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RESEARCH ARTICLE

Toxic dinoflagellates (*Alexandrium fundyense* and *A. catenella*) have minimal apparent effects on oyster hemocytes

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Abstract The possible effect of *Alexandrium* spp. containing paralytic shellfish poisoning (PSP) toxins on the hemocytes of oysters was tested experimentally. In one trial, eastern oysters, *Crassostrea virginica* Gmelin, were exposed to bloom concentrations of the sympatric dinoflagellate, *Alexandrium fundyense* Balech, alone and in a mixture with a non-toxic diatom, *Thalassiosira weissflogii* (Grun) Fryxell et Hasle. Subsequently, another experiment exposed Pacific oysters, *Crassostrea gigas* Thunberg, to a mixed suspension of the sympatric, toxic species *Alexandrium catenella* (Whedon et Kofoid) Balech, with *T. weissflogii*. Measurements of numbers of oyster hemocytes, percentages of different cell types, and functions (phagocytosis, reactive oxygen species (ROS) production, and mortality) were made using flow-cytometry. During and after exposure, almost no significant effects of *Alexandrium* spp. upon hemocyte numbers, morphology, or functions were detected, despite observations of adductor-muscle paralysis in *C. virginica* and measured toxin accumulation in *C. gigas*. The only significant correlation found was between toxin accumulation at one temperature and higher numbers of circulating live and dead hemocytes in *C. gigas*. The PSP toxins are known to interfere specifically with sodium-channel function; therefore, the finding that the toxins had no effect on measured hemocyte functions suggests that sodium-channel physiology is not important in these hemocyte functions. Finally, because oysters were exposed to the living algae, not purified toxins, there was no evidence of bioactive compounds other than PSP toxins affecting hemocytes in the two species of *Alexandrium* studied.

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Introduction

Paralytic shellfish poisoning (PSP) toxins are produced by a number of dinoflagellates including *Alexandrium* spp. (formerly included in the genus *Protogonyaulax* or *Gonyaulax*), *Gymnodinium catenatum*, and *Pyrodinium bahamense*. Suspension feeders, such as bivalve molluscs, are the principal vectors transferring these phytotoxins to humans and other consumers of shellfish, as bivalves accumulate toxins from the toxic algae they consume (Bricelj and Shumway 1998). Capacity to accumulate PSP toxin differs among bivalve species (Shumway et al. 1988; Gainey and Shumway 1988a; Bricelj and Shumway 1998; Bricelj et al. 2005). For example, when exposed simultaneously to a toxic algal bloom, oysters accumulated lower levels of toxins than did mussels (Lassus et al. 1999).

Despite apparent tolerance of accumulated PSP and other toxins in their soft tissues, some bivalves may have reduced survival and growth (Shumway 1990). Other studies provide evidence of sub-lethal effects, such as reduced clearance rates in juvenile bay scallops (*Argopecten irradians*

irradians), northern quahogs (*Mercenaria mercenaria*), soft-shell clams (*Mya arenaria*), blue mussels (*Mytilus edulis*), and flat oysters (*Ostrea edulis*) in the presence of toxic *Alexandrium* spp. cells (Lesser and Shumway 1993). Similarly, Lassus et al. (1996) reported reduced clearance rates in Pacific oysters (*Crassostrea gigas*) exposed to *A. tamarense* and Shumway and Cucci (1987) demonstrated its effects on shell-valve closure and mucus production in several bivalve species. Changes in heart rate of bivalves have also been observed in the presence of *A. tamarense* (Gainey and Shumway 1988b). Li et al. (2002) showed a decline in the clearance rate of the clam *Ruditapes philippinarum* exposed to highly toxic *A. tamarense*, and decreases in absorption efficiency of both clams (*R. philippinarum*) and mussels (*Perna viridis)* coincident with toxin accumulation. Depending upon the bivalve species, morphological effects of accumulated paralytic phycotoxins from *A. tamarense* are seen in different organs, especially in the digestive gland and the kidney, and less commonly in other organs, such as the adductor muscle and the gonad (Lassus et al. 1999; Bougrier et al. 2001; Choi et al. 2003). These diverse effects of PSP-containing dinoflagellates on bivalve molluscs suggest that other aspects of physiological processes may also be affected.

Invertebrates, including bivalves, have no acquired immunity and are therefore, limited to an innate immune response to pathogens, parasites, and physical injury (Janeway 1994), which is mediated by chemical agents and specialized cells called hemocytes circulating throughout the body. Hemocytes recognize and attempt to eliminate non-self particles within an open vascular system and tissues, but they do not "remember" a prior experience with a harmful agent and more effectively protect an individual in subsequent exposures. Recent studies in the laboratory and in the field (Hégaret et al. 2005a, b) of effects of the harmful dinoflagellate *Prorocentrum minimum* on bivalves were the first to show effects on hemocytes. These effects included changes in morphological and functional parameters, such as: concentrations and proportions of the different hemocyte types, production of reactive oxygen species, phagocytosis or the percentage of dead hemocytes in eastern oysters (*C. virginica*) and northern bay scallops (*A. irradians*). The bioactive compound or compounds in *P. minimum* responsible for these effects have not been identified. In the present study, we evaluated hemocyte characteristics and functions in: (1) *C. virginica* exposed to *A. fundyense*, two species that co-occur on the east coast of north America, and (2) *C. gigas* exposed to *A. catenella*, species that co-occur in western Europe. Experimental exposures used cultured algae and included additional variables, such as algal cell density, combination with non-toxic algae, and temperature. The two experiments were done independently and, in part, for different purposes. Thus, there were some differences in experimental design and protocols for hemocyte-parameter analyses.

Materials and methods

Two experiments were conducted in which oysters were exposed to cultures of toxin-producing strains of dinoflagellates, *Alexandrium* spp. and then analyzed for hemocyte parameter changes: one was conducted in May 2004 at the Milford Laboratory (Connecticut, USA), and another was done in June 2004 at the IFREMER facility in Nantes (France). Eastern oysters, *C. virginica* Gmelin, were studied in Milford, and Pacific oysters, *C. gigas* Thunberg, were studied in Nantes.

Experimental oysters

Eastern oysters, *C. virginica*, obtained from the F.M. Flower Hatchery (Oyster Bay, New York, USA), were used in the Milford experiment. Before being transferred to experimental basins, oysters (ca. 30 mm shell height) were conditioned in running, unfiltered seawater (17°C) in the Milford Laboratory. These oysters were not gametogenic.

Triploid Pacific oysters, *C. gigas*, used for the two experiments in Nantes were obtained from a producer in Le Croisic, France. These oysters were produced by the hatchery Vendée Naissain, and were grown in basins. The experimental oysters (9.1 ± 1.6 g wet weight, ca. 60 mm shell height) were acclimated in basins containing running seawater for 6 days at the experimental temperature of 16 ± 0.5°C and were transferred into experimental units after being cleaned. Triploid oysters were chosen because, compared to diploids, they are thought to have faster growth and better survival for aquaculture production (Nell 2002). Moreover, triploids do not produce gametes, which, if present at the time of the experiment, could interfere with hemocyte analyses by contaminating hemolymph samples.

Algal cultures

For the experiment in Milford, strains of *A. fundyense* Balech (BF 2) and non-toxic *T. weissflogii* (Grun.) Fryxell et Hasle (TW) were obtained from the Milford Laboratory microalgal culture collection. This strain of *A. fundyense* causes mortality in bay scallops after 24 h exposure to stationary-phase cells (Hégaret and Wikfors 2005a). Both BF 2 and TW contain bacterial contaminants, but cultures were managed aseptically to preclude contamination with additional microbes. Strain TW was cultured in the "E + Si Medium" seawater enrichment of Ukeles (1973) at a salinity of 15; BF 2 was cultured in E medium at 30. Carboy cultures, grown under continuous illumination (300 μE m^{-2} s^{-1}) in a

temperature controlled (18°C) room, were managed semi-continuously, with half the volume harvested and replaced aseptically every 7 days.

For the experiment in Nantes, strains of *A. catenella* (Whedon et Kofoid) (ATTL01) and *T. weissflogii* were batch cultured in 10-l carboys at 16°C, with a light intensity of 50 μE m^{-2} s^{-1} and a 12 h light:12 h dark cycle. *A. catenella* was cultured in f/2 medium (Guillard and Rhyther 1962), the *T. weissflogii* culture was grown in natural seawater filtered to 0.22 µm and enriched with ES nutrients (Provasoli 1966). The *A. catenella* strain ATTL01 was isolated from the Thau basin in 1998 (Lilly et al. 2002) and contained 5–15 pg saxitoxin equivalent (eq.STX) $cell^{-1}$ (Parkhill and Cembella 1999) at the end of the exponential growth phase in the present study. The non-toxic diatom, *T. weissflogii*, was chosen to represent the nanoplankton community that dominates during the presence of *A. catenella* in the Thau basin.

Experimental design

Crassostrea virginica exposed to A. fundyense

This experiment was conducted as part of a screening program for dinoflagellate effects on molluscs. Oysters were sorted randomly, eight into each of 12, 1.2-l basins (three replicates of four different microalgal treatments), with a continuous flow of 2 ml min^{-1} of: (1) 5×10^2 cells ml^{-1} *A. fundyense*, (2) 1×10^2 cells ml^{-1} *A. fundyense*, (3) 2.5×10^2 cells ml^{-1} *A. fundyense* plus 2.5×10^2 cells ml^{-1} *T. weissflogii*, or (4) 5×10^3 cells ml^{-1} *T. weissflogii.* All hemocyte parameters listed above were analyzed in triplicate for each group of oysters after 7 days of exposure to the different diets. No toxin analyses were done for these oysters.

Crassostrea gigas exposed to A. catenella

This experiment, conducted to determine toxin accumulation rates under various experimental conditions, presented an opportunity to conduct hemocyte analyses. Oysters were sorted randomly, one into each of 20 individual basins described in several previous studies (Bougrier et al. 2001, 2003), which were supplied with a continuous flow on a 24 h sequence of cycling microalgal flows: 1.5×10^3 cells ml^{-1} *T. weissflogii* and 75 cells ml^{-1} *A. catenella* for 14 h, and then 1.5×10^3 cells ml^{-1} *T. weissflogii* and 1.5×10^2 cells ml^{-1} *A. catenella*, for 10 h day^{-1} over 4 days. Cell concentrations, including diurnal fluctuations. of *A. catenella*, for these experiments were equivalent to those observed in natural blooms recorded on the Catalogne coast in the summers 1998 and 1999, wherein *A. catenella* cell concentrations varied between 10^4 and 10^6 cells l^{-1} (Vila et al. 2001). The concentrations of *A. catenella* and *T. weissflogii* were monitored with a Coulter Multisizer and a microscope (using an Utermohl counting chamber) every morning during two experiments conducted at 12 or 18°C with oysters pre-conditioned for 5 days to each temperature. These temperatures represent the coldest and the warmest temperatures generally observed in the Thau basin during blooms of *A. catenella*.

Hemocyte responses, as listed below, were analyzed in oysters sub-sampled from the basins as follows: (1) four individuals after a 1 day exposure, (2) four after a 2 day exposure, (3) four after a 3 day exposure, and (4) eight after a 4 day exposure. In addition, eight control oysters, which were not exposed to *A. catenella* and were not transferred to the basins, were analyzed; these are reported as "Day 0" in the analysis of results and on the figures.

Paralysis determination

Paralysis of oysters was assessed semi-quantitatively by observation of the oysters' shell gape. Gaping oysters were stimulated mechanically with a micropipette on the gills and adductor muscle. If oysters did not react with any movement, they were considered paralyzed. Viability of paralyzed oysters was determined by observation of water movement in the shell cavity and subsequent recovery of muscle function after removal from *A. fundyense* exposure.

Toxin analysis

Analysis for PSP toxins in *A. catenella* and in PSP-contaminated oysters was performed by reverse-phase, ion-pairing, high-performance liquid chromatography (IP-HPLC) according to the method of Oshima (1989). Total soft tissues of oysters were minced in HCl (0.1 N at 4°C). After centrifugation (3,000×*g*, 15 min, 4°C), the pH of extracts was adjusted to 3.0–3.5 with 12 N HCl. After half-dilution, supernatants were ultrafiltered (20,000 Da, Sartorius Centrisart) and stored at 4°C until analysis. Diluted solutions of each compound of PSP1-B reference material (MACSP, NRC-Halifax, Canada) were used as external standards for quantitative detection. In consideration of the dilution factors, the molar concentrations were converted to saxitoxin-equivalents, µg STX Eq. 100 g^{-1} of bivalve tissue by using the conversion factors of Oshima (1995), i.e. 297 µg STX.eq.µM for GTX3, 168 for GTX2, 48 for C1 and C2, 28 for B1; and 354 for GTX4. For *A. catenella* toxins, a few (B1, C1, C2) are quantified indirectly after acid hydrolysis (Masselin et al. 2001).

Hemocyte morphology and functions

Flow-cytometric analyses of hemocyte morphological characteristics and functions were conducted on hemolymph

extracted from the oysters. Prior to hemolymph sampling, a notch was ground in the ventral edges of *C. virginica* shells, and oysters were returned to flowing, ultrafiltered seawater flow for 1 or 2 days to clear shell pieces from the mantle cavity. For bleeding *C. gigas*, a notch was made in the shell with pliers immediately before hemolymph extraction. Hemolymph was withdrawn from the adductor muscle of each oyster using a needle and 1 ml syringe and stored temporarily in an Eppendorf microcentrifuge tube on ice to retard cell clumping.

In the Milford experiment, hemolymph from four oysters was pooled (Hégaret et al. 2003a) for the flow-cytometric analyses of all hemocyte parameters. In the Nantes experiments, hemolymph from each individual oyster was analyzed to examine the relationships between hemocyte parameters and toxin content.

Procedures for characterization of *C. virginica* hemocytes were modified slightly from those of Hégaret et al. (2003b) and for function (mortality, phagocytosis, and reactive oxygen species (ROS) production) according to the methods of Hégaret et al. (2003a). Concentrations and viability of sub-population hemocytes and phagocytic activity in *C. gigas* were measured according to Delaporte et al. (2003) and Delaporte (2005). Production of reactive oxygen species in *C. gigas* hemocytes was assessed in seawater according to Lambert et al. (2003). Either a FACScan (Milford) or FACSCalibur (Nantes) (BD Biosciences, San Diego, California; use of trade names does not imply endorsement) flow-cytometer was used for all hemocyte analyses.

Statistical analysis

Results of Milford and Nantes experiments were analyzed statistically using regression analyses, linear and rank correlation matrices, and ANOVA, with the hemocyte parameters as dependent variables and the feeding treatments, temperature, toxin accumulation, and days of exposure as independent variables. We used Statgraphics Plus statistical software (Manugistics, Inc, Rockville, MD, USA). Results were considered significant when the *P*-value was < 0.05 and highly significant if $P < 0.01$.

Results

Paralysis of the oysters

Adductor-muscle paralysis was observed in *C. virginica* fed *A. fundyense*. Indeed, after 4 days of exposure to the high concentration of *A. fundyense* and the mixed diet, the oysters did not close their shells when stimulated mechanically; 5- to 6-days exposures were necessary to paralyse the oysters fed the low *A. fundyense* diet. The oysters were agape, with gills extended, but they did not react to mechanical perturbation. Only hemolymph withdrawal with needle and syringe caused a slow and delayed shell-closure response. After 6 days of exposure, 40% of the oysters ($n = 10$) subjected to a high concentration of *A. fundyense* were paralyzed. Sixteen percent of the oysters ($n = 4$) fed the mixed diet of *A. fundyense* and *T. weissflogii*, and only 5% of the oysters ($n = 1$) fed the low concentration of *A. fundyense* were paralysed. In contrast, no paralysis was observed in oysters fed only *T. weissflogii* for 6 days.

Toxin accumulation

Toxin accumulation in the soft tissues of individual *C. gigas* in the presence of *A. catenella* was quantified; oysters held at 18°C accumulated significantly more toxin (ANOVA, $P < 0.001$) than those exposed to the same microalgae at 12°C (Fig. 1). Moreover, the amount of toxin present in the oysters increased with days of exposure (Fig. 1). A regression analysis of toxin accumulation with time of exposure shows a significant increase in toxin, measured as saxitoxin-equivalents, in the tissues of the oysters at 18°C ($P < 0.01$); the same regression was not statistically significant at 12°C.

Hemocyte parameter analysis

In the Milford experiment exposing *C. virginica* to several concentrations of *A. fundyense*, three different types of hemocytes were detected: granulocytes and small and large hyalinocytes. The type of algal exposure had no significant effect (ANOVA, $P > 0.05$) on any hemocyte characteristic measured after a 7 days exposure: hemocyte numbers and percentages (granulocytes and hyalinocytes), percentage of dead hemocytes, percentage of hemocyte phagocytosis, and

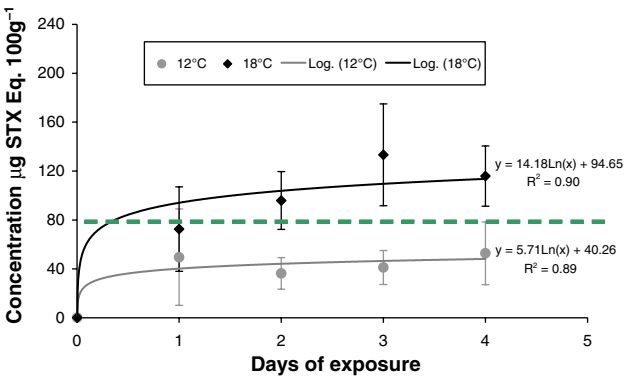

Fig. 1 *Crassostrea gigas.* Toxin accumulation, measured in STX equivalent in tissues of oysters according to days and the temperature of exposure. *Dashed line* threshold for human consumption limit

reactive oxygen species (ROS) production of the hemocytes (granulocytes and hyalinocytes).

The time of exposure of *C. gigas* to *A. catenella* had no statistically significant effect on hemocyte parameters. An ANOVA was run with time of exposure (0–4 days) as the independent variable and each hemocyte parameter as the dependent variable. These ANOVAs included all the data from all oysters tested: 12 and 18°C, control. The hemocyte functions and characteristics analyzed as the dependant variables were: (1) hemocyte concentrations and (2) percentages of the three populations of hemocytes detected, (i.e., granulocytes, hyalinocytes, and agranulocytes), (3) percentage of dead hemocytes, (4) percentage of phagocytic hemocytes, and (5) ROS production in the three hemocyte cell populations. There was no significant effect of the time of exposure of the oysters to *A. catenella* on any hemocyte parameter.

Relationships between toxin accumulation in individual *C. gigas* and the morphological or functional hemocyte measurements were also assessed using regression analysis, correlation analysis, and correlation analysis with ranked data at both temperatures combined or individually. Regression analyses were done on the oysters that had been exposed to *A. catenella* for 0, 1, 2, 3, or 4 days, and between toxin accumulation and each individual hemocyte parameter. Only one regression analysis showed a significant relationship between a hemocyte measure and tissue toxin content; in oysters held at 18°C, the percentage of dead hemocytes was positively correlated with tissue toxin accumulation (Fig. 2). This relationship was not significant at 12°C or when data from both temperatures were combined.

As most regression analyses of hemocyte parameters versus toxin accumulation in *C. gigas* tissues were not significant, less restrictive, simple correlation analyses were run with all hemocyte parameters and the toxin content in the tissues (Table 1). At 12°C, or with data from both temperatures combined, no correlation was significant. At 18°C, a significant, positive correlation was found between the percentage of dead hemocytes and toxin accumulation, as seen with regression analysis. To detect any non-linear relationships between toxin accumulation in the tissues and changes in hemocyte parameters, ranked correlations were tested (Table 1). Once again, at 18°C, there was a positive correlation between percentage of dead hemocytes and toxin content in the tissues. Moreover, when data from the two temperatures were combined, there was also a positive correlation between toxin accumulation and concentration of hemocytes ($P < 0.05$), especially the concentrations of granulocytes ($P < 0.01$) and small hyalinocytes ($P < 0.01$).

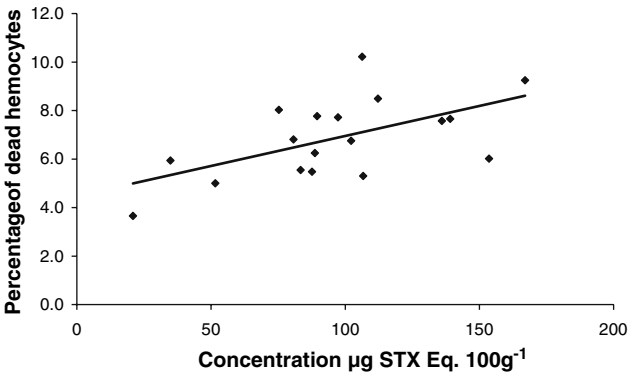

Fig. 2 *C. gigas.* Regression analysis (R^2 0.34, $P < 0.05$) of percentage of dead hemocytes in oysters exposed to *A. catenella* at 18°C with tissue toxin accumulation in STX Eq. 100 mg^{-1}

Table 1 *C. gigas.* Results of correlation analysis between several hemocyte parameters and toxin concentrations in tissues of oysters

Hemocyte parameters	Both temperatures	12°C	18°C
Concentration of hemocytes	NS	NS	NS
	0.36 (38) 0.04	NS	NS
Concentration of granulocytes	NS	NS	NS
	0.33 (38) 0.04	NS	NS
Percentage dead hemocytes	NS	NS	0.58 (18) 0.01
	NS	NS	0.50 (18) 0.04

First line represents correlation analysis with raw data; second line the correlation analysis with ranked data. The table includes correlation coefficient (R^2), number of observations (parentheses) and the *P*-value for tests. The following hemocyte parameters were analyzed and showed no significant difference under any circumstances: concentration of large hyalinocytes, percentage of granulocytes, percentage of large hyalinocytes, percentage of small hyalinocytes, production of ROS by granulocytes, production of ROS by large hyalinocytes, production of ROS by small hyalinocytes, and percentage of phagocytosis

Discussion

In the two experiments, there was almost no effect of *Alexandrium* spp. on the measured hemocyte parameters of *C. virginica* or *C. gigas.* Indeed, eastern oyster (*C. virginica*) hemocytes had the same characteristics after being fed *Thalassiosira weissflogii*, a mix of *T. weissflogii* and *A. fundyense*, or *A. fundyense* at two different concentrations. Despite this absence of effect on the hemocyte parameters, a clear physiological effect of *A. fundyense* on *C. virginica* was observed, specifically adductor-muscle paralysis of some oysters fed the higher concentration of *A. fundyense* after 4 days, and after 5–6 days for the oysters fed the mix and the low concentration.

Similarly, there was almost no apparent effect of *A. catenella* on the *C. gigas* hemocyte parameters tested. The hemocyte parameters appeared to be independent of the time of exposure to *A. catenella*. Moreover, in most cases

hemocyte parameters also appeared to be independent of the toxin accumulation in the tissues of the oysters. Based upon ranked correlation analysis, oysters that accumulated more toxins at 18°C tended to have a higher percentage of dead hemocytes, and also a higher concentration of circulating, live hemocytes. Because the numeric correlation was not significant, the changes in hemocytes were not quantitatively proportional to the magnitude of toxin accumulation in the tissues, although oysters with higher toxin contents also tended to be those with higher hemocyte concentrations and more dead hemocytes. Accumulation of dead cells and the increase in hemocyte number may indicate that hemocyte turnover and recycling was slowed as oysters accumulated more toxins, or it may indicate that oysters with higher concentrations of circulating hemocytes, including dead cells, were also those that tended to accumulate more toxin. Toxin accumulation is a function of both assimilation and depuration; therefore, differences in individual oyster metabolic rate could be related to variation in rates of both toxin depuration and hemocyte turnover. This relationship with metabolic rate is supported by the observation that the effect was significant at 18°C, but not 12°C and by a previous study demonstrating a positive relationship between heart rate and concentration of circulating hemocytes (Feng 1965).

PSP toxins are known to be neurotoxins that exert their effects by blocking sodium-channel function at neural synapses (Catterall 1980), and the adductor-muscle paralysis in *C. virginica* after feeding on *A. fundyense* is consistent with this knowledge. Moreover, our finding of no effect of PSP-containing *Alexandrium* spp. on the measured hemocyte characteristics may suggest that sodium-channel physiology does not play an important role in these particular functions.

A dinoflagellate species may produce a diverse array of bioactive chemical compounds, some functioning as allelochemicals affecting other phytoplankton (Tillmann and John 2002) and others that may serve as grazing deterrents (Delgado and Alcaraz 1999). Our findings of no effect of two different *Alexandrium* species upon certain hemocyte properties in two different oyster species exposed in the laboratory indicate that there are apparently no chemical products other than the known PSP toxins in these *Alexandrium* species that affect oyster hemocytes.

Practically, the finding of no effect of *Alexandrium* spp. on most hemocyte parameters indicates that oysters experiencing toxic *Alexandrium* blooms are likely to have functional hemocytes to respond to pathogens or parasites, at least during short-term (time scale of days) blooms. Chronic effects of long-term or repeated exposures of oysters to PSP toxins remain to be explored. Further, field studies of oysters during natural, toxic dinoflagellate blooms are needed to confirm these experimental data under environmental conditions that may present multiple stresses.

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