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Hélène Hégaret
University of Connecticut

Roxanna Smolowitz
Roger Williams University, rsmolowitz@rwu.edu

Inke Sunila
State of Connecticut, Department of Agriculture, Bureau of Aquaculture

Sandra E. Shumway
University of Connecticut

Jennifer Alix
Northeast Fisheries Science Center, National Marine Fisheries Service

See next page for additional authors

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Authors
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Combined effects of a parasite, QPX, and the harmful-alga, *Prorocentrum minimum* on northern quahogs, *Mercenaria mercenaria*

Hélène Hégaret [a,*], Roxanna M. Smolowitz [b], Inke Sunila [c], Sandra E. Shumway [a], Jennifer Alix [d], Mark Dixon [d], Gary H. Wikfors [d]

[a] University of Connecticut, Department of Marine Sciences, 1080 Shennecossett Road, Groton, CT 06340, USA
[b] Marine Biological Laboratory, MRC 213, 7 MBL Street, Woods Hole, MA 02543, USA
[c] State of Connecticut, Department of Agriculture, Bureau of Aquaculture, P.O. Box 97, Milford, CT 06460, USA
[d] Northeast Fisheries Science Center, National Marine Fisheries Service, NOAA, 212 Rogers Avenue, Milford, CT 06460, USA

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ABSTRACT

Northern quahogs, *Mercenaria mercenaria* (L.), frequently are infected with the parasite Quahog Parasite Unknown (QPX, Labyrintohomorpha, Thraustochytriales), which can cause morbidity and mortality of the quahogs. Possible interactions between this parasitic disease and exposure to the harmful dinoflagellate *Prorocentrum minimum* in *M. mercenaria* were studied experimentally. Quahogs from Massachusetts with variable intensity of QPX infection were exposed, under controlled laboratory conditions, to cultured *P. minimum* added to the natural plankton at a cell density equivalent to a natural bloom. After 5 days of exposure, individual clams were diagnosed histologically to assess prevalence and intensity of parasitic infection, as well as other pathological conditions. Further, cellular defense status of clams was evaluated by analyzing hemocyte parameters (morphological and functional) using flow-cytometry. Exposure of quahogs to *P. minimum* resulted in: a lower percentage of phagocytic hemocytes, higher production of reactive oxygen species (ROS), larger hemocyte size, more-numerous hemocytic aggregates, and increased numbers of hemocytes in gills accompanied by vacuolation and hyperplasia of the water-tubular epithelial cells of the gills. Quahogs had a low prevalence of QPX; by chance, the parasite was present only in quahogs exposed to *P. minimum*. Thus, the effect of QPX alone on the hemocyte parameters of quahogs could not be assessed in this experiment, but it was possible to assess different responses of infected *versus* non-infected quahogs to *P. minimum*. QPX-infected quahogs exposed to *P. minimum* had repressed percentage of phagocytic hemocytes, consistent with immuno-modulating effect of *P. minimum* upon several molluscan species, as well as smaller hemocytes and increased hemocyte infiltration throughout the soft tissues. This experiment demonstrates the importance of considering interactive effects of different factors on the immunology and histopathology of bivalve shellfish, and highlights the importance of considering the presence of parasites when bivalves are subjected to harmful-algal blooms.

1. Introduction

Northern quahogs, *Mercenaria mercenaria* (L.), from a portion of the east coast of North America (Virginia to Prince Edward Island) have demonstrated variable infection with the protozoan parasite, Quahog Parasite Unknown, QPX, Labyrintohomorpha, Thraustrochytriales (Smolowitz and Leavitt, 1997), in some locations resulting in variable and sometimes high mortalities (Smolowitz et al., 1998; Ford, 2001; Ford et al., 2002; Dahl et al., 2008). Pathogenesis of this disease begins with appearance of parasite cells in mantle and gill tissues, which induces hemocyte migration into the area of infection to isolate and destroy the QPX cells (Smolowitz et al., 1998). Progression of the disease may include large, focal lesions or multifocal, granulomatous, inflammatory responses induced by the parasites, which increase with the severity of infection (Smolowitz et al., 1998). Eventually, necrosis and bacterial/fungal decomposition of infected tissues occurs, implying that immune functions may become impaired by QPX infection. The prevalence of the parasite in quahogs correlates with the mortality rate of the animals, and also is related to the origin of the quahog broodstock, i.e., specific genotypes appear to have higher susceptibility to the parasite (Ragone Calvo et al., 2007).

* Corresponding author. Present address: Université de Bretagne Occidentale, IUEM, LEMAR CNRS UMR 6539, Place Nicolas Copernic, Technopôle Brest Iroise-29280 Plouzané, France.
E-mail address: helene.hegaret@univ-brest.fr (H. Hégaret).

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Studies of how QPX disease modifies the morphology and functions of hemocytes, circulating cells involved in defense, in quahogs have already been performed (Hégaret et al., 2008a; Perrigault and Allam, 2009). Another thraustochytrid protist, phylogenetically related to QPX but without the envelope of secreted mucoid material present around QPX, isolated from *M. mercenaria*, C9G, has been demonstrated to activate the hemocyte phagocytic response, but to have no effect on production of ROS in quahog hemocytes (Anderson et al., 2003a). This selective response suggests that hemocytes were able to kill the parasite, but without involving oxygen-dependant mechanisms (Anderson et al., 2003a). Other parasites also have demonstrated effects on bivalve hemocytes (Anderson et al., 1995; Allam et al., 2001; Cochennec-Laureau et al., 2003; Goedken et al., 2005a, b). Thus, the presence of a parasite can be expected to affect the immune status of a bivalve, modifying responses to other environmental changes such as harmful-algal blooms (HABs) (Hégaret et al., 2007a; da Silva et al., 2008). Harvell et al. (1999) listed HABs as one of the growing concerns that may enhance the impact of diseases and parasites on marine organisms and the food webs supporting them.

Harmful-algal blooms can have diverse, deleterious effects on bivalve species (reviewed in Shumway (1990) and Landsberg (2002)), resulting in morbidity to mortality. Several studies have highlighted effects of harmful-algal species upon bivalve hemocytes (Hégaret and Wikfors, 2005a, b; Hégaret et al., 2007a, b; da Silva et al., 2008; Ford et al., 2008; Galimany et al., 2008a, b). Specifically, the dinoflagellate *Prorocentrum minimum* has been shown to cause morphological and functional changes in hemocytes of several bivalve species (Hégaret and Wikfors, 2005a, b; Galimany et al., 2008a; Hégaret et al., 2008b, 2009). This dinoflagellate is present throughout the world (Heil et al., 2005) and has been reported to affect filtration, growth, survival, or organ and tissue development of northern quahogs, bay scallops and juvenile eastern oysters (Leibovitz et al., 1984; Shumway et al., 1985; Luckenbach et al., 1993; Wikfors and Smolowitz, 1993, 1995, reviewed in Wikfors (2005)). Blooms of *P. minimum* have been recorded on the East Coast of the United States (Freudenthal and Jijina, 1985), indicating that this phytoplankter is sympatric with QPX-infected quahogs. Possible combined effects of *P. minimum* and QPX on quahogs, however, have never been assessed.

Objectives of the present study were to determine (1) if *in vivo* exposure of northern quahogs, *M. mercenaria*, to *P. minimum* would impart immunological or pathological changes, (2) whether or not the presence of the parasite QPX could affect any responses observed following a harmful-algal exposure, and thus assessing if there could be any combined effect of these both stresses.

2. Materials and methods

2.1. Experimental clams

Northern quahogs, *M. mercenaria* (45–55 mm shell length), were collected on August 10th 2006 from a low-intertidal sand flat near Scudder's Lane in Barnstable, MA, a location where the prevalence of QPX varies from 30% to 70% in 2 year-old quahogs (Smolowitz, unpubl. obs.). Quahogs were acclimated in flow-through seawater tanks for one week before the experiment in unfiltered seawater containing natural plankton assemblages pumped from Vineyard Sound, just off shore of Woods Hole, MA. The natural plankton assemblage was examined micro scopically to ensure the absence of any natural, harmful-algal bloom. The dinoflagellate *P. minimum* is not known to occur at bloom levels in this area. All the water coming out of the flow through system was collected, treated with sodium hypochlorite and discarded into the fresh water sewer system to prevent the potential spreading of the disease and the harmful-alga.

2.2. Algal cultures

The *P. minimum* (Pavillard) Schiller strain JA-98-01 (isolated from the Choptank River, Chesapeake Bay, Maryland, USA), was obtained from the Milford Microalgal Culture Collection. As inconsistent responses of bivalves to *P. minimum* have been observed in nature and in experiments (Wikfors, 2005), the strain JA-98-01 was chosen for its toxicity to juvenile bay scallops, *Argopecten irradians*, used as a bioassay to test algal toxicity; mortality of juvenile bay scallops occurs after 24 h exposure to stationary-phase cells, but log-phase cells are less toxic (Hégaret and Wikfors, 2005a; Galimany et al., 2008a). Cultures of *P. minimum* were grown in EDL7 medium, a modified version of the enriched-seawater E-medium (Ukeles, 1973) that contains L-1 trace metals, double the EDTA of the standard E formulation, KNO_3 rather than $NaNO_3$, and soil extract. The microalga was cultured in 20 L glass carboy assemblies using aseptic technique (Ukeles, 1973). Cultures were maintained at 20 °C with 24 h light, and harvested semi-continuously to maintain consistency in culture quality over the course of the study. Cells were harvested in stationary phase, usually approaching a concentration of $1–5 \times 10^5$ cells mL^{-1}. Algal cell densities were determined by hemocytometer counts under a light microscope.

2.3. Experimental design

Sixty quahogs were distributed randomly into twelve 1 L basins, i.e. five clams per basin. Six replicates of two different treatments were done in this experiment:

(1) Clams fed only the natural plankton, a community of 2–5 μm cyanobacteria and non-motile eukaryotic cells at approximately 10^4 cells ml^{-1}.
(2) Clams fed *P. minimum* at 2×10^4 cells ml^{-1}, added to the natural plankton.

Each replicate group of clams was fed continuously 5 mL min^{-1} for 5 days using a self-contained, integrated apparatus for exposing aquatic organisms to different water sources (Smith et al., 2006). Briefly, this integrated apparatus contains 12 flow meters, feeding twelve 1 L basins, which are themselves contained in a much larger 80 L basin. The overflow of this basin is collected by one-single drain to be treated before disposal. The twelve 1 L basins connected to individual flow meters can each receive a different algal mix. In this experiment, the two algal mixes were fed to the clams using gravity; the flow was controlled using 12 individual float-ball flow meters (Cole-Parmer). The algal suspension was provided to each basin, six receiving the natural plankton, and the other six the natural plankton to which *P. minimum* had been added. As the algal mixes were continuously added to the basins, they overflowed into the large basin and treated with Chlorox. Previous experiments showed that effects of *P. minimum* on eastern oysters hemocytes could be observed after 5 days of exposure (Hégaret and Wikfors, 2005a). Thus, after 5 days of exposure, the clams were removed from the apparatus; hemocytes were analyzed using flow-cytometric methods, and presence of Quahog Parasite Unknown (QPX) and other pathological conditions were assessed by histology.

2.4. Analysis of hemocyte parameters

Hemolymph was withdrawn with a needle and 1 mL syringe from the adductor muscle of each quahog, filtered through 75 μm mesh, and stored temporarily before use in an Eppendorf

microcentrifuge tube held on ice. Hemocyte analyses were conducted on hemolymph collected from individual bivalves.

Hemato-immunological parameters measured were: the concentration of circulating hemocytes (=total hemocyte counts – THC, cells ml^{-1}) as well as hemocyte characterization, in terms of size using the FSC detector and granularity or internal complexity using the SSC detector, according to Hégaret et al. (2003a). The immune functions measured were:

(a) Hemocyte mortality, as percentage of dead hemocytes, using Propidium Iodide (Sigma, final concentration 20 μg ml^{-1}) according to Hégaret et al. (2003b);
(b) Phagocytosis of fluorescent microbeads (Fluoresbrite YG Microspheres, 2.00 μm, Polysciences) by hemocytes, as percentage of highly-phagocytic (>2 beads) hemocytes according to Hégaret et al. (2003b) and by the number of beads phagocytized by each hemocyte ($G_{mean(>2\ beads)}/G_{mean(1\ bead)}$);
(c) Hemocyte production of reactive oxygen species (ROS) with potential to kill non-self, engulfed particles was assessed using 2 V,7 V-dichlorofluorescein diacetate (DCFH-DA, Sigma) described in Buggé et al. (2007).
(d) Adhesion of the hemocytes was measured by assessing the proportion of hemocytes that detach from the surface of experimental chambers after incubation with potential toxins as previously described for clams by Choquet et al. (2003). The assay was conducted in 24-well plates.
(e) Apoptosis of hemocytes was measured according to Goedken et al. (2005b), using Propidium Iodide (Sigma) and Annexin V (Fluoroprobes).

A FACScan (BD Biosciences, San Jose, CA) flow-cytometer was used for all hemocyte analyses.

2.5. Histopathological observations

Clams were examined macro- and micro-scopically to confirm the presence of the parasite and to detect other tissue damage. Quahogs were shucked and examined grossly for any abnormal swellings or nodules in the mantle, which are commonly found in QPX-infected, sub-market sized, aquacultured clams in Massachusetts, USA. Tissues from each quahog were prepared for histological sectioning by excising two diagonally-slanted cross-sections of tissue through the clam, which included all organs and foot. Additionally, a small section of mantle adjacent to the siphon (where nodules are often found), and any nodules/swelling in the mantle were sampled. Tissues were fixed in 10% formalin in seawater, processed in paraffin (in one cassette), sectioned at 6 μm, and stained with Harris' hematoxylin and eosin as per standard methods (Humason, 1979; Howard et al., 2004).

The slides were read blind, and histopathological features, such as inflammation of the organs, tubular-epithelium hyperplasia, water-tubule vacuolation, and hemocyte aggregates, were assessed under a light microscope and categorized for further analyses as 0 when the histopathological lesion was absent and 1 when present.

2.6. Detection and quantification of QPX infection in M. mercenaria

Tissue from each clam was evaluated and scored for presence and intensity of QPX parasite infections, the amount of associated hemocytic infiltration, and the amount of mucus production by the QPX organisms, visible as small, clear halos around QPX cells, indicating the presence of mucus before tissue processing (Smolowitz et al., 1998). The presence and intensity of QPX was scored according to Cheville (1983) as focal (localized area within a tissue or an organ), multifocal (several foci within a tissue or an organ), focally extensive (extension of the focus to involve a considerably-larger area within a tissue or an organ), and diffuse (distributed throughout all tissues examined). QPX presence and intensity were assessed for each of the following organs within an individual section: mantle, gill, dorsal tissues (heart, kidney, pericardium, and dorsal intestine), ventral tissues (ventral intestine, foot, ganglion, and sinus), and visceral mass. These evaluation methods are modifications of those developed by Ragone Calvo et al. (2007).

2.7. Statistical analysis

Results were analyzed statistically using *t*-tests and ANOVAs to assess effects of experimental treatments upon the individual response variables. Chi-square tests were also performed to assess the effect of the independent variables (algal exposure and parasite infection) on the histological features of the clams. We used Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA).

3. Results

Both groups of quahogs produced feces and pseudofeces during the course of the experiment. The natural phytoplankton to which *P. minimum* culture was added consisted of small (5 μm) *Thalassiosira* spp. and even smaller (1–2 μm) cyanobacteria. Quahogs exposed to *P. minimum* produced more biodeposits than clams from the control diet. The presence of intact and partially-digested cells of *P. minimum* in the biodeposits also clearly indicated that quahogs filtered and consumed the harmful-alga.

3.1. Presence of QPX in the tissues of quahogs

In the experiment, QPX was detected in 8 of the 60 quahogs. Only one QPX-infected clam was in the group exposed to natural plankton; this clam showed a multifocal QPX infection located in the mantle and at the base of the siphon, with a very large number of QPX cells. Seven QPX-infected clams were in the group exposed to *P. minimum*. QPX infection was most often observed (5 of 7 clams) in the mantle at the base of the siphon, where multifocal and focally extensive QPX infection associated with intense hemocytic infiltration was observed. Most QPX cells observed in these infiltrated areas of the mantle were dead before processing and were observed as dark-pink stippling within light-pink, round cells. These round cells showed indistinct or poorly-distinguished cell walls surrounded by hemocytes or within the cytoplasm of hemocytes, as described in Ragone Calvo et al. (1998). Two clams (of 7) showed QPX infections accompanied by hemocytic infiltration within the gills and connective tissues around the digestive tubules. Such infections in the connective tissues surrounding the digestive tubules and in sinusoids of the gills, in addition to the mantle, usually indicate an overall more-extensive infection of the clam, as compared to infection in the mantle alone (Smolowitz et al., 1998).

Microscopic observations did not show evidence of mucus production by QPX in the tissues, except for one clam, which had small, clear halos around QPX cells. This clam was the only individual in which QPX had infected the gonadal tubules.

3.2. Hemocyte analyses following exposure to P. minimum or natural plankton (in non-infected clams)

The effects of *P. minimum* upon hemocyte parameters of the clams were assessed, contrasting the non-infected quahogs exposed to the natural plankton or to *P. minimum* added to the natural plankton (Table 1). Exposure of quahogs to *P. minimum* for 5 days caused increases in mean size of hemocytes and production

Table 1
Effects of harmful-algal exposure on quahog, *Mercenaria mercenaria*, immunological parameters after 5 days of exposure to two algal treatments: *Prorocentrum minimum* plus natural plankton, or a control of natural plankton alone. ($N = 51$, including only the clams not infected with QPX: 29 fed the natural plankton and 22 fed *Prorocentrum minimum*; *t*-test: significant differences $^{*}P < 0.05$ and $^{**}P < 0.01$ as well as non significant (NS) differences are presented). AU: arbitrary units.

Hematological parameters	*P*-value	Natural phytoplankton	Prorocentrum minimum + Natural phytoplankton
		Mean (SE)	
Hemocyte counts (AU, 30 s acquisition)	NS	2269 (379)	2922 (435)
Size of hemocytes (AU)	**	421.1 (4.6)	444.0 (4.0)
Complexity of hemocytes (AU)	NS	44.7 (1.1)	45.1 (1.3)
% Necrotic hemocytes	NS	5.8 (0.7)	4.4 (0.6)
% of phagocytic hemocytes	*	31.5 (1.7)	25.2 (2.4)
Number of beads phagocytized by hemocytes	NS	5.71 (0.09)	5.53 (0.10)
Production of ROS (AU)	*	118.8 (12.8)	181.5 (12.2)
% Apoptotic hemocytes	NS	13.6 (1.4)	12.4 (1.1)
% Adhered hemocytes	NS	85.4 (2.6)	86.1 (2.27)

of reactive oxygen species, as well as a decrease in the percentage of phagocytic hemocytes (Table 1).

3.3. Effect of QPX infection in hemocyte responses of clams to an exposure to P. minimum

A lack of infected animals fed the natural plankton prevented assessment of any effect of QPX alone on the circulating hemocytes. Conversely, 7 of the 8 infected quahogs were exposed to *P. minimum*; thus, it was possible to assess the effects of QPX infection on the hemocyte responses of clams exposed to *P. minimum*. Results indicated that hemocytes of quahogs exposed to *P. minimum* were smaller in the QPX-infected quahogs than in those not infected (Fig. 1A). The percentage of phagocytic hemocytes and the number of beads phagocytized by each hemocyte in infected clams exposed to *P. minimum* were also lower (Fig. 1B and C). Differences in the percentage of phagocytic hemocytes and number of beads phagocytized by each hemocyte could be observed between the four groups of quahogs: exposed or not to *P. minimum* and infected or not with QPX. Only one quahog exposed to the control diet (natural plankton) was infected by QPX, which did not permit inclusion of this variable in the statistical analysis. Consequently, the one-way ANOVA calculated included only the three other treatments: quahogs fed the natural plankton and infected with QPX, quahogs fed *P. minimum*, infected with QPX, and quahogs fed *P. minimum*, not infected with QPX. Results (Fig. 1B and C) indicate a significant difference between the three treatments, with a decrease of the percentage of phagocytic hemocytes and the number of beads phagocytized by each hemocyte in quahogs fed *P. minimum*, which was even more pronounced in quahogs infected with the parasite. Neither *P. minimum*, nor QPX affected the percentage of apoptotic or dead (but not by apoptosis) hemocytes in circulating hemolymph, which, respectively, averaged 13% and 5%.

3.4. Histopathological analyses following exposure to P. minimum or natural plankton (in non-infected clams)

Exposure of non-infected clams to *P. minimum* resulted in mild to moderate hyperplasia (Table 2, Fig. 2, Chi-square, $P < 0.01$) and vacuolation (Table 2, Fig. 2, Chi-square, $P < 0.01$) of the water-tubular epithelial cells of the gills (Table 2; Fig. 2). Hemocyte infiltration and aggregates (granuloma/encapsulation) were observed only in quahogs exposed to *P. minimum* (Table 2, Figs. 2 and 3, Chi-square, $P < 0.01$) and were present in the connective tissues and sinuses of different organs: kidney, gills, mantle, foot, heart, and pericardial sac.

3.5. Histopathological effects of QPX infection in clams exposed to P. minimum

Further, histological sections revealed that effects of QPX, in quahogs exposed to *P. minimum*, included intense hemocytic

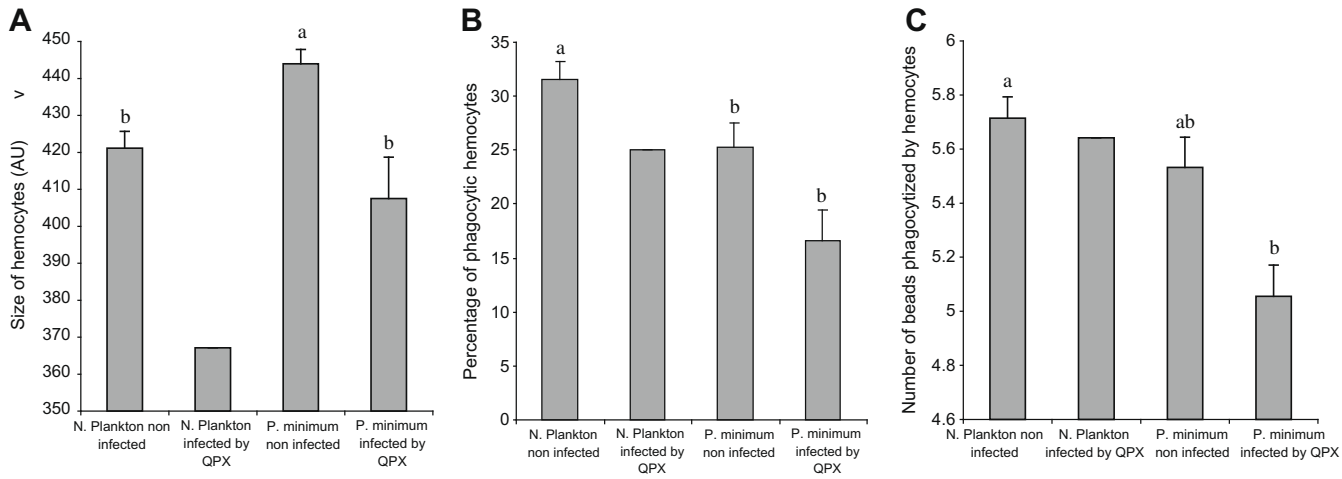

Fig. 1. Size (A) and percentage of highly phagocytic (B) and number of beads phagocytized by hemocyte (C) of hemocytes from quahogs, *Mercenaria mercenaria*, with or without QPX infection, exposed for 5 days to a control diet of Natural Plankton or to *Prorocentrum minimum* added to the natural plankton (*P. minimum*). ($N = 59$, ANOVA including only three groups: quahogs fed *P. minimum* infected ($n = 7$) or not ($n = 23$) with QPX, and quahogs fed the natural plankton and not infected ($n = 29$) with QPX; letters indicate significant differences ($P < 0.01$) between the three groups. As only one quahog fed the natural plankton diet was infected with QPX, these data were not included in the statistical analysis.) AU: arbitrary units.

Table 2
Effects of harmful-algal, *Prorocentrum minimum*, exposure upon non-QPX-infected quahog, *Mercenaria mercenaria*, histological parameters after 5 days of exposure ($N = 52$, 29 exposed to the natural plankton and 23 exposed to *Prorocentrum minimum*). The data were categorized as 0 or 1, according to the absence or presence of the observed character; Chi-square tests were performed (NS: not significant; $^{*}P < 0.05$; $^{**}P < 0.01$).

Histological features	*P*-value	Number of individuals showing histological features
	Natural plankton	*P. minimum*
Inflammation of the organs	NS	9 8
Tubular-epithelium hyperplasia	**	0 15
Water-tubule vacuolation	**	6 18
Hemocyte aggregates	**	0 8

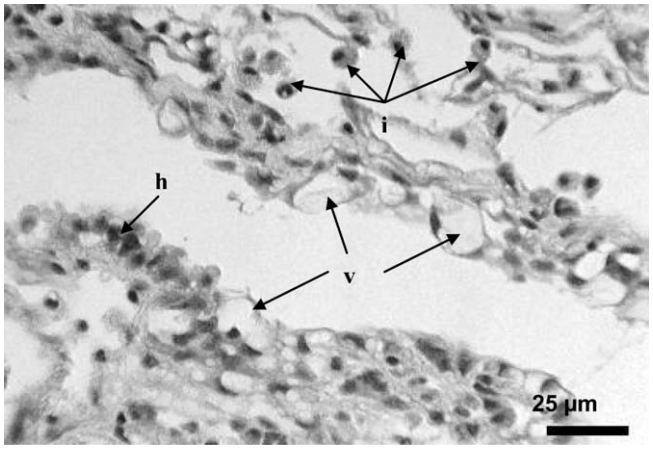

Fig. 2. Hyperplasia (*h*) of water-tubular epithelium, with individual-cell vacuolation (*v*) and hemocyte infiltration (*i*) in gills of quahogs *Mercenaria mercenaria* exposed to *Prorocentrum minimum*.

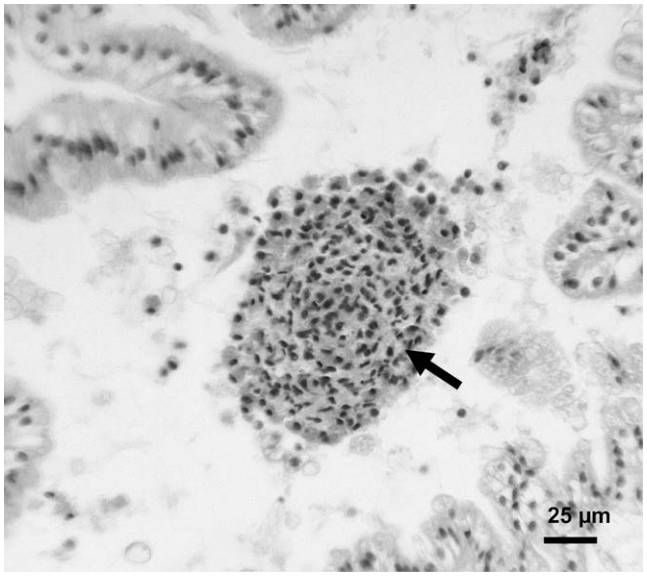

Fig. 3. Hemocyte aggregation, or granuloma (arrow) in the kidney lumen of a quahog, *Mercenaria mercenaria*, exposed to *Prorocentrum minimum*.

infiltration into the connective tissues of several organs including mantle (Fig. 4) and gills; 100% of quahogs, both infected and exposed to the harmful-alga, had hemocyte infiltration in the tissues, which was only observed in 36% of the cases of un-infected quahogs exposed to *P. minimum* (Chi-square, $P < 0.01$), indicating combined effects of both stressors. In addition, quahogs exposed to *P. minimum* and infected with QPX showed a mild increase in hemocytes within the gills (Chi-square, $P < 0.01$).

4. Discussion

This experiment explored the combined effects of two potential stressors, *P. minimum*, a HAB species, and a parasite (QPX), upon hemocyte parameters, and histopathological condition of quahogs (*M. mercenaria*).

Exposure to *P. minimum* caused an increase in mean size of hemocytes, a decrease in the percentage of phagocytic hemocytes and the number of beads phagocytized by each hemocyte, and higher production of ROS by quahog hemocytes. Quahogs exposed to *P. minimum* also showed presence of hemocytic infiltration and formation of granulomas in various tissues. Similar hemocyte responses to *P. minimum* have been observed in other bivalves. Oysters and scallops exposed to an artificial bloom of *P. minimum* had higher mean percentages of dead hemocytes and production of ROS, along with lower concentrations of circulating hemocytes and repressed phagocytosis in the case of scallops (Hégaret and Wikfors, 2005a). Manila clams, *Ruditapes philippinarum*, exposed to *P. minimum* also contained a lower percentage of phagocytic hemocytes (Hégaret et al., 2009). Thus, repression of hemocyte phagocytosis (in number of beads phagocytized and percentage) is a consistent, immuno-modulating effect of *P. minimum* upon several molluscan species, including clams and scallops.

Histological observations indicated that clams exposed to *P. minimum* had several pathological conditions attributable to the harmful-alga. The gills of quahogs exposed to *P. minimum* had significant water-tubular epithelial cell vacuolation that can lead to rupture and necrosis of these cells. Gills of *P. minimum*-exposed quahogs also often showed hyperplasia of the water-tubular epithelium, resulting in regenerative cell proliferation by the remaining epithelium and suggesting potential mild epithelium lysis. Hemocyte aggregates (thrombi/clots/granuloma) were also noted in the sinuses of the gills, kidney, pericardial sac, heart and foot. Hemocyte infiltration and granuloma in the tissues have also been observed in other bivalves, such as bay scallops *A. irradians*, Pacific oysters *Crassostrea gigas*, blue mussels *Mytilus edulis* and Manila clams *R. philippinarum* exposed to *Prorocentrum* spp. (Wikfors and Smolowitz, 1993; Pearce et al., 2005; Galimany et al., 2008a; Hégaret et al., 2009). Wikfors and Smolowitz (1993) also reported degeneration of the digestive gland and presence of hemocyte aggregates (granulomas) in several tissues of bay scallops exposed to *P. minimum*. Indeed, as bivalve molluscs feed, hemocytes can interact with harmful-alga, their toxins, or metabolites within the digestive diverticula, but also in other tissues, such as gills or mantle. Thus, observation of hemocytic infiltration or aggregation in the intestines, gills, gonadal follicles, etc. of *P. minimum*-exposed quahogs indicates that hemocytes probably reacted to the presence of the harmful-algae or its toxins throughout tissues.

Toxic effects of *P. minimum* have been demonstrated several times with scallop bioassays (Hégaret and Wikfors, 2005a; Galimany et al., 2008a), even though the toxic agent is not clearly identified at this time. The dinoflagellate *P. minimum* may produce some toxic compound responsible for epithelial cell necrosis (Grzebyk et al., 1997) generating hemocyte infiltration or aggregation to accomplish tissue repair or to isolate the tissues from the algal cells. The toxins produced by the entire *P. minimum* cells may also have direct effects on the epithelium, damaging the tissues, which could also explain the swelling of the water-tubular cells and hemocyte inflammation and aggregation. These aggregates could

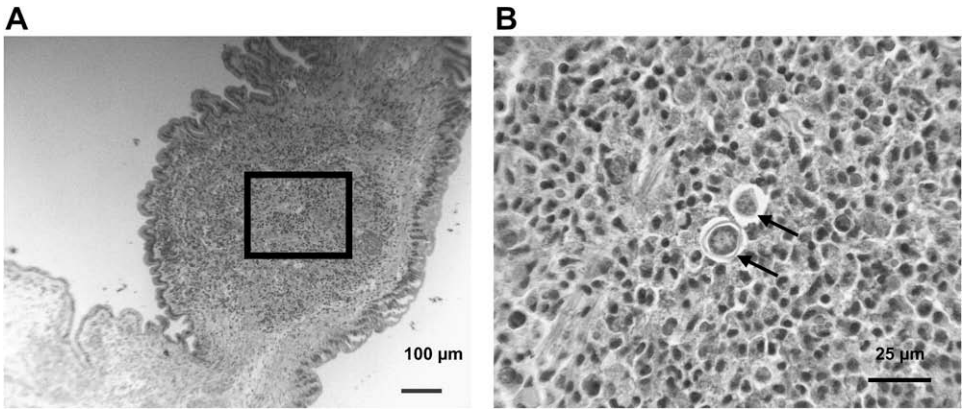

Fig. 4. Quahog Parasite Unknown (QPX, arrow) surrounded by hemocyte infiltration in the mantle of a quahog, *Mercenaria mercenaria* exposed to *Prorocentrum minimum*. Fig 4B (scale = 25 µm) represents the black square visible in Fig. 4A (scale = 100 µm).

reflect the response of encapsulation of the *P. minimum* cells by hemocytes, as suggested by Galimany et al. (2008a). Galimany and co-workers also suggested that the hemocyte responses of blue mussels *M. edulis* to *P. minimum* appear to be similar to the responses to a parasite infection, such as *Perkinsus* sp. or QPX. Encapsulation of *P. minimum* cells associated with a decrease in phagocytic capability was also observed *in vitro* when hemocytes of quahogs were incubated with *P. minimum* cells (Hégaret et al., 2008b). Moreover, no chlorophyll fluorescence was observed within hemocytes exposed to *P. minimum in vitro*; whereas, fluorescence could be seen when hemocytes were incubated with other algal species. These findings support the hypothesis of Galimany et al. (2008a) that quahog hemocytes do not engulf *P. minimum* algal cells, but rather isolate them by encapsulation, the same response elicited by parasites in several bivalve species.

The number of circulating hemocytes remained the same in all treatments, indicating that quahogs were able to overcome such stresses and produced new hemocytes to replace those involved in the response to the harmful-alga or the parasite. Similar observations were made by Galimany et al. (2008a) and Hégaret et al. (2009) who reported that blue mussels, *M. edulis*, and Manila clams *R. philippinarum*, exposed to *P. minimum*; despite intense hemocyte infiltration into the tissues and diapedesis into the alimentary canal, maintained a constant hemocyte concentration in the circulating hemolymph. In both studies, a large amount of bacteria was also observed in the intestine, surrounded by hemocytes, which had undergone diapedesis. Conversely, bacterial multiplication in intestine or alimentary canal of quahogs could not be seen in this study. Previous work demonstrated the ability of quahogs *M. mercenaria* exposed to bacteria to clear them by rapidly sedimenting them into feces or pseudofeces (Hartland and Timoney, 1979; Timoney and Abston, 1984). Northern quahogs may have a better antibacterial defense mechanism or may be better prepared to react against an exposure to *P. minimum*.

Infection of quahogs with QPX was not well distributed in experimental groups; only eight clams of 60 were infected by QPX. Unfortunately, the infection by QPX of each individual clams could not be assessed previous to the experiment, as the method of determination for the presence of the parasite is lethal. By chance, seven infected clams were in groups exposed to *P. minimum*. Thus, we were able to observe how infection with QPX can modify the response of quahogs to *P. minimum*, but not effects of QPX alone. Results indicated that the mean size of hemocytes in QPX-infected quahogs tended to decrease following exposure to *P. minimum*; whereas, hemocytes of non-infected quahogs were larger when exposed to *P. minimum* for 5 days. One hypothesis explaining the decrease in the size of hemocytes could be that new, smaller hemocytes were produced in response to the increasing movement of hemocytes into tissues infected with QPX, as observed by the high increase of hemocytic infiltration surrounding the parasites. QPX has been shown to induce hemocytic infiltrations in tissues (Smolowitz and Leavitt, 1997; Smolowitz et al., 1998; Dove et al., 2004; Ragone Calvo et al., 2007), which were indeed much more intense in *P. minimum*-exposed quahogs infected with QPX, than in the un-infected clams. Moreover, the presence of QPX is often associated with phagocytic hemocytes (Ragone Calvo et al., 1998; Smolowitz et al., 1998). It is not possible with flow-cytometry to distinguish granular from agranular hemocytes in quahogs; however, we hypothesize that highly-phagocytic hemocytes, usually more granular, would be directed from the open vascular system towards the infected tissues. This hemocyte migration would be consistent with, the reduction in size of the circulating hemocytes, as well as in percentage of phagocytic hemocytes in the overall circulating hemocyte population.

Mucoid secretion by QPX has been shown to represent a virulence factor (Anderson et al., 2003b). In this study, only one QPX-infected quahog showed presence of QPX in the gonadal tubules, less often infected than other organs (MacCallum and McGladdery, 2000; Dove et al., 2004) and which represents a later and more-severe stage of infection. The QPX parasites from this clam had halos, indicating the production of mucus, which may be at the origin of the heavier infection stage of this clam. This was confirmed by histological observations showing more intense water-tubule vacuolation, tubular-epithelium hyperplasia, and high hemocytic infiltration in these tissues.

The present study shows that QPX disease in quahog clams can modify the response of quahogs to environmental stressors, such as harmful-algal blooms. These results are consistent with the findings of Hégaret et al. (2007b), who demonstrated combined effects of *P. olseni* and the harmful-alga *Karenia selliformis* on concentration of circulating hemocytes and hemocyte phagocytic activity in Manila clams. Indeed, when quahogs were subjected to both stressors, combined infection with QPX and exposure to the HAB, hemocyte size decreased, indicating an increase in impact when both stressors were combined. Similarly, non-infected quahogs exposed to *P. minimum* had depressed phagocytosis, which was more intense when the quahogs were infected, indicating another combined effect of both types of stressors. These results also confirm that repressed phagocytosis (in percentage and number of beads phagocytized) could be used as evidence of physiologically-active HAB effects upon bivalves, as it is already been applied in the case of pollutants, such as heavy metals, hydrocarbons, pesticides, etc. (Cheng and Sullivan, 1984; Alvarez and Friedl, 1992; Brousseau et al., 2000; Hamoutene et al., 2004; Gagnaire et al., 2006).

5. Conclusion

The harmful dinoflagellate *P. minimum* had a low-grade but significant toxic effect on the tissues of quahogs, causing vacuolation and hyperplasia of the water-tubular epithelium of the gills as well as hemocyte aggregates (granuloma/encapsulation) in the sinuses of various organs. Exposure to *P. minimum* also modified quahog circulating hemocytes, reducing the percentage of highly-phagocytic hemocytes, as well as the number of beads phagocytized by each hemocyte, activating production of ROS, and slightly modifying hemocyte morphology. This study also showed that an existing QPX infection in clams can modify response of quahogs to HAB exposure, indicating combined effects of these stressors upon the immunology and histopathology of bivalve shellfish.

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