Pathology and immune response of the blue mussel (Mytilus edulis L.) after an exposure to the harmful dinoflagellate Prorocentrum minimum

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Abstract

The harmful dinoflagellate Prorocentrum minimum has different effects upon various species of grazing bivalves, and these effects also vary with life-history stage. Possible effects of this dinoflagellate upon mussels have not been reported; therefore, experiments exposing adult blue mussels, Mytilus edulis, to P. minimum were conducted. Mussels were exposed to cultures of toxic P. minimum or benign Rhodomonas sp. in glass aquaria. After a short period of acclimation, samples were collected on day 0 (before the exposure) and after 3, 6, and 9 days of continuous-exposure experiment. Hemolymph was extracted for flow-cytometric analyses of hemocyte, immune-response functions, and soft tissues were excised for histopathology. Mussels responded to P. minimum exposure with diapedesis of hemocytes into the intestine, presumably to isolate P. minimum cells within the gut, thereby minimizing damage to other tissues. This immune response appeared to have been sustained throughout the 9-day exposure period, as circulating hemocytes retained hematological and functional properties. Bacteria proliferated in the intestines of the P. minimum-exposed mussels. Hemocytes within the intestine appeared to be either overwhelmed by the large number of bacteria or fully occupied in the encapsulating response to P. minimum cells; when hemocytes reached the intestine lumina, they underwent apoptosis and bacterial degradation. This experiment demonstrated that M. edulis is affected by ingestion of toxic P. minimum; however, the specific responses observed in the blue mussel differed from those reported for other bivalve species. This finding highlights the need to study effects of HABs on different bivalve species, rather than inferring that results from one species reflect the exposure responses of all bivalves.

Key words: Harmful algal blooms, hemocyte, histopathology, immunology, Mytilus edulis, Prorocentrum minimum.

1. Introduction

Mussels (Mytilus sp.) are suspension-feeding bivalves that are harvested for human consumption around the world (Figueiras et al., 2002, Mortensen et al., 2006). As mussels, whether from wild fisheries or aquaculture, are grown and harvested under natural conditions, chemical and biological quality of growing waters is very important for survival and growth, hence quantity and quality of the yield. Mussels can be affected by different types of toxins, from both chemical (Dyrynda et al., 2000, Parry and Pipe, 2004) and biological sources (Galimany et al., 2008).

Biological effects of toxic microalgae upon bivalve mollusks can include mortality (Wikfors and Smolowitz, 1993), tissue damage (Pearce et al., 2005), cellular dysfunction (Hégaret and Wikfors, 2005), and reproductive failure (Granmo et al., 1988). In addition, edible tissues can become contaminated with chemical or biological toxins, rendering them unfit for human consumption (EUROHAB, 1998, Heil et al., 2005). Harmfull algal blooms (HABs) appear to be increasing in geographic distribution and intensity (Hallegraeff, 2003). This has led to a growing concern about effects of HABs upon shellfish resources, in terms of both seafood safety and production efficiency.

Inimical effects of HABs upon grazing animals have raised questions about the evolutionary and ecological relevance of toxic or noxious properties in these microorganisms. Do toxins or harmful properties of HABs confer protection from grazing? It is very common for forage species of land plants to protect themselves from grazing by producing noxious properties or toxicity (Agrawal et al., 1999, Agrawal,

2007). In terrestrial ecosystems, it is not uncommon for grazing species to have developed tolerance to toxins from specific forage species (De Mazancourt et al., 2001, Karban and Agrawal, 2002). Recently, growing evidence for similar, grazing-deterrent functions of toxins in marine dinoflagellates have been reported (Selander et al., 2006). As in terrestrial ecosystems, susceptibility of grazers, including bivalve mollusks, to specific grazing-deterrent chemicals produced by HAB taxa appear to be species-specific (Landsberg, 2002).

The microalgal species Prorocentrum minimum (Pavillard) Schiller is a dinoflagellate with the capacity to express toxicity (Grzebyk et al., 1997; Wikfors, 2005) that is now considered a "HAB" species (Heil et al., 2005). Toxicity of P. minimum varies among different strains studied (Grzebyk et al., 1997, Denardou-Queneherve et al., 1999) and has been found to fluctuate according to growth phase, i.e., stationary-phase populations are more toxic than actively-growing populations (Grzebyk et al., 1997). Despite this variability, P. minimum has been demonstrated to be toxic to molluscan shellfish, causing a wide diversity of symptoms, including pseudofeces production in several species of oyster and clam (Hégaret et al., 2007), tissue damage and developmental abnormalities in young stages of the Eastern oyster, Crassostrea virginica (Wikfors and Smolowitz, 1995a), changes in immune parameters of Eastern oysters and bay scallops, Argopecten irradians irradians (Hégaret and Wikfors, 2005) and mortality (Shumway and Cucci, 1987; Shumway, 1990, Luckenbach et al., 1993). Most studies of P. minimum effects upon mollusks reported to date have been with oysters and clams, but not with mussels, although it has been demonstrated that mussels can accumulate toxicity (Denardou-Queneherve at el., 1999). Mussels are known to have unusual tolerances to microalgal biotoxins (Wootton et al., 2003), such as saxitoxin, that cause clear biological effects in other molluscan species

(Landsberg, 2002, Heil et al., 2005). Thus, possible effects of P. minimum upon mussels cannot be deduced from findings with other molluscan species.

The present study, therefore, investigated the effects of P. minimum upon adult blue mussels, Mytilus edulis, under experimental conditions, focusing on immune functions and histopathology, both of which have been shown to respond to P. minimum exposure in other molluscan species.

2. Materials and methods

2.1 Experimental animals

Mussels, Mytilus edulis (47.6-73.9 mm shell length) for this experiment were collected from Westcott Cove, Stamford, Connecticut, USA, from an intertidal beach on the north shore of Long Island Sound in June of 2007. Mussels were acclimated for 4 days before the experiment, the first 3 days in the experimental tanks with filtered seawater, and the fourth day fed with Rhodomonas sp. (RHODO, see below) at a concentration of $1x10^4$ cells ml^{-1}.

2.2. Algal cultures

The Prorocentrum minimum strain used for the experiment was obtained from the Milford Microalgal Culture Collection, strain JA-98-01 (isolated from the Choptank, River, Chesapeake Bay, Maryland, U.S.A.). In addition, the RHODO strain of the cryptophyte Rhodomonas sp. was used as a non-toxic, control alga.

The microalgae were cultured in 20-l glass carboy assemblies using aseptic technique (Ukeles, 1973). Cultures were harvested semi-continuously to maintain consistency in culture quality over the course of the study and were harvested in late-log or early-stationary phase. Cultures of Prorocentrum minimum were grown in EDL7 medium, a modified version of the enriched-seawater E-medium (Ukeles, 1973) that

contains L-1 trace metals (Guillard and Hargraves, 1993), double the EDTA of the standard E formulation, KNO_3 rather than $NaNO_3$, and 10 ml l^{-1} soil extract. Rhodomonas sp. was cultured in E-medium. Both cultures were maintained at 20ºC with 24-h light. Algal cell densities were determined by hemocytometer counts with a light microscope.

The toxicity of the P. minimum culture used in the mussel-exposure experiment was tested with a scallop bioassay (Rosetta and McManus, 2003). Five northern bay scallops, Argopecten irradians irradians, were placed in each of 12 1-l beakers; the following 4 treatments were tested in triplicate beakers: 1) filtered sea water, 2) filtered sea water diluted with distilled water to equal salinity of algal treatments, 3) Rhodomonas sp. at $1.9x10^5$ cells ml^{-1}, and 4) P. minimum at the same cell density. Observations of scallop activity and mortality were made periodically for 20 hr. Dissolved oxygen, pH and salinity were measured at the beginning and at the end of the exposure.

2.3. Experimental design

The main experiment tested the effects of cultured P. minimum, upon the immunology and histopathology of mussels, M. edulis. Two-hundred and seventy (270) mussels were distributed randomly into 6 20-l glass-aquaria, i.e. 45 mussels per aquarium. Three replicates of each treatment were done in this experiment: Rhodomonas sp. or P. minimum, each at $1x10^4$ cells ml^{-1}, were given with a regime of 16 feedings per day, every 90 min (285 ml per day), using the cover of a rearing-chamber system incorporating computer-automated valves to add microalgal culture as programmed (Smith and Wikfors, 1998).

Samples of mussels were collected on day 0, before exposures, and after 3, 6 and 9 days of exposure to the experimental, microalgal treatments. At each sampling time,

mussels were removed from the basins and analyzed for hemocyte parameters, stomach contents, and histopathology. Feces and pseudofeces were also examined microscopically throughout the experiment.

2.3.1. Stomach contents, feces and pseudofeces

Stomach contents of mussels were removed with a 25-gauge needle and a 1-ml syringe. Samples were preserved in 300 µl of 1% glutaraldehyde and observed with a light microscope. Feces and pseudofeces were collected from the tanks with a pipette and observed directly with a light microscope.

2.3.2. Immunological analysis - Hemocytes

Hemolymph was withdrawn with a 21-gauge needle and a 1-ml syringe from the adductor muscle of each individual mussel and stored temporarily in an Eppendorf microcentrifuge tube on ice. Analyses of hemocyte morphology and function were done on hemolymph extracted from each individual mussel.

Procedures for characterization of the hemocytes, cell density of circulating hemocytes (cells ml^{-1}), size, and internal complexity of the hemocytes, and immunological functions (listed below) were adapted from Delaporte et al. (2003), Hégaret et al. (2003a, 2003b), Lambert et al. (2003), Soudant et al. (2004) and Buggé et al. (2007). Hemocyte apoptosis was also assessed according to a protocol adapted from Goedken et al. (2005a). For the hemocyte analyses, a FACScan flow-cytometer (BD Biosciences, San Jose, CA) was used.

The five functional hemocyte parameters measured were:

a) hemocyte mortality, as percentage of dead hemocytes
b) phagocytosis of fluorescent microbeads by hemocytes, which simulates the engulfment of non-self particles

c) respiratory-burst response in hemocytes, that measures reactive oxygen species' potential to kill non-self particles previously engulfed

d) percentage of adhering hemocytes

e) percentage of apoptotic hemocytes

2.3.3. Histopathology

A 4-mm cross-section of each mussel, including digestive diverticulum, gills, mantle, kidneys, plicate membranes, and the byssus gland, was dissected and fixed in Davidson's fixative for 48 h at 4ºC. The tissues were then rinsed in 50% ethanol in filtered seawater, and transferred to 70% ethanol. Samples were dehydrated and embedded in paraffin. After processing, 5-µm sections were stained using a hematoxylin-eosin staining procedure (Howard et al., 2004) and examined under a light microscope. Blocks containing four mussels with bacteria subsequently were resectioned and stained with Gram-stain according to Howard et al. (2004).

2.4. Statistical analysis

Results for the hemocyte assays and total number of pathological changes were analyzed statistically using Correlation analysis and Multifactor Analysis of Variance (MANOVA) to assess effects of experimental treatments upon the response variables. Results for single pathological changes (bacteria in the intestine, migration of hemocytes to the stomach and intestine, and hemocytes around gonadal follicles) and stomach contents were analyzed using Chi-square tests for each sampling time. The statistical software used was Statgraphics Plus (Manugistics, Inc., Rockville, MD, USA).

3. Results

3.1 Prorocentrum minimum toxicity

At the beginning of the bay-scallop exposure, the media had pH values in the range of 7.5 – 8.0, oxygen saturation ranged between 92.3 and 94.4%, and salinity was 25‰ in the filtered sea water beakers but 13‰ in all other containers. After 4h of exposure, all scallops were open and alive. 20h after beginning the exposures, all scallops exposed to P. minimum were dead, and dissolved oxygen had decreased to a mean value of 35% in these beakers. One third of the scallops exposed to Rhodomonas sp. also died, but pH, oxygen saturation, and salinity of the alga media tested remained constant. We note that lower salinity in beakers containing algal cultures probably stressed scallops, but we chose to maintain the salinity used to culture the algae, rather than add salt or brine to increase salinity to that of the source water for the scallops. We interpret the finding of 100% mortality in scallops exposed to P. minimum, compared to 33% mortality in Rhodomonas sp.- exposed scallops, as an indication that the P. minimum culture was bioactive against a bivalve model at the time it was used – an important consideration in light of previous findings of fluctuating toxicity in P. minimum cultures.

3.2 Stomach contents, feces and pseudofeces

Cells of the alga provided, either Prorocentrum minimum or Rhodomonas sp., were present in the stomach, feces and pseudofeces of the exposed mussels. After 3 days of exposure to P. minimum two of the mussels exposed to the toxic algae had large quantities of bacteria included in the stomach contents ($p>0.05$). This observation differed significantly between treatments at the end of the experiment ($p<0.05$). High numbers of hemocytes were observed in the feces of P. minimum-exposed mussels, usually forming aggregations around P. minimum cells.

3.3 Immunological analyses

Results from both hemocyte characterization and immunological functions are shown in Table 1. Main effects and interactions were not significantly different for most parameters studied when analyzed with the MANOVA. The only hemocyte characterization that showed significant differences throughout the experiment was internal complexity of hemocytes ($p<0.05$) (Fig. 1). Neither cell density of the hemocytes nor cell size varied with time or treatment. The immunological functions also did not show significant differences throughout the experiment.

3.4 Histopathology

Pathological changes found in the M. edulis tissues in the day-0 sample consisted of focal inflammatory responses (prevalence in the day-0 sample 2/30, 7%; in the entire experiment 33/210, 15%), phagocytes in follicles (7%/1%), kidney stones (3%/2%), and ceroid (3%/13%). Parasites present were trematodes Proctoeces maculatus (3%/3%), and trematodes from the family Gymnophallidae (50%/57%). The ciliate Ancistrum mytili was present on the gills at a low prevalence (0%/1%). The presence of focal inflammatory responses in the digestive diverticula or the connective tissue of the mantle, and pearls (0%/2%) were associated with Gymnophallidaes. Ceroidosis occurred with a prevalence of 11% in P. minimum-exposed mussels, and 19% in Rhodomonas sp.-exposed mussels. There was a 2% prevalence of abscesses in the mussels: two cases occurred in P. minimum-exposed mussels and three in Rhodomonas sp.-exposed mussels. The above pathological changes occurred independently of the algal exposure or time, and are considered to be background pathology, or "noise" in this experiment.

Mussels exposed to the harmful dinoflagellate P. minimum showed significant pathological differences compared to those exposed to the non-toxic Rhodomonas sp. (Figs 2, 3). P. minimum-exposed mussels had significantly more cases of elevated

quantities of bacteria in the stomach and intestine ($p<0.01$), and migration of hemocytes into the stomach and intestine ($p<0.01$). Sixty percent of mussels exposed to P. minimum showed abnormally-high numbers of bacteria in the alimentary canal, starting in the first sampling following initial exposure. Gram-negative, rod-shaped bacteria appeared as large, grey colonies in hematoxylin-eosin stained sections and red colonies in Gram-stained sections in the lumina of the stomachs and intestines (Fig 4) forming a biofilm in the middle of the alimentary canal. Cilia in the intestine epithelium were surrounded by bacteria, and in some areas appeared to be beating asyncronously while caught in the biofilm. During the following samplings, the percentages of mussels with gut-bacterial proliferation remained near 54%. None of the mussels exposed to Rhodomonas sp. showed this anomaly.

Mussels exposed to P. minimum also had hemocytes in the stomachs and intestines (Figs 2, 5). Healthy hemocytes surrounded the intestine and stomach epithelia, and were seen migrating through epithelia into the lumina of the alimentary canal (diapedesis). The prevalence of mussels with this anomaly reached 80% in the first sampling after exposure and increased to 93% after six days and 90% nine days after the beginning of the experiment (Fig. 2). Once in the alimentary canal lumina, hemocytes engulfed some bacteria, but were generally overwhelmed and instead succumbed to bacterial degradation (Fig 6). Some hemocytes died by apoptosis, distinguished by marginal, crescent-shaped, condensed chromatin inside hemocyte nuclei, but some hemocytes also were necrotic. Hemocyte nuclei and remnants of hemocytes were observed within the bacterial mass during degradation. This condition was also present in some individuals from the control tanks, but at significantly lower prevalences (Fig. 2).

After 9 days of exposure to P. minimum, but not after an exposure to Rhodomonas sp., some mussels had aggregations of hemocytes in the connective tissue between the gonadal follicles ($p<0.05$) (Fig 7). This pathological change appeared also in one mussel after 3 days of exposure to P. minimum, but this observation was not statistically significant ($p>0.05$).

The total number of pathological changes increased in the M. edulis exposed to P. minimum during the experiment (Fig. 3). Significant differences were found between the algae ($p<0.01$), time ($p<0.01$) and the interaction of these two factors ($p<0.01$).

Discussion

The scallop bioassay to test P. minimum toxicity showed mortality in all scallops exposed; thus it can be concluded that the algal culture was toxic. Although this method does not quantify the toxicity in any way, it showed harmful effects of the dinoflagellate upon bivalves as anticipated. These findings agree with those of Hégaret and Wikfors (2005) who also found 100% mortality of scallops exposed to the same P. minimum strain. One third of the scallops exposed to Rhodomonas sp. also died by the end of the bioassay; this may be a consequence of ammonia accumulation resulting from metabolism of scallops in the beakers (Widman et al., in press).

Histology revealed that the immune response of mussels to P. minimum was a massive migration of hemocytes into the stomach and intestine (diapedesis) to protect the tissues from exposure to the toxic algae. Similar results were found by Hégaret et al. (in prep) when Manila clams were exposed to P. minimum. Hemocytes appear to encapsulate the toxic cells in the alimentary canal and form hemocyte-P. minimum aggregates to remove P. minimum cells from the bivalves, thereby minimizing contact with other tissues. Histological sections of bay scallops after exposure to P. minimum

(Wikfors and Smolowitz, 1993) showed increased numbers of hemocytes in the connective tissue surrounding digestive diverticula and other pathological changes, in contrast to findings of the present experiment. It has been suggested that the site or tissue within the animal where P. minimum is digested may play a role in determining response to this dinoflagellate (Wikfors and Smolowitz, 1995b).

The immunological analyses done for the hemocytes showed different results than those found for oysters and scallops (Hégaret and Wikfors, 2005), wherein hemocyte numbers and functions were affected by the toxic-algal exposure. In the present study, the immune parameters analyzed did not show significant differences throughout the experiment. The hemocytes did not phagocytose P. minimum but seemed to encapsulate it. Phagocytosis is a process that can be linked to a subsequent respiratory-burst response (Pipe, 1992, Carballal et el., 1997a). In the present study, neither the phagocytic capacity of the hemocytes nor the respiratory burst response showed differences throughout the experiment. Adhesion of circulating hemocytes remained above 80% throughout the experiment; this hemocyte characteristic did not diminish, and P. minimum did not affect this function in the circulating hemocytes (Chen and Bayne, 1995). This observation is consistent with the effective adherence and aggregation of the hemocytes within the digestive system. Neither hemocyte mortality nor apoptosis showed significant differences with flow-cytometry throughout the experiment.

The mean number of circulating hemocytes did not vary throughout the exposure, although many hemocytes were removed from circulation into and through the alimentary canal. The explanation for this phenomenon is that new hemocytes were produced to replace those responding to the presence of P. minimum and bacteria in the intestine and stomach. The site of hematopoiesis in bivalves is not known (Bachère et

al., 2004); data from cytograms or histological samples did not reveal a specific anatomical location where the hemocytes were dividing.

The complexity of the hemocytes was the only hemocyte characteristic that showed significant differences between the algal exposures and time. Complexity of hemocytes in mussels exposed to P. minimum showed a progressive decrease from the beginning of the experiment until day 6. This decline in hemocyte complexity could indicate degranulation, as part of the immune response (Carballal et al., 1997b), or a consequence of dilution of the existing granules as cells divided faster than they created new granules. We favor the second hypothesis, as none of the other immune characteristics were altered, but hemocytes apparently were dividing quickly to maintain the number of circulating hemocytes. On day 9 of the experiment, hemocyte complexity from both treatments decreased and reached the same value, suggesting a possible general response to deteriorating conditions in the aquaria.

Elevated concentrations of bacteria were observed in intestines and stomachs of mussels exposed to P. minimum. As bacteria are too small to be efficiently filtered by the mussels (Jørgensen, 1975, Riisgård et al., 1996), and considering the different results found for each algal exposure, we hypothesize that there was bacterial growth in the alimentary canal. Although the P. minimum culture was not bacteria-free, the numbers of bacteria relative to P. minimum cells were not sufficiently high to account for the mass in the alimentary canal. Alternately, it is possible that these bacteria were normal fauna within the intestine that proliferated abnormally because digestive functions of the mussels were impaired. Bacteria in the P. minimum culture were indistinguishable, morphologically and according to Gram stain, from each other; therefore the origin of these bacteria remains unknown. Within the intestines of

mussels exposed to P. minimum, hemocytes eventually were degraded by bacteria and died by both apoptosic and necrotic processes.

Chemical toxins can suppress immune function in bivalves (Gagnaire et al., 2004, Ordás et al., 2007) but potential pathogens or parasites can activate hemocyte responses (Cáceres-Martínes et al., 2000, Villalba et al., 2004). In our study, the lack of immunosuppression, but rather activation of a protective immune response in mussels exposed to P. minimum, suggests that the mussels perceive this dinoflagellate as a potential invader, rather than experiencing physiological impairment from a chemical toxin in P. minimum. It is noteworthy that the mussels did not have the same response to Rhodomonas sp. cells within the digestive system. Mussels thus appear to perceive P. minimum as not only non-self, but a threat, and activate internal defense mechanisms: recognition, migration, diapedesis, and isolation by encapsulation. Several authors have reported the close phylogenetic relationship between protozoan parasites from the Family Perkinsidae and dinoflagellates (Goggin and Barker, 1993, Leander and Keeling, 2004). We hypothesize that mussels could recognize P. minimum as a threat because it is similar to P. marinus in some chemical or cell-surface factor. This is supported by the findings of Bushek et al. (2002) who found nearly identical reciprocal labeling of epitopes on P. marinus and seven parasitic dinoflagellates. No cryptophytes have been identified as bivalve parasites, thus, this difference in phylogeny between P. minimum and Rhodomonas sp. could explain the different response of the mussels to these two phytoplankters, i.e., there has been no evolutionary selective pressure for an immune response to cryptophytes, but there has been for perkinsus-like protists. As the innate-immune system of invertebrates is non-adaptive, i.e., there is no antibody "memory" of prior exposures to a specific pathogen or parasite, the only mechanism by which recognition of threatening non-self organisms can be improved is by selective

survival of high-responding individuals in a population experiencing disease pressure (Janeway et al., 2004). Thus, the findings of this study suggest the hypothesis that blue mussels may have evolved an effective immune response to P. minimum, through survival of individuals in populations experiencing P. minimum blooms, or of individuals surviving Perkinsus species with similar immune-recognition characteristics (Medzhitov and Janeway, 2002).

The immune response activated in P. minimum-exposed mussels was, however, different from what has been observed in oysters exposed to Perkinsus marinus, wherein phagocytosis occurs (Goedken et al., 2005b). The immune response observed in this experiment is much more similar to the clam hemocyte response to QPX (Quahog Parasite Unknown, Tharaustochytridae, Labyrinthulomycota) than the oyster hemocyte response to Perkinsus marinus. Hemocytes of hard clams (Mercenaria mercenaria) are unable to engulf the QPX parasite but encapsulate the parasite to isolate it (Smolowitz et al., 1998). Small particles, such as Perkinsus marinus (2 μm), can be engulfed by bivalve hemocytes, but large particles are isolated by encapsulation. P. minimum has a similar size to QPX (thalli and sporangia ~20 μm), which could explain the similarity of immune responses between mussels to P. minimum and clams to QPX. Indeed, phagocytosis of bacterial and viral particles, but aggregation and encapsulation around larger intrusions, are general characteristics of the innate immune response in bivalves (Bayne, 1983). Recent experiments investigating in vitro interactions between P. minimum cells and hemocytes of bay scallops and quahogs yielded similar aggregation and encapsulation responses of the hemocytes (Hégaret et al. in prep.).

Conclusions

This is the first study that has combined immune and histopathological techniques to determine the effects of the harmful dinoflagellate P. minimum upon the blue mussel, M. edulis. Results showed the importance of using different analyses to achieve a more-complete understanding of the effects of a HAB species upon a bivalve. Mussel hemocyte responses to P. minimum were diapedesis, aggregation and encapsulation; these responses were activated within the mussel alimentary canal. No putative microalgal toxins affected the hemocytes in circulation because the first line of defense occurred outside the epithelial barriers in the stomach and intestine. Healthy hemocytes left circulation and became impaired in the intestine when encountering P. minimum. Bacteria proliferated in the stomach and intestine as hemocytes were responding to the P. minimum ingested and were unable to control the bacterial growth.

The close phylogenetic relationship between Perkinsus and some dinoflagellates suggest that the genera may share cell-surface factors that make them recognizable to the bivalve host as non-self and a potential threat. But because of the large size of P. minimum relative to hemocytes, the aggregation and encapsulation response is more effective than phagocytosis.

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Figure legends

Figure 1: Hemocyte complexity throughout the experiment. Black line: mussels exposed to Rhodomonas sp., Dotted line: mussels exposed to the toxic dinoflagellate P. minimum. SE shown in graph.

Figure 2: Percentage of mussels showing bacteria (B) in the intestine or hemocytes (H) in the stomach and intestine during exposure to P. minimum and the control algae Rhodomonas sp. throughout the experiment.

Figure 3: Total pathological changes per algal exposure throughout the experiment. Numbers refer to the sum of observations/individual expressed as mean ± SE.

Figure 4: Bacteria in the intestine of a mussel Mytilus edulis exposed to P. minimum. Hematoxylin-eosin stained paraffin section. B: large colony of bacteria S: intestine epithelium or typhlosole.

Figure 5: Hemocytes in the stomach of a mussel Mytilus edulis exposed to P. minimum. Hematoxylin-eosin stained paraffin section. G: gastric shield, H: hemocytes, S: stomach epithelium.

Figure 6: Diapedesis of hemocytes into the intestine of a mussel Mytilus edulis exposed to P. minimum. After diapedesis hemocytes migrate to the bacterial mass in the lumen of the intestine, but appear to go through apoptosis and end up being degraded by the bacteria. Hematoxylin-eosin stained paraffin section S: Intestine epithelium, B: bacteria. Arrows point to hemocytes in diapedesis, broken arrows to apoptotic hemocytes.

Figure 7: Aggregations of hemocytes in connective tissue of a mussel, Mytilus edulis exposed to P. minimum. Hematoxylin-eosin stained paraffin section. G: gonadal follicle, H: hemocytes.

Figure 1

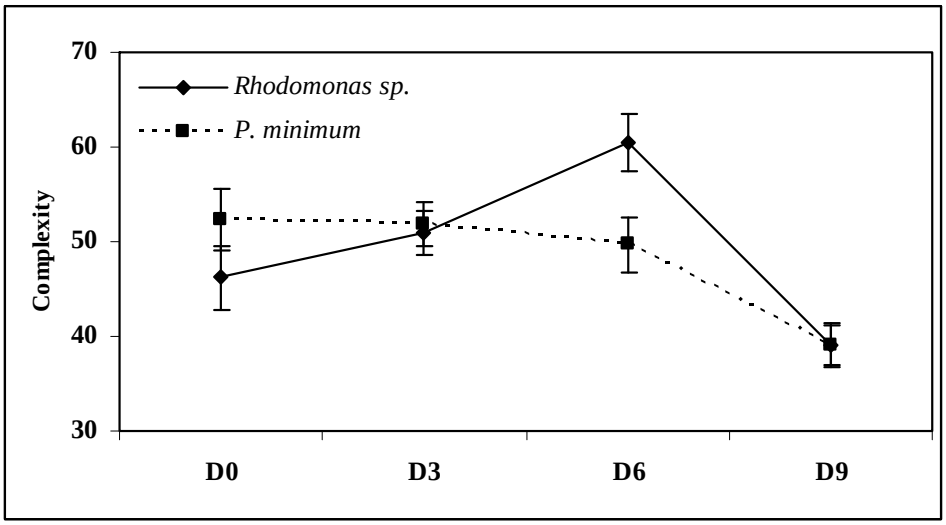
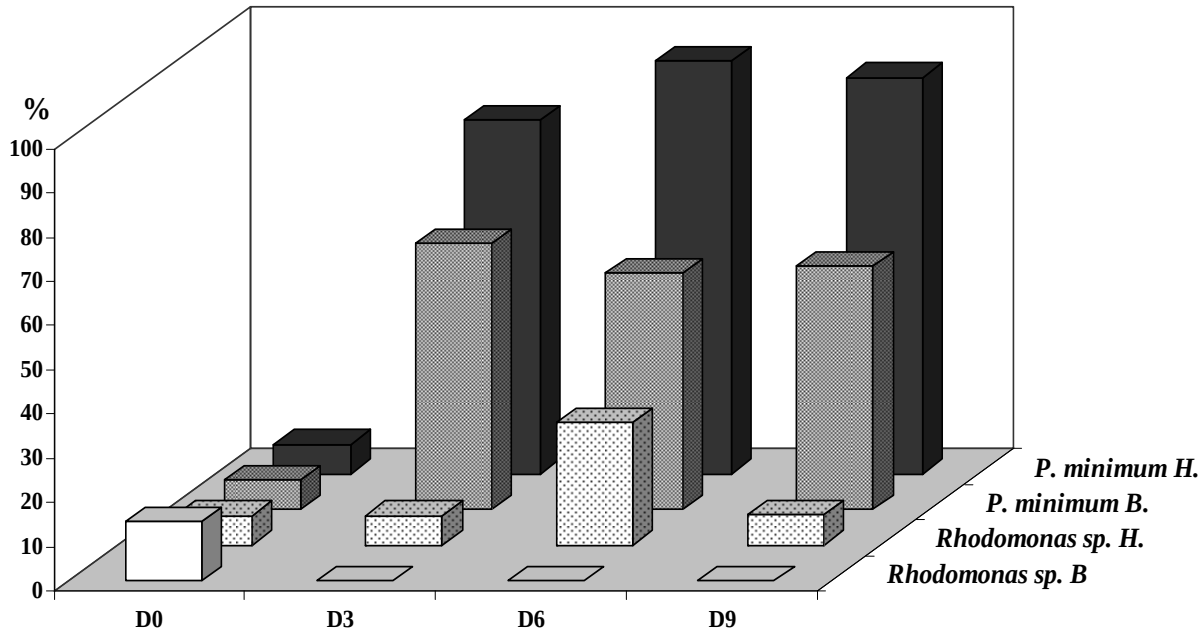


Figure 2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P. minimum B.
Rhodomonas sp. H.
Rhodomonas sp. B

Figure 3

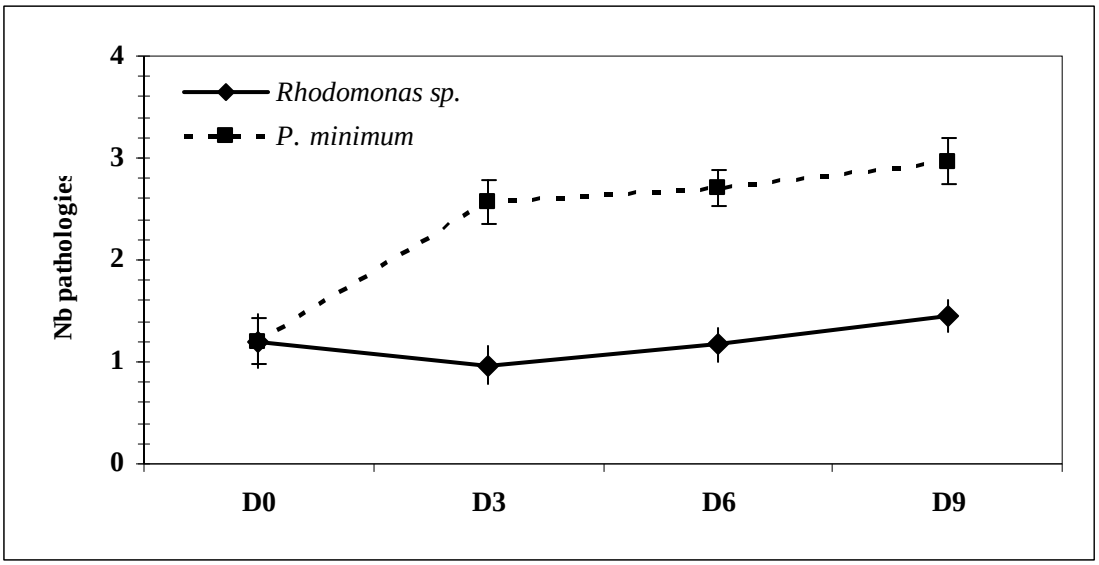
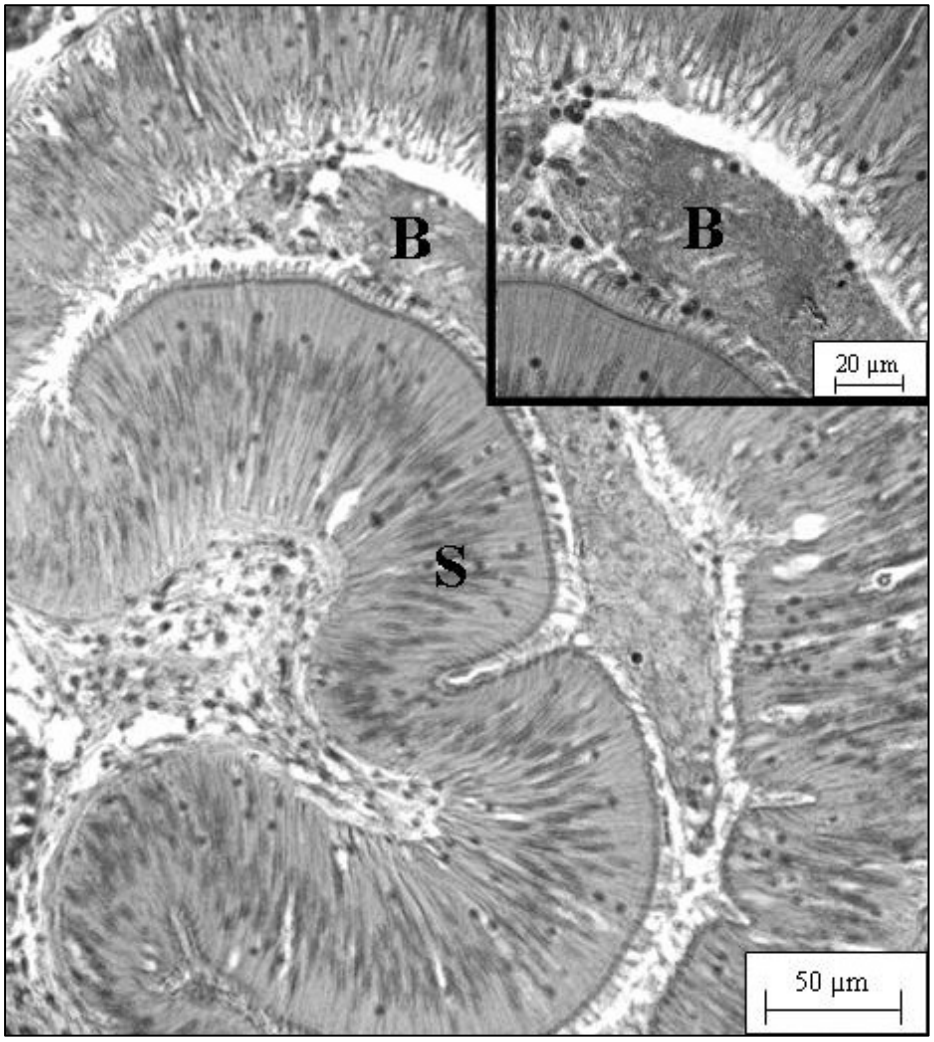


Figure 4



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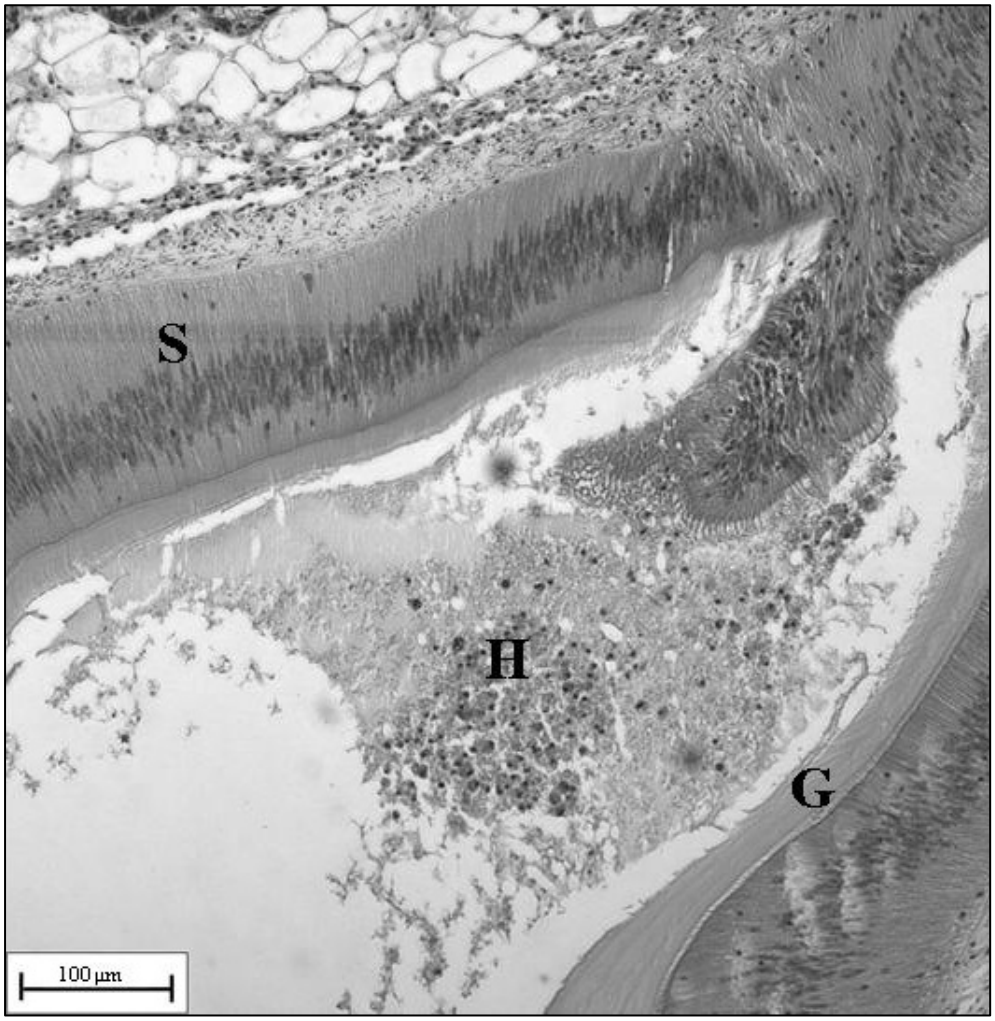

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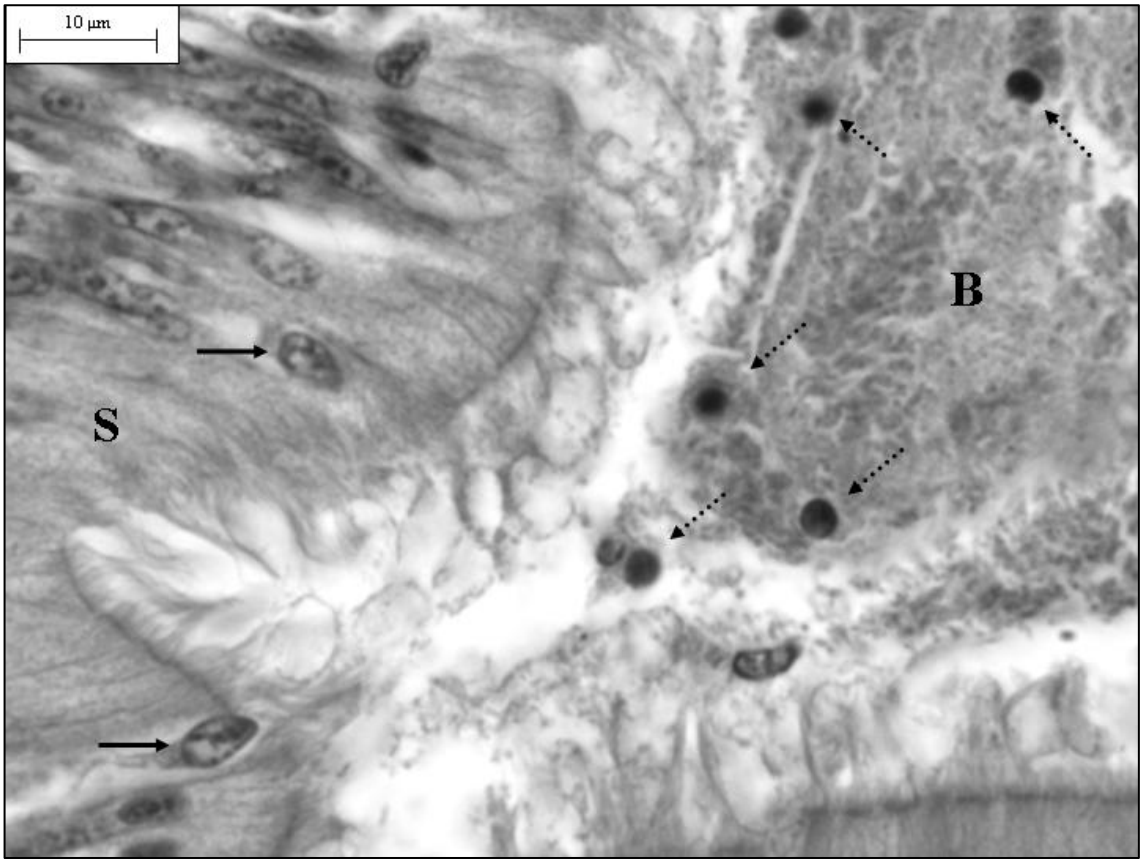

Figure 7

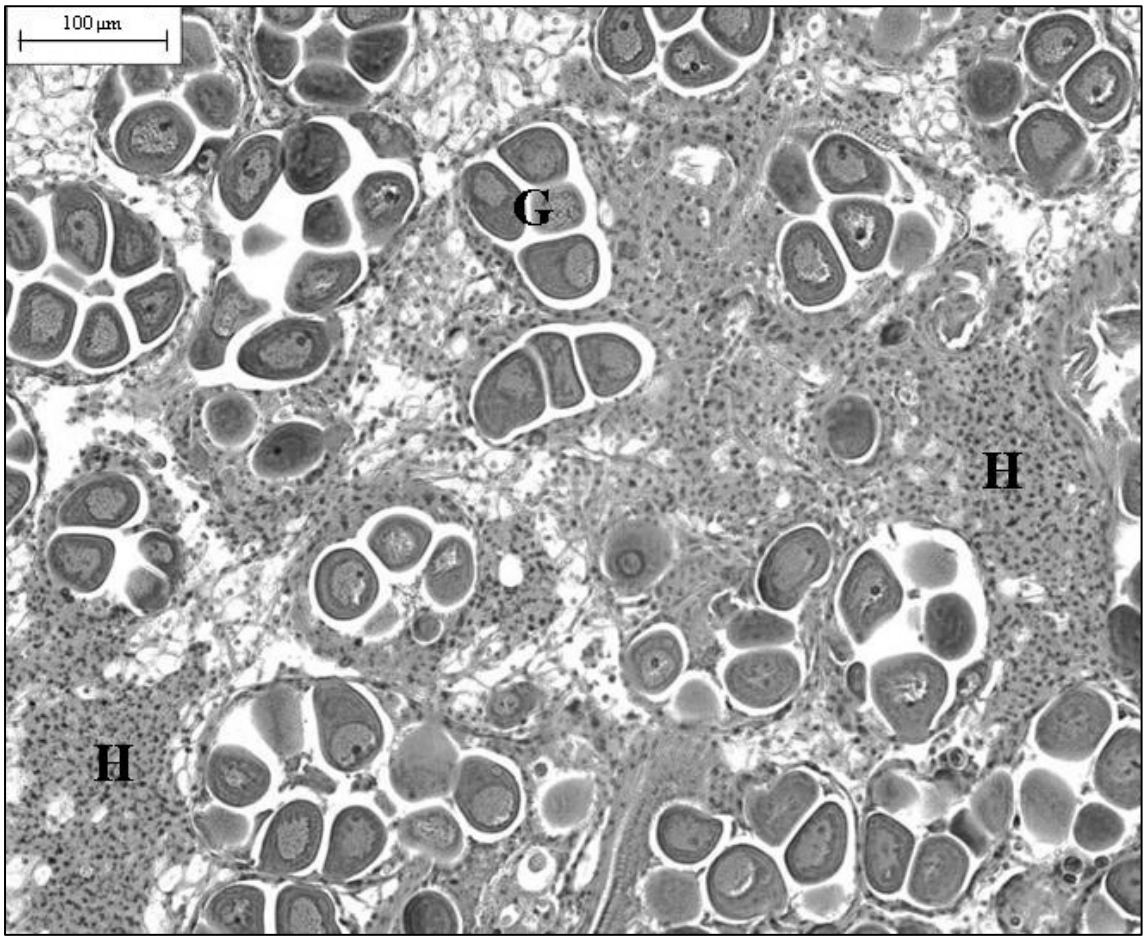

Table 1: Mean values (± standard error) for hemocyte characterization and immune functions for mussels exposed to both algae throughout the experiment. Size and complexity of hemocytes, as well as their production of ROS (Respiratory burst) are presented as arbitrary units. Percentage values were Arcsin(sqrt) transformed for analyses.

	D0		**D3**		**D6**		**D9**	
Hemocyte characterization	*Rhodomonas sp.*	*P. minimum*	*Rhodomonas sp.*	*P. minimum*	*Rhodomonas sp.*	*P. minimum*	*Rhodomonas sp.*	*P. minimum*
Cell density (cells ml^{-1})	1.05 ± 0.17 x 10^6	1.54 ± 0.17 x 10^6	1.07 ± 0.010 x 10^6	9.80 ± 1.00 x 10^5	7.89 ± 0.71 x 10^5	6.41 ± 0.68 x 10^5	8.52 ± 0.89 x 10^6	7.33 ± 0.89 x 10^6
Size	278 ± 7.24	293 ± 7.24	285 ± 4.80	282 ± 4.88	315 ± 5.84	298 ± 5.64	229 ± 5.12	229 ± 5.12
Internal complexity	46.2 ± 3.36	52.3 ± 3.24	50.9 ± 2.28	51.8 ± 2.32	60.4 ± 3.08	49.7 ± 2.91	39.1 ± 2.18	39.0 ± 2.18
Hemocyte functions	*Rhodomonas sp.*	*P. minimum*	*Rhodomonas sp.*	*P. minimum*	*Rhodomonas sp.*	*P. minimum*	*Rhodomonas sp.*	*P. minimum*
Phagocytosis (%)	28.93 ± 2.01	22.77 ± 2.01	49.34 ± 2.83	53.82 ± 2.88	37.51 ± 2.02	36.70 ± 1.95	48.48 ± 2.56	47.19 ± 2.56
Respiratory burst	127 ± 13.99	107 ± 13.99	173 ± 21.13	164 ± 21.49	96 ± 15.99	105 ± 15.43	90 ± 13.12	56 ± 13.12
Adhesion (%)	83.2 ± 2.31	83.8 ± 2.31	80.9 ± 2.33	81.4 ± 2.33	90.7 ± 2.32	84.9 ± 2.24	72.5 ± 2.63	68.3 ± 2.68
Mortality (%)	1.79 ± 0.27	2.06 ± 0.27	1.32 ± 0.15	1.69 ± 0.15	2.57 ± 0.21	2.33 ± 0.20	1.90 ± 0.18	1.11 ± 0.18
Apoptosis (%)			3.08 ± 0.44	2.60 ± 0.42	3.03 ± 0.40	3.05 ± 0.40	3.02 ± 0.40	3.58 ± 0.41