Vol. 66: 199–209, 2012
doi: 10.3354/ame01570

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online May 31

Reactive oxygen species are linked to the toxicity of the dinoflagellate *Alexandrium* spp. to protists

Hayley S. Flores[1,3], Gary H. Wikfors[2], Hans G. Dam[1,*]

[1]Department of Marine Sciences, University of Connecticut, 1080 Shennecossett Road, Groton, Connecticut 06340, USA
[2]Northeast Fisheries Science Center, National Marine Fisheries Service, 212 Rogers Avenue, Milford, Connecticut 06460, USA
[3]*Present address:* Algenol Biofuels, 16121 Lee Road, Ft. Myers, Florida 33912, USA

ABSTRACT: Short-term experiments were conducted to examine the response of the ciliate *Tiarina fusus* and the heterotrophic dinoflagellate *Polykrikos kofoidii* to 3 strains in the *Alexandrium tamarense* species complex, each with a different paralytic shellfish toxin (PST) content. Both protist species fed on all 3 *Alexandrium* strains, but significant mortality occurred within 24 h of initial exposure to high densities of each dinoflagellate isolate. Protist mortality was not related, however, to the PST content of the *Alexandrium* strains, indicating a different mechanism of toxicity. Exposure of *T. fusus* to cell-free culture filtrates or cell extracts did not cause significant ciliate mortality. In contrast, significant mortality occurred when ciliates were separated physically from a live *Alexandrium* sp. culture by a 10 µm nylon mesh, suggesting that the toxicity is dependent upon the viability of the *Alexandrium* spp. cells but does not require physical contact or ingestion. Addition of antioxidant compounds significantly increased the survival of both protist species when exposed to *Alexandrium*, suggesting that reactive oxygen species and/or the secondary compounds produced by ROS-induced lipid peroxidation are involved in the toxicity of *Alexandrium* spp. to ciliates and heterotrophic dinoflagellates. This mechanism of toxicity is previously unknown for *Alexandrium* spp. and may play an important role in bloom dynamics and toxin transfer within the food web.

KEY WORDS: *Alexandrium* · Ciliate · Harmful algae · Heterotrophic dinoflagellate · *Polykrikos kofoidii* · Reactive oxygen species · *Tiarina fusus*

INTRODUCTION

Harmful algal blooms (HABs) in marine ecosystems are increasing worldwide, presenting a scientifically complex and economically significant challenge to the management of coastal waters (Smayda 1990, Hallegraeff 1993). Among HAB species, dinoflagellates in the genus *Alexandrium* are among the most ecologically important because some species produce neurotoxins referred to as paralytic shellfish toxins (PSTs). PSTs can be accumulated by filter-feeding shellfish and other grazers and transferred to humans and other animals, leading to severe illness and possibly death (White 1980, Shumway 1990, Durbin et al. 2002). Given the potential economic and human health risks associated with blooms of toxic *Alexandrium* spp., it is important to understand the mechanisms controlling the population dynamics of these harmful dinoflagellates.

Grazing is thought to be an important biological factor influencing the formation and termination of HABs (Buskey et al. 1997, Turner & Tester 1997, Colin & Dam 2007, Smayda 2008). Microzooplankton, particularly ciliates and heterotrophic dinoflagellates, are often the most active grazers of phytoplankton, consuming 60 to 70% of daily planktonic primary production (Sherr & Sherr 2002, Calbet & Landry 2004, Calbet 2008). Although high abun-

*Corresponding author. Email: hans.dam@uconn.edu

dances of ciliates and heterotrophic dinoflagellates have been observed during blooms of *Alexandrium* spp. (Needler 1949, Prakash 1963, Watras et al. 1985, Carreto et al. 1986), the interactions of these protists with *Alexandrium* spp. are not well understood. Several heterotrophic protist species have been reported to ingest *Alexandrium* spp. with no apparent adverse effects (Stoecker et al. 1981, Hansen 1992, Matsuoka et al. 2000, Kamiyama et al. 2005), but other protists exhibit altered swimming behavior, reduced ingestion, growth inhibition, or mortality (Hansen 1989, Hansen et al. 1992, Tillmann & John 2002, Fistarol et al. 2004, Fulco 2007, Tillmann et al. 2007, 2008). The disparate results from previous research cannot be attributed to differences in the PST content of the algal isolates (Tillmann & John 2002, Tillmann et al. 2008, 2009). Instead, it appears that uncharacterized metabolites produced by *Alexandrium* spp. are responsible for the toxicity of these dinoflagellates to protists (Tillmann & John 2002, Fistarol et al. 2004). These harmful compounds are often referred to as 'allelochemicals,' i.e. secondary metabolites that inhibit the growth of competing organisms (Legrand et al. 2003, Granéli & Hansen 2006). In marine microbial ecology, the category of allelochemicals can include compounds that incapacitate or deter grazers (Cembella 2003, Granéli & Hansen 2006).

The bioactive, allelochemical compounds produced by *Alexandrium* spp. affect the structure and function of cell membranes, causing immobilization of co-occurring protist cells, followed by cell swelling and lysis (Hansen 1989, Tillmann & John 2002). The specific mode of action, however, is currently unknown. Emura et al. (2004) speculated that a protein-like toxin is responsible for the lytic activity of *Alexandrium* spp. Further research by Ma et al. (2009) has suggested that the allelochemicals may be amphipathic compounds that form large aggregates or macromolecular complexes. Elevated concentrations of reactive oxygen species (ROS) can disrupt a variety of cellular processes, including cell membrane integrity (Halliwell & Gutteridge 1985). Although these compounds have been linked to the toxicity of other HAB species to aquatic organisms (Yang et al. 1995, Ishimatsu et al. 1997, Kim et al. 1999, Tang & Gobler 2009), their possible role in the toxicity of *Alexandrium* spp. to grazers has not been examined. Determination of the mechanism of toxicity is needed to better understand the interactions between heterotrophic protists and *Alexandrium* spp. and to evaluate the role of these grazers in the formation and termination of blooms.

In the present study, we investigated the effects of 3 isolates in the *Alexandrium tamarense* species complex on the survival of the ciliate *Tiarina fusus* and the heterotrophic dinoflagellate *Polykrikos kofoidii*. Further, we tested a hypothesis that ROS are linked to the toxicity of *Alexandrium* spp. to protists by examining the effect of free-radical-scavenging enzymes on the survival of *T. fusus* and *P. kofodii* exposed to *Alexandrium*.

MATERIALS AND METHODS

Experimental cultures

Three strains in the *Alexandrium tamarense* species complex (Table 1; hereafter referred to as *Alexandrium* spp.) and one strain each of the dinoflagellates *Lingulodinium polyedra* and *Scrippsiella trochoidea* were maintained in f/2 medium without silicate (Guillard 1975) at 18°C on a 12:12 h light:dark cycle. The cultures were transferred biweekly to fresh medium and were in exponential growth for all experiments. The cultures were not axenic, but aseptic techniques were used to minimize additional microbial contamination. Prior to experimentation, *Alexandrium* spp. strains were examined for the production of PSTs. Triplicate samples were extracted according to Anderson et al. (1994) and analyzed using high performance liquid chromatography for saxitoxin (STX), neosaxitoxin (NEO), and gonyautoxins I-IV (GTX1-4) using the methods of Oshima et al. (1989). Toxin standards were obtained from the National Research Council, Marine Analytical Chemistry Standards Program, Halifax, Nova Scotia, Canada. Based upon the analysis, the 3 *Alexandrium* sp. isolates were designated 'High PST' (NB-05), 'Low PST' (CB-307), and 'No PST' (CCMP115) (Table 1).

Table 1. *Alexandrium* spp. strains, source location, isolation date, and toxin content. STX eq: saxitoxin equivalent. –: no measurable toxin content

Strain name	Source location	Isolation date	Average toxin content (pg STX eq. cell^{-1})
NB-05	Bay of Fundy, NB	2001	22.25
CB-307	Casco Bay, ME	2001	11.98
CCMP115	Tamar Estuary, UK	1957	–

The ciliate *Tiarina fusus* was isolated from Long Island Sound off Avery Point, CT, in June 2008. Ciliate cultures were maintained in 25 cm^2 polystyrene tissue-culture flasks containing 20 ml of f/2 medium, to which the dinoflagellate *Lingulodinium polyedra* was added as a food source. The heterotrophic dinoflagellate *Polykrikos kofoidii* was isolated from Northport Bay, located on the north shore of Long Island, NY, during a bloom of *Alexandrium* spp. in May 2009. *P. kofoidii* cultures were maintained in 6 well polystyrene tissue-culture plates and were fed a mixture of *L. polyedra* and *Scrippsiella trochoidea*. All heterotrophic protist cultures were incubated at 18°C with a 12:12 h light:dark cycle and were transferred weekly or biweekly into fresh medium containing prey.

Interactions between *Alexandrium* spp. and heterotrophic protists

Observational experiments were conducted to qualitatively examine the effects of each *Alexandrium* spp. strain upon *Tiarina fusus* and *Polykrikos kofoidii*. Groups of 25 *T. fusus* or *P. kofoidii* cells were transferred by micropipette into individual wells of 12 well polystyrene tissue-culture plates containing 2 ml of 0.2 µm filtered seawater (FSW). Both of the heterotrophic protist species were starved for 24 h prior to experimentation to ensure digestion of any recently ingested *Lingulodinium polyedra* or *Scrippsiella trochoidea* cells from the stock cultures. Following starvation, aliquots of each *Alexandrium* spp. culture were added to the wells containing *T. fusus* or *P. kofoidii*. For each *Alexandrium* spp. strain, cell densities of 200 and 2000 cells ml^{-1} were tested. Controls consisted of FSW and *L. polyedra* (200 or 2000 cells ml^{-1}). The behavior of individual *T. fusus* and *P. kofoidii* cells was observed under a stereomicroscope at 15 min intervals for 2 h.

Effect of *Alexandrium* sp. cell density

Results from the observational experiments indicated that exposure of *Tiarina fusus* and *Polykrikos kofoidii* to all 3 *Alexandrium* spp. strains caused noticeable protist mortality. Therefore, a quantitative experiment was conducted to examine the effect of *Alexandrium* spp. cell densities on *T. fusus* and *P. kofoidii* survival. *Alexandrium* spp. cultures (High, Low, and No PST strains; 1900 to 3000 cells ml^{-1}) were diluted with f/2 medium to yield 5 cell densities, ranging from 63 to 1000 cells ml^{-1}. From each dilution, 5 ml (in triplicate) was added to each well of 12 well polystyrene tissue-culture plates. Groups of 15 ciliates or heterotrophic dinoflagellates were added to each experimental well, and treatments were incubated for 24 h at 18°C on a 12:12 h light:dark cycle. Following incubation, acidic Lugol's solution (2% final concentration) was added to each well, and intact *T. fusus* or *P. kofoidii* cells were enumerated by light microscopy. Controls consisted of the dinoflagellate *Lingulodinium polyedra* (63 to 1000 cells ml^{-1}) and 0.2 µm filtered seawater (FSW) and were also conducted in triplicate.

Culture filtrates and extracts

Additional experiments were conducted with *Tiarina fusus* to examine the effects of cell-free *Alexandrium* spp. culture filtrates and extracts upon ciliate survival. *Alexandrium* spp. cultures (High, Low, and No PST strains; 1700 to 3300 cells ml^{-1}) were diluted with f/2 medium to a density of 1000 cells ml^{-1}. An aliquot (20 ml) of each *Alexandrium* spp. culture was filtered gently through a 0.2 µm syringe filter, resulting in a filtrate free of both *Alexandrium* spp. cells and bacteria. To examine the possible effects of bacteria present in the *Alexandrium* spp. cultures on *T. fusus* survival, additional aliquots (20 ml) from each *Alexandrium* spp. culture were filtered through 5.0 µm syringe filters, allowing bacteria, but not *Alexandrium* spp. cells, to pass through the filter. Cell extracts from *Alexandrium* spp. cultures were prepared by sonicating *Alexandrium* spp. culture aliquots (20 ml), on ice, with a Fisher model 100 sonic dismembrator until cells were completely disrupted (as confirmed by microscopy). Following sonication, the extracted samples were filtered through a 0.2 µm syringe filter to remove cell debris. Filtrates (0.2 to 5.0 µm filtered) and extracts were added (5 ml; in triplicate) to individual wells of a 12 well polystyrene tissue-culture plate. Controls consisted of intact *Alexandrium* spp. cultures (1000 cells ml^{-1}) and FSW. A total of 15 ciliates were added to each experimental well, and treatments were incubated and enumerated as described above.

Physical separation from live *Alexandrium* spp. cultures

To determine if the observed mortality of *Tiarina fusus* exposed to *Alexandrium* spp. was a result of physical contact with and/or ingestion of the dinoflagellate, groups of 15 *T. fusus* cells were placed into

individual wells of 12 well polystyrene tissue-culture plates containing FSW (2.5 ml). A culture plate insert with a 10 µm nylon mesh bottom was added to each experimental well. Aliquots (1.5 ml) of each *Alexandrium* spp. culture (High and No PST; ~2800 cells ml^{-1}) were added to each culture insert, resulting in a final concentration of dissolved compounds in the treatment equivalent to a ~1000 cells ml^{-1} *Alexandrium* spp. culture. The 10 µm mesh separating the *Alexandrium* spp. culture from the *T. fusus* cells prevented physical contact between the species while permitting exchange of dissolved compounds. Controls consisted of *Alexandrium* spp. cultures (1000 cells ml^{-1}) in direct contact with *T. fusus* as well as FSW. All of the experimental treatments and controls were conducted in triplicate and were incubated and enumerated as described in the above experiments.

Mitigation of toxicity

To test the hypothesis that reactive oxygen species play a role in the toxicity of *Alexandrium* spp. to heterotrophic protists, an experiment was conducted to examine the effects of scavengers of reactive oxygen species on *Tiarina fusus* and *Polykrikos kofoidii* survival when exposed to *Alexandrium* spp. The antioxidant enzymes peroxidase (MP Biomedicals, #191370), catalase (MP Biomedicals, #100429), and superoxide dismutase (MP Biomedicals, #190117) were prepared as aqueous solutions according to manufacturer specifications. All of the solutions were used within 1 h of preparation or were frozen immediately (–20°C) and thawed just before use. Protein-like compounds are thought to play a role in the toxicity of a related species, *Alexandrium taylori*, to mammalian cells (Emura et al. 2004). For this reason, an additional treatment testing the protease trypsin was included to examine the possibility that protein or protein-like compounds are responsible for the toxicity of *Alexandrium* spp. to protists. *Alexandrium* spp. cultures (High, Low, and No PST; 1500 to 2800 cells ml^{-1}) were diluted with f/2 medium to a density of 1000 cells ml^{-1}. Each *Alexandrium* spp. culture was subdivided, and peroxidase (1.25 µg ml^{-1}), catalase (2 U ml^{-1}), superoxide dismutase (5 U ml^{-1}), or trypsin (500 µg ml^{-1}) was added. Similar concentrations of these compounds were shown to mitigate the toxicity of the dinoflagellate *Cochlodinium polykrikoides* to the sheepshead minnow *Cyprinodon variegates* (Tang & Gobler 2009). Aliquots (5 ml, in triplicate) of each culture were added to individual wells of 12 well polystyrene tissue-culture plates. A group of 15 *T. fusus* or *P. kofoidii* cells was added to each experimental well, and treatments were incubated and enumerated as described above. Controls consisted of ciliates and heterotrophic dinoflagellates exposed to *Alexandrium* spp. cultures without the addition of the enzymes and also FSW with the addition of each enzyme.

Statistics

Differences among the treatments were assessed using 1-way or 2-way ANOVA. Post hoc comparisons employed the Tukey-Kramer method. In all cases, significance levels were set at $p < 0.05$.

RESULTS

Effects on *Tiarina fusus* and *Polykrikos kofoidii*

When exposed to low densities (200 cells ml^{-1}) of each *Alexandrium* spp. strain (High, Low, and No PST), *Tiarina fusus* and *Polykrikos kofoidii* cells continued to swim normally in a forward direction, and individuals were observed feeding on the dinoflagellate with no apparent adverse effects. Following the 2 h incubation, most ciliates and heterotrophic dinoflagellates contained 1 to 2 ingested *Alexandrium* spp. cells. In contrast, exposure to a high cell density of each *Alexandrium* spp. strain (2000 cells ml^{-1}) caused many *T. fusus* cells to start swimming backward within 5 to 10 min, and feeding attempts were not observed. For most ciliates, complete loss of motility followed by cell lysis occurred within 15 to 30 min. The response of *P. kofoidii* to a high cell density of *Alexandrium* spp. was nearly identical to that of *T. fusus,* except that no backward swimming was observed. No negative effects were observed when *T. fusus* or *P. kofoidii* was exposed to the dinoflagellate *Lingulodinium polyedra*, regardless of the cell concentration.

Effect of *Alexandrium* spp. cell density

The survival of *Tiarina fusus* was dependent on both the *Alexandrium* spp. strain and the cell density (Fig. 1) (2-way ANOVA, $p < 0.001$). The High PST and Low PST *Alexandrium* spp. strains caused significant *T. fusus* mortality at densities ≥250 cells ml^{-1}, relative to the *Lingulodinium polyedra* and FSW controls ($p < 0.001$). Cell densities of 500 cells ml^{-1} were required to cause significant lysis of *T. fusus* cells exposed to the

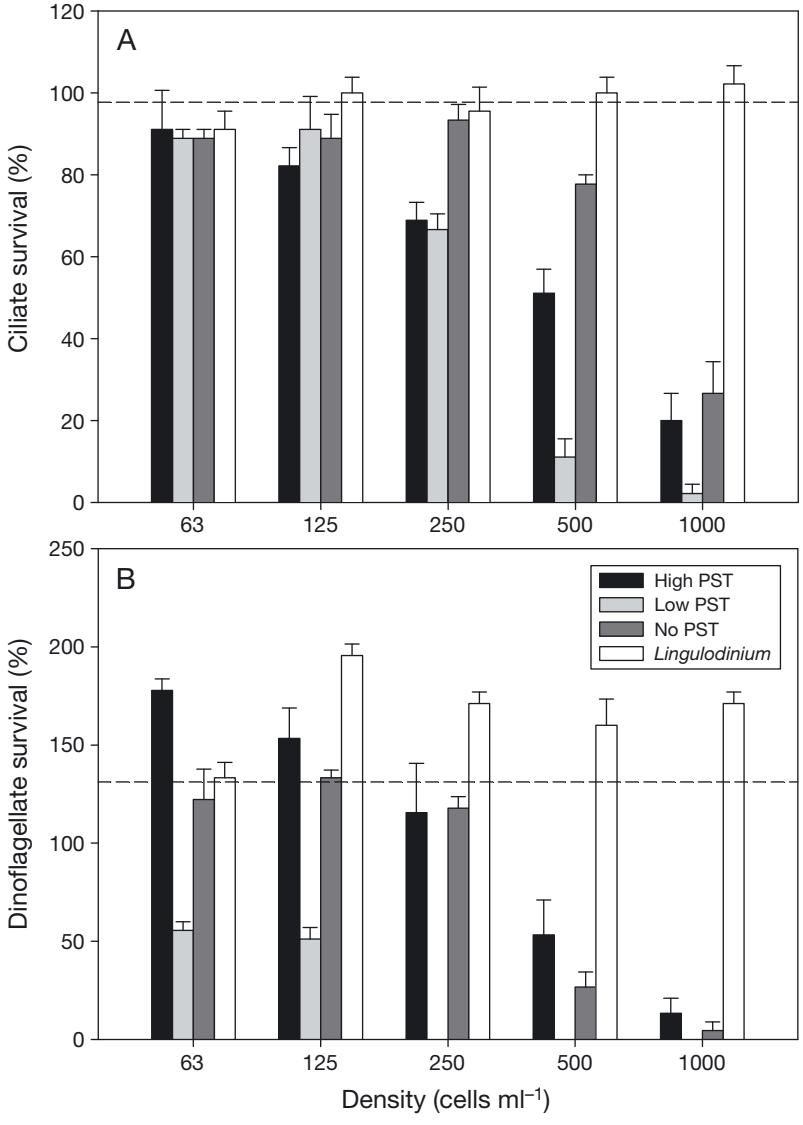

Fig. 1. Survival as a function of *Alexandrium* sp. cell density for (A) the ciliate *Tiarina fusus* and (B) the heterotrophic dinoflagellate *Polykrikos kofoidii* following 24 h exposure to strains with different paralytic shellfish toxin (PST) contents (High, Low, No PST) or a nontoxic dinoflagellate *Lingulodinium polyedra*. Values exceeding 100% on the *y*-axis represent both survival and growth of protists. Dashed line represents mean survival in the filtered seawater (FSW) control. Data are means ± SE (n = 3 per treatment)

No PST *Alexandrium* sp. isolate (Fig. 1A; $p = 0.002$). *L. polyedra* did not cause ciliate cell lysis, and *T. fusus* survival was not affected by the density of *L. polyedra* cells (Fig. 1A; $p > 0.1$). *Polykrikos kofoidii* survival following exposure to *Alexandrium* spp. also varied significantly depending on the isolate and the cell density (Fig. 1; $p < 0.001$). When *P. kofoidii* was exposed to the low density (63 cells ml^{-1}) of the *Alexandrium* sp. strains, the survival was statistically similar to the *L. polyedra* and FSW treatments ($p > 0.05$); however, cell densities ≥500 cells ml^{-1} resulted in significant lysis of *P. kofoidii* cells (Fig. 1B; $p < 0.001$). The Low PST *Alexandrium* sp. was toxic to *P. kofoidii* at all tested cell densities ($p < 0.001$). The survival of *P. kofoidii* exposed to 63 to 250 cells ml^{-1} of the No PST strain was not different from that in the FSW control ($p > 0.9$), but at densities ≥125 cells ml^{-1}, survival was significantly lower than in the *L. polyedra* treatment ($p < 0.02$). High densities (≥500 cells ml^{-1}) of the No PST *Alexandrium* sp. strain resulted in significant *P. kofoidii* mortality, relative to both the *L. polyedra* and FSW controls ($p < 0.001$). Exposure to *L. polyedra* did not cause *P. kofoidii* mortality at any of the tested cell densities.

Culture filtrates and extracts

The *Alexandrium* spp. culture filtrates (0.2 µm) and sonicated cell extracts (High, Low, and No PST) did not cause lysis of *Tiarina fusus* cells, and the ciliate survival was significantly higher than in the live, intact *Alexandrium* spp. treatments (Fig. 2) (1-way ANOVA, $p < 0.001$). Similarly, the 5.0 µm filtrate from the High and No PST *Alexandrium* spp. cultures did not cause significant *T. fusus* mortality ($p < 0.001$). Significant mortality occurred when ciliates were exposed to the 5.0 µm filtrate from the Low PST *Alexandrium* sp. strain ($p < 0.001$); however, survival was significantly higher in this treatment than in the live, intact, Low PST *Alexandrium* sp. treatment (Fig. 2) ($p < 0.001$).

Physical separation

Exposure of *Tiarina fusus* to live *Alexandrium* spp. cultures (High PST and No PST) that were physically separated from the ciliates by a 10 µm nylon mesh resulted in significant ciliate mortality relative to the filtered seawater (FSW) controls (Fig. 3) (1-way ANOVA, $p < 0.001$). *T. fusus* survival in the mesh treatments was, however, significantly higher than in treatments in which ciliates were in direct contact with *Alexandrium* spp. cells ($p < 0.05$).

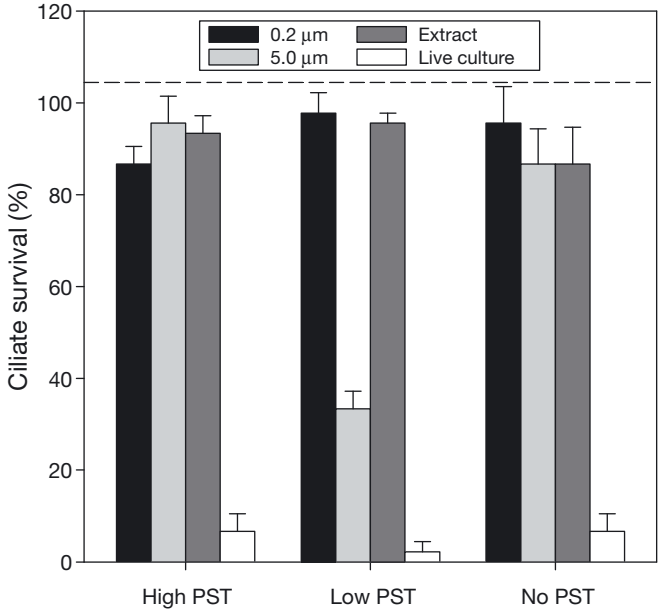

Fig. 2. *Tiarina fusus.* Survival of the ciliate following 24 h exposure to 0.2 µm and 5.0 µm filtrates or sonicated cell extracts from *Alexandrium* spp. cultures. Live, intact *Alexandrium* spp. cells served as controls. Dashed line represents mean survival in the filtered seawater (FSW) control. Data are means ± SE (n = 3 per treatment). PST: paralytic shellfish toxin

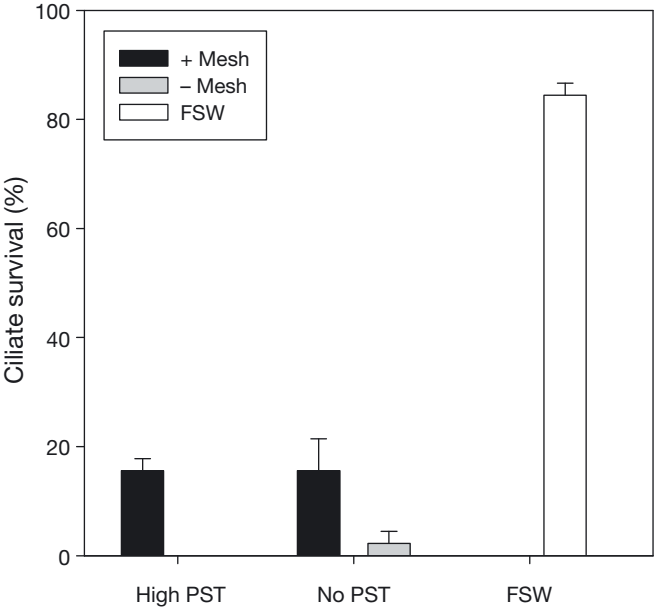

Fig. 3. *Tiarina fusus.* Survival of the ciliate when separated from living *Alexandrium* spp. cultures by a 10 µm mesh (+ mesh). Controls included exposure to live *Alexandrium* spp. cultures without separation (–mesh) and filtered seawater (FSW). Data are means ± SE (n = 3 per treatment). PST: paralytic shellfish toxin

Mitigation of toxicity

The addition of the enzyme peroxidase significantly increased the survival of both *Tiarina fusus* and *Polykrikos kofoidii* exposed to the High PST and No PST *Alexandrium* spp. strains relative to the no-addition control, which consisted of *Alexandrium* spp. without the addition of any enzymes (Fig. 4) (1-way ANOVA; High PST, $p < 0.001$ for both *T. fusus* and *P. kofoidii*; No PST, $p = 0.04$ for *T. fusus* and

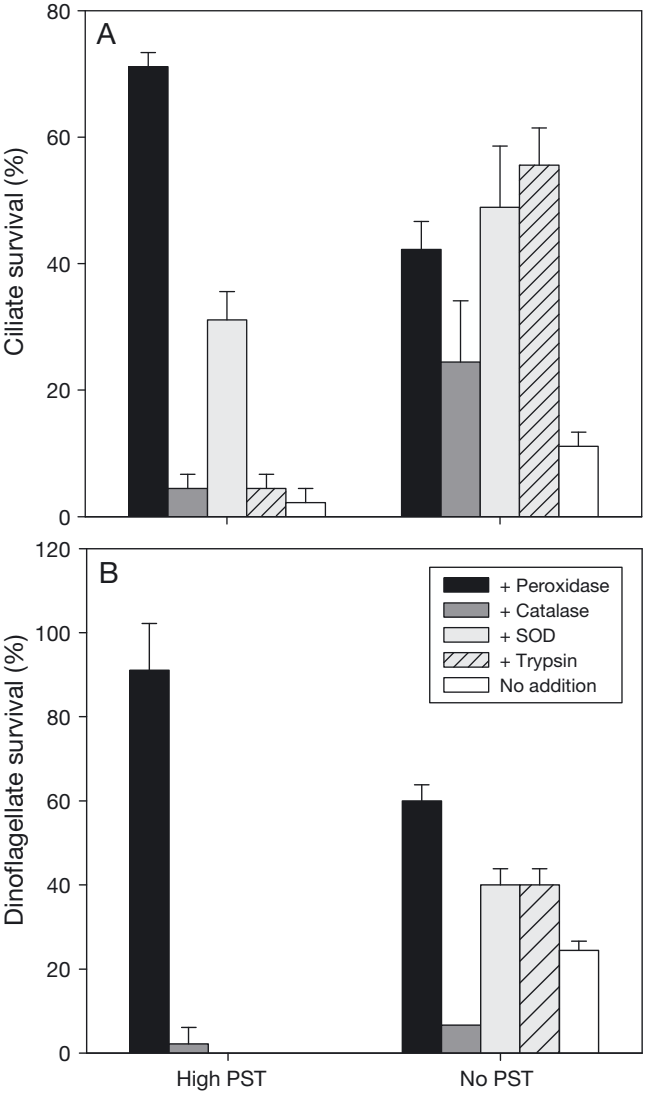

Fig. 4. Survival of (A) the ciliate *Tiarina fusus* and (B) the heterotrophic dinoflagellate *Polykrikos kofoidii* following 24 h exposure to live *Alexandrium* spp. cultures with the addition of the enzymes peroxidase, catalase, superoxide dismutase (SOD), or trypsin. Controls consisted of exposure to live *Alexandrium* spp. cultures without the addition of enzymes. Data are means ± SE (n = 3 per treatment). PST: paralytic shellfish toxin

$p < 0.001$ for *P. kofoidii*). Superoxide dismutase also reduced the mortality of *T. fusus* cells exposed to the High PST and No PST *Alexandrium* spp. (High PST, $p < 0.001$; No PST, $p = 0.02$) but only increased the survival of *P. kofoidii* in the No PST *Alexandrium* sp. treatment (Fig. 4; $p = 0.036$). Catalase significantly increased the survival of *T. fusus* when exposed to the No PST *Alexandrium* sp. ($p = 0.017$); however, the enzyme did not mitigate the toxicity of the High PST isolate (Fig. 4; $p = 0.998$). Further, catalase did not increase the survival of *P. kofoidii* significantly in any experimental treatment ($p > 0.6$). In contrast to the variable effectiveness of ROS scavengers tested, the protease trypsin significantly increased the survival of both *T. fusus* and *P. kofoidii* cells exposed to the No PST *Alexandrium* sp. ($p = 0.008$ [*T. fusus*], $p = 0.036$ [*P. kofoidii*]) but did not affect protist survival in the High PST treatment (Fig. 4; $p > 0.5$). The toxicity of the Low PST *Alexandrium* spp. culture to *T. fusus* and *P. kofoidii* was not mitigated by any of the tested compounds, and 100% mortality was observed in all experimental treatments (data not shown).

DISCUSSION

The production of allelopathic compounds appears to be common in the genus *Alexandrium*, affecting a wide variety of heterotrophic and autotrophic protists (Hansen 1989, Hansen et al. 1992, Arzul et al. 1999, Matsuoka et al. 2000, Tillmann & John 2002, Fistarol et al. 2004, Tillmann et al. 2007, 2008). In the present study, 3 strains in the *A. tamarense* species complex caused immobilization and cell lysis of the ciliate *Tiarina fusus* and the heterotrophic dinoflagellate *Polykrikos kofoidii*. The lytic activity of *Alexandrium* spp., however, could not be attributed to the PST toxin content of the algal isolates, as the No PST strain also caused significant mortality of both protist species. Further, *T. fusus* survival was not affected by cell extracts from any of the tested *Alexandrium* spp. strains, including those with detectable PST toxins. The PST toxins produced by *Alexandrium* spp. are water-soluble and heat-stable (Wang 2008) and therefore are not destroyed by the sonication method used to prepare the cell extracts. If PST toxins were responsible for the toxicity of *Alexandrium* spp. to protists, cell extracts from the High and Low PST strains should have caused significant ciliate mortality. These results are consistent with previous studies that have examined the allelopathic effect of *Alexandrium* spp. on other protist species (Tillmann & John 2002, Fistarol et al. 2004).

The high survival of *Tiarina fusus* following exposure to cell-free (0.2 µm filtered) *Alexandrium* spp. culture filtrates suggested that toxicity may occur only after physical contact with or ingestion of the dinoflagellate cells. Significant ciliate mortality occurred, however, when *T. fusus* was separated from living *Alexandrium* spp. cells by a 10 µm mesh, indicating that the bioactive compounds produced by *Alexandrium* spp. are released extracellularly, and physical contact and/or ingestion is not required to affect the protists. It is possible that the lytic compounds produced by *Alexandrium* spp. are relatively labile, which would explain the conflicting results between the 2 experiments. Continuous production by live *Alexandrium* sp. cells might be needed for toxicity to be observed. The present results are in contrast to previous research reporting abnormal swimming behavior and mortality within 1 h of exposure to *Alexandrium* spp. filtrates (Tillmann & John 2002, Tillmann et al. 2007). In those studies, however, the toxicity of the filtrate decreased over time and, depending upon the protist species being tested, it was no longer effective within hours to several days (Tillmann et al. 2007), suggesting that the bioactive compounds are, indeed, labile. The ineffectiveness of the *Alexandrium* sp. filtrates in the present study may be attributable to variability in the amount of lytic compounds produced by various *Alexandrium* sp. isolates (Tillmann et al. 2009) and/or differences in the sensitivity of *T. fusus* to these allelochemicals. Recent research by Ma et al. (2009), however, indicated that the allelochemicals produced by *Alexandrium* spp. are not labile and that the temporal stability of the compound(s) is actually high. These researchers were able to restore the lytic activity of an *A. tamarense* culture filtrate by vigorous shaking, providing support for the hypothesis that amphipathic compounds play a role in the toxicity of *Alexandrium* spp. to protists (Ma et al. 2009) while possibly also explaining the 'loss' of lytic activity observed in the present and previous studies.

The 5.0 µm filtrate from the Low PSP *Alexandrium* sp. strain caused significant *Tiarina fusus* mortality, suggesting that bacteria present in that particular algal culture may produce compounds that are toxic to heterotrophic protists. Tillmann & John (2002) and Tillmann et al. (2007) performed similar experiments with filtrates from several *Alexandrium* sp. cultures and concluded that the bacteria present in those cultures were not responsible for the observed lytic effects on protists. The production of lytic compounds by bacteria appears to be relatively common (Holmström & Kjelleberg 1999), and it is possible that the

bacteria alone are responsible for the harmful effects of the Low PSP *Alexandrium* sp. culture on protists. However, the survival of *T. fusus* exposed to the 5.0 µm filtrate was significantly higher than that in the live *Alexandrium* sp. treatment, suggesting that both bacteria and the dinoflagellate play a role in the toxicity. Further research is needed to resolve the specific contributions of *Alexandrium* sp. and bacteria to the toxicity of this culture to protists.

Although the harmful effects of *Alexandrium* spp. upon protists are thought to be attributable to extracellular, lytic compounds and not to PSTs, the specific mechanism of toxicity remains unknown. The addition of peroxidase, superoxide dismutase, or catalase mitigated the toxicity of the High PST and the No PST *Alexandrium* sp. strains, although the effectiveness of each specific enzyme varied depending upon the particular algal isolate and the target protist species. Superoxide dismutase transforms the superoxide radical (O_2^-) into the reactive oxygen compound, hydrogen peroxide (H_2O_2), and peroxidase and catalase convert H_2O_2 into water (Apel & Hirt 2004). Some peroxidases can also interact with a variety of organic peroxides, including cholesterol and long-chain fatty acid peroxides (Arthur 2000). The increased survival of *Tiarina fusus* and *Polykrikos kofoidii* in treatments containing these antioxidants suggests that reactive oxygen species and/or products of lipid oxidation are likely involved in the toxicity of *Alexandrium* spp. to protists.

Reactive oxygen species are generated by eukaryotic and prokaryotic cells as by-products of cell metabolism; however, elevated concentrations of these compounds can cause oxidative damage to cellular macromolecules, including DNA, proteins, and lipids (Halliwell & Gutteridge 1985, Apel & Hirt 2004). Several HAB species produce relatively high levels of ROS in comparison to other algae (Oda et al. 1997, Kim et al. 1999, Marshall et al. 2005); therefore, it has been speculated that ROS are responsible for the toxicity of these species to other aquatic organisms. Kim et al. (1999) proposed that ROS generated by the dinoflagellate *Cochlodinium polykrikoides* are involved in the toxicity of this HAB species to fish. Similarly, Yang et al. (1995) found that the addition of superoxide dismutase and/or catalase increased the survival of juvenile rainbow trout *Oncorhynchus mykiss* when exposed to the raphidophyte *Heterosigma carterae* and suggested that ROS were the causative ichthyotoxic compounds. Further, increased production of O_2^- by another raphidophyte species, *Chattonella marina*, was thought to be induced by fish mucous, resulting in ROS-mediated gill tissue damage in yellowtail *Seriola quinqueradiata* (Ishimatsu et al. 1997, Kim et al. 2001). Subsequent research, however, has noted discrepancies between the amount of ROS produced by these flagellates and the concentrations required to cause fish death, and the specific involvement of ROS in the toxicity of these HAB species remains a subject of debate (Twiner et al. 2001, Kim et al. 2002, Tang et al. 2005).

Although *Alexandrium* spp. can produce moderate levels of ROS (Kim et al. 1999, Marshall et al. 2005), recent research by Ma et al. (2009) has provided evidence that the extracellular allelochemicals in *A. tamarense* are large, amphipathic macromolecules. Amphipathic compounds have both hydrophilic and lipophilic properties, and examples of these compounds include most membrane lipids. It is possible that the ROS produced by *Alexandrium* spp. may not be directly responsible for the toxicity of this species to protists but alternatively are involved in lipid oxidation pathways that produce toxic secondary metabolites. Marshall et al. (2003) reported that the raphidophyte *Chattonella marina* contains high amounts of the polyunsaturated fatty acid eicosapentaenoic acid (EPA) and proposed that the mechanism of ichthyotoxicity in this species is the ROS-mediated oxidation of EPA. Jüttner (2001) demonstrated that the free fatty-acid form of EPA released from diatom biofilms can be toxic to a zooplankter. In a review, Ikawa (2004) concluded that microalgal PUFA oxidation products appear to be bioactive agents of allelopathic and grazer-defense interactions of many microalgal taxa. Wu et al. (2006) demonstrated membrane disruption of microalgal cells by free fatty acids, leading to potassium and phycobiliprotein leakage. The effects were more severe from fatty acids with a higher degree of saturation. These authors further postulated that relatively insoluble, free fatty acids may form micelles in aqueous solution that then bind to membranes of target cells. Although a possible mechanism for the release of membrane-bound ROS has not yet been examined in *Alexandrium* spp., cells incubated with 2′,7′-dichlorfluorescein-diacetate, a fluorogenic probe used to detect ROS, show bright fluorescence at the membrane surface, indicating oxidation of the probe at this site (data not shown). Further, some *Alexandrium* spp. strains appear to have high concentrations of EPA in the glycolipids associated with the cell membrane, relative to other dinoflagellate species (Leblond & Chapman 2000). Accordingly, *Alexandrium* spp. toxicity to protists may be a result of ROS-mediated oxidation of cell membrane lipids (e.g. glycolipids) or free fatty acids that are rich in EPA or other polyunsaturated fatty acids.

The protease trypsin increased the survival of both *Tiarina fusus* and *Polykrikos kofoidii* when exposed to the No PST *Alexandrium* sp. strain, suggesting that protein-like toxins also are produced by this isolate. Emura et al. (2004) provided similar evidence for a proteinaceous exotoxin in a related dinoflagellate, *Alexandrium taylori*, and suggested that the hemolytic compound was responsible for the toxicity of this species to the brine shrimp *Artemia*. Trypsin, however, did not mitigate the toxicity of the High PST or Low PST strains for either heterotrophic protist species. Trypsin was tested at one concentration (500 µg l^{-1}) in the present research, and it is possible that a higher concentration would have increased protist survival. Alternatively, some *Alexandrium* spp. strains may not produce these toxins. Variation in toxin production among closely related dinoflagellate species and even within a species is relatively common (summarized by Burkholder & Glibert 2006).

The ecological significance of the allelochemicals produced by *Alexandrium* spp. presently is not well understood; however, recent studies by Weissbach et al. (2010, 2011) suggest that the cytolytic effects of these bioactive compounds may influence the structure and dynamics of plankton communities. *Alexandrium* spp. generally constitute a small fraction of the total phytoplankton biomass during blooms, but cell densities approaching or exceeding 1×10^6 cells l^{-1} have been reported (Carreto et al. 1986, Cembella et al. 2002, Hattenrath et al. 2010). In the present study, similar *Alexandrium* spp. densities caused significant protist mortality within 24 h. It is possible that once a bloom reaches a sufficient cell density, the lytic compounds produced by *Alexandrium* spp. substantially reduce protist grazing, allowing the bloom to persist for a longer period. Portune et al. (2010) found that the raphidophyte *Heterosigma akashiwo* produced high concentrations of O_2^- at high cell densities and suggested that this species might release a significant amount of this potentially harmful radical during the formation of dense algal blooms. The sublethal effects of low densities of *Alexandrium* spp. upon protists have not been well studied. Both *Tiarina fusus* and *Polykrikos kofoidii* readily ingested *Alexandrium* spp. cells when provided at a low density, suggesting that a certain concentration of the bioactive compound(s) is required to deter or prevent grazing. Grazing experiments examining the response of heterotrophic protists to low densities of *Alexandrium* spp., with or without the addition of antioxidants, could improve the understanding of the possible impact of these cytolytic compounds upon grazers during early bloom development. In addition, characterization of the chemical structure(s) of *Alexandrium* spp. allelochemicals is needed to assess the roles of these compounds in natural plankton communities.

Acknowledgements. We thank G. McManus for the *Tiarina fusus* culture. Funding was provided by grants from NOAA, including an Oceans and Human Health Initiative grant for the Interdisciplinary Research and Training Initiative on Coastal Ecosystems and Human Health (I-RICH), which provided a postdoctoral fellowship to H.S.F., and grant (NA06NOS4780249). Support during the writing phase of this project was also provided by NSF grants (OCE-0648126 and OCE-1130284). This is ECOHAB contribution number 686.

LITERATURE CITED

Anderson DM, Kulis DM, Doucette GJ, Gallagher JC, Balech E (1994) Biogeography of toxic dinoflagellates in the genus *Alexandrium* from the northeastern United States and Canada. Mar Biol 120:467–478

Apel K, Hirt H (2004) Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373–399

Arthur JR (2000) The glutathione peroxidases. Cell Mol Life Sci 57:1825–1835

Arzul G, Seguel M, Guzman L, Erard-Le Denn E (1999) Comparison of allelopathic properties in three toxic *Alexandrium* species. J Exp Mar Biol Ecol 232:285–295

Burkholder JM, Glibert PM (2006) Intraspecific variability: an important consideration in forming generalisations about toxigenic algal species. Afr J Mar Sci 28:177–180

Buskey EJ, Montagna PA, Amos AF, Whitledge TE (1997) Disruption of grazer populations as a contributing factor to the initiation of the Texas brown tide algal bloom. Limnol Oceanogr 42:1215–1222

Calbet A (2008) The trophic role of microzooplankton in marine systems. ICES J Mar Sci 65:325–331

Calbet A, Landry MR (2004) Phytoplankton growth, microzooplankton grazing, and carbon cycling in marine systems. Limnol Oceanogr 49:51–57

Carreto JI, Benavides HR, Negri RM, Glorioso PD (1986) Toxic red-tide in the Argentine Sea. Phytoplankton distribution and survival of the toxic dinoflagellate *Gonyaulax excavata* in a frontal area. J Plankton Res 8:15–28

Cembella AD (2003) Chemical ecology of eukaryotic microalgae in marine systems. Phycologia 42:420–447

Cembella AD, Quilliam MA, Lewis NI, Bauder AG and others (2002) The toxigenic marine dinoflagellate *Alexandrium tamarense* as the probable cause of mortality of caged salmon in Nova Scotia. Harmful Algae 1:313–325

Colin SP, Dam HG (2007) Comparison of the functional and numerical responses of resistant versus non-resistant populations of the copepod *Acartia hudsonica* fed the toxic dinoflagellate *Alexandrium tamarense.* Harmful Algae 6:875–882

Durbin E, Teegarden G, Campbell R, Cembella A, Baumgartner MF, Mate BR (2002) North Atlantic right whales, *Eubalaena glacialis*, exposed to paralytic shellfish poisoning (PSP) toxins via a zooplankton vector *Calanus finmarchicus.* Harmful Algae 1:243–251

Emura A, Matsuyama Y, Oda T (2004) Evidence for the production of a novel proteinaceous hemolytic exotoxin by

dinoflagellate *Alexandrium taylori.* Harmful Algae 3: 29–37

Fistarol GO, Legrand C, Selander E, Hummert C, Stolte W, Granéli E (2004) Allelopathy in *Alexandrium* spp.: effect on a natural plankton community and on algal monocultures. Aquat Microb Ecol 35:45–56

Fulco VK (2007) Harmful effects of the toxic dinoflagellate *Alexandrium tamarense* on the tintinnids *Favella taraikaensis* and *Eutintinnus* sp. J Mar Biol Assoc UK 87: 1085–1088

Granéli E, Hansen PJ (2006) Allelopathy in harmful algae: a mechanism to compete for resources? In: Graneli E, Turner JT (eds) Ecology of harmful algae. Springer-Verlag, Berlin, p 189–201

Guillard RR (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanley MH (eds) Culture of marine invertebrate animals. Plenum Press, New York, NY, p 29–60

Hallegraeff GM (1993) A review of harmful algal blooms and their apparent global increase. Phycologia 32:79–99

Halliwell B, Gutteridge JM (1985) Free radicals in biology and medicine. Clarendon Press, Oxford

Hansen PJ (1989) The red tide dinoflagellate *Alexandrium tamarense*: effects on behavior and growth of a tintinnid ciliate. Mar Ecol Prog Ser 53:105–116

Hansen PJ (1992) Prey size selection, feeding rates and growth dynamics of heterotrophic dinoflagellates with special emphasis on *Gyrodinium spirale.* Mar Biol 114: 327–334

Hansen PJ, Cembella AD, Moestrup O (1992) The marine dinoflagellate *Alexandrium ostenfeldii*: paralytic shellfish toxin concentration, composition, and toxicity to a tintinnid ciliate. J Phycol 28:597–603

Hattenrath TK, Anderson DM, Gobler CJ (2010) The influence of anthropogenic nitrogen loading and meteorological conditions on the dynamics and toxicity of *Alexandrium fundyense* blooms in a New York (USA) estuary. Harmful Algae 9:402–412

Holmström C, Kjelleberg S (1999) Marine *Pseudoalteromonas* species are associated with higher organisms and produce biologically active extracellular agents. FEMS Microbiol Ecol 30:285–293

Ikawa M (2004) Algal polyunsaturated fatty acids and effects on plankton ecology and other organisms. UNH Cent Freshw Biol Res 6:17–44

Ishimatsu A, Maruta H, Oda T, Ozaki M (1997) A comparison of physiological responses in yellowtail to fatal environmental hypoxia and exposure to *C. marina.* Fish Sci 63:557–562

Jüttner F (2001) Liberation of 5,8,11,14,17-eicosapentaenoic acid and other polyunsaturated fatty acids from lipids as a grazer defense reaction in epilithic diatom biofilms. J Phycol 37:744–755

Kamiyama T, Tsujino M, Matsuyama Y, Uchida T (2005) Growth and grazing rates of the tintinnid ciliate *Favella taraikaensis* on the toxic dinoflagellate *Alexandrium tamarense.* Mar Biol 147:989–997

Kim CS, Lee SG, Lee CK, Kim HG, Jung J (1999) Reactive oxygen species as causative agents in the icthyotoxicity of the red tide dinoflagellate *Cochlodinium polykrikoides.* J Plankton Res 21:2105–2115

Kim D, Okamota T, Oda T, Tachibana K and others (2001) Possible involvement of the glycocalyx in the ichthyotoxicity of *C. marina* (Raphidophyceae): immunological approach using anti-serum against cell surface structures of the flagellate. Mar Biol 139:625–632

Kim D, Oda T, Muramatsu T, Kim D, Matsuyama Y, Honjo T (2002) Possible factors responsible for the toxicity *of Cochlodinium polykrikoides*, a red tide phytoplankton. Comp Biochem Physiol C 132:415–423

Leblond JD, Chapman PJ (2000) Lipid class distribution of highly unsaturated long chain fatty acids in marine dinoflagellates. J Phycol 36:1103–1108

Legrand C, Rengefors K, Fistarol GO, Granéli E (2003) Allelopathy in phytoplankton - biochemical, ecological and evolutionary aspects. Phycologia 42:406–419

Ma H, Krock B, Tillmann U, Cembella A (2009) Preliminary characterization of extracellular allelochemicals of the toxin marine dinoflagellate *Alexandrium tamarense* using a *Rhodomonas salina* bioassay. Mar Drugs 7: 497–522

Marshall JA, Nichols PD, Hamilton B, Lewis RJ, Hallegraeff GM (2003) Ichthyotoxicity of *Chattonella marina* (Raphidophyceae) to damselfish (*Acanthochromis polycanthus*): the synergistic role of reactive oxygen species and free fatty acids. Harmful Algae 2:273–281

Marshall JA, de Salas M, Oda T, Hallegraeff G (2005) Superoxide production by marine microalgae. I. Survey of 37 species from 6 classes. Mar Biol 147:533–540

Matsuoka K, Cho HJ, Jacobson DM (2000) Observation of the feeding behavior and growth rates of the heterotrophic dinoflagellate *Polykrikos kofoidii* (Polykrikaceae, Dinophyceae). Phycologia 39:82–86

Needler AB (1949) Paralytic shellfish poisoning and Gonyaulax tamarensis. J Fish Res Board Can 7:490–504

Oda T, Nakamura A, Shikayama M, Kawano I, Ishimatsu A, Muramatsu T (1997) Generation of reactive oxygen species by raphidophycean phytoplankton. Biosci Biotechnol Biochem 61:1658–1662

Oshima Y, Sugino K, Yasumoto T (1989) Latest advances in HPLC analysis of paralytic shellfish toxins. In: Natori S, Hashimoto K, Ueno Y (eds) Mycotoxins and phycotoxins. Elsevier, Amsterdam, p 319–326

Portune KJ, Cary SC, Warner ME (2010) Antioxidant enzymes response and reactive oxygen species production in marine raphidophytes. J Phycol 46:1161–1171

Prakash A (1963) Source of paralytic shellfish toxin in the Bay of Fundy. J Fish Res Board Can 20:983–996

Sherr EB, Sherr BF (2002) Significance of predation by protists in aquatic microbial food webs. Antonie van Leeuwenhoek 81:293–308

Shumway SE (1990) A review of the effects of algal blooms on shellfish and aquaculture. J World Aquacult Soc 21: 65–105

Smayda TJ (1990) Novel and nuisance phytoplankton blooms in the sea: evidence for a global epidemic. In: Granéli E, Sunderström B, Elder L, Anderson DM (eds) Toxic marine phytoplankton. Elsevier, New York, NY, p 29–40

Smayda TJ (2008) Complexity in the eutrophication-harmful algal bloom relationship, with comment on the importance of grazing. Harmful Algae 8:140–151

Stoecker D, Guillard RRL, Kavee RM (1981) Selective predation by *Favella ehrenbergii* (Tintinnia) on and among dinoflagellates. Biol Bull 160:136–145

Tang YZ, Gobler CJ (2009) Characterization of the toxicity of *Cochlodinium polykrikoides* isolates from Northeast US estuaries to finfish and shellfish. Harmful Algae 8: 454–462

Tang JYM, Anderson DM, Au DWT (2005) Hydrogen peroxide is not the cause of fish kills associated with *Chat-*

tonella marina: cytological and physiological evidence. Aquat Toxicol 72:351–360

Tillmann U, John U (2002) Toxic effects of *Alexandrium* spp. on heterotrophic dinoflagellates: an allelochemical defence mechanism independent of PSP-toxin content. Mar Ecol Prog Ser 230:47–58

Tillmann U, John U, Cembella A (2007) On the allelochemical potency of the marine dinoflagellate *Alexandrium ostenfeldii* against heterotrophic and autotrophic protists. J Plankton Res 29:527–543

Tillmann U, Alpermann T, John U, Cembella A (2008) Allelochemical interactions and short-term effects of the dinoflagellate *Alexandrium* on selected photoautotrophic and heterotrophic protists. Harmful Algae 7: 52–64

Tillmann U, Alpermann TL, da Purificação RC, Krock B, Cembella A (2009) Intra-population clonal variability in allelochemical potency of the toxigenic dinoflagellate *Alexandrium tamarense.* Harmful Algae 8:759–769

Turner JT, Tester PA (1997) Toxic marine phytoplankton, zooplankton grazers, and pelagic food webs. Limnol Oceanogr 42:1203–1214

Twiner MJ, Dixon SJ, Trick CG (2001) Toxic effects of *Heterosigma akashiwo* do not appear to be mediated by hydrogen peroxide. Limnol Oceanogr 46:1400–1405

Wang DZ (2008) Neurotoxins from marine dinoflagellates: a brief review. Mar Drugs 6:349–371

Watras CJ, Garcon VC, Olson RJ, Chisholm SW, Anderson DM (1985) The effect of zooplankton grazing on estuarine blooms of the toxic dinoflagellate *Gonyaulax tamarensis.* J Plankton Res 7:891–908

Weissbach A, Tillman U, Legrand C (2010) Allelopathic potential of the dinoflagellate *Alexandrium tamarense* on marine microbial communities. Harmful Algae 10: 9–18

Weissbach A, Rudström M, Olofsson M, Béchemin C and others (2011) Phytoplankton allelochemical interactions change microbial food web dynamics. Limnol Oceanogr 56:899–909

White AW (1980) Recurrence of kills of Atlantic herring (*Clupea harengus harengus*) caused by dinoflagellate toxins transferred through herbivorous zooplankton. Can J Fish Aquat Sci 37:2262–2265

Wu JT, Chiang YR, Huang WY, Jane WJ (2006) Cytotoxic effects of free fatty acids on phytoplankton algae and cyanobacteria. Aquat Toxicol 80:338–345

Yang CZ, Albright LJ, Yousif AN (1995) Oxygen-radical-mediated effects of the toxic phytoplankter *Heterosigma carterae* on juvenile rainbow trout *Oncorhynchus mykiss*. Dis Aquat Org 23:101–108

Editorial responsibility: Patricia Glibert, Cambridge, Maryland, USA

Submitted: January 11, 2011; Accepted: April 14, 2012
Proofs received from author(s): May 28, 2012