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Hemocyte responses of Manila clams, *Ruditapes philippinarum*, with varying parasite, *Perkinsus olseni*, severity to toxic-algal exposures

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Abstract

This study assessed the possible combined effects of harmful algae and parasite infection on hemocyte and hemolymph parameters of a bivalve mollusc. Manila clams *Ruditapes philippinarum*, were exposed for 1 week, under controlled laboratory conditions, to bloom concentrations of two cultured dinoflagellates: *Karenia selliformis*, and *Karenia mikimotoi*, with demonstrated, sub-lethal, pathological effects upon these bivalves. Each dinoflagellate treatment was added to a basal diet of *Chaetoceros neogracile*; controls consisted of clams fed only *C. neogracile*.

Hemocyte characteristics measured with flow-cytometric analyses, and agglutination titer, condition index, and prevalence and intensity of *Perkinsus olseni*, were assessed for individual clams before and after 3 and 6 days of microalgal exposure. Multifactor analysis of variance tests were conducted to determine possible effects of the harmful algae, time of exposure, and *P. olseni* intensity, as well as interactions between these three factors, upon each physiological variable measured.

There was no relationship between *P. olseni* intensity and hemolymph measures. Both *Karenia* species, however, had a significant effect upon hemocyte profiles of the clams, and this effect was dependent upon duration of exposure; 3 days of exposure to the dinoflagellates generally was sufficient to resolve the effects on the clams. *K. selliformis* had a stronger effect than *K. mikimotoi*, which was intermediate between *K. selliformis* and clams fed the non-toxic control, *C. neogracile*. Total hemocyte counts increased in clams exposed to the harmful algae, while the percentage of dead hemocytes, as well as hemocyte size and complexity, decreased. Furthermore, these immunomodulating effects of *K. selliformis* were significantly more extreme in clams with a high parasite burden, compared with lightly infected clams.

This report is, to our knowledge, the first study assessing the combined effects of harmful algae and parasite infection on a physiological function (hemocyte and hemolymph parameters) of a bivalve mollusc. These findings demonstrate that clams maintain hemocyte function when infected with *P. olseni*, that the clam immune system responds to harmful or toxic algal exposure, and that this response is modified by parasite infection.

Keywords: Bivalve; Clam; Dinoflagellate; HAB; Harmful algae; *Gymnodinium*; *Karenia selliformis*; *Karenia mikimotoi*; *Perkinsus olseni*; Hemocyte

1. Introduction

All living organisms are confronted with multiple and simultaneous threats in the natural environment. Physiological and immunological responses to these threats are attempts by the animals to maintain homeostasis of anatomy and function. In bivalve mollusks, after physical and chemical variations in temperature, salinity, and dissolved oxygen, parasitic, pathogenic, and toxic microorganisms represent some of the more persistent challenges to animal health. Pathogenic bacteria and protozoan parasites are often cited as factors limiting production of bivalve species harvested for human consumption (Lauckner, 1983; Lees, 2000; Formiga-Cruz et al., 2002; Potasman et al., 2002; OIE, 2006). Similarly, harmful and toxic microalgae are increasingly recognized as stressors of bivalve populations (Shumway and Cucci, 1987; reviewed in Shumway, 1990; Landsberg, 2002). Moreover, an increase in harmful algal blooms (HABs) has been listed as one of the growing concerns that may contribute to increased impacts of diseases and parasites on important marine resource species and the food webs that

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support them (Landsberg, 1996; Harvell et al., 1999). Accordingly, studies on physiological and immunological changes induced by parasites and harmful algae provide clues about the importance of these stressors in population biology and thus, production of commercially important bivalves.

Parasitic diseases can have lethal effects on bivalves depending upon the severity of infection. Protozoan parasites have been shown to affect the hemocytes of bivalves; effects described include direct infection of hemocytes by several *Bonamia ostreae* cells resulting ultimately in lysis of hemocytes (da Silva and Villalba, 2004; Cochennec-Laureau et al., 2003). Similar cell damage also occurs in other bonamiosis-affected oyster species. Symptoms of Dermo disease, caused by *Perkinsus marinus*, in eastern oysters, *Crassostrea virginica* (Mackin et al., 1950), include repressed oxidative burst in oyster hemocytes infected with *P. marinus* meronts (Anderson et al., 1995). Moreover, *P. marinus* also affects phagocytosis, oxidative burst, and apoptosis of hemocytes in *C. virginica* (Yee et al., 2005; Goedken et al., 2005a,b). By contrast, *Perkinsus olseni* (= *Perkinsus atlanticus*) does not infect clam hemocytes, but inhabits connective tissues within different organs of *Ruditapes decussatus* and *Ruditapes philippinarum*, provoking intense hemocytic infiltration and parasite encapsulation (Casas, 2002; Choi et al., 2005).

Manila clams *R. philippinarum* are infaunal suspension feeders that were introduced into the Atlantic coast of France for aquaculture purposes (Flassch and Leborgne, 1992). In Brittany (NW France) naturalized populations of Manila clams have been reported to have a high prevalence of the parasite *P. olseni* (Lassalle et al., 2007). In Brittany, however, *P. olseni* prevalence and intensity in Manila clams does not show any recognizable seasonal pattern (our data not published), as observed in *P. olseni* in clams in Galicia (Villalba et al., 2005), which present a seasonal pattern in prevalence and intensity over the annual temperature cycle. The hemocyte response in clams to isolate and encapsulate *P. olseni* within affected tissues suggested the possibility that intensity of *P. olseni* infection in Manila clams could modify clam physiology and damage hemocytes, thereby impairing immune response to other environmental stressors, such as harmful algal blooms (HABs).

Blooms of the harmful alga, *Karenia mikimotoi,* have been reported from Norway to the Galician cost of Spain (Gentien, 1998) resulting in ichthyotoxic effects (Matsuyama et al., 1999; Sola et al., 1999; Jenkinson and Arzul, 2000; Sellem et al., 2000). In 1995, mass mortality of mussels on the Atlantic coast of France was reported coincident with a bloom of *K. mikimotoi* (Gentien, 1998). Blooms of *K. mikimotoi* also occur recurrently on the coast of Brittany in France; therefore, the risk of *R. philippinarum* being exposed to *K. mikimotoi* is always present. The dinoflagellate *K. mikimotoi* has been shown to produce exotoxins (Gentien and Arzul, 1990) and also glycolipids, rich in polyunsaturated fatty acid (PUFAs), which could be responsible for toxicity (Arzul et al., 1995). This species has also shown some hemolytic activity (Jenkinson and Arzul, 2000). A second dinoflagellate from the same genus, *Karenia selliformis*, was also tested in the present study, as it showed a higher hemolytic activity than *K. mikimotoi* (Jenkinson and Arzul, 2000). This species does not occur in Brittany, but has triggered massive fish kills in Tunisia (Arzul et al., 1995) and in the Arabian Sea (Heil et al., 2001) and was responsible for shellfish contamination in New Zealand (MacKenzie et al., 1995). Indeed, *K. selliformis* produces a toxin, gymnodimine (Seki et al., 1995; Miles et al., 2000, 2003) that accumulates in the tissues of bivalves feeding upon it (MacKenzie et al., 1995; Biré et al., 2002). Both of these harmful algae have demonstrated impacts on marine organisms and occur in areas where Manila clams are located.

Many blooms of harmful algae occur in the spring or summer when high temperatures result in proliferation of *P. olseni* within the tissues of infected clams. Thus, we chose harmful-algal exposure as a secondary stressor because previous studies have demonstrated clear changes in hemocyte morphology and function associated with HAB exposure (Hégaret and Wikfors, 2005a,b; Hégaret et al., 2007). No previous studies, however, have explored the possible physiological and immunological effects of HABs on Manila clams. Furthermore, since HABs have become more widespread and frequent phenomena (Hallagraeff, 1993), blooms now are more likely to occur in areas where parasite-stressed animals are located. As both parasites and harmful algae may modify hemocyte characteristics in affected bivalves, it is possible that combined effects of both stressors could be more severe than either condition individually. Thus, the objective of our study was to assess the effects of *P. olseni* and HAB exposure, alone and combined, upon physiological and hemocyte responses of Manila clams.

2. Materials and methods

2.1. Experimental animals

One hundred Manila clams, *R. philippinarum* (35–45 mm shell length), used for this experiment were collected from the Island of Bailleron in the Morbihan Golfe in Brittany (NW France), where prevalence of *P. olseni* varies from 30 to 70% (Lassalle et al., 2007). Clams were collected on 17th October 2005, the temperature at the date of collection was 17 °C. The clams were conditioned for 1 week before the experiment in an open-seawater system, where temperature was maintained at 18 °C—a temperature compatible with the development of *P. olseni* infection.

2.2. Algal cultures

A diatom, *Chaetoceros neogracile*, commonly provided as an aquaculture feed to bivalves, was used as the control and basal diet. The algae were cultured in 300-l open tanks in the IFREMER hatchery at Argenton. The *K. selliformis* strain (GM94GAB), formerly referred to as *Gymnodinium maguelonnense* or *Karenia* sp. (Guillou et al., 2002, Shao et al., 2004), was isolated from Gulf of Gabès (Tunisia), after an episode of fish mass mortality (Hansen et al., 2004), by E. Erard-Le Denn, who identified it as *Gymnodinium* sp. (Arzul et al., 1995). This alga was chosen for its marked cytotoxicity (Jenkinson and Arzul, 2000), which was tested prior to these experiments (see Section 2.3). The alga *K. mikimotoi* (= *Gymnodinium mikimotoi*,

GATIN95) used in this study, was isolated in 1995 by E. Erard-Le-Denn during a bloom in the Bay of Brest, in Brittany (NW France). Cultures of both *K. selliformis* and *K. mikimotoi* used in this study were obtained from the Dyneco Department of IFREMER (Brest, France).

The dinoflagellates were cultured in sterile, 6-l carboys. Cultures were grown in F/2-enriched seawater from the Argenton hatchery (Guillard and Ryther, 1962; Guillard, 1975), filtered to 1 μm, and autoclaved. Algal cultures were grown at 20 °C in a 12 h light/12 h dark cycle. Algal cell density was determined by Malassez cell or Nageotte cell counts with the light microscope.

2.3. Preliminary experiments: hemolytic tests

To select HAB strains and concentrations for focused experiments, we conducted preliminary exposure experiments, according to the protocol developed by Arzul et al. (1994) using horse red blood cells to determine the hemolytic activity of algae, as horse red blood cells show a very high sensitivity to toxins, and standardized protocols have been established using these commercially available cells. Several concentrations and ages of cultures of *K. selliformis* and *K. mikimotoi* were tested to select the characteristics of the cultures necessary to obtain the highest toxicity for clam exposures. Concentrations tested for *K. selliformis* and *K. mikimotoi* were 10^2 and 10^3, respectively and ages of culture tested were 7, 10 and 12 days for *K. selliformis*, and 9 and 10 days for *K. mikimotoi*.

2.4. Dependent variables measured

Hemocyte and hemolymph analyses were done on individual clams to permit correlation with the *P. olseni* status of each clam.

2.4.1. Immunological analysis—hemocytes

Hemolymph was withdrawn from the adductor muscle of each individual clam with a 25-gauge needle attached to a 1-ml syringe, then passed through 80-μm mesh, and stored temporarily in a microcentrifuge tube on crushed ice to retard cell clumping.

Procedures for characterization and function of clam hemocytes were adapted from those of Soudant et al. (2004), Delaporte et al. (2003), Hégaret et al. (2003a,b) and Lambert et al. (2003). We used a FACScalibur (BD Biosciences, San Jose, CA) flow-cytometer for all hemocyte analyses. Hemato-immunological parameters measured were: number of hemocytes detected during a set sampling time (an estimate of hemocyte counts per millilitre) as well as hemocyte characterization, in terms of size and internal complexity. The several immune functions measured were:

(a) hemocyte viability, as percentage of dead hemocytes;
(b) phagocytosis of fluorescent beads by hemocytes, which simulates the engulfment of non-self particles;
(c) hemocyte reactive oxygen species (ROS) production, that measures the potential to kill non-self, engulfed particles;
(d) adhesion of the hemocytes.

2.4.2. Immunological analysis—plasma

Two plasma parameters were measured: agglutination titer, indicative of the presence of lectins, and protein concentration. For both assays, hemolymph from each clam was centrifuged (800 × *g*, 5 min, 4 °C), and the supernatant was frozen immediately (−20 °C) until use. Quantification of agglutination titer was performed according to the protocol from Barracco et al. (1999), using horse red blood cells. Briefly, 50-μl plasma samples were added to U-shaped wells of 96-well-microtiter plates, and a two-fold, serial dilution was prepared using Tris-buffered saline (containing 0.15 M NaCl) as the diluent. The same volume of a 2% suspension of horse red blood cells in TBS was added to each well and incubated for 2 h at room temperature. In controls, plasma was replaced by TBS. The agglutination titer was expressed as the reciprocal of the highest dilution showing a positive pattern of agglutination. The protein concentration of the clam plasma was determined with the method of Bradford (1976), using bovine serum albumin as the standard protein.

2.4.3. Detection and quantification of infection by P. olseni

Each clam was shucked, and the two branchial lamellae of one side were excised, weighed, and incubated in Ray's fluid-thioglycollate medium (RFTM, Ray, 1966) for 7 days in the dark at room temperature. Gill tissues incubated in RFTM were removed after 7 days and prepared according to the protocol of Choi et al. (1989). Briefly, gill tissues were centrifuged (800 × *g*, 10 min) to remove medium, digested with NaOH (2N, 1 h at 60 °C, twice), followed by two washes in PBS (0.1 M). Finally, the pellet was re-suspended in 1 ml of PBS. Ten microlitres of Lugol's solution were added, and the number of *P. olseni* hypnospores was assessed using a Nageotte chamber under the light microscope.

For data presentation, prevalence of *P. olseni* is presented as the percentage of clams infected with *P. olseni* from the total number of clams analyzed on each date and treatment. The level of infection by *P. olseni* was calculated as the number of hypnospores per gram of gill tissue. The concentration was log-transformed (number of *P. olseni* cells per gram of gill tissue of clam + 1) = $\log_{10}(X+1)$. To run Multifactor ANOVAs, the level of infection of clams by *P. olseni* was also divided into two classes: one called "Null-Light," representing the animals with no parasite or low level of infection (0–1000 *P. olseni* cells per gram of gill tissue or $\log_{10}(X+1) < 3$), and class "Moderate-Heavy," that included animals severely infected (more than 1000 *P. olseni* cells per gram of gill tissue or $\log_{10}(X+1) > 3$).

2.4.4. Condition index

To assess the energy status of clams during the experiment, the condition index (CI) was calculated using the dry meat weight (DMW) in relation to dry shell weight (DSW): CI = DMW × 100/DSW.

2.5. Microalgal-exposure experiments

A self-contained, integrated apparatus for exposing aquatic organisms to different water sources developed in the NOAA-NMFS Milford Laboratory by B.C. Smith, was shipped to France

for these experiments. This system provided clams with constant flows of different microalgal suspensions or seawater, and constant temperature (18 °C) during the experiment. Seventy-two clams were distributed randomly into 12 l-L basins, i.e., six animals per basin. Four replicates of three different treatments (detailed below) were done in this experiment. Clams were fed continuously 5 ml min^{-1} using a flow-through system. To adjust the concentrations, coarsely filtered seawater from the Bay of Brest was pumped and kept at a temperature of 18 °C. Twenty animals were analyzed as a control, i.e., initial status, at the beginning of the experiment.

After 3 days of exposure, one half of the clams from each basin (36 clams, 12 for each condition) were analyzed for hemocyte parameters, plasma protein concentration, agglutination titer, condition index, and presence of *P. olseni* and other pathological conditions, as described above. After 6 days of exposure, the remaining clams were analyzed for the same parameters.

2.5.1. Exposure of clams to K. selliformis and K. mikimotoi

The effects of the two harmful algae, *K. selliformis* and *K. mikimotoi*, were tested on the physiological and immunological characteristics of clams (listed above). Experimental treatments (each in quadruplicate) were as follows:

(a) unialgal *C. neogracile*, at a constant quantity at 4×10^8 cells per clam per day, which corresponded to a maintenance ration (4% of clam dry weight in algal dry weight per day);
(b) *C. neogracile* at the same quantity plus *K. mikimotoi* at 10^3 cells ml^{-1};
(c) *C. neogracile* at the same quantity plus *K. selliformis* at 500 cells ml^{-1}.

2.6. Statistical analysis

All variables were analyzed statistically using Multifactor ANOVA to evaluate possible interactive effects of microalgal treatments, time of exposure, and parasite-infection intensity. The percentages of phagocytic hemocytes, and dead hemocytes, were Arcsin-transformed to meet homogeneity requirements before multifactor ANOVAs. The differences in prevalence of *P. olseni* between treatments were analyzed using a Friedman test, in which the treatments were the algae and blocks were the days. We used Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA) for most analyses, except for the Friedman test, for which we used MINITAB 13.1 software (Minitab Inc., Pennsylvania State University, USA).

3. Results

3.1. Hemolytic tests

According to bioassays with horse red blood cells, *K. selliformis* appeared always to be more toxic than *K. mikimotoi*, for all concentrations and culture-ages tested. Ten-day-old cultures showed the highest toxicity, independent of the concentration, when compared with cultures harvested after 7 or 12 days of growth. These hemolytic tests allowed us to choose the age and the concentration of the cultures to be used for the following clam-exposure experiments. Thus, based upon these results, 9–11-day-old cultures at concentrations of 500 cells ml^{-1} for *K. selliformis* and 10^3 cells ml^{-1} for *K. mikimotoi*, were used. These concentrations were, coincidentally, consistent with population densities in natural blooms.

3.2. Microalgae

The *C. neogracile* batch cultures were harvested after 3–6 days of growth, usually at a concentration approaching 4×10^6 cells ml^{-1}. The *K. mikimotoi* and *K. selliformis* batch cultures harvested between 9 and 11 days contained vastly different cell densities. The culture of *K. mikimotoi* reached concentrations of 5×10^4 cells ml^{-1}; whereas, concentrations of *K. selliformis* reached only $\sim 10^4$ cells ml^{-1}. The dinoflagellates were usually in late-exponential or early stationary-phase upon harvest.

3.3. Prevalence and intensity of perkinsosis

To assess any possible effects of perkinsosis upon response variables, we established a categorization of animals according to parasite-gill burden. This categorization of clams by the level of infection into these two classes "Null-Light" and "Moderate-Heavy" essentially divided the population in half. The "Null-Light" class contained 45 clams, with a mean of 154 parasites per gram of gill wet weight ranging from 0 to 881. The "Moderate-Heavy" class contained 47 clams, with a mean of 142,000 parasites per gram of gill wet weight, ranging from 1020 to 1,870,000.

3.4. Effect of infection by P. olseni upon immunological and physiological parameters before exposure

The possible effects of *P. olseni* upon immunological and physiological parameters of clams were evaluated at T_0, before the experiment started, i.e., before the clams were exposed to any algal diet. As seen in Table 1, there was no difference in any of the tested parameters attributable to level of infection of clams by *P. olseni*.

3.5. P. olseni prevalence and algal treatment

The prevalence of *P. olseni* recorded in clams at the beginning of the experiment was 55% (Table 2). During the experiment, no differences of *P. olseni* prevalence among treatments were significant with the Friedman test. Prevalence of *P. olseni* ranged from 70 to 83% in clams sampled after 3 days of exposure and ranged from 66.7 to 90% after 6 days.

3.6. Effect of the algal treatment and infection by P. olseni upon hemocyte parameters and condition index

To assess the combined effects of the harmful algae, *P. olseni* infection, and the length of time the clams were exposed to the

Table 1
Hemocyte parameters and condition index of Null-Light and Moderate-Heavy classes of *P. olseni* infection prior to algal treatments

	Null-Light ($n=12$)		Moderate-Heavy ($n=8$)		*T*-test
	Mean	S.E.	Mean	S.E.	
Size (arbitrary units)	401	9.5	405	9.1	NS
Complexity (arbitrary units)	235	11.0	226	16.0	NS
THC (cells ml^{-1})	733,000	119,000	879,000	233,000	NS
% of dead hemocytes	4.2	1.1	3.7	0.8	NS
% of phagocytic hemocytes	28.4	2.1	27.4	2.9	NS
Log (agglutination titer)	5.0	1.2	3.3	0.9	NS
Protein content ($\mu g\ ml^{-1}$)	196	10.7	186	16.6	NS
Condition Index	5.6	0.5	4.8	0.3	NS

NS indicates no significant difference ($P>0.05$) between the two infection classes (*T*-test, $P<0.05$). THC: total hemocyte count.

Table 2
Prevalence (%) and intensity (mean and S.E.) of *P. olseni* infection in Manila clams, *R. philippinarum*, exposed to different microalgae, and percentages of clams in Null-Light and Moderate-Heavy categories of infection by *P. olseni*

Time	Algal treatment	Prevalence of *P. olseni* (%)	Infection by *P. olseni* mean (S.E.)	Null-Light (%)	Moderate–Heavy (%)	*N*
T_0	Original population	55.0	1.99 (0.45)	60	40	20
T_3	*C. neogracile*	70.0	1.42 (0.64)	58.3	41.7	12
	C. neogracile + *K. mikimotoi*	75.0	1.50 (0.60)	50	50	12
	C. neogracile + *K. selliformis*	83.3	1.50 (0.57)	50	50	12
T_6	*C. neogracile*	66.7	1.58 (0.58)	41.7	58.3	12
	C. neogracile + *K. mikimotoi*	90.9	1.85 (0.45)	16.7	83.3	12
	C. neogracile + *K. selliformis*	83.3	1.42 (0.40)	58.3	41.7	12

N: number of clams in each class. Infection intensity results are expressed as $\log_{10}(X+1)$, where *X* is the number of *P. olseni* cells per gram gill wet weight.

algae, multifactor analyses of variance (Multifactor ANOVA) were run with the time of exposure (3 or 6 days), the two classes of *P. olseni* infection (Null-Light and Moderate-Heavy), and the algae to which the clams had been exposed (*C. neogracile*, *K. mikimotoi*, *K. selliformis*) as the independent variables (Table 3). Results indicate that *P. olseni* alone did not have any significant effect on any hemocyte parameter tested, which is consistent with the results at T_0. The parasite *P. olseni* caused significant effects only when combined with the additional experimental stressor—exposure to a harmful algal culture.

Except for the phagocytic activity of hemocytes and the total protein content of the hemolymph, the physiological and hemocyte responses were similar between 3-day and 6-day exposures. Percentage of phagocytic hemocytes (mean ± S.E.) increased significantly from 42.7 ± 1.5 to $53.6 \pm 1.4\%$, and total protein content increased from 159.7 ± 7.2 to $205.9 \pm 10.7\ \mu g\ ml^{-1}$ from day 3 to day 6.

The following main effects on the hemocyte parameters were attributable to exposure to the harmful algal cells: internal complexity and size of the hemocytes exposed to *K. selliformis* (a) were lower ($P<0.05$) than in clams exposed to *K. mikimotoi* (ab) and to *C. neogracile* (b) (Fig. 1). Percentages of dead hemocytes in clams exposed to *K. selliformis* and *K. mikimotoi* were also significantly lower ($P<0.05$) than in clams exposed to

Table 3
Effect of harmful-algal exposure on Manila clam, *R. philippinarum*, hemocyte parameters and condition index during 6 days of exposure to three different algal treatments: *K. selliformis* plus *C. neogracile*, *K. mikimotoi* plus *C. neogracile* or *C. neogracile* alone

	Time	Parasite	HAB	Time/parasite	Time/HAB	Parasite/HAB	Time/parasite/HAB
Size of hemocytes	NS	NS	*	NS	NS	NS	NS
Complexity of hemocytes	NS	NS	*	NS	NS	NS	NS
Concentration of hemocytes	NS	NS	*	NS	*	*	NS
% of dead hemocytes	NS	NS	*	NS	NS	NS	NS
% of phagocytic hemocytes	**	NS	NS	NS	NS	*	*
ROS production	NS	NS	NS	NS	NS	NS	NS
Log (agglutination titer)	NS	NS	NS	NS	NS	NS	NS
Total protein content	**	NS	NS	NS	NS	NS	NS
Condition Index	NS	NS	NS	NS	NS	NS	NS

Agglutination activity presented as the $\log_2(\text{titer}+1)$.
* Significant differences $P<0.05$ as well as non-significant (NS) differences are presented in this table.
** Significant differences $P<0.01$ as well as non-significant (NS) differences are presented in this table.

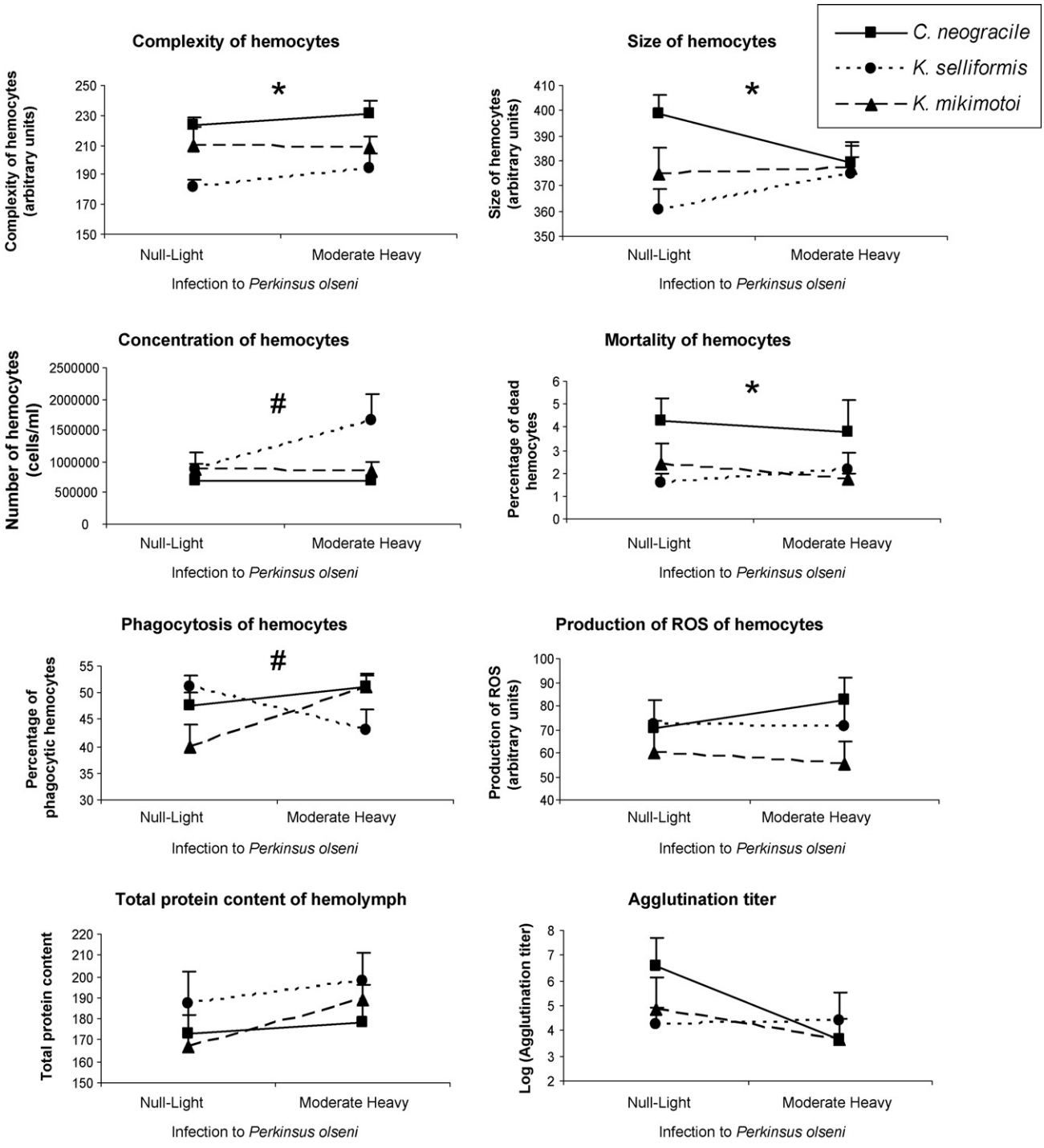

Fig. 1. Effect of harmful-algal exposure on Manila clam, *R. philippinarum*, hemocyte parameters (means ± S.E., $n = 72$) during 6 days of exposure to three different algae treatments; *K. selliformis* plus *C. neogracile*, *K. mikimotoi* plus *C. neogracile* or *C. neogracile* alone. Agglutination activity presented as the $\log_2(\text{titer} + 1)$. Significant differences ($P < 0.05$) between diets *, and significant interactions ($P < 0.05$) between the algal diet and the *P. olseni* infection # (three-way ANOVA). (■: *C. neogracile*, ●: *K. selliformis*, ▲: *K. mikimotoi*).

C. neogracile (Fig. 1). The concentration of circulating hemocytes in clams was also affected by the harmful algae, but as described above, these changes were also affected by the interaction between the parasite infection and the algal exposure.

Indeed, some interactions between the algal exposures and the level of infection of the clams with *P. olseni* indicated a possible modulation of hemocyte responses to the harmful algae by *P. olseni*. Clams heavily infected with *P. olseni* and exposed to *K. selliformis* showed a much higher concentration of circulating hemocytes, as compared to clams with no or a low level of *P. olseni* infection exposed to the same harmful alga, or than in clams fed *C. neogracile* and *K. mikimotoi*, regardless of *P. olseni* infection level (Fig. 1). This observation was especially true after 3 days of exposure; indeed, after 6 days of exposure the level

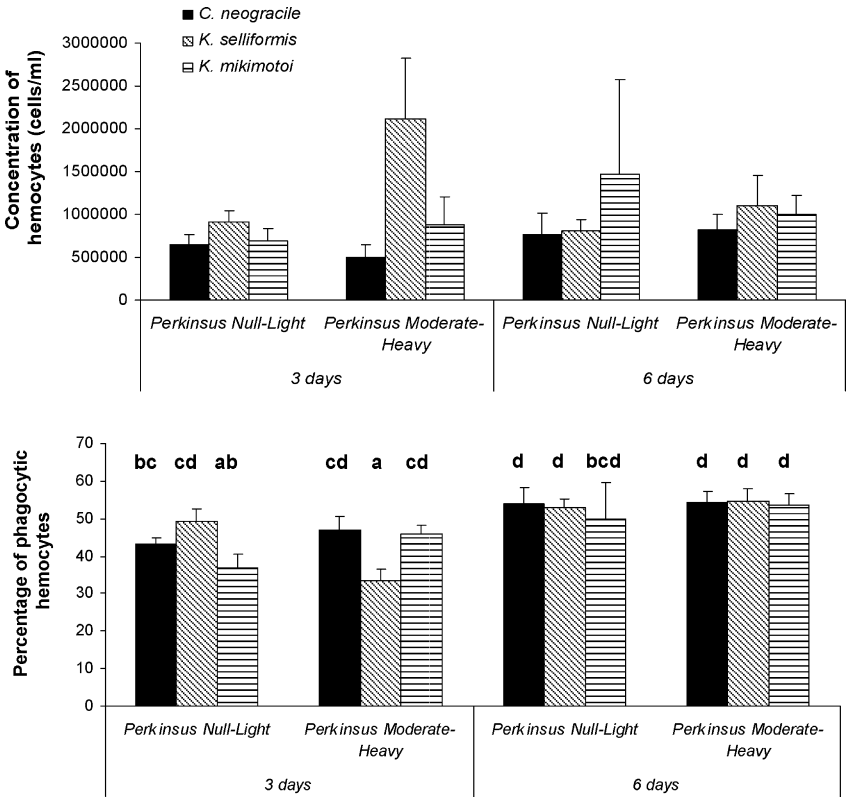

Fig. 2. Effect of harmful-algal exposure and *Perkinsus* infection (Null-Light and Moderate-Heavy) on the number of circulating hemocytes (A) and the percentage of phagocytic hemocytes (B) of Manila clams *R. philippinarum* (means ± S.E.) after 3 and 6 days of exposure to three different algal treatments: *K. selliformis* plus *C. neogracile*, *K. mikimotoi* plus *C. neogracile*, or *C. neogracile* alone.

of circulating hemocytes in moderate to heavily infected clams exposed to *K. selliformis* returned to the same level as the other treatments.

The percentage of phagocytic hemocytes was significantly ($P < 0.05$) affected by the interaction between the algal treatment and level of infection by *P. olseni* (Fig. 2), as well as by the three-way interaction between the time of incubation, the algal treatment, and level of infection by *P. olseni* (Fig. 2). After 3 days of exposure, clams heavily stressed, i.e., exposed to *K. selliformis* and heavily infected with *P. olseni*, presented the lowest hemocyte phagocytic activity. After 6 days of exposure, the percentage of phagocytic hemocytes was high again for all the treatments.

Condition index was not affected by *P. olseni* infection, algal treatment, or by interactions of these independent variables. Mean values (±S.E.) of condition index ranged from 4.71 ± 0.35 to 5.65 0.43 in all treatments, levels of infection, and times of exposure.

4. Discussion

The establishment of a stable, specific host–parasite relationship requires that the parasite acquire resources from the host without impacting host fitness too severely such that the parasite's resource base is eroded. In bivalve molluscs, however, parasites can be significant regulators of population biology, with notable examples including bonamiosis, which practically decimated flat oyster populations in some European countries (Carnegie and Cochennec-Laureau, 2004; da Silva et al., 2005), and perkinsosis in several bivalve molluscs (Villalba et al., 2004). In considering possible interactions between other environmental stressors and parasite dynamics in molluscs, previous studies have assessed the effects of toxic pollutants on the resistance of oysters, *C. virginica,* to acquisition of *P. marinus* infections, or on the progression of the disease (Anderson et al., 1996; Fisher et al., 1999). da Silva (in preparation) recently assessed the effect of toxic-algal bloom exposure on the progression of *P. olseni* infection in Manila clams over a period of 6 weeks. The question of whether or not heavily parasitized individuals are more susceptible to, or respond differently to, toxins, has not however been addressed directly in bivalves. Reports of such modulations in host responses to toxins by parasitic diseases in other aquatic organisms are also rare. Sures (2006) reported that parasites may influence the metabolism of pollutants in fish; additionally, Kahn (1991) suggested that parasites may enhance the susceptibility of winter flounder to pollutants. Thus, the experiments reported in the present study are, to our knowledge, the first addressing directly the question of combined effects of toxic algae and parasites on physiological processes in a bivalve mollusc.

Clams selected randomly for different experimental treatments were assessed for prevalence and severity of infection of perkinsosis, a parasitic disease. Nearly even distributions between light and advanced perkinsosis in the clam population,

however, permitted statistical analyses of perkinsosis effects on the hemocyte variables and condition index.

The prevalence and intensity of *P. olseni*, assessed in Manila clam gills varied little during the course of the experiment. The results of the Friedman test showed no effect of the algal treatments on the intensity of *P. olseni* in the clams. Indeed, it is very unlikely that the prevalence can change in such a short period of time; thus, the observed small differences may simply be a consequence of the random selection of clams from the source population and a reflection of the highly variable parasite burden in natural clam populations.

The effect of the natural infection of the clams with *P. olseni* was assessed at the beginning of the experiment, before any exposure to harmful algae. Neither the hemocyte parameters nor the condition index were affected by severity of parasite infection. Consistently, during algal treatments, the Multifactor-ANOVAs testing the level of infection of clams by *P. olseni* as an independent variable showed no significant effects of *P. olseni* on the hemocyte parameters or on the condition index.

Previous studies have shown strong effects of *Perkinsus* spp. infection on various immunological parameters of bivalves, including those measured here. For example, Anderson et al. (1995) showed an increase in the number of circulating hemocytes in Eastern oysters, *C. virginica*, heavily infected with *P. marinus*. Goedken et al. (2005a) also demonstrated an increase of the percentage of phagocytic hemocytes in oysters *C. virginica* and *Crassostrea gigas* exposed to *P. marinus*. Unlike *P. marinus* in Eastern oysters, however, *P. olseni* does not infect clam hemocytes directly, but rather inhabits connective tissues (Casas, 2002; Choi et al., 2005). Apparently, once the hemocyte response to the parasite has accomplished isolation of the parasite, concentrations, morphologies, and activities of circulating hemocytes return to equilibrium. This may explain the absence of effect of natural *P. olseni* infection on the hemocyte parameters of the clams in our study.

In the present study, the quantification of agglutination titer, indicative of the presence of lectins in the hemolymph of Manila clams, was not affected by the algal exposure or by the level of infection by the parasite. This was unexpected, as recent studies have reported that naturally infected clams (*R. decussatus*) have a higher agglutination titer than uninfected clams (Ordás et al., 2000) and that the expression of lectin EST in hemocytes was shown to increase in clams exposed to *P. olseni* (Kang et al., 2006; Kim et al., 2006). Again, a return to homeostasis following effective immune response in clams used in the present study may explain the similar lectin conditions in heavily and lightly infected clams.

The effects of two cultured, toxic dinoflagellates (*K. selliformis* and *K. mikimotoi*) upon the plasma and hemocyte parameters of clams were assessed in the present experiment using multifactor ANOVAs. Overall, these tests generally showed little or no differences in hemocyte or physiological variables between clams exposed to each algal treatment for 3 or for 6 days, indicating rapid onset of effects. Similar findings of rapid response of a few days, in hemocytes to HAB exposure were reported by Hégaret and Wikfors (2005a) and Hégaret et al. (2007). Changes in hemocyte immune functions attributable to *K. selliformis* exposure were mainly observed in the concentration and morphology of hemocytes; and in percentages of dead cells. The *K. selliformis* algal exposure, compared to the control *C. neogracile*, triggered a significant decrease in hemocyte complexity and size, as well as a decrease in the percentage of dead hemocytes. Conversely, the number of circulating hemocytes increased in clams exposed to *K. selliformis*. Decrease in hemocyte size and complexity in clams exposed to *Karenia* spp. can thus be interpreted as a direct response to toxins produced by these algal species (Arzul and Gentien, 2005). Arzul et al. (1995) suggested that glycolipids, rich in polyunsaturated fatty acid (PUFAs), are responsible for the toxic effects (hemolytic activity) of *Karenia* spp.; these chemical compounds may also be responsible for the effects upon hemocytes.

Except for the percentage of dead hemocytes, which did not differ between the two harmful-algal exposures, exposure to *K. mikimotoi* generally resulted in an intermediate reaction in most parameters (complexity, size, and concentration of the hemocytes), between *C. neogracile* and *K. selliformis*. This same trend can also be observed for the production of ROS by hemocytes. This intermediate level of toxicity of *K. mikimotoi*, compared to *K. selliformis*, in all the immunological parameters measured, is consistent with the results of the haemolytic tests (Table 3), showing an intermediate toxicity of *K. mikimotoi* compared to *K. selliformis*. These results confirm the hypothesis that effects on hemocytes are attributable to a toxic compound, as the toxicity of *K. mikimotoi* appears to be lower than that of *K. selliformis*.

The concentration of circulating hemocytes was significantly affected by the interactions between harmful-algal exposure and level of infection of *Perkinsus* (Table 3). The concentration of circulating hemocytes in clams exposed to *K. selliformis* was much higher in clams heavily infected with *P. olseni*, compared to the lightly infected clams or the heavy-infected clams exposed to *C. neogracile* or *K. mikimotoi*. The first reaction of the host to *Perkinsus* infection is an increase in the number of circulating hemocytes (Anderson et al., 1995; Villalba et al., 2004). Casas (2002) also suggested that hemocytes could be massively transferred to the affected tissues for encapsulation and destruction of *P. olseni*. The increase in hemocyte concentration in the heavily infected clams exposed to *K. selliformis* in the present study appeared especially intense after 3 days of exposure; hemocyte concentration returned the same concentration as the control clams fed *C. neogracile* after 6 days of exposure. Our study also demonstrated an increase in the percentage of dead hemocytes when total counts of circulating hemocytes were depleted (P value < 0.01, data not shown) and *vice versa*. This has been observed previously in oysters, *Crassostrea virginica* (Hégaret and Wikfors, 2005a,b) and *C. gigas* (Hégaret et al., 2007). This may indicate that bivalve shellfish, after some stimulus, can rapidly produce large numbers of hemocytes. These hemocytes are possibly "young" cells less prone to mortality, decreasing the percentage of dead hemocytes in the hemocyte population. The site of hematopoiesis in bivalves is not firmly established, and thus cell renewal and maturation process are not well understood (Auffret, 1988; Hine, 1999).

A similar observation was made with the interactive effects of perkinsosis severity and HAB exposure on the percentage

of phagocytic hemocytes in clams. After 3 days of exposure, clams heavily stressed, i.e., exposed to *K. selliformis* and heavily infected with *P. olseni*, had depressed hemocyte phagocytic activity, which seemed to be overcome after 6 days, as the percentage of phagocytic hemocytes rebounded. Ordás et al. (2000) also reported a lower phagocytic rate in clams infected by *P. olseni* but the effect was not significant. Our findings support the expectation that an additional source of stress, such as a harmful algal exposure, can amplify the effect of a *P. olseni* infection. The increase in hemocyte densitiess coincident with the decrease in percentage of phagocytic hemocytes may indicate new production of non-phagocytic hemocytes when clam are exposed to toxic algae. Immunological responses to combined HAB and perkinsosis stressors, thus, were similar to those observed for repair of tissue damage, wherein newly produced hemocytes are mainly immature cells (Hine, 1999; Auffret, 1988) incapable of phagocytosis.

These data indicate that, when exposed to either of these stressors alone (high *P. olseni* infection or harmful-algal exposure), the clams are able to regulate immune parameters and do not seem to be adversely affected. As the combined stressors are more severe or diverse (heavy infection to a parasite plus exposure to a highly toxic, harmful-algal species), the clam immune system seems to be activated and responds with a change in hemocyte parameters.

In this study, infection of the clams by *P. olseni* did not have any direct effect on the condition index or on the hemocyte parameters of the clams, but findings demonstrate that an infection of the clams by *P. olseni* can affect the response of the clams to a harmful algal bloom. These observations are of practical importance, as HABs occur principally in the spring–summer in Brittany, which is also the time when the temperature of the water is warmest, favoring the highest presence of *P. olseni* in the clams (Villalba et al., 2005). Thus, as a HAB occurs in nature, clams infected by *P. olseni* are likely to experience at least short-term immunomodulation more severe than in less-infected clams. Moreover, the observation that the changes in hemocyte parameters occur after 3 days, and that the immune status returns to the original level after a longer exposure, suggests a possible adaptation of the clams to multiple stressors and an ability to respond after a fairly short time period. These findings also highlight the importance of considering multiple environmental factors when assessing the immunological status of bivalve molluscs, and confirm the suggestion by Harvell et al. (1999), that harmful algae may contribute to enhanced impacts of diseases and parasites on marine organisms.

5. Conclusions

Perkinsosis alone in this experiment caused no direct effects upon morphological or functional characteristics of clam hemocytes. The two species of toxic dinoflagellate (*Karenia selliformis* and *Karenia mikimotoi*) tested had immediate (after 3 days) effects upon hemocyte characteristics measured. Severity of effects of the two *Karenia* species was consistent with relative level of toxicity indicated by hemolytic tests. Severe perkinsosis modulated the response of clams to HAB exposure; clams more or less infected by *P. olseni* are not equally susceptible to effects of HABs; a severe infection having no effect under normal conditions can change the response of the clams when a HAB occurs. Similar studies of interactive effects of shellfish parasites and HABs, focused on parasites with more direct hemocyte interactions, could reveal additional combined effects.

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