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Sublethal effects of the toxic alga *Heterosigma akashiwo* on the southeastern oyster (*Crassostrea virginica*)

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Abstract

Over the last three years, several blooms of *Heterosigma akashiwo* (Raphidophyceae) were documented in South Carolina (SC) brackish waters, including areas containing extensive oyster (*Crassostrea virginica)* beds. This study examined the sublethal effects of *H. akashiwo* on *C. virginica*, based on cellular biomarker responses after exposure to laboratory cultures of *H. akashiwo* isolated from SC waters, and to water collected from two SC *H. akashiwo* blooms. Exposure to laboratory cultures or blooms of *H. akashiwo* significantly increased oyster hepatopancreas lysosomal destabilization rates, but had little effect on gill p-glycoprotein (p-gp) expression. Lysosomal destabilization in oysters continued to increase even after a 7-day recovery period in clean seawater, suggesting that *H. akashiwo* toxin or other cellular byproducts continued to damage the hepatopancreas. These results suggest that even short-term exposures of oysters to high cell densities of *H. akashiwo* could have long-term adverse physiological effects, and imply that oyster health may be compromised in areas where repetitive *H. akashiwo* blooms occur.
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Keywords: Crassostrea virginica; Harmful algal blooms; *Heterosigma akashiwo*; Lysosomal destabilization; P-glycoprotein; Raphidophytes; Shellfish; Sublethal effects

1. Introduction

Toxin-producing microalgae often form dense blooms (harmful algal blooms or HABs) that can be manifested in a number of ways, ranging from massive "red tides" (blooms that discolor the water) to dilute, inconspicuous concentrations of cells noticed only because of the harm caused by their toxins. Acute lethal and chronic sublethal effects of HABs on a wide

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variety of organisms have been documented, although little is known of the long-term impacts of chronic HAB exposure on ecosystem function or faunal and human health (Shumway et al., 1995; Burkholder, 1998; Landsberg, 2002). This issue is particularly relevant to the sustainability of shellfish populations, which are commonly distributed in waters frequented by HABs and are marked by high capacities for microalgal ingestion and toxin accumulation.

Numerous sublethal, behavioral and physiological effects of HABs on bivalves have been demonstrated, including reduced filtration and feeding rates (Lesser and Shumway, 1993; Matsuyama et al., 1999), decreases in byssus production and oxygen consumption (Shumway et al., 1985, 1987), and shell valve closure (Shumway and Cucci, 1987). The vast majority of these reports come from observed responses to dinoflagellate exposure, with additional references to diatom (*Pseudo-Nitzschia* spp., *Rhizosolenia chunii*), prymnesiophyte (*Chrysochromulina polylepis*, *Phaeocystis pouchetii*), pelagophyte (*Aureococcus anophagefferens*) and cyanobacteria (*Anabaena circinalis*) effects (Table 9 in Landsberg, 2002).

Noticeably lacking among harmful algae linked to adverse shellfish effects are the raphidophytes, a group globally associated with finfish kills (Okaichi, 1989; Chang et al., 1990; Taylor, 1993; Smayda, 1998; Landsberg, 2002). Several mechanisms of toxicity by raphidophytes have been proposed, including the production of brevetoxin-like compounds (Khan et al., 1996a,b, 1997; Bourdelais et al., 2002), mucus or lectin-like polysaccharides (Pratt, 1966; Chang et al., 1990), reactive oxygen species such as superoxide and hydrogen peroxide (Yang et al., 1995; Oda et al., 1997; Twiner and Trick, 2000) and hemaglutinating and hemolysing compounds (Onoue and Nozouwa, 1989; Ahmed et al., 1995). Among these potential stressors, the production of brevetoxin-like substances may have the greatest potential for chronic toxicity to shellfish if accumulation and metabolism of the toxin can occur; e.g. for *Karenia brevis* (Dinophyceae)-derived brevetoxin in oysters (Chen and Chou, 2001; Plakas et al., 2002).

Since 2001, numerous raphidophyte blooms from four species (*Heterosigma akashiwo*, *Chattonella subsalsa*, *C.* cf. *verruculosa*, *Fibrocapsa japonica*) have been documented in South Carolina (SC) coastal waters, primarily in lagoonal stormwater detention ponds (Lewitus and Holland, 2003; Lewitus et al., 2003, 2004). Some of these blooms were associated with measurable levels of brevetoxin-like substances (Lewitus and Holland, 2003), as determined by an ELISA developed at the University of North Carolina at Wilmington (Bourdelais et al., 2002). Because these ponds exchange water and HAB populations with adjacent tidal creeks (Lewitus et al., 2004, unpublished data), the potential exists that raphidophyte blooms derived from the ponds can affect shellfish (primarily oysters, *Crassostrea virginica)* in nearby creeks. Also, blooms of one species (*H. akashiwo*) had been documented in SC tidal creeks since 2001 (Lewitus et al., unpublished data, this study).

On 29 April 2003 and 25 June 2003, *H. akashiwo* blooms occurred in SC estuarine waters that included areas with rich oyster beds. The April bloom extended from inside Bulls Bay to 6–8 km offshore (Fig. 1). The bloom was estimated at ca. 207 km^2, and was associated with a large area of dead fish (estimated at ca. 1 × 10^4 fish). The June bloom occurred in Shem Creek, an inshore creek off of Charleston Harbor (Fig. 1). Bloom material from each of these events was used to evaluate the sublethal cellular effects in oysters exposed to high cell densities of *H. akashiwo*.

Sublethal effects of toxin exposure in oysters can be detected through the use of cellular biomarkers, thought to be among the most sensitive and earliest detectable responses (Ringwood et al., 1999). The biomarkers evaluated in this study represent both a cellular damage response (lysosomal destabilization) and a detoxification response (p-glycoprotein [p-gp] expression). Lysosomes, typically involved in cellular defense, tissue repair and nutrition, can become destabilized through exposure to a variety of stressors. When lysosomal membranes are destabilized (e.g. damaged), the contents leak into the cytoplasm of the cell, which ultimately causes cell death (Moore, 1988, 1990; Lowe, 1996). Recent studies have shown that exposure to purified brevetoxin (PbTx-3; Ringwood, unpublished data) and other harmful dinoflagellates (Lewitus et al., 2003) causes an increase in lysosomal destabilization. P-glycoprotein is a membrane-bound glycoprotein that acts as an efflux pump for organic xenobiotics. P-gp has been found to be over-expressed in bivalves collected at sites with high levels of organic pollution (Minier et al., 1993; Kurelec et al.,
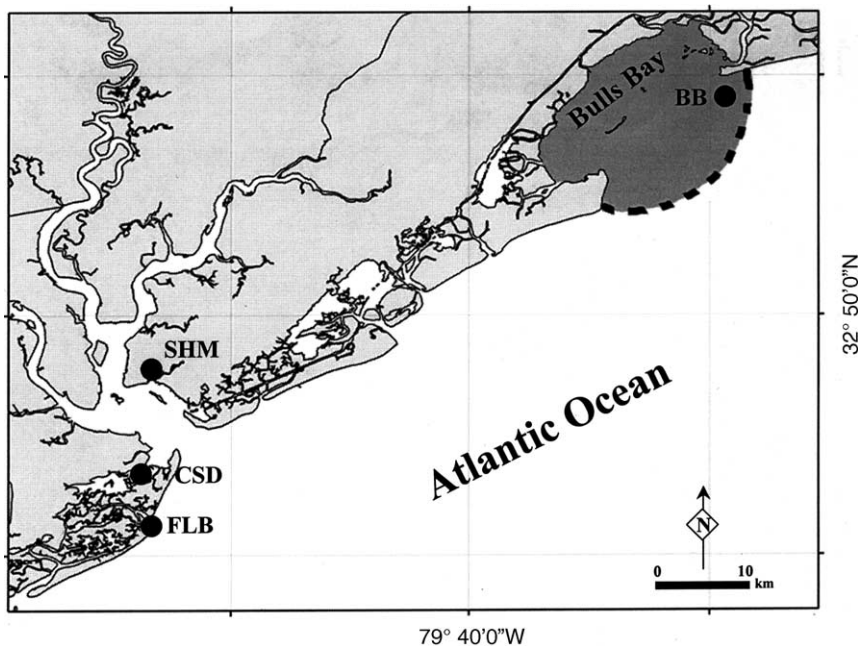
Fig. 1. *Heterosigma akashiwo* bloom and oyster collection sites in South Carolina. FLB (Folly Beach) and CSD (Clark Sound) are control sites, and SHM (Shem Creek) and BB (Bulls Bay) are *H. akashiwo* bloom sites. The shaded area and dotted line indicate the approximate area covered by the 29 April 2003 Bulls Bay bloom.

1996). Recent work has focused on the effects of natural stressors on p-gp expression and activity, because algal extracts have been shown to affect p-gp activity (Eufemia et al., 2002). The overall purpose of the present study was to determine the sublethal cellular effects of *H. akashiwo* exposure on oysters, *C. virginica*, based on lysosomal destabilization and p-gp responses.

2. Materials and methods

2.1. Bloom water

Heterosigma akashiwo bloom material was collected from surface waters offshore of Bulls Bay, SC (33° 00.115′ N, 79° 29.090′ W) on 29 April 2003. *H. akashiwo* abundance was estimated at 1.9 $\times$ 10^4 cells ml^{-1}, 34-fold higher than the next most abundant phytoplankton species, *Kryptoperidinium foliaceum* (Table 1). A second bloom of *H. akashiwo* was sampled from Shem Creek, SC (32° 48.393′ N, 79° 51.545′ W), on 25 June 2003, with *H. akashiwo*

abundance at 2.6×10^4 cells ml^{-1}, again the dominant phytoplankton species (Table 1).

2.2. Culture material

H. akashiwo strain CAAE 1663X was isolated by Cheng Zhang (Center for Applied Aquatic Ecology, NCSU) on 17 April 2001 from a sample collected from Hilton Head, SC, on 11 April 2001. Cultures were maintained on f/2–Si medium (Guillard, 1975) at a salinity of 20‰, on a 12 h light/12 h dark cycle (75 μE m^{-2} s^{-1}) at 25 °C.

2.3. Oyster cellular biomarker response experiments

The effects of *H. akashiwo* on oyster lysosomal destabilization and p-gp expression were determined by comparing these biomarker responses: (1) in field collected resident oysters from bloom waters versus "control oysters" collected from a reference site (described below); (2) in oysters exposed in the laboratory to bloom samples versus those exposed to control site water supplemented with a laboratory cultured alga,

Table 1
Phytoplankton composition of dominant species from two bloom samples.

Bloom	Date	Taxon	Cell (ml^{-1})
Bulls Bay	29 April 2003	*Heterosigma akashiwo* (Raphidophyceae)	19000
		Kryptoperidinium foliaceum (Dinophyceae)	556
		Karlodinium micrum (Dinophyceae)	185
		Katodinium glaucum (Dinophyceae)	165
		Gyrodinium pinque (Dinophyceae	21
		Prorocentrum minimum (Dinophyceae)	<1
		Heterocapsa rotundatum (Dinophyceae)	<1
		Skeletonema sp. (Bacillariophyceae)	<1
Shem Creek	25 June 2003	*Heterosigma akashiwo* (Raphidophyceae)	26000
		Scrippsiella sp. (Dinophyceae)	6757
		Cryptomonas sp. (Cryptophyceae)	2472
		Nitzschia sp. (Bacillariophyceae)	1895
		Navicula sp. (Bacillariophyceae)	1154
		Gymnodinium sp. (Dinophyceae)	412
		Heterocapsa sp. (Dinophyceae)	330
		Coscinodiscus sp. (Bacillariophyceae)	330

Isochrysis galbana; and (3) in oysters exposed to laboratory cultured *H. akashiwo* versus those exposed to laboratory cultured *I. galbana*.

2.3.1. Measurements on resident oysters

A minimum of 20 native oysters were collected from each of the sample sites with *H. akashiwo* blooms, brought to the Marine Resources Research Institute (MRRI) laboratory (Charleston, SC), held overnight in site water, and processed the following day for lysosomal destabilization and p-gp analyses. Oysters were also collected at the same time from control sites either on Folly Beach or Clark Sound, SC (Fig. 1), brought to the MRRI laboratory, held overnight in control site water and processed for the above biomarker measurements. Folly Beach and Clark Sound are considered control sites based on extensive sediment contaminant analyses, which confirmed the low or undetectable levels of metals, polycyclic aromatic hydrocarbons and pesticides at these sites (Hyland et al., 1998; Sanger et al., 1999a,b).

2.3.2. Laboratory exposures of control oysters to bloom water

The day following collection, oysters were scrubbed clean to remove sediment and epibionts, and randomly placed into 3.5 l control seawater and *H. akashiwo* treatments. Each treatment consisted of three replicate containers, with seven oysters in each container

($n = 21$ oysters per treatment). An initial subset of oysters was dissected at the beginning of the experiments to determine the cellular biomarker levels at day zero. The water was renewed after 48 h, i.e. 50% of the exposure water was poured off and replaced with fresh filtered seawater and algae. At the end of the 96-h exposure, oysters were dissected for immediate lysosomal destabilization analysis, and gill tissues were frozen at –80 °C for p-glycoprotein analysis. *Isochrysis galbana* (a non-toxic alga cultured in the laboratory) was diluted to match the *H. akashiwo* bloom concentrations of each exposure (Table 1) and added to the control treatments.

2.3.3. Laboratory exposures of control oysters to Heterosigma akashiwo culture

The laboratory exposures with *H. akashiwo* cultures (initial abundance $= 1.1 \times 10^4$ cell ml^{-1}) were similarly treated, with one additional component. Following the 4-day exposure, half of the oysters were processed for cellular analyses and the other half were put into clean seawater and fed only *I. galbana* for 7 days. Water from these samples was replaced twice over the remaining 7 days as described above. This "recovery period" was added to determine if the effects of *H. akashiwo* exposure were reversible. Oysters used in these experiments were collected on 7 July 2003.

To determine whether the oysters were consuming the algae, counts were conducted at the beginning and

end of the experiments, as well as before and after the water changes. The number of algae in the beakers decreased by roughly an order of magnitude during the initial 48 h (data not shown), suggesting that the algae were being consumed by the oysters, though at a relatively low rate.

2.4. Lysosomal destabilization

The lysosomal destabilization assay was conducted following the methods described in Ringwood et al. (1998). Briefly, digestive gland tissue was diced and incubated on a shaker in calcium/magnesium-free saline (CMFS). Trypsin (Type XIII; Sigma Chemical Company, St. Louis, MO) was added to each sample to complete cellular disaggregation, then cell suspensions were filtered (23 μm mesh) and centrifuged. The pellet was washed twice and resuspended in CMFS. This suspension was mixed 1:1 with a neutral red solution (NR; Sigma Chemical Company) and incubated in the dark at room temperature for 1 h. Digestive gland cells containing lysosomes were examined under a light microscope to evaluate NR retention. A minimum of 50 cells were scored as stable (NR retention in the lysosomes) or destabilized (NR leaking into the cytoplasm), and the data were expressed as the percentage of cells with destabilized lysosomes per oyster.

2.5. P-gp expression

P-gp expression was determined using methods described in Keppler and Ringwood (2001). Briefly, gill tissues were individually weighed and homogenized in lysis buffer and centrifuged. The resulting supernatant was recentrifuged and used for the total protein assay (Bio–Rad Protein Assay, Bio–Rad, Melville, NY) and dot blot analysis. Supernatant samples were standardized to a total protein concentration of 1 mg ml^{-1} by diluting with Tris-buffered saline (TBS). Two 250-μl replicates of each sample were loaded directly onto nitrocellulose paper (using the Bio–Rad Bio–Dot apparatus) and allowed to filter through, followed by a TBS wash. The nitrocellulose paper was then removed from the apparatus, washed in TBS, blocked in TRIS-BLOTTO, rewashed and incubated overnight in C219 primary antibody (mouse monoclonal antibody for mammalian p-gp, Signet Laboratories, Inc., Dedham, MA; 1:2000 in BLOTTO) at 4 °C. The blot was then washed in TBS, incubated in the secondary antibody (goat α-mouse IgG, whole molecule alkaline phosphatase conjugate; Sigma Immunochemicals, St. Louis, MO; 1:500 in BLOTTO) for 2 h, washed in TBS and detected using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazoleum substrate (Sigma Chemical Company). Each blot was analyzed using a Bio–Rad Versadoc 1000 to detect the relative differences in the densities of each dot, and the data are expressed as optical density units per gram protein (Odu g protein^{-1}).

2.6. Statistical analysis

Data were analyzed using Sigma Stat (Jandel Scientific, Chicago, IL). Differences between treatments were examined using *t*-tests or analysis of variance (ANOVA), with the Student–Newman–Keuls method used for multiple comparisons.

3. Results

3.1. Native oysters

Biomarker responses of native oysters collected at the time of a *H. akashiwo* bloom are shown in Fig. 2. Lysosomal destabilization was significantly elevated in native oysters from both sites when compared to oysters collected from a control site (Fig. 2A). P-gp expression was not significantly different between oysters from either site and the control oysters (Fig. 2B).

3.2. Laboratory exposure of oysters to bloom water

Although no mortality occurred, increases in lysosomal destabilization rates were evident in oysters after a 4-day exposure to *H. akashiwo* bloom water collected from both Bulls Bay and Shem Creek (Fig. 3A). Lysosomal destabilization rates were significantly higher than both the *I. galbana* control groups and the initial day zero group. Significant p-gp expression was only observed in oysters exposed to Bulls Bay bloom water (Fig. 3B).
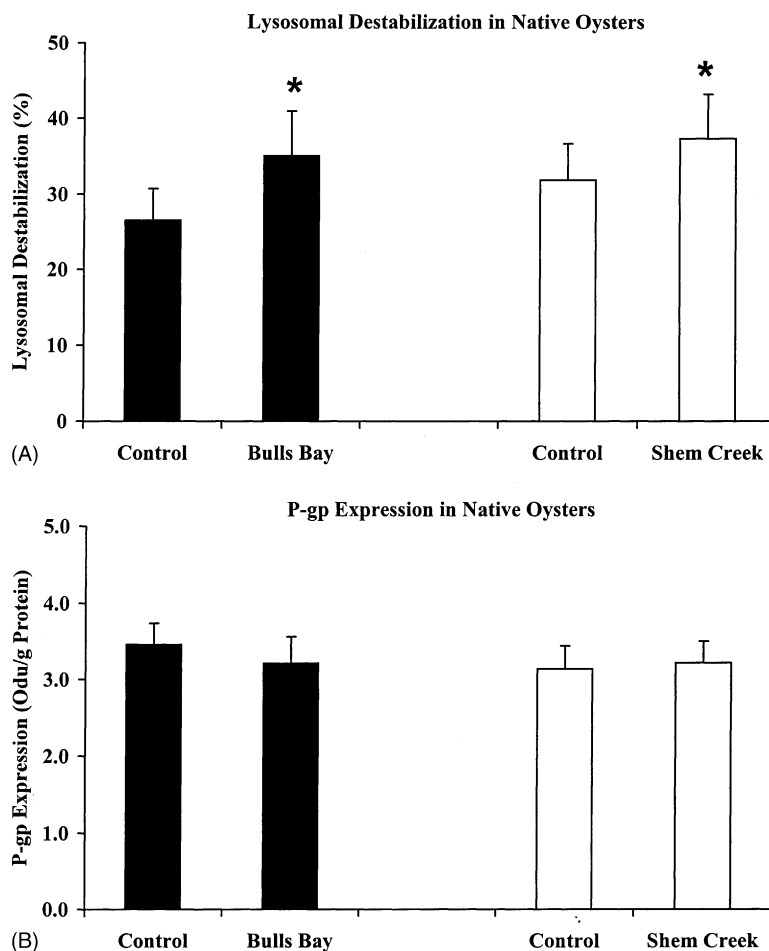
Fig. 2. Cellular biomarker responses in native oysters collected during the *H. akashiwo* blooms ("Bulls Bay", "Shem Creek") and the control site on corresponding dates ("Control"). (A) Lysosomal destabilization. (B) P-gp expression. Values are means + S.D. The symbol (✱) indicates a significant difference from the control oysters.

3.3. Laboratory exposure of oysters to cultured *Heterosigma akashiwo*

Similar to the field collected bloom water exposures, no oyster mortalities occurred over the course of the exposures. Lysosomal destabilization rates significantly increased in oysters exposed to laboratory cultured *H. akashiwo* for 4 days, and these rates continued to increase even after the 7-day recovery period in which the oysters were only exposed to *I. galbana* (Fig. 4A). The destabilization rates in the *H. akashiwo* exposed 7-day recovery oysters were also significantly higher than in the 4-day exposed oysters.

No change occurred in oyster p-gp expression after the 4-day exposure or 7-day recovery period (Fig. 4B).

4. Discussion

Lysosomal destabilization is a well-established and sensitive biomarker of cellular stress in oysters (Ringwood et al., 1999). In this study, laboratory exposures to *H. akashiwo* cultures and natural water samples collected during a *H. akashiwo*-dominated bloom always resulted in increased lysosomal
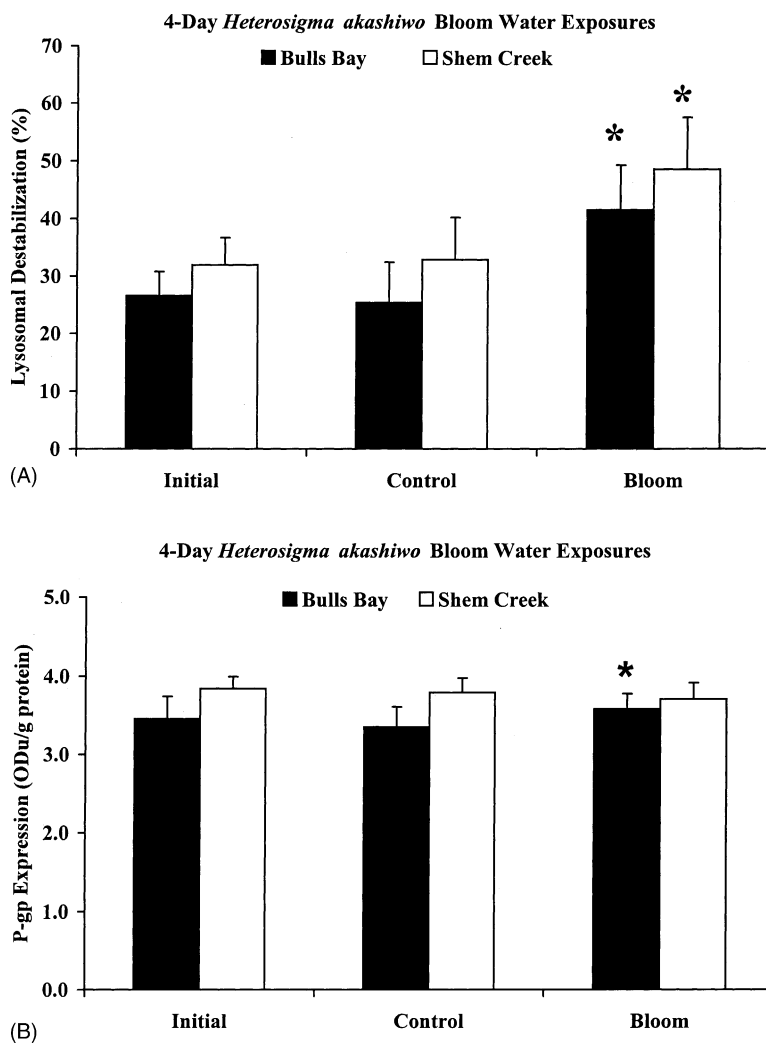
4-Day *Heterosigma akashiwo* Bloom Water Exposures

(A)

4-Day *Heterosigma akashiwo* Bloom Water Exposures

(B)

Fig. 3. Cellular biomarker responses in oysters taken initially ("Initial") and following 4-day exposure to control site water ("Control") or bloom water ("Bloom"). (A) Lysosomal destabilization. (B) P-gp expression. Values are means + S.D. The symbol (✱) indicates a significant difference from the control oysters.

destabilization rates. The similar responses of oysters to cultures and bloom water implicate *H. akashiwo*'s role in the bloom water effects; however, the contribution of other co-occurring phytoplankton cannot be discounted. For example, elevated lysosomal destabilization rates have also been demonstrated in oysters exposed to *Kryptoperidinium foliaceum*-dominated blooms (Lewitus et al., 2003). The levels of lysosomal destabilization measured in *H. akashiwo* culture- and bloom water-exposed oysters (>39% lysosomal destabilization) are indicative of pronounced malfunctioning of the hepatopancreas. Ringwood et al. (2002) demonstrated that the normal range of lysosomal destabilization in oysters is 20–30%, with rates >35% indicating significant stress. Moreover, recent studies have shown that parental rates of lysosomal destabilization >35% are associated with the production of gametes with significantly reduced viability and very poor rates of embryonic development (Ringwood et al., 2004).
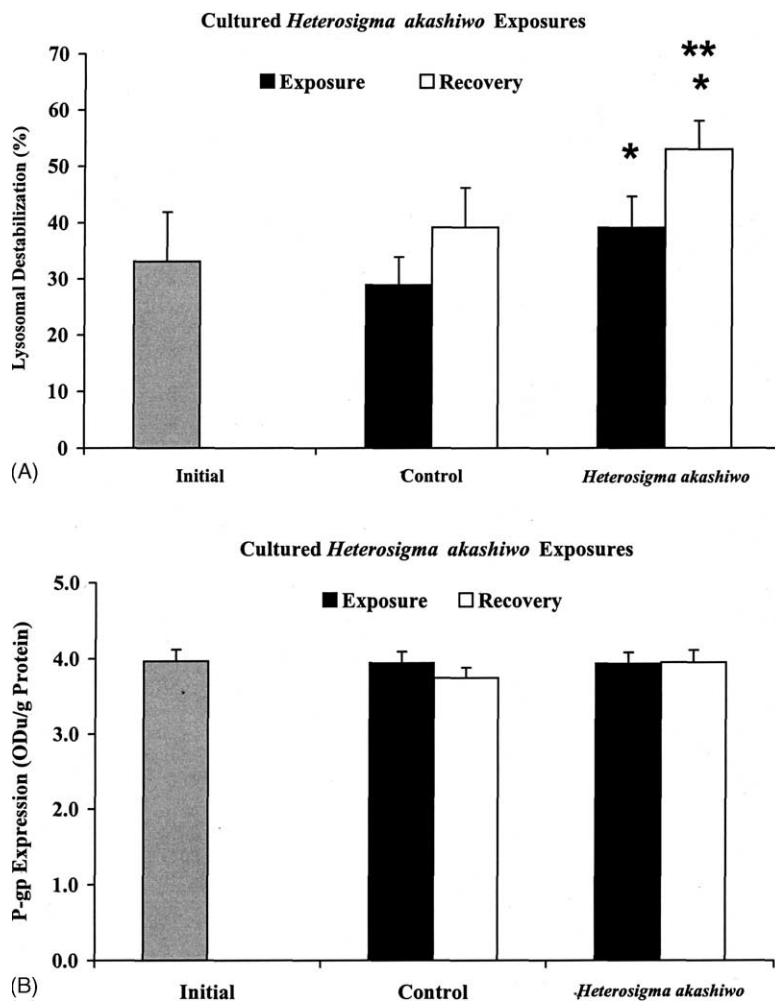
Cultured *Heterosigma akashiwo* Exposures

(A)

Cultured *Heterosigma akashiwo* Exposures

(B)

Fig. 4. Cellular biomarker responses in oysters taken initially ("Initial"), after a 4-day exposure to control site water with *Isochrysis galbana* ("Control") or *Heterosigma akashiwo* cultures (black columns), and after a subsequent 7-day recovery period in *I. galbana*-supplemented water (white columns). (A) Lysosomal destabilization. (B) P-gp expression. Values are means + S.D. The symbol (✱) indicates a significant difference from the control oysters, and (✱✱) indicates a significant difference between the 4-day exposure and 7-day recovery period response.

P-gp expression was not upregulated during the course of the laboratory cultured *H. akashiwo* exposure, nor was there any significant difference in p-gp expression between native oysters from bloom sites and those from control sites. Laboratory exposure of control site oysters to Bulls Bay bloom water increased p-gp expression, but exposure to Shem Creek bloom water had no significant effect. This discrepancy in bloom water effects may relate to seasonal variability

in p-gp expression. Because oyster p-gp activity increases in the summer (Keppler and Ringwood, 2001), we speculate that p-gp expression in oysters collected from the control site in the summer (i.e. during the Shem Creek bloom) was sufficient for detoxification after *H. akashiwo* exposure, but not in oysters collected in the spring (i.e. during the Bulls Bay bloom). The toxin associated with *H. akashiwo* has not been completely characterized, so it is difficult to predict

whether it would be expected to affect p-gp expression. Algal extracts (including seaweed and phytoplankton) and purified brevetoxin have previously been shown to affect p-gp activity, suggesting that algal products are possible p-gp substrates in marine bivalves (Eufemia et al., 2002; Keppler and Ringwood, 2002).

P-gp functions as an important detoxification mechanism, but if upregulation of the protein does not occur, or the toxin is not a substrate of p-gp, accumulation of the toxin could occur. The increase in lysosomal destabilization detected in the hepatopancreas of the "recovery period" oysters suggests that the toxin associated with *H. akashiwo* had not been transported out of the cells, but had accumulated within the cells and continued to cause damage to hepatopancreas tissues. It is also possible that the toxin was not metabolized. *C. virginica* has been shown to metabolize certain forms of brevetoxin (PbTx-2, in particular); however, PbTx-3 is not metabolized and can remain in oyster tissues for up to 2 weeks (Plakas et al., 2002). If the toxin associated with *H. akashiwo* is actually hemolytic or superoxide in nature, then an effect on p-gp expression would not necessarily be expected. Further research is needed to characterize the mechanisms associated with *H. akashiwo* toxicity.

The results from this study indicate that *H. akashiwo* cultures and *H. akashiwo*-associated blooms can cause significant stress to oysters. *H. akashiwo* and other raphidophyte blooms have recently been discovered to be prevalent in SC estuarine waters, including brackish stormwater detention ponds (Lewitus et al., 2003, 2004) and the tidal creeks that exchange water with these ponds (Lewitus, unpub. data). Furthermore, the extensive Bulls Bay bloom sampled in this study illustrates that raphidophyte blooms in SC are not restricted to detention ponds and upper tidal creeks. The extent of raphidophyte-induced sublethal effects on SC shellfish is unknown, but the present study suggests a potential for pronounced short-term effects and, given the continued increase in lysosomal destabilization during a 7-day recovery period, even a short-term exposure to *H. akashiwo* could have long-term adverse effects. The impacts of raphidophyte blooms on reproductive status of the abundant oyster populations in SC tidal creeks need to be explored.

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