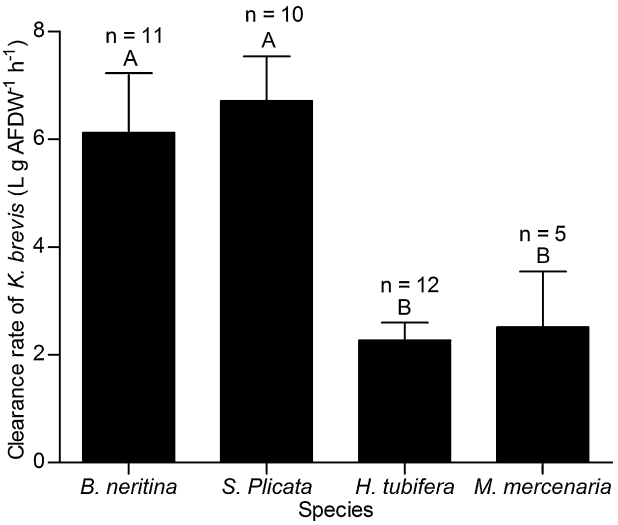

Fig. 4. Mean (±SE) clearance rates of cells of *K. brevis* by suspension feeding invertebrates. Different letters indicate significant differences ($p < 0.05$) in clearance rate between species (1-way ANOVA, Tukey's HSD test).

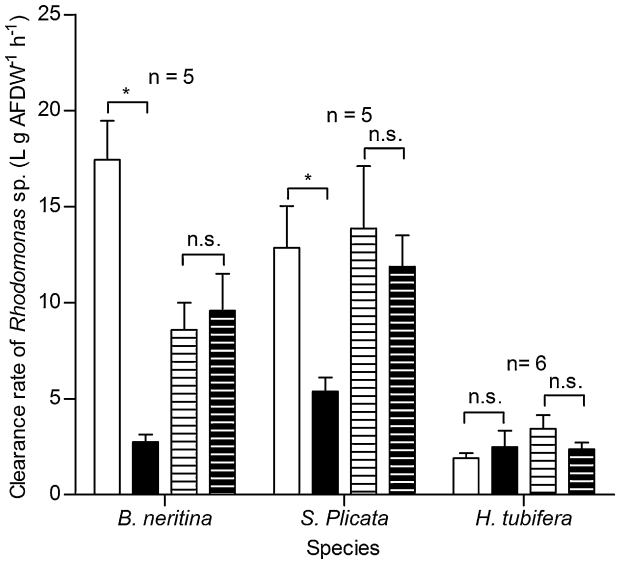

Fig. 5. Mean (±SE) pre- and post-recovery step clearance rates of cells of *Rhodomonas* sp. by suspension feeding invertebrates after 13 h (post-recovery step). (□), control (−*K. brevis*) pre-recovery; (■), treatment (+*K. brevis*) pre-recovery; (▤) control (−*K. brevis*) post-recovery; (▬) treatment (+*K. brevis*) post-recovery. Significant differences ($p < 0.05$) between control and treatment groups before and after recovery steps are indicated by * (Repeated measures 2-way ANOVA, Bonferroni corrected *T*-test).

brevis. The tissue toxicity of each species relative to the others changed between pre- and post-recovery steps indicating that they depurated toxins at different rates (Fig. 6). Mean brevetoxin concentrations in animals that had not experienced a recovery step ranged from 972 ± 262 ng Type B PbTx g BWM^{-1} in *H. tubifera* to 3703 ± 895 ng Type B PbTx g BWM^{-1} in *B. neritina; M. mercenaria* and *S. plicata* had intermediate toxin concentrations (Fig. 6). For animals that had experienced a recovery step, mean brevetoxin concentrations ranged from 171 ± 44 ng Type B PbTx g BWM^{-1} in *H. tubifera* to 1160 ± 202 ng Type B PbTx g BWM^{-1} in *S. plicata; M. mercenaria* and *B. neritina* had intermediate concentrations (Fig. 6). No brevetoxin was detected in the tissue of control animals.

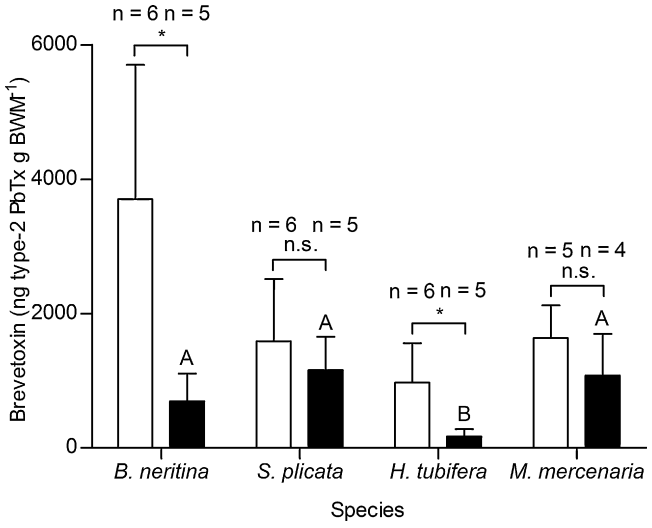

Fig. 6. Mean (±SE) brevetoxin type B concentrations in tissues of filter feeding invertebrates before and after 15 h recovery from exposure to *K. brevis* (pre- and post-recovery steps). (□), no recovery step; (■), with recovery step. Different letters indicate significant differences ($p < 0.05$) in brevetoxin concentrations between species within treatment post-recovery step. There were no significant differences ($p > 0.05$) between species pre-recovery step. Within species differences ($p < 0.05$) before and after recovery are represented by * (2-way ANOVA, Tukey's HSD test).

A two-factor ANOVA revealed that recovery step, species and interactive effects had significant effects ($p < 0.05$) on tissue brevetoxin concentrations. There were no significant differences in pre-recovery step tissue toxin concentrations between species (Tukey's HSD test). Tissue from *H. tubifera* had a significantly lower post-recovery step toxin concentration than other species (Tukey's HSD; $p < 0.05$). There were no significant differences between within species pre- and post-recovery step tissue toxin concentrations of *M. mercenaria* and *S. plicata* (Tukey's HSD test). There were significant differences in pre- and post-recovery step tissue toxin concentrations of *B. neritina* and *H. tubifera* (Tukey's HSD; $p < 0.05$). Toxin concentrations decreased by about 80% between recovery steps for both species (Fig. 6). No significant non-normality of residuals was found following statistical tests of CF data.

Several seawater samples taken from jars after 12 h recovery steps had PbTx Type B concentrations that were below detection limits. Samples that had detectable brevetoxin concentrations were close to the detection limit of the ELISA assay (~0.2 ng mL^{-1}). Seawater samples below the detection limit were counted as zero when calculating means. Toxin concentrations in seawater samples were 1.13 ± 0.67 ng Type B PbTx mL^{-1}, 1.55 ± 0.87 ng Type B PbTx mL^{-1}, 1.42 ± 0.62 ng Type B PbTx mL^{-1}, and 1.04 ± 0.34 ng Type B PbTx mL^{-1} for *H. tubifera*, *B. neritina*, *M. mercenaria*, and *S. plicata*, respectively.

4. Discussion

4.1. Effects of K. brevis on clearance rates of benthic suspension feeding invertebrates

The clearance rate values we obtained for *M. mercenaria*, *S. plicata*, and *H. tubifera* fell within the range of values for species in their respective taxa reported in previous studies (Coughlan and Ansell, 1964 [reported in Riisgard, 2001]; Fiala-Medioni, 1978; Leverone et al., 2007; Petersen et al., 2006; Riisgard, 1988; Riisgard et al., 1993b; Summerel, 2009; Turon et al., 1997). To our knowledge clearance rates of bryozoans were normalized to mass in only one previous study, in which the clearance rate of the bryozoan *Electra bellula* was measured (Lisbjerg and Petersen, 2000). The clearance rate measured for *B. neritina* in the present study is approximately 25% of the clearance rate of *E. bellula* (Lisbjerg and Petersen, 2000). The difference in clearance rates between studies may be due to the different morphologies of *E. belulla* (encrusting) and *B. neritina* (arborescent), and the fact that only replicates with the highest clearance rates were reported for *E. bellula*, possibly resulting in a skewed value.

Sponges, bryozoans, bivalve molluscs, and tunicates each have different mechanisms of particle capture (Armsworthy et al., 2001; Leys and Eerkes-Medrano, 2006; Riisgard, 2001; Riisgard and Manriquez, 1997; Riisgard and Larsen, 2000, 2005; Silverman et al., 1999). Sponges capture very small particles on the microvilli of specialized feeding cells called choanocytes (Leys and Eerkes-Medrano, 2006), but may also phagocytose particles trapped on surface cells that line the system of canals that run through the sponge body. Bryozoans use cilia on their tentacles to direct food particles to the mouths of the many zooids that make up a colony (Riisgard and Manriquez, 1997). Suspension feeding bivalves capture particles by forcing water through a mucus sheet that is transported by cilia on gills and moved to the mouth (Silverman et al., 1999). Like suspension feeding bivalves, tunicates capture particles on sheets on mucus sheets that extend over the surface of the branchial basket (Armsworthy et al., 2001).

Sponges retain 0.3–50 μm particles with approximately 80% efficiency (Reiswig, 1971). The bryozoan *Cellepora hyalina* filtered particles > 6 μm with close to 100% efficiency. Particles < 5 μm are filtered much less efficiently because they pass through the lophophore filter (Riisgard and Manriquez, 1997). The bivalve *M. mercenaria* captured particles > 4 μm with ~100% efficiency (Riisgard, 1988). The solitary tunicate *Holocynthia pyriformis* filtered particles from 5 to 6 μm with 80% efficiency (Armsworthy et al., 2001). Based on previous estimates of particle capture efficiency it may be assumed that all of the suspension feeding invertebrates in the present study filtered *Rhodomonas* sp. (~5–12 μm) and *K. brevis* (~15–27 μm) with high efficiency.

Sponge clearance rates of *K. brevis* had not been calculated prior to this study, however, clearance rates of the toxic okadaic acid-producing dinoflagellate *Prorocentrum hoffmanianum* (length × width = 45 μm × 35 μm) (Perez and Sulkin, 2005) were calculated for five species of Florida Bay sponges. Clearance rates for each species ranged from approximately 0.200–1.3 $L\,h^{-1}\,g\,DM^{-1}$ (Petersen et al., 2006). In the current study the mean clearance rate of *K. brevis* by *H. tubifera* was $1.26 \pm 0.18\,L\,h^{-1}\,g\,DM^{-1}$, which falls near the upper limit of the range of previously calculated values.

Exposure to *K. brevis* did not negatively affect the clearance rate of *H. tubifera*, indicating that the ability of this sponge to maintain water quality during blooms may not be affected by the presence of *K. brevis*. Sponges do not contain neuronal cells and therefore may be immune to the neurotoxic effects of brevetoxins. Brevetoxins interfere with the activity of voltage-sensing sodium channels that are responsible for propagating action potentials in the neuronal cells of animals (Gawley et al., 1995). These sodium–potassium driven action potentials were not observed in demosponges (Carpaneto et al., 2003), which would lead one to predict that sponges would not be affected by brevetoxins.

Both *B. neritina* and *S. plicata* had higher clearance rates of *K. brevis* than *H. tubifera* or *M. mercenaria*, indicating that bryozoans and tunicates can both be important in controlling populations of *K. brevis* during blooms. Despite relatively high clearance rates of *K. brevis*, both *B. neritina* and *S. plicata* had decreased clearance rates of *Rhodomonas* sp. when exposed to *K. brevis*. This feeding inhibition may decrease grazing pressure on *K. brevis* and act as a positive feedback for the bloom formation once cell densities are sufficiently high.

The negative effects of *K. brevis* on clearance rates of *B. neritina* and *S. plicata* represent the first demonstration of a physio-behavioral response of tunicates and bryozoans to harmful algae and environmental toxins presumably because of the neurotoxic effects of brevetoxins or feeding deterrent metabolites. Metabolites produced by *K. brevis* deterred feeding by planktonic rotifers (Kubanek, 2007). Determining whether decreases in clearance rates of *B. neritina* and *S. plicata* are caused by neurotoxic or feeding deterrent effects is a subject for future research, and might be accomplished by treating inert food particles with purified brevetoxins and cellular extracts of *K. brevis* as was done by Kubanek (2007).

The negative effects of the presence of *K. brevis* on clearance rates by *S. plicata* and *B. neritina* were transient, in that they regained normal clearance rates after a short 13 h recovery period. Although mean clearance rates of post-recovery period control and treatment groups of *B. neritina* were lower than the pre-recovery control group (Fig. 5), this is most likely a container effect and reflects a decrease in health of bryozoans during the 12 h recovery period. Therefore exposure to *K. brevis* may not permanently affect the ability of tunicates and bryozoans to perform ecosystem functions such as maintain water quality. Although recovery of clearance rates after exposure to *K. brevis* has not been tested in previous studies, the gastropod molluscs *Fasciolaria hunteria*, *Melongena corona*, and *Oliva sayana* (none of which are filter feeders) all recovered after exhibiting loss of muscle control after exposure to *K. brevis* (Roberts et al., 1979).

The clearance rate of *K. brevis* by *M. mercenaria* calculated by Leverone et al. (2007) was $18.25\,L\,h^{-1}\,g$ dry mass $(DM)^{-1}$, which is considerably higher than the clearance rate that we calculated: $2.04 \pm 0.91\,L\,h^{-1}\,g\,DM^{-1}$. Juvenile hatchery-raised *M. mercenaria* were used in the study conducted by Leverone et al. (2007) while the current study used adult clams that grew under natural conditions. The difference in size of clams and rearing conditions between the two studies may have resulted in the difference in clearance rate observed. Juvenile clams may have higher clearance rates than adult clams due to higher metabolic demands. Also, hatchery raised clams may be better acclimated to lab conditions because they were grown under artificial conditions, resulting in higher clearance rates.

Exposure to *K. brevis* did not negatively affect the clearance rate of *M. mercenaria*. In a previous study *M. mercenaria* had a negative clearance rate response when exposed to 1000 cells *K. brevis* mL^{-1} but not when exposed to 500 cells *K. brevis* mL^{-1} (Leverone et al., 2007). The cell concentration of *K. brevis* used in feeding assays in the current study was only ~700 cells mL^{-1}, which may be lower than the cell concentration that elicits a reduction in clearance rate of *M. mercenaria*. The tolerance thresholds of suspension feeders to exposure of *K. brevis* are of ecological significance. Identifying the concentrations of harmful algae at which suspension feeders experience reductions in clearance rates may be important in determining how well they are able to regulate phytoplankton biomass relative to the density of algal blooms.

It should be noted that the present study was only an initial step toward understanding the impacts of *K. brevis* on suspension feeding invertebrates under natural conditions. We examined the responses of suspension feeders to *K. brevis* during the course of 4 h, with cell concentrations equivalent to those found in a bloom of moderate intensity (Heil and Steidinger, 2009). Blooms in nature occur over the course of several weeks and cell densities may be an order of magnitude higher than those used in the current study and may fluctuate considerably on a daily basis (Pierce et al., 2008). The effect of *K. brevis* on clearance rates of shellfish depends on both exposure time and cell density (Leverone et al., 2007). Future studies should test the effects of longer-term exposure to different concentrations *K. brevis* using invertebrates that are collected from the southwest coast of Florida where *K. brevis* blooms are common.

4.2. Bioaccumulation of brevetoxins

All 4 invertebrate species used in this study accumulated significant concentrations of brevetoxins, representing the first demonstration of brevetoxin accumulation in non-bivalve benthic suspension feeding invertebrates. These results also raise the possibility that benthic suspension feeders may transfer toxins to higher trophic levels during blooms in nature. Different species of suspension feeders may accumulate toxins at different rates depending on the rates at which they clear *K. brevis* from seawater. However, we observed no statistically significant differences in pre-recovery period tissue toxin concentrations between species despite differences in clearance rates of *K. brevis*.

Among bivalve molluscs, the mean concentrations of brevetoxin in tissue (normalized to blotted wet mass) from pre-recovery period clams used in the present study was about 8 fold higher than brevetoxin concentrations in tissue (normalized to wet mass) of cockle (*Austrovenus stutchburyi*) and greenshell mussel (*Perna canaliculus*) exposed to similar concentrations of *K. brevis* cells (Ishida et al., 2004). Differences in tissue toxin concentrations between studies may be due to differences in body size measurements (blotted wet mass vs. wet mass), different methods for extracting and analyzing toxin content (ELISA vs.

liquid chromatography–mass spectometry) or species-specific differences in toxin accumulation.

Levels of tissue toxicity of *M. mercenaria* and *S. plicata* did not decrease significantly after a recovery period, while tissue toxicity of *H. tubifera* and *B. neritina* did. Bryozoans and sponges have simpler body plans than tunicates and clams, and lack longer gut tracts where algal cells and toxins may be processed for longer periods of time. As the length of time after bloom termination increases, bivalves and tunicates may be more important as vectors of brevetoxin to higher trophic levels than bryozoans and sponges. In fact, brevetoxins may be detectable in shellfish tissue several months following bloom termination (Pierce et al., 2004). Monitoring the toxicity levels of different taxa from benthic communities over a time-course following bloom termination would be an important topic for future research.

Shellfish depurate brevetoxins at different rates and through different metabolic pathways, producing metabolites that have variable levels of toxicity (Baden et al., 2005; Dechraoui et al., 2007). Hard clams (*M. mercenaria*) collected during a 2001–2002 bloom were analyzed by LC–MS and contained no PbTx-2 and PbTx-3; they did however, contain cysteine and cysteine-sulfate conjugates of PbTx-2 (Pierce et al., 2004). We were not able to differentiate between different congeners of brevetoxin and brevetoxin metabolites because the ELISA assay nonselectively binds to brevetoxin congeners with type B backbone structures (Naar et al., 2002). The rapid depuration of brevetoxins by *B. neritina* and *H. tubifera* may be due to rapid elimination of undigested cells of *K. brevis,* or these species may more efficiently metabolize brevetoxins than the clam and tunicate.

Differences in tissue composition among the invertebrates used in this study may have also contributed to differences in retention of brevetoxins between species, because brevetoxins accumulate differentially in tissue types. For example, the fishes *Lagodon rhomboides* and *Micropogonias undulates* had higher brevetoxin concentrations in their viscera relative to muscle immediately after brevetoxin exposure, but after a depuration period, toxin concentrations were higher in muscle (Naar et al., 2007).

In conclusion, our results demonstrate that brevetoxins accumulate in the tissues of sponges, tunicates, bryozoans, and bivalves when exposed to cell concentrations of *K. brevis* equivalent to those found in a harmful algal bloom. The same phenomenon is likely to occur under natural conditions, and toxins are likely to be transferred from benthic filter feeders to higher trophic levels. Field sampling and toxin analysis of benthic suspension feeders exposed to blooms of *K. brevis* would be an important next step to quantify bioaccumulation of brevetoxins under natural conditions. Gut content and toxin analysis of animals that consume benthic invertebrates would provide a better understanding of how toxins are transferred between different trophic groups. It is also important to determine how long brevetoxins remain in different species after bloom termination. Using alternate methods of toxin analysis, such as LC–MS or *in vivo* methods such as mouse or cell toxicity bioassays to analyze invertebrate tissues after blooms would provide a more comprehensive view of how toxins are metabolized and eliminated in different groups.

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Author contributions

Michael Echevarria performed all the experiments and statistical analyses as part of his MS thesis research. The tasks of experimental design and manuscript preparation were shared by all the authors.

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