In vitro interactions between several species of harmful algae and hemocytes of bivalve molluscs

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Abstract

Harmful algal blooms (HABs) can have noxious and sublethal impacts on shellfish. The northern quahog (= hard clam), *Mercenaria mercenaria*, can experience blooms of several HAB species, including *Prorocentrum minimum*, *Heterosigma akashiwo* and *Alexandrium fundyense*. To understand the possible roles of hemocytes, the immune-defense cells, in bivalve responses to HABs, and how the algal cells are affected by these responses, *in vitro* tests of interactions between those harmful algae and hemocytes of *M. mercenaria* were carried out. Possible differences in hemocyte parameters attributable to harmful algae and also the effect of hemocytes on the algae themselves were measured. Using microscopic and flow-cytometric observations, changes in hematology and physiology, including cell concentration, phagocytosis, adhesion, and oxidative burst response of hemocytes, were determined. Changes in the physiology and the characteristics of the algal cells, including mortality, size, chlorophyll fluorescence and internal complexity, were also determined. The results show a species-specific response of the hemocytes depending upon the harmful algae to which they were exposed. Thus, *in vitro* tests allow a better understanding of the role of the hemocytes and the hemolymph in the defense mechanisms protecting molluscan shellfish from harmful algal cells.

Introduction

Harmful algal blooms (HABs) are increasingly recognized as having profound effects upon the ecology of coastal seas (Burkholder 1998) and upon the economics of fisheries and aquaculture (Shumway 1990; Anderson *et al.* 2000). Bivalve shellfish are routinely exposed to harmful algal blooms (HABs). In these suspension feeders, feeding and respiration are accomplished by the same physiological and behavioural activities of the gills, and thus avoidance of contact with HABs is possible for only short periods of time. Defense mechanisms in molluscan shellfish, protecting tissues from noxious or pathogenic agents, are attributable to an immune system (Cheng 1996) mediated by circulating cells called hemocytes that are similar to white blood cells in vertebrates. As bivalves have an open vascular system, hemocytes can be present everywhere in the body and, thus, come in contact with the harmful algae.

Previous experiments have shown effects of *in vivo* exposure of bivalve shellfish to HABs on hemocyte parameters. Hégaret *et al.* (2006) showed minimal effects of *Alexandrium catenella* on the hemocyte parameters of the oyster *Crassostrea gigas*; whereas, Hégaret and Wikfors (2005 a, b) demonstrated that *Prorocentrum minimum* significantly affects the hemocyte parameters of scallops (*Argopecten irradians irradians*) and oysters (*Crassostrea virginica*), and therefore likely their susceptibility to infection by parasites or environmental stresses. Immuno-modulation by harmful algae has the potential to increase the susceptibility of bivalves to diseases and parasites, thereby impacting commercial production and ecosystem services of molluscan populations.

Experiments reported here demonstrate the interactions *in vitro* between hemocytes of the hard clam *Mercenaria mercenaria* and three harmful algal species. An understanding of these interactions *in vitro* can help identify the role of the hemocytes in defense mechanisms when bivalves are exposed to HABs in the environment.

Materials and Methods

The algal species to which clams were exposed were obtained from the NOAA, Milford Laboratory (CT USA) collection: *Alexandrium fundyense* (strain BF2), *Prorocentrum minimum* (strain JA-98-01), and *Heterosigma akashiwo* (strain OL). *Rhodomonas* sp. was used as control. Algal species were cultured aseptically in 20-L carboys. Algal sizes varied from about 20 µm diameter for *A. fundyense* and *P. minimum* to 15 µm for *H. akashiwo* and *Rhodomonas* sp., which corresponds to the size of the hemocytes as well.

Northern quahogs (=hard clams) *Mercenaria mercenaria* were collected in Milford Harbor and kept in running seawater. Hemolymph was withdrawn from individual clams using a 5-ml syringe and

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stored in microcentrifuge tubes on ice before use to limit clumping.

To analyze interactions between hemocytes and harmful algae, microscopic observations as well as flow-cytometric analyses, were done. Changes in shape, size and internal complexity of the algal cells, as well as variation in chlorophyll or cell numbers after exposure to hemocytes, were determined. Simultaneously, several hematological parameters were also assessed: internal complexity and size, but also adhesion, the percentage of phagocytic cells, production of reactive oxygen species, and percentages of apoptotic and dead cells. All analyses were done according to the protocols of Hégaret *et al.* (2003a, b). Clam hemocytes were exposed, in 5 replicates, to the three different species of harmful algae experimentally for 1, 2, 3 or 4 h in microplates for the test of adhesion and in flow cytometer tubes for the other assays, at cell densities ten times higher than a natural bloom, to simulate the concentration of cells that occurs during filtration: 10^4 cells ml^{-1} for *Alexandrium fundyense* (Shumway *et al.* 1988; Townsend *et al.* 2005), 10^5 cells ml^{-1} for *Heterosigma akashiwo* (Rensel and Whyte 2003), 10^5 cells ml^{-1} for *Prorocentrum minimum* (Hégaret and Wikfors 2005) and 10^5 cells ml^{-1} for *Rhodomonas* sp. as a control. Control analyses were also done on hemocytes in filtered seawater (FSW) only. Results were analyzed with Multifactor Analysis of Variance, with time and algal treatments as factors, using Statgraphics 5.0. The times of incubation often significantly affected the results and are presented with letters following One-Way ANOVA with time, but in this review, we focus mainly on the effects of the different algal treatments on the hemocytes, represented with an asterisk on the graphs, and only statistically significant results will be presented.

Results

We observed species-specific effects of the algae on the hemocytes and vice versa. In the presence of *Prorocentrum minimum*, the number of hemocytes in the tube decreased after 4 h of incubation (Fig. 1). Microscopic observations showed the presence of large agglomerates of hemocytes and *P. minimum* cells. These agglomerates were not observed with the two other species of harmful algae. Phagocytosis of yellow, fluorescent microbeads by the hemocytes was inhibited by *Heterosigma akashiwo* (Fig. 2). In addition, microscopic observations showed chlorophyll fluorescence in some hemocytes. Concurrently, *Heterosigma akashiwo* cells incubated with hemocytes were

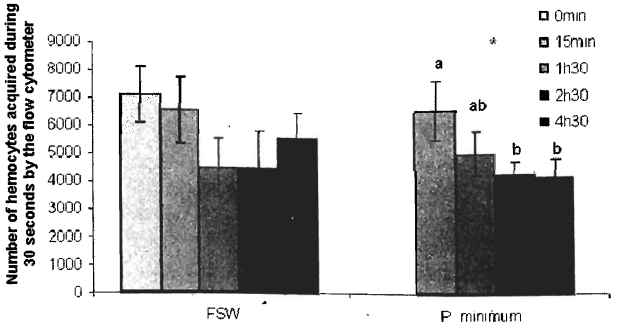

Figure 1. Effect of *Prorocentrum minimum* on the number of hemocytes from clams, *Mercenaria mercenaria*, over time (* indicates significant difference between FSW and *P. minimum* treatments; letters indicate significant differences between the times of incubation).

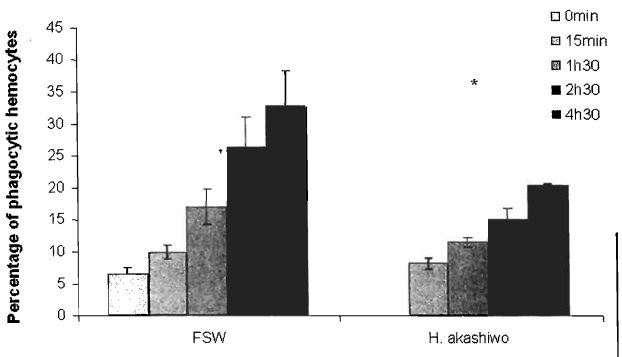

Figure 2. Effect of *Heterosigma akashiwo* on the percentage of phagocytic hemocytes from clams, *Mercenaria mercenaria*, over time (* indicates significant difference between FSW and *H. akashiwo*).

eliminated and degraded (achlorotic and fragmented). Phagocytosis was not affected with the two other species of harmful algae. Hemocytes in the presence of *Alexandrium fundyense* cells had much lower adhesion than controls (Fig. 3), as many freely-circulating hemocytes were left in the tube, but *A. fundyense* did not seem to affect any other immune parameter tested. Microscopic observations showed the transformation of *A. fundyense* cells exposed to hemocytes into temporary cysts. The two other species of harmful algae studied did not affect adhesion of hemocytes. Most of these changes were observable after 1 or 2 h, but continued to increase up to 4 h of incubation.

Discussion

The results demonstrate species-specific responses of the hemocytes to toxic algae and also that harmful algal cells are affected differently when exposed to hemocytes. Responses of hemocytes to harmful algal cells were very rapid; detectable within 1 h, but more extreme after 4 h. For future experiments, an incuba-

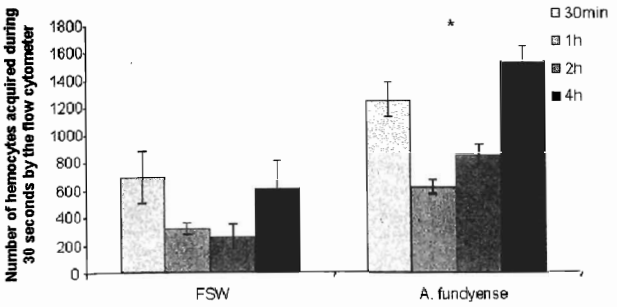

Figure 3. Effect of *Alexandrium fundyense* on the adhesion of clam, *Mercenaria mercenaria*, hemocytes over time (* indicates significant difference between FSW and *A. fundyense*).

tion time of 4 h would probably be sufficient to detect the effects of hemocytes on algae and vice versa.

Fewer free, single hemocytes were present in the tube incubated with the harmful alga *Prorocentrum minimum* (Fig.1), suggesting that hemocytes are aggregating together or around the *P. minimum* cells. Wikfors and Smolowitz (1993, 1995) reported the presence of hemocyte aggregates in gill and mantle tissues of oysters and scallops exposed to *P. minimum in vivo*. Thus, the *in vitro* finding in the present study is consistent with a protective response of the hemocytes to isolate the harmful algal cells from other tissues. *In vivo* experiments (Hégaret and Wikfors 2005 a, b) showed that *P. minimum* triggered an initial, rapid increase in circulating hemocyte concentration, followed by a decrease, which may be attributable to aggregation around the *P. minimum* cells.

Microscopic observation of hemocytes incubated with *Heterosigma akashiwo* showed the presence of hemocytes with chlorophyll fluorescence, indicating phagocytosis of the algal cells by the hemocytes. This activity may have been responsible for depressed phagocytosis of plastic microbeads (Fig. 2), but release of an inhibitory metabolite by *H. akashiwo* cannot be ruled out. In the presence of hemocytes, *H. akashiwo* cells lost fluorescence and shape. Further experiments should be conducted to assess the ability of *H. akashiwo* to produce chemical compounds, such as toxins or proteases, affecting the hemocyte functions.

Microscopic observations showed transformation of some *A. fundyense* cells into temporary cysts when in contact with the hemocytes. Moreover, adhesion of hemocytes was affected by the presence of *A. fundyense* cells (Fig. 3). Previous, *in vivo* experiments exposing oysters to *Alexandrium catenella* showed no effect of the algae on hemocyte concentration, phagocytosis, or production of ROS (Hégaret *et al.* 2006), consistent with the results of the present study. Unfortunately the previous study did not include measurements of hemocyte adhesion, the function affected *in vitro*.

Responses of hemocytes were very different according to the harmful algal species to which they were exposed. This could be attributable to different lectins on the surfaces of the different algal cells, which could elicit differences in hemocyte recognition and response. Protective hemocyte responses, such as phagocytosis, production of ROS, or adhesion and aggregation around the algal cells can follow. Hemocytes, however, also may release chemical compounds into the hemolymph (lysozymes); this response may explain degradation of *Heterosigma akashiwo* cells or transformation of *Alexandrium fundyense* into temporary cysts (Hégaret *et al.* this volume). Further analyses will be necessary to understand fully the functional roles of hemocytes in the defense mechanisms of bivalves exposed to HABs.

Finally, these *in vitro* assays are easier and much more efficient to perform than *in vivo* experiments, which require long-term exposure in the laboratory. The demonstration that *in vitro* responses correspond with *in vivo* interactions will facilitate future analyses of HAB-shellfish interactions.

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