Immunological responses of the Manila clam (*Ruditapes philippinarum*) with varying parasite (*Perkinsus olseni*) burden, during a long-term exposure to the harmful alga, *Karenia selliformis*, and possible interactions [☆]

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Abstract

The present study evaluated the possible effects of a toxic dinoflagellate, *Karenia selliformis*, upon immunological hemocyte functions of the Manila clam *Ruditapes philippinarum*, and on the progression of infection by *Perkinsus olseni*. Clams with variable levels of perkinsosis were exposed for 6 weeks to simulated blooms of cultured the *K. selliformis* (10^2 and 10^3 cell ml^{-1}). Samples were collected after 0, 2, 3, and 6 weeks of exposure. The following hemocyte parameters were measured by flow cytometry: percentage of dead cells, cell size and complexity, apoptosis, phagocytosis, and production of reactive oxygen species. Agglutination activities of *K. selliformis* on horse erythrocytes, serum protein concentration, and condition index of clams were also assessed.

The harmful alga *K. selliformis* caused a significant decrease in hemocyte size and percentage of apoptotic cells. In contrast, *P. olseni* did not affect clams strongly; the only significant effect was an increase in hemocyte size in heavily infected clams. After 2 and 3 weeks, the prevalence and burden of *P. olseni* decreased in clams exposed to *K. selliformis*, but after 6 weeks, and a diminution in *K. selliformis* cell density in the exposure, this effect disappeared. *In vitro* tests exposing *P. olseni* to *K. selliformis* showed direct algal toxicity to the parasite (increased percentage of dead cells and altered morphology). Initial exposure of *P. olseni*-infected clams to *K. selliformis* appeared to modify the host–parasite interaction by causing effects in both organisms.
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1. Introduction

Harmful-algal blooms (HABs), or "red tides," are global phenomena caused by a number of microalgal species, mainly dinoflagellates and diatoms, that produce biotoxins of various kinds (Hallegraeff, 1993; Smayda, 1997a, b). Suspension-feeding bivalves naturally ingest most dinoflagellate species (Gainey and Shumway, 1988; Lesser and Shumway, 1993) and are thus exposed to a variety of toxic components. Accumulation and persistence of toxicity in bivalves is species-dependent and varies according to the concentration of the bloom and rates of feeding and toxin elimination in the shellfish (see the review by Shumway, 1990).

Some HABs may cause pathologies and mortalities in the shellfish themselves (see reviews by Shumway, 1990; Landsberg, 2002). Most previous studies have addressed general physiological responses of bivalves, such as shell-valve closure, clearance and filtration rates, oxygen consumption, scope for growth, and mortality (Gainey and Shumway, 1988; Shumway, 1990, 1995; Lesser and Shumway, 1993; Landsberg, 2002; Leverone et al., 2007). A few studies have demonstrated deleterious effects of harmful algae upon bivalves at the cellular, tissue, and organ levels (Wikfors and Smolowitz, 1993, 1995; Smolowitz and Shumway, 1997; Pearce et al., 2005), or upon immunological function in bivalves (Hégaret and Wikfors, 2005a, b; Hégaret et al., 2007a, b; Galimany et al., 2008). Thus, the literature on interactions between filter-feeding molluscs and HABs demonstrates a wide diversity of effects, even though only a small number of potential HAB–bivalve pairs have been investigated. Moreover, the increase in HABs has been recognized as a factor that may contribute to acceleration of pathological conditions in aquatic animals (Landsberg, 1996; Harvell et al., 1999).

The dinoflagellate *Karenia selliformis* is thought to be responsible for mass mortalities of fish in the Gulf of Gabès, Tunisia, in 1994 (Arzul et al., 1995), and probably in Kuwait Bay, Arabian Sea (Heil et al., 2001). This dinoflagellate showed strong hemolytic activity against horse red blood cells (Jenkinson and Arzul, 2000). One toxin produced by *K. selliformis*, gymnodimine, has been characterized chemically (Seki et al., 1995; Miles et al., 2000, 2003). Gymnodimine was first characterized in New Zealand, where it is widely distributed but generally in low concentration (Stirling, 2001). The

presence of this toxin in Tunisia, concentrated in digestive-gland tissues of clams, has been reported (Biré et al., 2002). This species is, thus, a good model for studying the effects of HABs upon bivalves because it occurs naturally in molluscs and has demonstrated a variety of toxic effects.

When a bivalve is exposed to a toxic or noxious particle, shell-valve closure and reduced filtration may constitute the first responses and may serve to minimize contact of the soft tissues (Gainey and Shumway, 1988). As bivalves need to move water through the shell for respiration, these protective responses are temporary. Moreover, shell-valve closure and reduced pumping are not always completely effective in isolating soft tissues from harmful algae; therefore, internal defense responses may be stimulated (Hégaret et al., 2007c). Cellular and humoral responses comprise the internal defense mechanisms in bivalves. The cellular immune response is attributed to the hemocytes, usually exhibiting two morphological types: granulocytes and hyalinocytes (Cheng, 1981). Hemocytes play an important role in recognition and destruction of invading organisms, mainly by phagocytosis, the most effective mechanism of defense, but also by producing immune factors such as lectins, enzymes, antimicrobial peptides, etc. The impairment of these cells could result in higher disease susceptibility. As harmful algae cells can pass intact and viable into the bivalve digestive system (Bricelj et al., 1993; Scarratt et al., 1993; Laabir and Gentien, 1999; Bauder and Cembella, 2000; Hégaret et al., in press), the algal cells are likely to be in contact with hemocytes in the digestive system or in other tissues. Possible impairment of bivalve hemocytes by harmful algae is poorly understood.

Parasites of the genus *Perkinsus* infect marine molluscs throughout the world causing, in some cases, mass mortalities (Villalba et al., 2004). The parasite *Perkinsus olseni* occurs in Europe, including France (Goggin, 1992; Lassalle et al., 2007), within two species of clams, *Ruditapes decussatus* and *Ruditapes philippinarum*. As hemocytes in molluscs can be affected by both parasitic diseases and harmful algae, understanding the relationship between immune function in bivalves and harmful algae can contribute to more informed interpretation and management of consequences resulting from simultaneous effects of both parasites and HABs upon shellfish populations.

The present study employed a long-term (6-week) exposure of Manila clams, *R. philippinarum*, to an ichthyotoxic alga, *K. selliformis*, to elucidate possible relationships between three biological components: the harmful alga, the clam, and the parasite. The experiment tested whether or not infection by *P. olseni*, or exposure to *K. selliformis*, is associated with changes in immune characteristics of Manila clams, and whether or not one phenomenon can amplify the effect of the other.

2. Material and methods

2.1. Experimental animals

In October 2005, approximately 300 Manila clams, *R. philippinarum* (35–45 mm shell length), were collected from a natural population affected by perkinsosis near Bailleron Island, Golfe of Morbihan (Brittany, NW France). The clams were conditioned for 1 week prior to the experiment in a recirculating-seawater system wherein temperature was raised progressively from 16 to 18 °C, a temperature that can sustain development of *P. olseni* infections.

2.2. Algal cultures

The GM94GAB strain of *K. selliformis* used was obtained from the Department of Dyneco, IFREMER (Brest, France). This strain was isolated from Gulf of Gabès (Tunisia), after a massive fish mortality event, by E. Erard-Le Denn, who identified it as *Gymnodinium* sp. (Arzul et al., 1995). The species was recently re-described by Haywood et al. (2002) as *K. selliformis*, having been formerly referred to as *Gymnodinium maguelonnense* or *Karenia* sp. (Guillou et al., 2002; Shao et al., 2004). This strain has been maintained in the IFREMER culture collection for 12 years since its isolation. This alga was chosen for its marked cytotoxicity (Hégaret et al., 2007b). This dinoflagellate was cultured in 6-l carboys with F/2-enriched (Guillard and Ryther, 1962; Guillard, 1975), filtered (1 μm), autoclaved seawater. Algal cultures were maintained in a 12 h/12 h light–dark cycle and reached a maximum cell density of 10^4 cells ml^{-1}. Batch cultures of *K. selliformis* were harvested in late-exponential or early stationary phase after 9–11 days of growth.

The non-toxic alga *Chaetoceros neogracile* was cultured in 300-l tanks in the IFREMER hatchery in Argenton (France). Batch cultures of *C. neogra-cile* were harvested after 3–6 days of growth, usually at a concentration approaching 4×10^6 cells ml^{-1}.

Algal-cell density was quantified using a Malassez (*C. neogracile*) or Nageotte chamber (*K. selliformis*), depending upon algal species.

2.3. Dependent variables measured

2.3.1. Immunological analysis—hemocytes and plasma

Hemocytes and hemolymph analyses were done on individual clams to allow correlation of dependent variables with the *P. olseni* status of each clam. Hemocytes and plasma were collected and prepared as described in Hégaret et al. (2007a,b).

Hemocyte parameters include number and characterization (size, complexity) of hemocytes, percentage of dead hemocytes, percentage of apoptotic hemocytes, phagocytosis activity, and reactive oxygen species production. Hemocytes were analyzed according to the procedures described in Hégaret et al. (2007a,b) using a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA). Plasma parameters—agglutination titer and protein concentration—were determined according to Hégaret et al. (2007a,b).

2.3.2. Detection and quantification of P. olseni infection

P. olseni was detected and quantified according to Choi et al. (1989). The number of hypnospores per g of gill tissue obtained was log_{10} transformed. To facilitate statistical analysis, clams were categorized into two classes of *P. olseni* infection intensity: null–light ($\leqslant 1000$ hypnospores per g gill wet weight) and moderate–heavy (> 1000 hypnospores per g gill wet weight).

2.3.3. Condition index

Condition index (CI; Mann, 1978) was calculated using the dry meat weight (DMW) in relation with dry shell weight (DSW) as follows: $CI = DMW \times 100/DSW$.

2.4. Exposure of Manila clams R. philippinarum to K. selliformis

One-hundred and sixty clams were distributed into eight 10-l tanks (20 clams per tank) and 20 clams were used as an initial control. Experimental treatments were as follows:

(A) Unialgal *C. neogracile*, at 4×10^8 cells clam^{-1} day^{-1}, which corresponded to a maintenance

ration (4% of clam dry weight in algal dry weight per day; $n = 4$)).

(B) *C. neogracile* at the same quantity as above plus *K. selliformis* at 10^2 cells ml^{-1} (Hansen et al., 2004) for the first 2 weeks and the last 3 weeks of experiment, to simulate the beginning and the end of a bloom, and 10^3 cells ml^{-1} during the third week to simulate the peak of an algal bloom ($n = 4$).

A peristaltic pump was used to distribute algal suspensions and to ensure a continuous seawater flow, with renewal of the total tank volume in 2 days. Sampling was carried out at different times: before the exposure started (T0), after 2 weeks of low concentration (T2), after the peak bloom (T3), and at the end of the experiment (T6). At each time, 5 clams were collected from each replicate tank to analyze 20 clams per treatment.

2.5. In vitro challenge of P. olseni with K. selliformis

An additional experiment was carried out to test the hypothesis that *K. selliformis* can affect the viability and morphology of *P. olseni*.

2.5.1. P. olseni culture

The *P. olseni* isolate used (PJg5F) was established from gills of infected *R. decussatus* collected in Galicia (NW Spain). *P. olseni* cells were maintained in DMEM:HAM's F-12 culture medium (Gauthier and Vasta, 1993) at 1000 mOsm kg^{-1} and 18 °C. Trophozoites of *P. olseni* in log growth phase were used for the experiment. The culture was passed three times through a 23-G needle with a 5 ml syringe to break up cell aggregates, centrifuged (800g, 10 min) to eliminate the medium, and resuspended in filter-sterilized seawater (FSSW). To ascertain the quality of the culture before the experiment, cell viability and counts were assessed by staining with neutral red solution (50 mg l^{-1}). The *P. olseni* concentration used was approximately 10^6 cells ml^{-1}.

2.5.2. K. selliformis culture

The *K. selliformis* culture (as specified in Section 2.2) was tested as the complete culture and as cell-free medium prepared as follows: 30 ml of culture was dispensed into each of three tubes. The tubes were centrifuged (360g, 5 min); the supernatant was transferred to other tubes, and the process was repeated twice with the last centrifuga-

tion at 800g for 15 min to ensure complete removal of algae. Uninoculated medium was used as a control. The Nageotte chamber was used for microscope counts. The age and concentration of *K. selliformis* culture used in the experiments were 9 days and 4×10^3 cells ml^{-1}, respectively. The *K. selliformis*:*P. olseni* cell ratio was approximately 1:250; this corresponds to a 1:10 ratio by biovolume.

2.5.3. In vitro exposure of P. olseni to K. selliformis

The experiment was performed using five different culture flasks of *P. olseni* as replicates. Treatments were as follows: (A) *P. olseni* alone (PA); (B) *P. olseni* plus uninoculated medium (PUM); (C) *P. olseni* plus surpernatant of culture of *K. selliformis* (PSK); (D) *P. olseni* plus complete culture of *K. selliformis* (PCK). After 30 min, 1 h 30 min, and 3 h 30 min of incubation in flow-cytometer tubes, viability, size, and complexity of *P. olseni* cells were assessed by flow cytometry as described in Soudant et al. (2005).

2.6. Statistical analysis

All clam measurements made were analyzed by a multifactor analysis of variance (multifactor ANOVA), wherein algal diet and the level of infection by *P. olseni* (two classes established) were the two factors, and the time of exposure was run as a co-variable. Multifactor ANOVAs did not include the first sampling (T0). The percentages of phagocytic, dead, and apoptotic hemocytes were arcsin-transformed to meet homogeneity and normal-distribution requirements before multifactor ANOVAs. The differences in prevalence of *P. olseni* were analyzed using a Friedman test, in which the treatments were the algal diet and the blocks the weeks. A *T*-test was used to analyze the differences between means for all parameters, contrasting the two classes of *P. olseni* intensity at T0. To analyze differences in *P. olseni* infection intensity between treatments, a one-way ANOVA was used at each time of sampling (except T0). Percentage of dead cells, and size, and complexity of *P. olseni* were analyzed using a multifactor ANOVA (treatment and time of incubation as main factors). Statgraphics Plus statistical software was used for all analysis, except for the Friedman test for which Minitab 14 was used.

3. Results

3.1. Exposure of Manila clams R. philippinarum to K. selliformis

3.1.1. Variation in prevalence and intensity of infection with P. olseni

No clam mortality was observed during the entire experiment in any of the treatments. The RFTM analysis revealed numerous positive samples for *P. olseni*, with different parasite burdens. Of 140 clams analyzed, 98 were infected and less than half did not show the presence of the parasite. The distribution of clams into the two classes of *P. olseni* infection is shown in Table 1: 92 clams were categorized as having a "Null or Light" infection, and 48 a "Moderate to Heavy" infection, which represents sufficient numbers of clams in both categories to run the following statistical analyses (multifactor ANOVAs).

The prevalence of infection by *P. olseni* was the highest (90%) at the beginning of the experiment, and began to decrease after 2 weeks in both treatments (*K. selliformis* 65%; *C. neogracile* 70%); the trend was more pronounced in the clams exposed to *K. selliformis*, although the differences were not significant (Friedman test, $P = 0.083$). In the latter group, the lowest prevalence was observed after 3 weeks, i.e. after 2 weeks of exposure to 10^2 cells ml^{-1} followed by 1 week with 10^3 cells ml^{-1} *K. selliformis* (*K. selliformis* 50%; *C. neogracile* 70%). After 6 weeks, parasite prevalence increased to levels similar to values in clams fed *C. neogracile* (*K. selliformis* 70%, *C. neogracile* 75%). A similar pattern in parasite burden to that of *P. olseni* prevalence was observed, i.e. intensity was high at the beginning of the experiment, decreased afterward for both treatments, and was lower in clams exposed to *K. selliformis* for 2 weeks (significant

difference, ANOVA, $P = 0.007$) and after 3 weeks (no significant difference) than clams fed *C. neogracile* (Fig. 1).

3.1.2. Effect of K. selliformis, infection with P. olseni, and their interaction, upon hemocyte immune parameters in Manila clams R. philippinarum

The effect of infection by the protozoan parasite *P. olseni* upon hemocyte parameters of clams was tested before the experiment started, i.e. before exposing clams to any cultured microalgae. No effect of a natural infection of clams with *P. olseni* on any clam parameter was observed (data not shown). Likewise, during the 6 weeks of exposure to *K. selliformis*, the impact of the parasite was quite mild; only one parameter was affected strongly—hemocyte size, which was significantly ($P = 0.016$) higher in clams moderately to heavily infected with *P. olseni* than in lightly infected clams (Fig. 2).

The effect of the long-term exposure of *R. philippinarum* to *K. selliformis* was mainly observed on two hemocyte parameters: hemocyte size ($P = 0.022$) and percentage of apoptotic hemocytes ($P = 0.021$), which were severely reduced with respect to the control during the entire exposure (Fig. 3). Moreover, some tendencies were observed in the clams fed *K. selliformis*. An increase in agglutination activity was observed, starting at the beginning of the exposure and intensifying after 3 weeks of exposure; clams exposed to *K. selliformis*

Table 1
Categorization of *P. olseni* infection intensity according to the number of *P. olseni* cells in clam gills (X). Two categories are defined as null–light and moderate–heavy, respectively, with less or more that 1000 parasites per g of gill wet weight. $\text{Log}_{10}(X+1)$ is indicated between parentheses. The results include clams before the exposure and after 2, 3 and 6 weeks of exposure. n = number of clams in each class

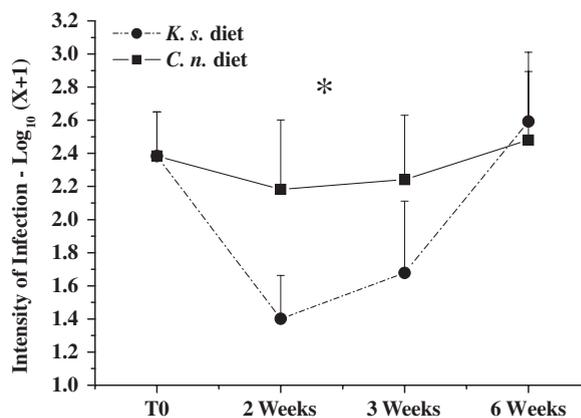| Infection classes	Mean	Minimum	Maximum	n
Null–light	146 (1.15)	0 (0.00)	971 (2.99)	92
Moderate–heavy	46 927 (4.03)	1257 (3.10)	894 640 (5.95)	48

Fig. 1. Infection intensity (mean ± SE) of *P. olseni* in Manila clams, *R. philippinarum*, during the 6 weeks of exposure to two different algal treatments; *K. selliformis* plus *C. neogracile* (*K. s.*, $n = 60$) or *C. neogracile* alone (*C. n.*, $n = 60$) (T0, $n = 20$). Asterisk indicates where algal treatment affected significantly the *P. olseni* burden (ANOVA, $P < 0.05$). Results are expressed as $\log_{10}(X+1)$ where X is the number of hypnospores of *P. olseni* per g of gill wet weight.

tended to have a much higher agglutination activity than control clams (Fig. 3). Fewer circulating hemocytes were observed in clams fed *K. selliformis* compared with clams fed *C. neogracile*, especially after the third week (Fig. 3). No differences in condition index between treatments were observed, although values were slightly lower for clams fed
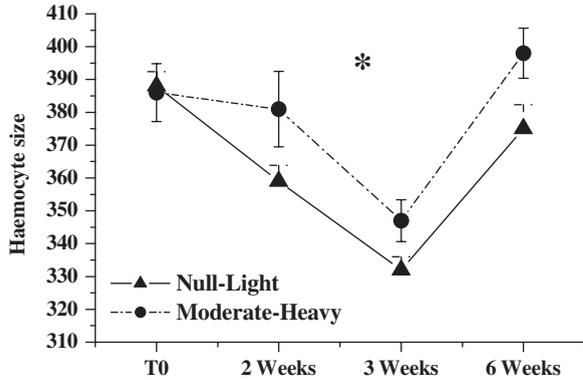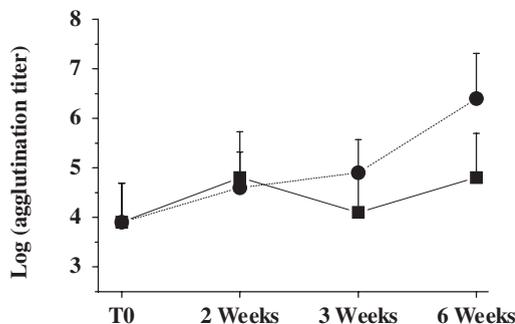
K. selliformis (T2: 4.6 ± 0.21; T3: 4.7 ± 0.20; and T6: 4.7 ± 0.20), in comparison to control groups fed *C. neogracile* (T2: 4.8 ± 0.26; T3: 4.8 ± 0.22; and T6: 5.0 ± 0.30).

Variation over time of exposure, for each individual parameter, was often significant, and was quite similar for both microalgal treatments (Fig. 3). Hemocyte size, complexity, and percentages of dead and phagocytic hemocytes showed similar, strong decreases ($P < 0.05$) after 3 weeks, but increased thereafter (except for percentage of dead hemocytes). Conversely, percentages of apoptotic hemocytes increased ($P < 0.001$) after 3 weeks of exposure (Fig. 3). Production of ROS by clam hemocytes was highest ($P = 0.034$) at the beginning of the exposure and decreased over time. Total hemocyte count (THC), agglutination titer, protein concentration, and condition index were not affected by time of exposure.

3.3. In vitro exposure of P. olseni to K. selliformis

As the severity of *P. olseni* infection decreased in clams fed *K. selliformis*, one question that arose was whether or not the harmful alga had toxic effects
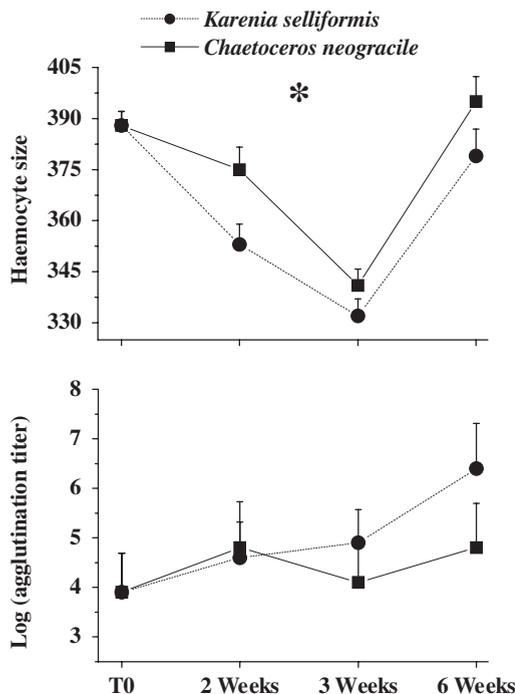
Fig. 2. Effect of *P. olseni* on Manila clam *R. philippinarum* hemocyte size (in arbitrary units, mean ± SE). Asterisks indicate significant differences in hemocyte size between classes of infection by *P. olseni* (null–light, $n = 92$; moderate–heavy, $n = 48$) (ANOVA, $P < 0.05$).
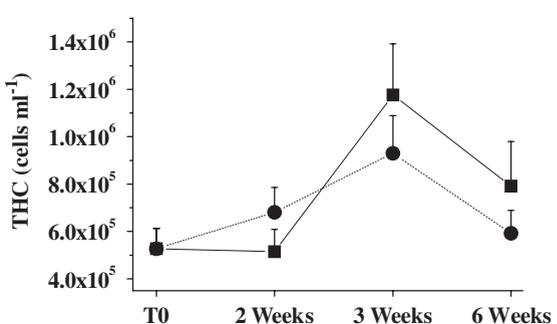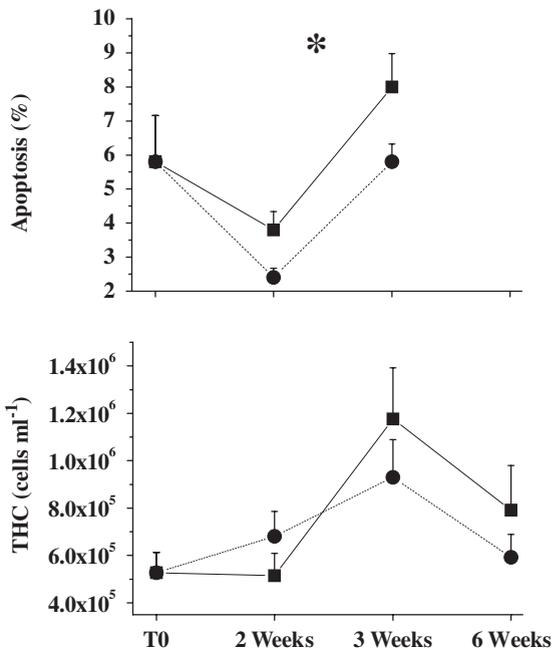
Fig. 3. Effect of *K. selliformis* on Manila clam, *R. philippinarum*, hemocyte parameters (means ± SE) during the 6 weeks of exposure to two different algal treatments; *K. selliformis* plus *C. neogracile* (*K. s.*) or *C. neogracile* solely (*C. n.*). Hemocyte size is in arbitrary units. Apoptosis values are missing for 6 weeks. Asterisks indicate significant differences between algal treatments (ANOVA, $P < 0.05$). Apoptosis: *K. s.*, $n = 20$, *C. n.*, $n = 24$; hemocyte size, agglutination and THC: *K. s.*, $n = 60$; *C. n.*, $n = 60$; T0: $n = 20$.

upon the parasite itself. Thus, *in vitro* studies were conducted co-incubating *K. selliformis* and *P. olseni*. The trophozoites of *P. olseni* were greatly affected by the presence of *K. selliformis* cells. Percentages of dead parasite cells increased approximately 10 times ($P < 0.001$) and trophozoites of *P. olseni* showed significant reduction in cell complexity ($P < 0.001$) (Fig. 4). Parasite cell size was also reduced, but differences were not significant ($P = 0.320$) (Fig. 4). The effect of cultured *K. selliformis* upon the trophozoites of *P. olseni* was found only with the complete culture, not with cell-free medium, indicating that the presence of the algal cells is necessary to induce toxic effects upon *P. olseni*.
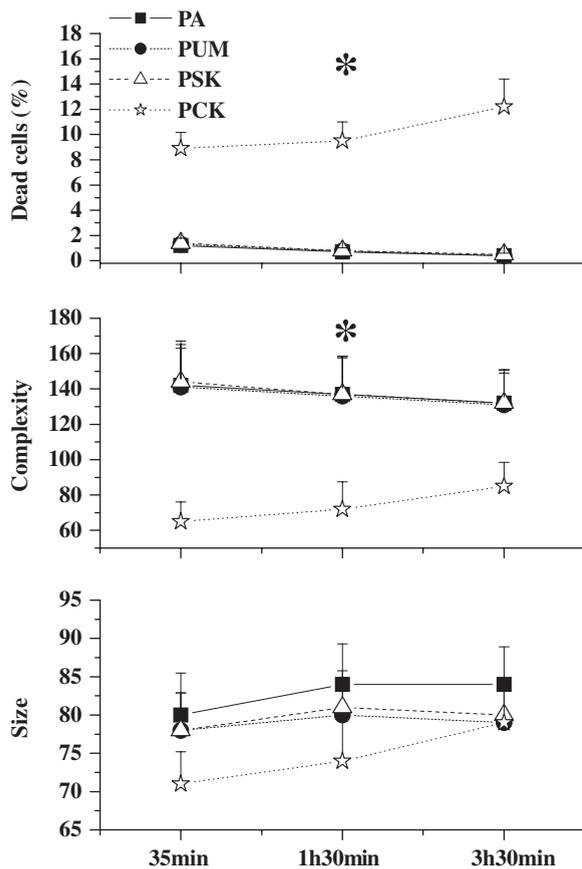

Fig. 4. Effects of *K. selliformis* on *P. olseni* cell parameters (mean ± SE, $n = 5$ per treatment at each time): the percentage of dead cells, size and complexity (in arbitrary units), after different times of *in vitro* incubation with cultured algal samples. PA: *Perkinsus olseni* alone; PUM: *Perkinsus olseni* plus uninoculated medium; PSK: *Perkinsus olseni* plus supernatant of culture of *Karenia selliformis*; PCK: *Perkinsus olseni* plus complete culture of *Karenia selliformis*. Asterisks indicate significant differences between *P. olseni* incubated with complete culture of *K. selliformis* and other treatments (ANOVA, $P < 0.05$).

4. Discussion

The main goal of the present study was to understand the combined effects of both parasitic infection and a simulated HAB on hemocyte parameters and condition index of Manila clam, thereby establishing a linkage between the three components of the biological-interaction model: harmful alga–clam–parasite.

As there was no interactive effect of perkinsosis and the HAB exposure on any of the hemocyte parameters or condition index, the effect of *K. selliformis* upon clam hemocytes will be discussed independently of the effect of infection by *P. olseni*. Clam hemocyte size was diminished when *K. selliformis* was present. Results from short-term, Manila clam–hemocyte exposure experiments with *K. selliformis* and *K. mikimotoi* also showed reduction in hemocyte size and complexity (Hégaret et al., 2007b). The effects of harmful-algal cells upon morphology of bivalve hemocytes, recorded in the literature, are very variable, probably because of the variety of mechanisms of toxicity specific to each algal species. The presence of yessotoxin in mussel hemocyte cytoplasm has also been associated with hemocyte shape changes, including reduction in size (Franchini et al., 2003; Malagoli and Ottaviani, 2004), which is attributed by the authors to an increase in intracellular Ca^{2+}. On the contrary, significant increases in hemocyte size and complexity have been reported in *Crassostrea virginica* after a *Prorocentrum minimum* bloom (Hégaret and Wikfors, 2005a), but no effects were found on oyster hemocytes (*C. virginica* and *C. gigas*) with other toxic dinoflagellates, *Alexandrium fundyense* and *A. catenella* (Hégaret et al., 2007a). The first studies on the effects of a harmful dinoflagellate upon bivalve hemocyte characteristics were reported by Hégaret and Wikfors (2005a, b); main effects observed were on concentrations and proportions of the different hemocyte types, production of reactive oxygen species, phagocytosis and hemocyte mortality in both *C. virginica* and *Argopecten irradians* after simulated and natural blooms of *P. minimum*.

In the present work, *K. selliformis* triggered a decrease in the percentage of apoptotic hemocytes. In contrast, phycotoxin-induced apoptosis has been reported in several human cell types exposed to algal toxins, such as okadaic acid (Cabado et al., 2003; Lago et al., 2005) or yessotoxin (Leira et al., 2002). Apoptosis eliminates over-abundant and potentially harmful cells; thus, apoptosis and cell

proliferation are coupled to maintain homeostasis, as shown for human lymphocytes (Fortner and Budd, 2005). Thus far, in bivalves, apoptosis is little studied; mechanisms and possible inducers could be quite different from those in vertebrate organisms. Some contaminants, such as cadmium, can induce apoptosis in oyster hemocytes (Sokolova et al., 2004). Goedken et al. (2005) showed that two oyster species, *C. virginica* and *C. gigas*, had repressed hemocyte apoptosis resulting from *P. marinus* infection in hemocytes. These authors also demonstrated, however, that *C. gigas*, which is resistant to *P. marinus*, seemed to overcome that suppression faster than *C. virginica*.

The *K. selliformis* phycotoxin, gymnodimine (Seki et al., 1995), apparently persists over the long term (even years) in mussel and oyster tissues (Stirling, 2001; MacKenzie et al., 2002). Gymnodimine accumulates in the digestive gland as well as in adjacent tissues (MacKenzie et al., 2002). Thus, this toxin may be expected to affect all organs and tissues, including hemocytes. Franchini et al. (2003) reported that yessotoxin is found in abundance in mussel hemocyte cytoplasm, which led these authors to hypothesize that hemocytes probably are the main vectors transporting these toxins throughout shellfish tissues. It is, however, still unknown whether or not this mechanism may be active for gymnodimine.

The effects of the parasite *P. olseni* upon clam–hemocyte parameters and condition index were also assessed. Categories of *P. olseni* infection intensity established in the present study paralleled the Mackin scale for *P. marinus* infection in eastern oysters, *C. virginica* (Ray, 1954). Our categories were shown to be very useful for experimental studies wherein the number of samples does not permit categorization of the clams into more than 2 groups (Hégaret et al., 2007b). In the present study, moderate–heavy *P. olseni* infection had little effect upon the hemocyte parameters and condition index of the Manila clam, as it was associated with an increase in hemocyte size only. The higher hemocyte size in clams with moderate–heavy infections of *P. olseni* is possibly attributable to the engulfment of tissue debris resulting from heavy infection, which provokes tissue disruption and organ dysfunction (Villalba et al., 2004; Kim et al., 2006). The engulfment of *P. olseni* by phagocytosis is believed to be rare in clams; *Perkinsus* spp. are instead encapsulated by hemocytes (Villalba et al., 2005). The low impact of *P. olseni* on the clam immune system was a bit surprising. Ordás et al. (2000) had reported a significant increase in lectins (agglutination titer) in carpet shell clams, *R. decussatus*, naturally infected with *P. olseni*. Lectins have been shown to be induced selectively in Manila clams upon *P. olseni* infection (Kim et al., 2006). In contrast, herein *P. olseni* did not induce lectin production.

The length of the experiment seemed to affect both groups of clams, exposed or not to the harmful algae, indicating that the clams may have been responding to external factors, such as the effect of the containers. This experiment did not highlight any specific changes in the immune parameters over time attributable to the exposure to *K. selliformis*.

The most striking result obtained in this study suggests an immediate toxic effect of the harmful alga *K. selliformis* upon *P. olseni* infection; indeed, the intensity and prevalence of *P. olseni* in the clams decreased after 2 and 3 weeks when the simulated HAB was the highest ($10^3 \, \text{cell ml}^{-1}$). It is unlikely that *K. selliformis* affects *P. olseni* development by stimulating a clam immune response against *P. olseni* as no major changes were observed in the functional hemocyte parameters of infected or uninfected clams. The hypothesis of a direct effect of *K. selliformis* upon *P. olseni* within exposed clams implies some mechanism whereby *K. selliformis* toxin is transported to *P. olseni* cells within clam tissues. As mentioned above, the toxin of *K. selliformis* accumulates in the digestive gland, as well as in adjacent tissues (MacKenzie et al., 2002). This tissue transfer of toxin may favor contact between parasite cells and toxin in clam tissues. It seems, however, that the effects of *K. selliformis* on *P. olseni* also depend upon the intensity of infection. Thus, we propose the hypothesis that heavily infected clams would have a higher probability of contact between parasites and toxins. The highest *P. olseni* intensity was observed at the start of experiment; subsequently, and not surprisingly, perkinsosis intensity declined quickly, leaving less-heavily infected clams present at the time when the clams were exposed to the higher concentration of *K. selliformis*. Finally, intensity of *P. olseni* re-increased at the end of the experiment, as the concentration of *K. selliformis* to which clams were exposed decreased.

To test the hypothesis described above on the potential toxicity of *K. selliformis* to *P. olseni* cells, *in vitro* tests were performed. The results showed that a culture of *K. selliformis* in its culture medium

is toxic to *P. olseni* cells, triggering cell mortality, and decreases in complexity of *P. olseni* trophozoites. Moreover, the intensity of *P. olseni* in the clams exposed to the HAB was strongly reduced from the first week of exposure throughout the bloom exposure, likely because of microalgal toxicity.

The exact mode of action of gymnodimine from *K. selliformis* has not been elucidated (Kong et al., 2005); therefore, it is not possible to attribute the increased mortality and the reduction in parasite complexity and size observed to a specific, chemical effect. Thus, other experiments using pure extracts of gymnodimine or other biotoxins from *K. selliformis* would provide insights into understanding the biochemical and physiological mechanism by which *K. selliformis* exerts toxic effects upon *P. olseni* cells.

We conclude from the present study that initial impairment of *P. olseni* infection in clams feeding on *K. selliformis* is short-lived. This phenomenon may reflect an ancestral interaction between phylogenetically close, unicellular organisms—Perkinsae and dinoflagellates (Leander and Keeling, 2004). Results also highlight that *P. olseni* alone had minimal impacts upon the immunological and physiological functions of clams that we measured. Conversely, *K. selliformis* did affect hemocytes and other physiological functions within Manila clams.

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