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Physiological responses of Manila clams *Venerupis* (=*Ruditapes*) *philippinarum* with varying parasite *Perkinsus olseni* burden to toxic algal *Alexandrium ostenfeldii* exposure

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ABSTRACT

Manila clam stock from Arcachon Bay, France, is declining, as is commercial harvest. To understand the role of environmental biotic interactions in this decrease, effects of a toxic dinoflagellate, *Alexandrium ostenfeldii*, which blooms regularly in Arcachon bay, and the interaction with perkinsosis on clam physiology were investigated. Manila clams from Arcachon Bay, with variable natural levels of perkinsosis, were exposed for seven days to a mix of the nutritious microalga T-*Iso* and the toxic dinoflagellate *A. ostenfeldii*, a producer of spirolides, followed by seven days of depuration fed only T-*Iso*. Following sacrifice and quantification of protozoan parasite *Perkinsus olseni* burden, clams were divided into two groups according to intensity of the infection ("Light–Moderate" and "Moderate–Heavy"). Hemocyte and plasma responses, digestive enzyme activities, antioxidant enzyme activities in gills, and histopathological responses were analyzed. Reactive oxygen species (ROS) production in hemocytes and catalase (CAT) activity in gills increased with *P. olseni* intensity of infection in control clams fed T-*Iso*, but did not vary among *A. ostenfeldii*-exposed clams. Exposure to *A. ostenfeldii* caused tissue alterations associated with an inflammatory response and modifications in hemocyte morphology. In the gills, superoxide dismutase (SOD) activity decreased, and an increase in brown cell occurrence was seen, suggesting oxidative stress. Observations of hemocytes and brown cells in tissues during exposure and depuration suggest involvement of both cell types in detoxication processes. Results suggest that exposure to *A. ostenfeldii* disrupted the pro-/anti-oxidant response of clams to heavy *P. olseni* intensity. In addition, depressed mitochondrial membrane potential (MMP) in hemocytes of clams exposed to *A. ostenfeldii* suggests that mitochondrial functions are regulated to maintain homeostasis of digestive enzyme activity and condition index.

1. Introduction

The Manila clam *Venerupis* (=*Ruditapes*) *philippinarum* is one of the most exploited bivalves in the world. Endemic to Indo-Pacific waters, it was introduced to the coast of France in the early 1970s for commercial purpose (Flassch and Leborgne, 1992). The Manila clam population from Arcachon Bay ranked first in France until 2012 (among the surveyed stocks) in terms of biomass (7200 t) and exploitable stock (720 t) (Sanchez et al., 2013). The exploitable stock, however, is declining (1159 t in 2008, 916 t in 2010), associated with decreasing harvests (Sanchez et al., 2013), causing a crisis for commercial fishermen. In this context, studies were conducted to understand the reason for this decrease and improve

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stock management. Although fishing is partially responsible (Dang et al., 2010b), effects of environmental factors remain to be investigated.

This population of clams is infected by the protozoan parasite *Perkinsus olseni*, with prevalences reaching high values (>90%) (Dang et al., 2010a). The parasite *P. olseni* resides in the clam connective tissue and usually induces hemocytic infiltration and parasite encapsulation that may lead to milky-white cysts or nodules in heavily-infected clams (Choi et al., 2002; Lee et al., 2001). Immune responses of clams *V. philippinarum* to *P. olseni* infection involve changes in humoral and hemocyte characteristics (Soudant et al., 2013). Although *P. olseni* infection was associated with massive mortalities in cultured and wild Manila clams in South Korea (Choi and Park, 2010, 1997; Park and Choi, 2001), this parasite was not reported to affect clam populations along the French Atlantic coast severely (Dang, 2009).

Concurrently, Arcachon Bay is a site of recurring Harmful Algal Blooms (HABs). The "French Phytoplankton and Phycotoxin Monitoring Network" (REPHY, Ifremer) detects the repeated presence of *Dinophysis* sp., *Pseudo-nitzschia* sp. and *Alexandrium* sp., associated to DSP (Diarrheic Shellfish Poison), ASP (Amnesic Shellfish Poison), and PSP (Paralytic Shellfish Poison) toxins, respectively. For the first time in 2005, spirolides, a group of macrocyclic imine toxins, were detected in shellfish from Arcachon Bay (Amzil et al., 2007). These toxins were coincident with *Alexandrium ostenfeldii* blooms (data from REPHY). Despite the absence of toxic effects of spirolides reported to date in humans, these toxins are considered to be "fast-acting toxins" because of acute toxicity to mice following intra-peritoneal injection (Hu et al., 1995). Although mechanisms of spirolide action are not fully elucidated, studies focusing mainly on 13-desmethyl-C spirolide suggested that these compounds are antagonists of cholinergic nicotinic receptors in mammalian systems (Bourne et al., 2010; Gill et al., 2003; Hauser et al., 2012; Wandscheer et al., 2010), causing neurotoxic symptoms (Gill et al., 2003; Munday et al., 2012; Otero et al., 2012; Richard et al., 2001). To date, only one study documented the effect of *A. ostenfeldii* exposure on bivalves, showing that ingestion of this dinoflagellate by the Pacific oyster *Crassostrea gigas* caused an inflammatory response in the digestive gland, which is the main organ accumulating spirolides (Medhioub et al., 2012). Effects of other HAB species upon bivalves have been reported at different biological levels. Various effects upon general physiological processes (Cucci et al., 1985; Gainey and Shumway, 1988a,b; Landsberg, 2002; Leverone et al., 2007; Shumway, 1990), on behavior (Gainey and Shumway, 1988b; Haberkorn et al., 2011; Hégaret et al., 2012; Shumway and Cucci, 1987; Tran et al., 2010), at tissue and cellular levels (da Silva et al., 2008; Galimany et al., 2008a,b,c; Haberkorn et al., 2010a,b; Hégaret et al., 2007a,b,c), and more recently at the molecular level (Estrada et al., 2007; Fabioux et al., submitted; Mello et al., 2013, 2012; Romero-Geraldo et al., 2012) have been observed. The first possible defense of bivalves against toxic or noxious particles in water is to close the valves and reduce filtration to minimize contact between microalgae and tissues (Gainey and Shumway, 1988b). There is, however, a balance between this protective response and the need for respiration and nutrition; consequently shell closure can be but a temporary response. The next line of defense in molluscan shellfish to noxious, harmful, or pathogenic agents is the immune system, involving humoral and cellular hemocyte responses which mediate internal defense mechanisms (Cheng, 1996; Song et al., 2010). Effects of HABs upon hemocyte morphology and functions have been reported (Hégaret and Wikfors, 2005a,b; Hégaret et al., 2007a,c; da Silva et al., 2008; Galimany et al., 2008a,b,c; Haberkorn et al., 2010a). Only few studies, however, have reported the effects of simultaneous exposures to HABs and pathogens upon bivalve physiology, although this phenomenon is common in the natural environment. Modifications of the host–pathogen interaction were observed after exposure of parasitized bivalves to toxic dinoflagellates (da Silva et al., 2008; Hégaret et al., 2009; Bricelj et al., 2011). In addition, the interaction of HABs and pathogens could lead to synergistic outcomes in bivalve physiology, affecting hemocyte functions (Bricelj et al., 2011; Hégaret et al., 2009, 2007a), but also inducing higher bivalve mortality rate, suggesting defeat of the defense system (Bricelj et al., 2011).

Manila clams from Arcachon Bay are likely to be subjected simultaneously to both perkinsosis and *A. ostenfeldii* exposure. This situation raises three questions: (i) What are the effects of different perkinsosis intensities? (ii) How does exposure to *A. ostenfeldii* affect clam physiology? (iii) Does exposure to *A. ostenfeldii* modifiy the host–pathogen interaction? To answer these questions, the effects upon different physiological responses of Manila clams caused by (i) varying natural *P. olseni* infection levels; (ii) experimental exposure of clams to *A. ostenfeldii*; and (iii) the combined effects of both biotic factors, were investigated. To describe the physiological responses of clams and identify the mechanisms underlying these responses, different biological levels and functions were studied, from individual to gene, with specific focus on digestive, immune, and mitochondrial functions, and pro-/anti-oxidative mechanisms.

2. Materials and methods

2.1. Experimental clams

Manila clams, *V. philippinarum* of 32.7 ± 0.3 mm shell length and 8.8 ± 0.2 g live weight (mean ± SE), were collected from "Estey de Tessillat" station in Arcachon Bay, France, where high prevalence of *P. olseni* was recorded previously (Dang, 2009). Manila clams were collected on May 17th, 2010 and transferred the next day to IUEM, Plouzané, France, where the experiment was performed.

2.2. Algal cultures

The microalga *Tisochrysis lutea* (El M. Bendif and I. Probert) (T-*Iso*), commonly provided as an aquaculture food for bivalves, was obtained from the Argenton hatchery (Ifremer, France) and was used as a control and a complementary diet for the experiment. T-*Iso* was cultured in f/2 medium (Guillard and Ryther, 1962) in 300-L open tanks, at 18 °C with 24-h light. Batch cultures of T-*Iso* were harvested after 3 to 5 days of growth, usually at a cell density approaching 5×10^6 cells mL^{-1}.

The Phycotoxin laboratory, Ifremer, Nantes (France), provided the toxic dinoflagellate *Alexandrium ostenfeldii*, strain CCMP1773 (isolated from Denmark), which is known to produce exclusively one type of toxin: spirolides (Otero et al., 2010). This strain was grown in L1 medium with soil extract (Guillard and Hargraves, 1993), and cultures were maintained at 16 °C with 12-h light in 20-L carboys. Cells were harvested in stationary phase, usually at a cell density approaching $5–7 \times 10^3$ cells mL^{-1}.

Algal cell densities were determined by counts using Nageotte cells under a light microscope.

2.3. Experimental design and sampling

One-hundred and twenty-eight clams were distributed randomly in eight 20-L tanks (16 clams per tank) containing a 7-cm sediment layer of ultra-pure SILAQ silica sand (granulometry from 0.8 to 1.4 mm diameter) to allow clams to burrow. Before starting the experiment, clams were acclimated for one week, fed T-*Iso* (5×10^5 cells mL^{-1}), in the 1-μm filtered, flow-through seawater system used for the experiment (15 L per 24 h per tank, *i.e.*, 10 mL min^{-1}), at 16 ± 1 °C, a temperature compatible with the

transmission and development of infection by *P. olseni* (Villalba et al., 2004).

Acclimation was followed by seven days of exposure to two different treatments with four replicates (*i.e.*, 4 tanks) per treatment: (i) control clams, fed only T-*Iso*, at a concentration of 5×10^5 cells mL^{-1}; (ii) HAB-exposed clams fed T-*Iso* at the same concentration with an addition of *A. ostenfeldii* at 10^3 cells mL^{-1}. Following this, a depuration period of seven days was applied during which clams were fed only T-*Iso* at 5×10^5 cells mL^{-1}. Six clams per tank were sampled (i) at the end of exposure ($T = 14$); and (ii) at the end of depuration ($T = 21$).

Hemocyte and plasma variables, condition index, histopathological condition, infection with *P. olseni*, activity of antioxidant and digestive enzymes, and toxin accumulation were analyzed at each sampling time.

2.4. Analysis of hemocyte and plasma variables

Hemolymph was withdrawn from the adductor muscle of each clam using a needle and a 1-mL syringe, filtered through an 80-μm mesh, and stored temporarily in an Eppendorf microcentrifuge tube held on ice.

Hemocyte morphology and functions were analyzed on hemolymph extracted from each clam. Procedures for characterization of clam hemocytes, concentration (=total hemocyte counts – THC, cells mL^{-1}), size, internal complexity and mortality of circulating hemocytes, as well as for functional responses, such as phagocytosis, unstimulated hemocyte production of reactive oxygen species (ROS) (specifically $O_2^{\bullet -}$ and H_2O_2), and mitochondrial membrane potential (MMP) were adapted from Delaporte et al. (2003), Soudant et al. (2004) and Donaghy et al. (2012). Analyses of hemocytes were performed with a FACScalibur flow-cytometer (BD Biosciences, San Jose, CA).

In addition, remaining hemolymph was centrifuged ($800 \times g$, 5 min, 4 °C) to be separated into two fractions: plasma (supernatant) and hemocytes (pellet), prior to freezing (−20 °C). Plasma was used for agglutination and hemolysis tests with horse red blood cells, following the protocol of Barracco et al. (1999), and expressed as the log (base 2) of the reciprocal of the highest dilution showing a positive pattern of agglutination or hemolysis of erythrocytes, respectively.

2.5. Condition index (CI)

Condition index (CI) of the Manila clams was calculated using the wet flesh weight (WFW) in relation to dry shell weight (DSW): CI = (WFW/DSW) × 100 (adapted from Bodoy et al., 1986).

2.6. Histopathological observations

For histology, each clam was shucked, and two diagonally-slanted, 5-mm sections of soft tissue, including gills, mantle, digestive gland, intestine, and gonad, were excised. Additionally, a section of adductor muscle was sampled. Tissues were fixed immediately in Davidson's solution (Shaw and Battle, 1957) for 24 h. Tissues then were transferred into 70% ethanol, dehydrated in ascending ethanol solutions, cleared with Claral®, and embedded in paraffin wax. Five-μm sections were stained with Harris' hematoxylin and eosin (Howard et al., 2004) and observed under a light microscope.

A six-level, semi-quantitative scale was established to assess intensity of each histopathological condition observed, from 0 to 2.5, as described in Table 1.

Table 1
Semi-quantitative scale categorizing intensity of histopathological conditions observed.

Level intensity	Occurrence of the pathological condition in the examined tissue area
0	Absence
0.5	Very low (1–5 total occurrence)
1	Low (>5 occurrence/presence in all fields at magnification 10×)
1.5	Moderate (presence in all fields at magnification 20×/covering about 10% of the tissue)
2	High (presence in all fields at magnification 40×/covering about 20% of the tissue)
2.5	Very high (presence in all fields at magnification 60×/covering about 30% or above of the tissue)

2.7. Detection and quantification of infection with P. olseni in V. philippinarum

From each clam, after removing the section for histological studies, a portion of gills was excised and weighed. Gills were incubated in Ray's fluid-thioglycollate medium (RFTM, Ray, 1966) for 7 days in the dark at room temperature and prepared to assess the parasite burden according to the protocol developed by Choi et al. (1989), as described in Hégaret et al. (2009). Briefly, gills were centrifuged ($800 \times g$, 10 min) to remove medium and digested with NaOH (2 N, 1 h at 60 °C, twice), followed by two washes in phosphate-buffered saline (PBS) (0.1 M). Finally, the pellet was re-suspended in 1 mL of PBS. Ten microlitres of Lugol's solution was added, and the number of *P. olseni* hypnospores was counted using a Nageotte chamber under a light microscope.

The intensity of infection with *P. olseni* was calculated as the number of hypnospores per gram of wet weight of gill. To run multifactor statistical tests, the level of infection with *P. olseni* was divided into two equally-abundant classes: "Very light–Light" infection class ($<4.2 \times 10^4$ *P. olseni* cells g^{-1} wet gill) and "Moderate–High" infection class ($\geq 4.2 \times 10^4$ *P. olseni* cells g^{-1} wet gills).

2.8. Activity of antioxidant enzymes

The rest of the gills were removed and frozen immediately in liquid nitrogen after dissection, and stored at −80 °C. Gill tissues were ground using a "Dangoumeau"-type ball grinder into liquid nitrogen.

2.8.1. Protein extraction

One fraction of 50 mg of ground gills was homogenized, using an ultra-turrax, in 250 μL of cold lysis buffer prepared following Guévélou et al. (2013) [NaCl 150 mM, Tris 10 mM, pH 7.4, EDTA 1 mM, EGTA 1 mM, Phosphatase inhibitor cocktail II (Sigma-Aldrich) 0.01%, Triton X-100 1% v/v, CA-630 Igepal (Sigma-Aldrich) 0.5%, 1 tablet of complete EDTA-free protease inhibitor cocktail (Roche) were added extemporaneously per 25 mL buffer; pH 8.8 at 4 °C] and centrifuged at 10,000*g* for 45 min at 4 °C. The supernatant then was stored at −80 °C until further analysis.

2.8.2. Protein assay

Total protein content in gills was determined using the Bio-Rad DC™ Protein Assay, based upon the method of Lowry et al. (1951) and appropriate for protein extracts containing detergents such as Triton X-100. Bovine serum albumin (Sigma-Aldrich) was used as protein standard. Absorbance at 750 nm was determined after 15 min of incubation at room temperature.

2.8.3. Activity of total superoxide dismutase (SOD), Mn SOD and Cu/Zn SOD

Total superoxide dismutase (SOD; EC 1.15.1.1) activity in gills was measured using an SOD Assay Kit (Sigma-Aldrich) according to manufacturer's instructions. Protein extracts were diluted 40 times

and absorbance at 440 nm was recorded after 20 min incubation at 25 °C. A standard inhibition curve was constructed using SOD from bovine erythrocytes (Sigma-Aldrich).

Mn SOD (=mitochondrial SOD) activity in gills was determined using the SOD Assay Kit and KCN (5 mM final concentration), a known inhibitor of the Cu/Zn SOD (=cytoplasmic SOD) (Fridovich, 1975; Manduzio et al., 2005). Protein extracts were diluted 5 times, and absorbance at 440 nm was recorded after 20 min of incubation at 25 °C.

Cu/Zn SOD activity was evaluated as the difference between total SOD activity and Mn SOD activity.

Total SOD, Mn SOD and Cu/Zn SOD specific activities were expressed in U mg^{-1} total proteins, one unit being defined as the amount of enzyme inhibiting by 50% the xanthine/xanthine oxidase complex.

2.8.4. Activity of catalase (CAT)

Catalase (CAT; EC 1.11.1.6) activity in gills was determined following the method of Aebi (1984). Decrease in absorbance at 240 nm attributable to the decomposition of H_2O_2 (10 mM) was recorded at 9-s intervals for 90 s. Protein extracts were diluted 200 times and CAT-specific activities were expressed in U mg^{-1} total proteins, one unit of CAT being defined as the amount of enzyme catalyzing 1 μmol of H_2O_2 min^{-1}.

2.9. Activity of digestive enzymes

2.9.1. Protein extraction

Digestive gland stored at −80 °C immediately after sampling was homogenized using a Potter in phosphate buffer 0.02 M (0.02 M NaH_2PO_4/0.02 M Na_2HPO_4) (100 mg wet weight mL^{-1}), and centrifuged at 4000 × *g* for 30 min. Supernatant was stored at −80 °C until further analyses.

2.9.2. Protein assay

To assess specific activity of digestive enzymes, protein extracts were diluted at 1/20 in phosphate buffer 0.02 M, and total protein content was determined with a Bio-Rad Protein Assay, based on the Bradford (1976) method using Bovine Serum Albumin as a protein standard.

2.9.3. Activity of amylase, cellulase and laminarinase

Activities of digestive enzymes were assessed using the method of Bernfeld (1955) slightly modified. Activities of amylase, cellulase and laminarinase were measured, by incubating diluted protein supernatants at 37 °C (dilution in phosphate buffer 0.02 M: 1/20 for amylase, 1/2 for cellulase, 1/4 for laminarinase) with the sugar they reduce: starch (30 min of incubation), cellulose (1 h), and laminarin (1 h), respectively. After the reaction was stopped by adding 250 μL of 3,5-dinitrosalicylic acid (3,5-DNS), samples were boiled for 15 min to enable the reduction of 3,5-DNS in 3-amino-5-nitrosalicylic acid, producing a coloration proportional to the quantity of sugar reduced, and absorbance was read spectrophotometrically at 540 nm. Specific activity was determined using maltose standards ranging from 0.2 to 1.0 mg mL^{-1} and expressed in mg of maltose liberated mg^{-1} proteins h^{-1}.

2.10. Toxin accumulation

Accumulation of spirolides was measured at each sampling time on 8 exposed individuals (2 per tank) and 2 control individuals in two fractions of soft tissue: digestive gland and remaining tissues (including siphon, foot, gills, adductor muscle and mantle), that had been frozen in liquid nitrogen immediately after sampling, then stored at −80 °C. Lipophilic toxins were extracted in methanol/water (95/5) and analyzed by LC–MS/MS at the EMP/PHYC laboratory of Ifremer, Nantes, France, as described in Amzil et al. (2007) and Medhioub et al. (2012).

2.11. Statistical analyses

T-test ($\alpha = 0.05$) was used to compare total spirolide accumulation between clams from algal treatments (T-*Iso vs. A. ostenfeldii*), sampling times (exposure *vs.* depuration), or tissues (digestive gland *vs.* remaining tissues), and one-way ANOVA followed by Tukey HSD post-hoc test ($\alpha = 0.05$) was used to assess differences in accumulation between the three spirolide analogues.

To assess the effect of time of experiment (exposure or depuration), algal treatment, and level of infection with *P. olseni* and their interactions on hemocyte variables and enzyme activities, a 3-way ANOVA (type 3 Sum of Squares) was performed, in which above factors were independents. After determining that the effect of the time during experiment was not significant, data from algal treatments and perkinsosis categories were analyzed using values from all sampling times of the experiment combined. In addition, to identify specific responses upon exposure or depuration, data were analyzed separately at each sampling time, using a 2-way ANOVA (type 3 Sum of Squares), in which algal treatment and level of infection with *P. olseni* were independent factors. When the effect of the interaction was significant, the Tukey HSD post-hoc test was used to identify differences between treatment means. Effects attributable to *P. olseni* burden were combined from the duration of the experiment.

When needed, data were transformed (log or $1/X$) prior to analysis to meet homoscedasticity and normality assumptions.

For histopathology results, a unilateral Mann–Whitney *U* test ($\alpha = 0.1$) was used to assess the effect of *P. olseni* burden over the duration of the experiment, and to assess the effect of algal treatment over the entire time of experiment, and separately after exposure and after depuration period.

Statistics were performed with Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA).

3. Results

3.1. Perkinsus olseni burden in clam gills

Prevalence of perkinsosis in experimental clams was 100%, and mean intensity of infection was $7.7 \pm 0.9 \times 10^4$ cells g^{-1} wet gills (mean ± SE). The mean intensity of infection of each class, "Very light–Light" (46 clams) and "Moderate–High" (45 clams), was $1.3 \pm 0.2 \times 10^4$ and $14 \pm 1 \times 10^4$ *P. olseni* cells g^{-1} wet gills (mean ± SE), respectively.

P. olseni burden was not significantly affected by exposure to *A. ostenfeldii* but showed a decreasing trend. The mean intensity of infection of all clams sampled was $6.6 \pm 1.0 \times 10^4$ cells g^{-1} wet gills (47 clams) in *A. ostenfeldii* exposed individuals, and was $8.8 \pm 1.6 \times 10^4$ cells g^{-1} wet gills (44 clams) in control clams (mean ± SE).

3.2. Toxin quantification

Three spirolide analogues were identified and quantified within the tissues: 13,19-didesmethyl-C (13,19-didesMeC), 13-desmethyl-C (13-desMeC) and 13-desmethyl-D (13-desMeD). Mean accumulation of total spirolides was significantly higher in the digestive gland than in the remaining tissues at both sampling times ($p < 0.001$). In both digestive gland and remaining tissues, and at both sampling times, accumulation of total spirolides was significantly higher in dinoflagellate-exposed individuals ($4.0 \pm 0.7 \times 10^2$ μg 13-desMeC eq. kg^{-1} in digestive gland

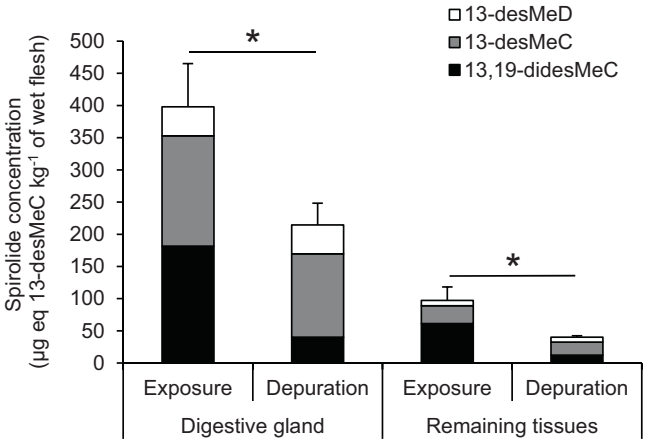

Fig. 1. Spirolide analogue content in digestive gland and in the remaing tissues of Manila clams *V. philippinarum* exposed to a mix of T-*Iso* and *A. ostenfeldii* for 7 days ("Exposure"), followed by 7 days of depuration fed T-*Iso* ("Depuration"). Error bars represent standard errors of total spirolide content. Significant differences in total spirolide content between exposure *vs.* depuration in each tissue are indicated by *: $p < 0.05$ (*t*-test). $N = 8$ in each group.

and $2.1 \pm 0.3 \times 10^2$ µg 13-desMeC eq. kg^{-1} in remaining tissues) compared to controls, in which only 13-desMeC was detected ($2.9 \pm 0.1 \times 10^1$ µg 13-desMeC eq. kg^{-1} in digestive gland and $2.6 \pm 0.2 \times 10^1$ µg 13-desMeC eq. kg^{-1} in remaining tissues) ($p < 0.05$ in digestive gland at both sampling times; $p < 0.01$ after exposure in remaining tissues; $p < 0.001$ after depuration in remaining tissues). Among spirolide analogues, only the 13,19-didesMeC decreased significantly after seven days of depuration in both digestive gland and remaining tissues of *A. ostenfeldii*-exposed clams ($p < 0.001$ in digestive gland; $p < 0.01$ in remaining tissues), leading to a significant decrease in total spirolide content in both digestive gland and remaining tissues ($p < 0.05$) (Fig. 1).

3.3. Condition index, hemocyte and plasma variables, digestive and antioxidant enzyme specific activities

Results of statistical analyses are summarized in Table 2. Effects of algal treatment are presented over the entire experiment (both sampling times combined), after 7 days exposure, and after 7 days of depuration. Effects attributable to *P. olseni* burden are presented over the entire experiment as well as effects of algal treatment and *P. olseni* burden interaction (because no significant effect was detected at each sampling time).

3.3.1. Condition index

Condition index of experimental clams did not vary with algal treatment or intensity of *P. olseni* infection.

3.3.2. Hemocyte and plasma analyses

Although granulocytes and hyalinocytes have already been described by cytochemical assays in Manila clams (Cima et al., 2000), morphological distinction between clam hemocyte types using flow cytometry is not always successful (Donaghy et al., 2009). Consequently, in this study, granulocytes and hyalinocytes were analyzed together.

Total hemocyte count (THC), internal complexity of hemocytes, phagocytosis, hemocyte mortality, agglutination and hemolysis titer did not vary significantly between the different experimental treatments.

Exposure to *A. ostenfeldii* led to an increase in the mean size of hemocytes, which was significant after exposure and over the duration of the experiment (Fig. 2A). Mean size of hemocytes also increased significantly with *P. olseni* burden, from 133 ± 1 a.u. (arbitrary units) in "Very lightly–Lightly" infected clams to 137 ± 1 a.u. in "Moderately–Highly" infected clams (mean ± SE).

Mitochondrial membrane potential (MMP) of hemocytes decreased significantly in clams exposed for 7 days to *A. ostenfeldii* (Fig. 2B).

A significant interaction of both algal treatment and *P. olseni* burden was detected throughout the experiment for ROS production in hemocytes. In individuals exposed to the non-toxic T-*Iso*, ROS production in "Very lightly–Lightly" infected clams was significantly lower than in "Moderately–Highly" infected clams (Fig. 2C).

3.3.3. Digestive enzyme specific activities

No significant effect was observed on specific activities of amylase, cellulase or laminarinase for algal treatment or intensity of *P. olseni* infection.

3.3.4. Antioxidant enzyme activities in gills

Total SOD specific activity was significantly lower in gills of clams exposed to *A. ostenfeldii* over the entire experiment, mostly attributable to the significant difference detected after depuration between control and exposed animals (Fig. 3A). The specific activites of both SOD isoforms Cu/Zn and Mn SOD, comprised in total SOD, were also significantly lower in gills of clams exposed to *A. ostenfeldii* throughout the experiment.

A significant effect of the interaction of *P. olseni* burden and algal treatment was identified (Fig. 3B) upon specific activity of CAT in gills. After exposure to T-*Iso*, CAT specific activity was higher in "Moderately–Highly" infected clams than in "Very lightly–Lightly" infected ones, but this response was not observed in *A. ostenfeldii*-exposed clams.

3.4. Histopathological effects

Effects of algal treatment over the entire experiment (both sampling times combined), after seven days of exposure and after seven days of depuration, and effects of *P. olseni* burden upon histological features of experimental clams are reported in Table 3.

To identify effects of *A. ostenfeldii* exposure upon clam tissues, intensities of pathologies first were compared between all clams exposed to *A. ostenfeldii* and all control clams sampled throughout the experiment. Exposure to *A. ostenfeldii* caused a decrease in hemocyte infiltration into the digestive gland ($p < 0.05$), as well as an increase in edema in the mantle ($p < 0.05$), vacuolation in the gills ($p < 0.1$) (Fig. 4A and B), hemocyte diapedesis into the intestine ($p < 0.05$) (Fig. 4D and E), and epithelium sloughing into the stomach lumen ($p < 0.1$). These pathologies were associated with observations of *A. ostenfeldii* cells within the intestinal lumen (Fig. 4E) after both exposure and depuration in exposed clams.

After seven days of exposure to *A. ostenfeldii*, hemocyte infiltration in gills ($p < 0.05$) (Fig. 5A), edema in the mantle ($p < 0.05$) (Fig. 5B), and epithelium sloughing into the intestinal lumen increased ($p < 0.05$) (Fig. 5C), while hemocyte infiltration into the digestive gland decreased ($p < 0.01$). In addition, *A. ostenfeldii* cells were observed in digestive tubules of one individual after seven days of exposure (Fig. 4F). After seven days of depuration, an increased intensity of cell vacuolation in gill filaments ($p < 0.1$) (Figs. 4A and B and 5D) and of brown cells ($p < 0.1$) (Figs. 4A and C and 5E), as well as an increased number of hemocytes in the intestinal lumen (Fig. 5F), were observed in *A. ostenfeldii*-exposed clams.

Histopathological observations showed that clams with "Moderate–Heavy" *P. olseni* burden, had more intense vacuolation in digestive tubules ($p < 0.1$) (Fig. 4G and H) compared to "Very lightly–Lightly" infected individuals, as well as more hemocytic encapsulation bodies in gonadal tissue ($p < 0.1$) and in the digestive gland ($p < 0.1$) (Fig. 4I). These pathologies were associated with a

Table 2
Comparison of physiological variables measured in *V. philippinarum* (i) over the entire experiment, (ii) after 7 days of exposure to *A. ostenfeldii* or to control T-*Iso*, and (iii) after 7 days of depuration (fed only T-*Iso*). NS: no significant difference; * Significant difference indicated by $p < 0.05$; ** Significant difference indicated by $p < 0.01$ (2-way ANOVA); "–": no analysis over the entire experiment because there was a significant effect of time of sampling (exposure *vs.* depuration); between brackets: significant effect after exposure and after depuration are indicated separately because of the absence of analysis over the entire experiment since there was a significant effect of time of sampling (exposure *vs.* depuration).

Physiological parameters	Over the entire experiment						After seven days of exposure		After seven days of depuration	
	Factor algal treatment		Factor *P. olseni* burden		Interaction		Factor algal treatment		Factor algal treatment	
	p	*N*	*p*	*N*	*p*	*n*	*p*	*n*	*p*	*N*
Condition index	NS	44	NS	44	NS	21–23	NS	23–24	NS	20–21
Hemocyte and plasma parameters										
Phagocytosis (% of phagocyting hemocytes)	–	–	(NS; NS)	(18–27; 16–26)	(NS; NS)	(8–16; 8–13)	NS	21–24	NS	21
Mortality of hemocytes	–	–	(NS; NS)	(16–27; 16–27)	(NS; NS)	(8–16; 8–15)	NS	19–24	NS	20–23
Hemocyte ROS production	NS	31–47	*	38–40	*	17–24	NS	15–24	NS	16–23
Hemocyte MMP	–	–	(NS; NS)	(18–29; 16–28)	(NS; NS)	(8–16; 8–15)	**	23–24	NS	21–23
THC	NS	40–47	NS	43–44	NS	19–24	NS	20–24	NS	20–23
Size of hemocytes	**	44–47	*	45–46	NS	21–24	*	23–24	NS	21–23
Complexity of hemocytes	NS	44–47	NS	45–46	NS	21–24	NS	23–24	NS	21–23
Agglutination titer	NS	32–43	NS	37–38	NS	15–23	NS	17–23	NS	15–20
Hemolysis titer	–	–	(NS; NS)	(16–24; 14–21)	(NS; NS)	(8–16; 6–12)	NS	17–23	NS	15–20
Antioxidant enzymes specific activity (gills)										
CAT	NS	20	NS	20	*	9–11	NS	9–10	NS	10–11
Total SOD	**	19–21	NS	19–21	NS	9–12	NS	9–10	*	10–11
Cu/Zn SOD	*	17–20	NS	17–20	NS	8–11	NS	8–9	NS	9–11
Mn SOD	**	17–20	NS	17–20	NS	8–11	NS	8–9	*	9–11
Digestive enzyme specific activity (digestive gland)										
Amylase	NS	12–14	NS	11–15	NS	5–9	NS	6–7	NS	8–5
Cellulase	NS	12–14	NS	11–15	NS	5–9	NS	6–7	NS	8–5
Laminarinase	NS	12–14	NS	11–15	NS	5–9	NS	6–7	NS	8–5

ROS: reactive oxygen species; MMP: mitochondrial membrane potential; THC: total hemocyte count; CAT: catalase; SOD: superoxide dismutase.

higher number of *Perkinsus* cells observed in the digestive gland ($p < 0.05$) (Fig. 4I).

Exposure to *A. ostenfeldii* resulted in a decrease in *Perkinsus* cells observed in clam tissues. Indeed, significantly fewer *Perkinsus* cells were observed in gills of clams throughout the experiment ($p < 0.1$), in mantle ($p < 0.01$) after seven days of exposure, and in adductor muscle ($p < 0.1$) after the depuration period, compared to clams fed to T-*Iso*.

4. Discussion

This study revealed physiological and histological consequences of variable parasite, *Perkinsus olseni*, infestation intensities upon Manila clams, *Venerupis philippinarum*, effects of exposure to the toxic dinoflagellate *Alexandrium ostenfeldii* upon the clams, and interactive effects as well. Consideration of two microbiological interactions in individual clams highlights the fact that these interactions occur in nature, although they may not be considered in controlled experiments that are designed to eliminate potentially confounding independent variables. Considering parasite and harmful algal effects individually and combined provided a better indication of the compound challenges that confront clams in nature, but this consideration also makes untangling causes and effects a complex task. Accordingly, we proceed by discussing individual responses of clams to parasitism or to harmful-algal-exposure, finally interpreting interactive responses.

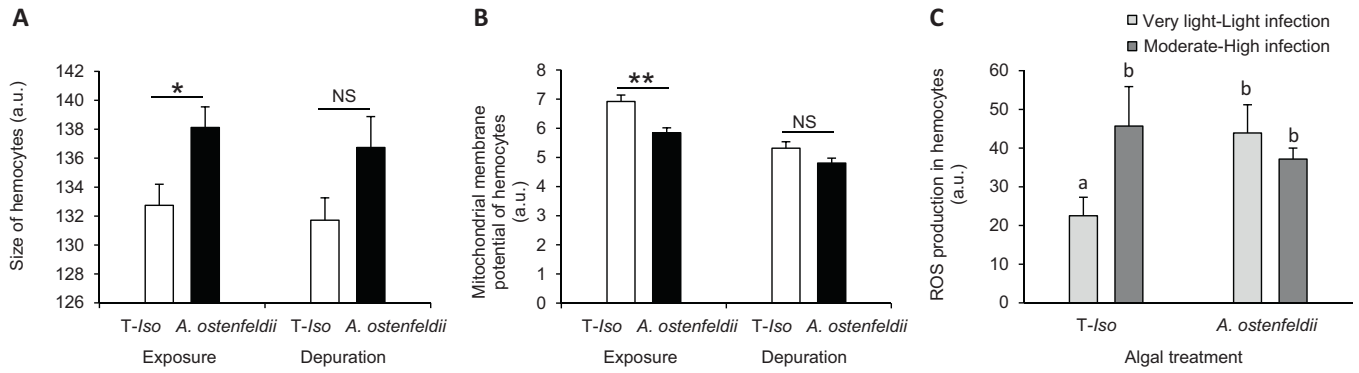

Fig. 2. Circulating hemocyte variables of Manila clams *V. philippinarum* exposed to T-*Iso* (control) or to a mix of T-*Iso* and *A. ostenfeldii*. (A) Size of hemocytes ($n = 21–24$ per group) and (B) mitochondrial membrane potential (MMP; $n = 8–16$ per group) after 7 days of dinoflagellate exposure ("Exposure") and after 7 days of depuration with the diet T-*Iso* ("Depuration"); (C) hemocyte ROS production according to algal treatment and to *P. olseni* burden ("Very light–Light" or "Moderate–Heavy"), over 7 days of dinoflagellate exposure followed by 7 days of depuration with the diet T-*Iso* ($n = 17–24$ per group). Error bars represent standard errors. * And letters indicate significant difference between groups, NS indicates no significant difference (ANOVA followed by post-hoc Tukey HSD test, $\alpha = 0.05$). a.u.: Arbitrary units.

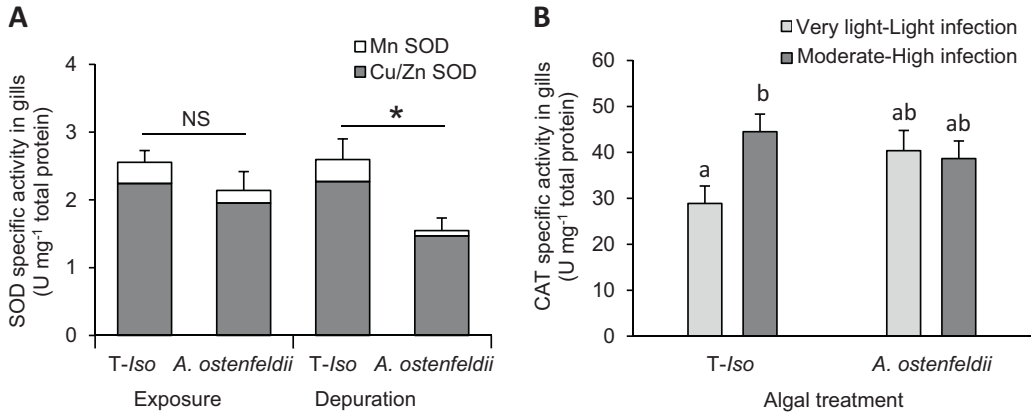

Fig. 3. Specific activity of antioxidant enzymes of Manila clams *V. philippinarum* exposed to T-*Iso* (control) or to a mix of T-*Iso* and *A. ostenfeldii*. (A) SOD specific activity after exposure and after depuration ($n = 8$–11 per group); (B) CAT specific activity according to algal treatment and *P. olseni* burden ("Very light–Light" or "Moderate–Heavy"), over 7 days of algal exposure followed by 7 days of depuration with T-*Iso* ($n = 9$–11 per group); Error bars represent standard errors. * And letters indicate significant difference between groups, NS indicates no significant difference (ANOVA followed by post-hoc Tukey HSD test, $\alpha = 0.05$).

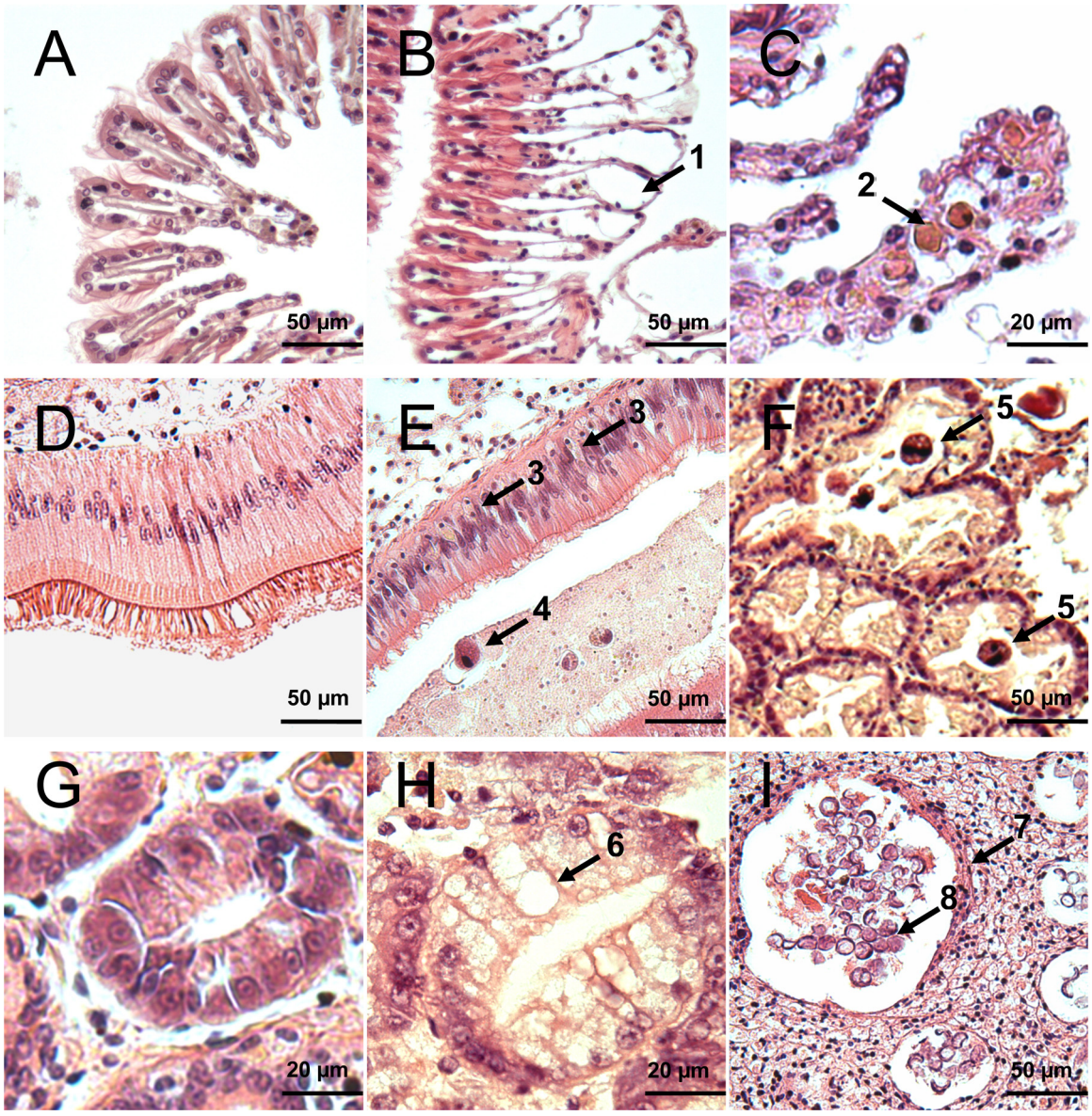

Fig. 4. Histopathological conditions. (A) Gills of control clam; (B) vacuolation (1) of gill filaments in *A. ostenfeldii*-exposed clam; (C) brown cells (2) in gills of *A. ostenfeldii*-exposed clam; (D) intestinal epithelium of control clam; (E) diapedesis of hemocytes (3) in intestinal epithelium and *A. ostenfeldii* cell (4) in intestinal lumen of *A. ostenfeldii*-exposed clam; (F) *A. ostenfeldii* cells (5) in digestive tubule lumen of one *A. ostenfeldii*-exposed clam; (G) digestive tubule of a control clam; (H) vacuolation (6) in the digestive tubule of "Moderately–Heavily" *P. olseni*-infected clam; (I) hemocytic encapsulation (7) of *P. olseni* trophozoites (8) in connective tissue of the digestive gland.

Table 3
Comparison of histological features in experimental clams, according to *P. olseni* burden categories ("Very light–Light" *vs* "Moderate–High"), and upon algal treatment (mix of T-*Iso* and *A. ostenfeldii vs* control T-*Iso*) after 7 days of exposure ("Exposure"), after 7 days of depuration (fed only T-*Iso*) ("Depuration"), and during the entire course of the experiment (Exposure + Depuration). NS: no significant difference. Significant differences indicated by #: $p < 0.1$; *: $p < 0.05$; **: $p < 0.01$ (Mann–Whitney test). †: A single clam showed this characteristic. NA: not available (not enough data). "–": No analysis over the entire course of the experiment because there was a significant effect of time of sampling (exposure *vs.* depuration).

Histological features	Differences between *P. olseni* burdens		Differences between algal treatments					
			Exposure		Depuration		Over the entire experiment	
	p	*n*	*p*	*n*	*p*	*n*	*p*	*n*
Hemocyte infiltration:								
Gills	NS	12	*	5–7	NS	5–7	–	–
Mantle	NS	11	NS	6	NS	4–6	–	–
Digestive gland	NS	13	#	5–7	NS	6–8	*	11–15
Digestive epithelium	NS	12–14	NS	5–7	NS	6–8	NS	11–15
Gonadal tissue	NS	13–14	NS	6–7	NS	6–8	NS	12–15
Adductor muscle	NS	6–7	NA		NS	3–6	NS	5–8
Hemocyte encapsulation:					NS			
Gills	NS	11–13	NS	6	NS	5–7	NS	11–13
Mantle	NS	9–11	NS	5	NS	4–6	NS	9–11
Digestive gland	#	12–14	NS	5–7	NS	5–8	NS	11–15
Digestive epithelium	NS	13	NS	6–7	NS	5–8	NS	11–15
Gonadal tissue	#	13	NS	6–7	NS	5–8	NS	11–15
Adductor muscle	NS	8–10	NS	3–5	NA		NS	8–10
Mantle: edema	NS	11–12	*	6	NS	5–6	*	11–12
Gills:								
Edema	NS	12–13	NS	6–7	NS	5–7	NS	11–14
Brown cells	NS	12–13	NS	6–7	#	5–7	NS	11–14
Vacuolation	NS	12–13	NS	6–7	#	5–7	#	11–14
Stomach: epithelium sloughing into the lumen	NS	13	NS	5–7	NS	6–8	#	11–15
Intestine:								
Hemocyte diapedesis	NS	12–13	NS	4–7	NS	6–8	*	10–15
Brown cells through the epithelium	NS	12–13	NS	4–7	NS	6–8	–	–
A. ostenfeldii cells in the lumen	NS	12–13	*	4–7	#	6–8	**	10–15
T-*Iso* cells in the lumen	NS	12–13	NS	4–7	NS	6–8	NS	10–15
Brown cells in the lumen	NS	12–13	NS	4–7	NS	6–8	–	–
Epithelium sloughing into the lumen	NS	12–13	*	4–7	NS	6–8	–	–
Hemocytes in the lumen	NS	12–13	NS	4–7	#	6–8	NS	10–15
Digestive tubules:								
Hemocyte diapedesis	NS	10–11	NS	4–5	NS	5–7	NS	9–12
Brown cells through the epithelium	NS	10–11	NS	4–5	NS	5–7	–	–
A. ostenfeldii cells in lumen	NS	10–11	NS †	4–5	NS	5–7	NS †	9–12
Brown cells in the lumen	NS	10–11	NS	4–5	NS	5–7	–	–
Epithelium sloughing into the lumen	NS	10–11	NS	4–5	NS	5–7	NS	9–12
Hemocytes in the lumen	NS	10–11	NS	4–5	NS	5–7	NS	9–12
Vesicles	NS	10–11	NS	4–5	NS	5–7	NS	9–12
Vacuolation	#	10–11	NS	4–5	NS	5–7	NS	9–12
P. olseni cells:								
Gills	NS	12–13	NS	6–7	NS	5–7	#	11–14
Mantle	NS	11–12	**	6	NS	4–7	–	–
Digestive gland	*	13–14	NS	6–7	NS	6–8	NS	12–15
Gonadal tissue	NS	13–14	NS	6–7	NS	6–8	NS	12–15
Adductor muscle	NS	6–7	NA		#	3–6	#	5–8

4.1. Effects of P. olseni

Overall, effects of higher *P. olseni* infections were relatively light and did not affect important physiological functions of clams. Although the digestive gland showed evidence of tissue damage, *i.e.*, vacuolation in digestive tubules, digestive functions were maintained, as shown by the representative digestive enzyme activities analyzed. Furthermore, presence of encapsulation bodies highlighted the effectiveness of the immune response, allowing other cellular and humoral immune responses, such as phagocytosis, hemolytic activity or agglutination titer, to occur. Encapsulation is indeed believed to be an important mechanism by which clams defend against *P. olseni* (Chagot et al., 1987; Montes et al., 1996, 1995; Navas et al., 1992; Ordás et al., 2001; Park and Choi, 2001; Sagrista et al., 1995). The increase in size of circulating hemocytes detected in our study could reflect cell differentiation for this immune response. These light effects can be attributed to the relatively low intensity of *P. olseni* infection compared to other studies. Waki and Yoshinaga (2013) and Waki et al. (2012) suggested that *P. olseni* pathogenicity to the Manila clam was considerable at a level of $\sim 10^6$ cells g^{-1} soft tissues, above which mortality occurred. Although such intensity levels are frequent in Manila clam populations in Asia (Choi and Park, 2010), mean *P. olseni* burdens reported in French populations usually do not exceed 10^5 cells g^{-1} of soft tissue (Binias et al., 2014, 2013; da Silva et al., 2008; Dang et al., 2013; De Montaudouin et al., 2010; Hégaret et al., 2007a), with no seasonal variation (Binias et al., 2014; Dang et al., 2013, 2010a), in contrast to other locations such as Gomso Bay, Korea (Yang et al., 2012) or Galicia, Spain (Villalba et al., 2005). Accordingly, Hégaret et al. (2007a, 2009) and da Silva et al. (2008), did not observe significant effects of *P. olseni* upon most of the hemocytic variables they measured in French Manila clams. For this intensity of infection, an explanation suggested by Hégaret et al. (2007a) is that, once the host hemocytes have accomplished isolation of the parasite, the remaining circulating hemocytes resume the main functions. No effects of parasite burden upon condition index or

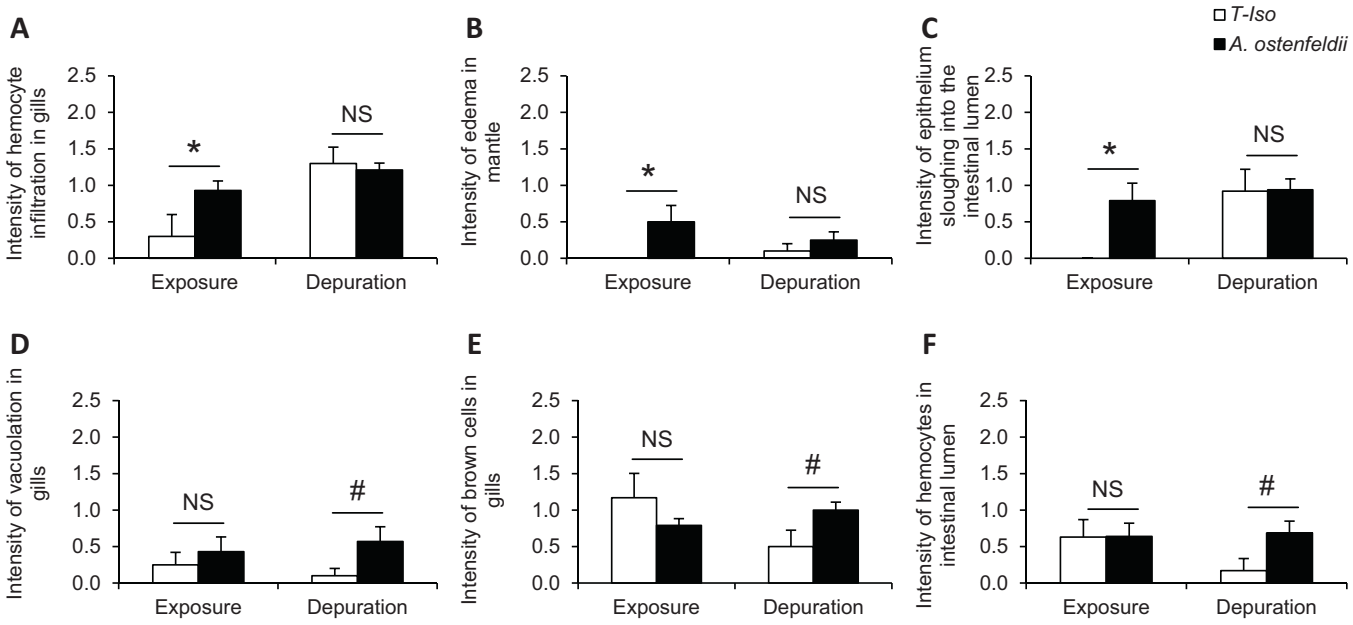

Fig. 5. Intensity of histopathological features (semi-quantitative scale) in tissues of Manila clams *V. philippinarum*, from two different algal treatments, T-*Iso* (control) or a mix of T-*Iso* and *A. ostenfeldii*, after 7 days of exposure and after 7 more days of depuration. Error bars represent standard errors. Significant differences between control ("T-*Iso*") and exposed clams ("*A. ostenfeldii*") are indicated by #: $p < 0.1$ and *: $p < 0.05$, and NS indicates no significant difference (Mann–Whitney test). $N = 4–8$ per group. (A) Hemocyte infiltration in gills; (B) edema in mantle; (C) epithelium sloughing into the intestinal lumen; (D) vacuolation in gill filaments; (E) brown cells in gills; (F) hemocytes in intestinal lumen.

mortality of clams were observed, thus confirming that the global host-pathogen interaction remained in equilibrium.

4.2. Effects of A. ostenfeldii exposure

After exposure of experimental Manila clams to the toxic dinoflagellate *A. ostenfeldii*, spirolide analogues accumulated preferentially in the digestive gland, as reported in oysters by Medhioub et al. (2012), but also in the remaining tissues including siphon, foot, gills, muscle and mantle. In addition, the 13-desmethyl-C spirolide was detected at very low levels in control clams, showing that these clams had been exposed to *A. ostenfeldii* in the field. Indeed, since 2005, spirolides have been detected recurrently by the "French Phytoplankton and Phycotoxin monitoring Network" (REPHY) in tissues of oysters and mussels from Arcachon Bay and were attributed to *A. ostenfeldii* blooms.

After seven days exposure, overall effects of exposure to *A. ostenfeldii* on clam "external" (mantle and gills) and "internal" organs (digestive gland) were identified. As clams are filter feeders, the first contact of microalgae with tissues occurs through siphons (not investigated in this study), mantle, and gills. Contact with *A. ostenfeldii* led to edema in the mantle, associated with hemocyte infiltration in the gills, indicating an inflammatory response. We suggest that the increase in hemocyte size is attributed to a differentiation process associated to this inflammatory response. These effects may be induced partly by the spirolides released after lysis of *A. ostenfeldii* cells during digestion and accumulated in tissues. As mantle and gills were in contact with living *A. ostenfeldii* cells, however, these tissues also may have been exposed to algal extracellular compounds. Indeed, independently from spirolides, *A. ostenfeldii* is able to produce extracellular compounds known to provoke immobilization and cell lysis of protists (Tillmann et al., 2007). Other *Alexandrium* species were reported to produce extracellular compounds with allelopathic, hemolytic, and ichthyotoxic properties (Arzul et al., 1999; Lelong et al., 2011; Ogata and Kodama, 1986; Simonsen et al., 1995) or oxidative properties (Flores et al., 2012). These compounds also can be deleterious to bivalves, as demonstrated by Ford et al. (2008) upon hemocyte functions. Ingestion of *A. ostenfeldii* cells, as observed on histological slides, was followed by deleterious effects upon internal organs, such as digestive epithelium sloughing into the intestinal lumen. In addition, the presence of intact *A. ostenfeldii* cells in the digestive tubules, where at least partially digested particles normally are found, and observation of *A. ostenfeldii* within the intestinal lumen after seven days of depuration, suggest partial dysfunction of digestive processes. Medhioub et al. (2012) also observed failure to digest *A. ostenfeldii* cells by oysters *C. gigas* and hemocyte diapedesis through the digestive epithelium. A role of hemocyte diapedesis after harmful algal exposure was suggested by other authors as a mechanism to eliminate toxin through the lumen of the intestine and/or to isolate *Alexandrium* cells (Galimany et al., 2008c; Haberkorn et al., 2010b; Hégaret et al., 2009). The detection of yessotoxin (another phycotoxin) in hemocytes of contaminated mussels by Franchini et al. (2003) strengthens the hypothesis that these cells could transport toxins out of the tissues as a detoxication process.

After a seven-day depuration time, spirolide content in digestive glands and remaining tissues of *A. ostenfeldii*-exposed clams decreased. Toxins, mainly 13-desmethyl-C spirolide, however, still were present in tissues, which could explain observations again of hemocytes undergoing diapedesis into the intestine and present in the lumen, consistent with our hypothesis that hemocytes participate in the detoxication process. In clam gill filaments, the harmful-algal exposure followed by a depuration period induced cell-vacuolation that probably affected gill functions, reflecting a persistent deleterious effect of *A. ostenfeldii* toxic compounds, extracellular compounds or spirolides. Alterations in gills were associated with higher amounts of brown cells (also referred to as ceroid bodies), suggesting oxidative stress, as these cells contain lipofuscin-like pigments constituted of non-degradable material, resulting mainly from the accumulation of oxidized proteins and lipids (Yin, 1996). Other authors also observed induction of brown cells in bivalve tissues following harmful-algal exposure (Galimany et al., 2008b), but also in response to organic pollutant exposure

(Lehmann et al., 2007; Morado and Mooney, 1997; Smolowitz and Leavitt, 1996). In addition, brown cells from the "red gland" or pericardial gland of bivalves have been shown to play a role in accumulation, detoxication, and degradation of pollutants (Zaroogian and Anderson, 1995; Zaroogian and Yevich, 1994; Zaroogian et al., 1989). In the present study, we suggest that the brown cells observed within gills indicated oxidative stress, and these cells may in addition participate in detoxication. Another indicator of possible oxidative stress in gills was the depression of total SOD specific activity, an antioxidant enzyme, as reported by Estrada et al. (2007) in gills of *Nodipecten subnodosus* exposed to the toxic dinoflagellate *Gymnodinium catenatum* and associated with lipid peroxidation. Exposure to *A. minutum* also presumably provoked oxidative stress in *C. gigas* gills, detected by measurement of antioxidant enzyme transcript levels (Fabioux et al., submitted). In this study, the decrease in mitochondrial SOD (Mn SOD) activity measured in gills of *A. ostenfeldii*-exposed clams may indicate mitochondrial metabolic adjustments to maintain homeostasis, as suggested by Romero-Geraldo et al. (2012). These authors observed an overall repression in antioxidant enzyme transcript levels, including SOD, in *C. gigas* for the first 14 days of exposure to toxic dinoflagellate *Prorocentrum lima*. The decrease of MMP in hemocytes in our study also supports the involvement of mitochondrial adjustments.

4.3. Modulation of *P. olseni* burden responses in clam pro-/anti-oxidative mechanisms by exposure to *A. ostenfeldii*

Exposure to *A. ostenfeldii* led to higher ROS production in hemocytes of "Very lightly–Lightly" infected clams. In addition, ROS production also increased in hemocytes of clams fed T-*Iso* as a response to the higher intensity of *P. olseni* infection. Changes in CAT activity within gills were concomitant with changes in circulating hemocyte ROS production, suggesting a protective response against higher production of ROS. These ROS compounds measured in unstimulated hemocytes probably originate from mitochondria, as shown in unstimulated hemocytes of *C. gigas* (Donaghy et al., 2012). ROS produced by mitochondria are involved in the modulation of many cellular processes such as cell signaling, immune responses, apoptosis, or ion-channel functions (Bartosz, 2009; Stowe and Camara, 2009). The increase in ROS production observed in our experiment may thus either play a role in the response to *P. olseni* infection and/or to *A. ostenfeldii* exposure by inducing modifications in cellular processes or it may be a consequence of physiologically-related mitochondrial changes (Stowe and Camara, 2009) induced by the parasitic infection and/or the algal exposure. No variation in ROS production, however, was observed in hemocytes of *A. ostenfeldii*-exposed clams with higher *P. olseni* infestation, suggesting that ROS production reached a threshold or that exposure to *A. ostenfeldii* is able to neutralize the increase in ROS stimulated by *P. olseni*.

4.4. Effect of *A. ostenfeldii* exposure on *P. olseni* burden in tissues of Manila clams

Although exposure to *A. ostenfeldii* did not modify *P. olseni* gill burden in experimental clams, a decreasing trend was observed, which also was confirmed by histological observations. *P. olseni* observations significantly decreased in gills, mantle and adductor muscle, suggesting a deleterious effect of *A. ostenfeldii* upon the parasite. In another study exposing Manila clams to the harmful alga *Karenia selliformis*, *P. olseni* infection intensity regressed in toxic algal-exposed clams, suggesting a negative effect of this alga upon *P. olseni* (da Silva et al., 2008). *In vitro* experiments confirmed that *K. selliformis* had negative effects upon *P. olseni* viability, modifying the host–pathogen interaction (da Silva et al., 2008). *In vitro* experiments also demonstrated detrimental effects of another dinoflagellate, *P. minimum* and its exudates, upon *P. olseni* cells (Hégaret et al., 2009). It would thus be interesting to assess the direct effect of *A. ostenfeldii* on *P. olseni* cells to understand the observed effects of *A. ostenfeldii* exposure upon the parasite occurrence within tissues.

5. Conclusions

Overall, hemocyte immune functions of Manila clams remained relatively unchanged according to perkinsosis level and *A. ostenfeldii* exposure, highlighting the capacity of clams to maintain homeostasis. Indeed, despite histological damage and partial dysfunction of the digestive gland caused by *A. ostenfeldii* exposure, digestive enzyme activities were not affected, showing that clams were able to maintain these main nutritional functions. Modifications in mitochondrial metabolism and detoxication processes probably participated in maintaining homeostasis. From this study, it appears that the interaction of *A. ostenfeldii* blooms and perkinsosis does not likely affect survival of Manila clam stocks from Arcachon Bay. Considering the roles of hemocytes in immunity, however, the question of a potential consequence of changes in hemocyte morphology and MMP induced by exposure to *A. ostenfeldii* on clam capacity to defend against other more virulent pathogens or other environmental stressors cannot be excluded.

Conflict of interest

Authors have nothing to declare.

Author contribution

Conceived and designed the experiment: PS, NLG, CL, XM, CF, HH. Performed the analyses: ML, PS, GR, NH, WM, PMS, AD, MB, NLG, CL, CF, HH. Analyzed the data: ML, PS, GR, NH, HH. Wrote the paper: ML, PS, PMS, CF, HH.

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