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Exposure to the toxic dinoflagellate *Alexandrium catenella* modulates juvenile oyster *Crassostrea gigas* hemocyte variables subjected to different biotic conditions

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Abstract :

The Pacific oyster *Crassostrea gigas* is an important commercial species cultured throughout the world. Oyster production practices often include transfers of animals into new environments that can be stressful, especially at young ages. This study was undertaken to determine if a toxic *Alexandrium* bloom, occurring repeatedly in French oyster beds, could modulate juvenile oyster cellular immune responses (i.e. hemocyte variables). We simulated planting on commercial beds by conducting a cohabitation exposure of juvenile, "specific pathogen-free" (SPF) oysters (naïve from the environment) with previously field-exposed oysters to induce interactions with new microorganisms. Indeed, toxic *Alexandrium* spp. exposures have been reported to modulate bivalve interaction with specific pathogens, as well as physiological and immunological variables in bivalves. In summary, SPF oysters were subjected to an artificial bloom of *A. catenella*, simultaneously with a cohabitation challenge.

Exposure to *A. catenella*, and thus to the paralytic shellfish toxins (PSTs) and extracellular bioactive compounds produced by this alga, induced higher concentration, size, complexity and reactive oxygen species (ROS) production of circulating hemocytes. Challenge by cohabitation with field-exposed oysters also activated these hemocyte responses, suggesting a defense response to new microorganism exposure. These hemocyte responses to cohabitation challenge, however, were partially inhibited by *A. catenella* exposure, which enhanced hemocyte mortality, suggesting either detrimental effects of the interaction of both stressors on immune capacity, or the implementation of an alternative immune strategy through apoptosis. Indeed, no infection with specific pathogens (herpesvirus OsHV-1 or *Vibrio aesturianus*) was detected. Additionally, lower PST accumulation in challenged oysters

suggests a physiological impairment through alteration of feeding-related processes. Overall, results of this study show that a short-term exposure to *A. catenella* combined with an exposure to a modified microbial community inhibited some hemocyte responses, and likely compromised physiological condition of the juvenile oysters.

Highlights

► Naïve juvenile oysters were exposed to a microbial challenge plus to *A. catenella* ► Hemocyte responded to the microbial challenge alone (induced by cohabitation with field-exposed oysters) ► These hemocyte responses were partially inhibited by *A. catenella* exposure ► Increased hemocyte mortality suggested either an immune strategy (apoptosis), or cytotoxicity ► Lower PST accumulation suggested alteration of feeding-related processes

Keywords : Crassostrea gigas, Alexandrium, harmful algal blooms, hemocyte, microbial challenge

1 Introduction

Infectious diseases have caused recurrent losses in shellfish stocks that have decreased the profitability of the aquaculture industry over the past decades [1–3]. Specific, pathogenic micro-organisms have been involved in major diseases of bivalves, including the herpesvirus OsHV-1µVar that is associated with recent, massive mortalities of Pacific oyster spat and juveniles [4–11], and several bacterial species and strains from the *Vibrio* genus, particularly *V. aesturianus* [12–15].

The Pacific oyster *Crassostrea gigas* is the most exploited bivalve species, with a worldwide production estimated over 1.9 million tons in 2013 [16–21]. Oyster farming practices usually include numerous transfers of oysters at all life stages, especially of spat and juveniles grown in hatchery and nursery systems before being transferred to oyster farming areas in open seawater [22]. Along with these farming practices, oysters must adapt to new abiotic and biotic environments. Biotic changes include interactions with new micro-organisms, potentially pathogenic or toxic, which can enter oysters *via* filtration and feeding processes.

In addition to pathogens, phytoplankton biotic interactions can change during and after transplanting. Natural phytoplankton constitute a main component of the oyster diet, but deleterious effects can occur when harmful algae are present. Dinoflagellates are the most represented group causing harmful algal blooms (HABs), with species in the Genus *Alexandrium* producing paralytic shellfish toxins (PSTs) and / or spirolides, both neurotoxic. *Alexandrium* spp. exposures can alter physiological processes and tissue integrity of bivalves [23–31].

Experimental exposures to *Alexandrium* spp. were reported to modulate host-pathogen interactions in oysters [32–34], possibly by altering immune function, as suggested by the alteration of hemocyte characteristics [33]. Several other studies also reported effects of

Alexandrium spp. exposure upon bivalve hemocytes [35–37]. Hemocytes, present in the tissues and in the circulating hemolymph, are the cellular mediators of immune responses in bivalves, which also include humoral factors. Hemocytes are involved in phagocytosis or encapsulation to achieve pathogen degradation through release of hydrolytic enzymes and oxidative compounds [38,39].

Alexandrium catenella recurrently blooms along the French Mediterranean coast [40,41] where major oyster farming activities occur. Considering the effects of *Alexandrium* spp. upon host-pathogen interactions and upon physiological and immunological variables, we hypothesized that exposure to *A. catenella* (producing PSTs) could compromise immune status of juvenile oysters (supposedly more sensitive than adults and commonly victims of massive mortality events). In addition, to assess if *A. catenella* exposure could render juvenile oysters more susceptible to opportunistic infection, spat produced under controlled conditions were put in contact with field-exposed oysters to introduce microorganisms from the environment.

This study thus investigated the possible interactions between juvenile oysters *C. gigas*, grown in hatchery, and a new biotic environment, defined by (i) an artificial bloom of *A. catenella*, and (ii) a modification of the microbial community induced by cohabitation with oysters previously exposed to the field, a process known to release and transmit pathogens [34,42]. Upon exposure to these biotic changes, hemocyte responses, toxin accumulation, OsHV-1 and *V. aesturianus* burdens (two pathogens monitored during massive mortality events by the French Monitoring Network for Shellfish Farming, RESCO[43,44]) as well as total *Vibrio* loads, were assessed after 4 and 9 days of exposure.

2 Material and methods

2.1 Algal cultures

Tisochrysis lutea (Bendif & Probert) (T-*Iso*) was fed to oysters during acclimation and maintenance stages at 5×10^5 cells mL^{-1}. T-*Iso* was cultured in 300-L cylinders containing seawater enriched with Conway medium [45] at 20°C with continuous light (200 µmol photons m^{-2} s^{-1}). T-*Iso* was harvested after 3 to 5 days of growth, at a cell density approaching 1×10^7 cells mL^{-1}.

The dinoflagellate *Alexandrium catenella* (Whedon & Kofoid) strain VGO676, a paralytic shellfish toxin (PST) producer [46], isolated in 2003 from the Thau lagoon (France), was used for toxic algal exposure, and *Heterocapsa triquetra* (Ehrenberg) Stein, strain HT99PZ (isolated from Penzé Bay, France in 1999), was used as a control, non-toxic dinoflagellate. Both strains were provided by the Phycotoxin Laboratory, Ifremer, Nantes (France). Both dinoflagellate cultures were grown in L1 medium [47] at 17°C with a light:dark cycle of 12:12h and were harvested during the exponential growth phase at a cell density approaching 5×10^4 cells mL^{-1}.

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

2.2 Specific Pathogen-Free (SPF) oysters

The Pacific oysters, *Crassostrea gigas* (Thunberg), used in this study all came from a single cohort produced in April 2011 in the Argenton Ifremer facilities (France) following a

116 standardized procedure to obtain OsHV-1-free diploid oysters described by Petton et al. [42].
117 Screening for OsHV-1 DNA was conducted by qPCR (following the standard procedure
118 described in Pépin et al. [48] a first time during D-larval stage and at 3 months of age
119 following thermal challenge, and all tested negative (analyses by IDHESA, Quimper, France).
120 At the beginning of the experiment (September 2011), oysters were 5 months of age,
121 measured 30 to 40 mm shell height and total wet weight (soft tissues and shell) was 3.2 ± 0.2
122 g (mean ± SE).

123

124 2.3 Field-exposure

125 On September 1, 2011, a subsample of the SPF oysters was transferred to an oyster farming
126 area in the Bay of Brest, at Pointe du Chateau (48° 20' 06.19" N, 4° 19' 06.37" W). Although
127 mass mortality events associated with OsHV-1 recurrently occur in this location, low
128 mortality was reported during this period (5 to 26% mortality in one month of juvenile, SPF
129 oysters, RESCO: http://wwz.ifremer.fr/observatoire_conchylicole/Resultats-
130 nationaux/Resultats-nationaux-2011/Mortalite-par-site-et-par-classe-d-age; B. Petton, pers.
131 com.). No harmful algal blooms were detected during this period (data monitored by
132 VELYGER and RESCO networks, Ifremer). After 2 weeks in the field (i.e. on September 19,
133 2011), oysters were transferred to the experimental facilities and were used to challenge SPF
134 oysters by cohabitation, as described below.

135

136 2.4 Experimental design

137 On September 15, 2011, 420 SPF oysters were distributed into twelve 15-L tanks (35 SPF
138 oysters per tank) and acclimated for 4 days fed continuously with T-*Iso* at 3-5 × 10^5 cell mL^{-1}.

139 At the end of this acclimation period, on September 19, 10 field-exposed oysters per tank,
140 held in a net, were added to six of the 12 experimental tanks. The SPF oysters that were thus
141 maintained in cohabitation with these field-exposed oysters were designated as “challenged”.
142 In the six other tanks, 10 other SPF oysters per tank, held in a net, were added to obtain the
143 same number of oysters in all tanks. The oysters in these tanks were then designated as
144 “unchallenged”. In addition, 3 “challenged” tanks and 3 “unchallenged” tanks were exposed
145 continuously to 1×10^2 cell mL^{-1} of the toxic dinoflagellate *A. catenella*; whereas, the other
146 tanks were exposed to the same concentration of the control, non-toxic dinoflagellate, *H.*
147 *triquetra.*

148 Experimental design is summarized in Figure 1. Four experimental conditions were used: *A.*
149 *catenella* (toxic algae) exposure and challenged oysters (condition 1); *A. catenella* (toxic
150 algae) exposure and unchallenged oysters (condition 2); non-toxic algal exposure and
151 challenged oysters (condition 3); non-toxic algal exposure and unchallenged oysters
152 (condition 4).

153 During the entire experiment, 1-µm-filtered and UV-sterilized seawater was supplied to the
154 tanks (10 mL min^{-1}, i.e. one tank renewal every 24h) with aeration at 20°C, close to the
155 temperature in the field at the time (18.5°C, VELYGER and RESCO networks, Ifremer,
156 http://wwz.ifremer.fr/observatoire_conchylicole/Resultats-nationaux/Resultats-nationaux-
157 2011/Mortalite-par-site-et-par-classe-d-age).

158

159 2.5 Sampling

160 Oysters were sampled at the end of the acclimation period, i.e. before the beginning of the
161 experiment (T_0), and after 4 days (T_4) and 9 days (T_9) of exposure.

At T_0, 2 SPF oysters per tank and 10 field-exposed oysters were sampled and mantles were dissected for OsHV-1, *V. aesturianus* and total *Vibrio* analyses.

At T_4 and T_9, 12 SPF oysters per tank were sampled: in 4 oysters per tank, transversal sections were cut for parasite detection using histological analysis; in the 8 other oysters per tank, hemolymph was withdrawn from the adductor muscle (the edge of the shell near the adductor muscle was carefully broken with tweezers, enabling to access the adductor muscle with the needle of a syringe) for hemocyte variable analyses, and digestive gland and mantle were dissected and frozen in liquid nitrogen before being stored at -80°C prior to analyses (PST in the digestive gland, and OsHV-1, *V. aesturianus* and total *Vibrio* quantification in the mantle).

Additionally, 3 field-exposed oysters per tank were sampled at T_9 for OsHV-1, *V. aesturianus* and total *Vibrio* analyses.

All analyses were run individually.

No mortality occurred during the experiment.

2.6 DNA extraction and OsHV-1, total *Vibrio* and *Vibrio aesturianus* quantification

DNA extraction was performed with the QIAamp DNA Mini Kit (QIAgen), from 20 mg of wet mantle, following the manufacturer instructions. 100 µL of molecular biology grade water (DNase-free) were used for elution. Nucleic acid concentration was measured with a NanoDrop® ND-1000 UV-Vis Spectrophotometer (conversion factor: 1 OD = 50 µg mL^{-1} DNA) at 260 nm, and purity was checked using the 260 / 230 nm and 260 / 280 nm ratios.

DNA detection and quantification of OsHV-1 and bacteria related to the *Vibrio* Genus were performed in the mantles of two SPF oysters per tank (n=24) and ten field-exposed oysters at

T_0, three SPF oysters per tank at T_4, and three SPF oysters and three field-exposed oysters per tank at T_9 (n=9 per condition at T_4 and T_9). Analyses were conducted by qPCR according to the methods specified in Table 1.

Each reaction was performed in triplicate (OsHV-1 and total *Vibrio* quantification) or in duplicate (*V. aesturianus* quantification). Each run included a no template control (water), a positive control for OsHV-1 and *V. aesturianus* quantification and standards prepared by successive ten-fold dilutions of stock solutions of OsHV-1 or *V. aesturianus* purified DNA. The standard curve obtained was used to calculate the percentage of amplification efficiency (% E) described in equation (1), which was between 85% and 110% for all analyses, and quantification of the samples was determined by comparing Ct values.

(1) $\% E = (10^{-1/a} - 1) \times 100$; with a = slope of the linear regression calculated from Ct = f($\log_{10}$ of dilution).

The specificity of the PCR products obtained using the SYBR Green chemistry was checked systematically with the melting temperature (Tm) value calculated from the dissociation curve.

For total *Vibrio*, results were expressed as number of DNA copies mg^{-1} of wet mantle, upon which statistical analyses were performed. Total *Vibrio* DNA copies mg^{-1} of wet mantle was confirmed to follow a linear, significant relationship with total *Vibrio* DNA copies ng^{-1} total DNA (Pearson product moment correlation, correlation coefficient=0.96, $p<0.001$, n=114). Total *Vibrio* detection was considered positive when Ct<38, associated with a Tm corresponding to *Vibrio* sp. DNA amplicon (81.5-83.5°C). Moreover, when the Ct was higher than the Ct of the most-diluted standard, although sample was considered positive for total *Vibrio* detection, quantification could not be determined and was thus estimated as 0.

208 2.7 Parasite detection by histology

209 Two diagonally-slanted, 5-mm sections of soft tissue, including gills, mantle, digestive gland,
210 intestine and gonad, were excised and a section of adductor muscle was sampled from four
211 SPF oysters per tank at T_4 and T_9 (n=12 per condition at each sampling time). Tissues were
212 fixed immediately in Davidson's solution [49] for 24 h. Tissues then were transferred into
213 70% ethanol, dehydrated in ascending ethanol solutions, cleared with Claral®, and embedded
214 in paraffin wax. Five-µm sections were stained with Harris' hematoxylin and eosin [50], and
215 observed under a light microscope for any parasitic infestation visible in histological slides.

216

217 2.8 PST accumulation

218 PST accumulation was measured individually in the digestive gland (eight SPF oysters per
219 tank exposed to *A. catenella*; one SPF oyster per tank exposed to the control alga *H. triquetra*)
220 with the PSP ELISA kit (Abraxis) as described in Lassudrie et al. [33]. PST extraction was
221 performed following manufacturer instructions: digestive gland tissue was homogenized in
222 HCl 0.1 M (1:1, w:v) using a Precellys®24 beads-grinder, then boiled for 5 min. Dilutions of
223 this homogenate were used in the ELISA assay. Toxicity was expressed as µg of saxitoxin
224 (STX) kg^{-1} of wet digestive gland weight.

225

226 2.9 Analysis of hemocyte variables

227 Hemolymph withdrawn from adductor muscle was stored temporarily in Eppendorf
228 microcentrifuge tubes held on ice before flow-cytometric analysis. Characteristics determined
229 in live circulating hemocytes: total (THC) and differential hemocyte counts (granulocytes and
230 agranular hemocytes) (in cell mL^{-1}), size, and internal complexity (in arbitrary units, a.u.), as

well as mortality (percentage of dead hemocytes) – were assessed following Hégaret et al. [51]. Functional responses, i.e. production of reactive oxygen species (ROS) (specifically H_2O_2 and $O_2^{\bullet-}$) by unstimulated hemocytes was determined as described in Delaporte et al. [52] and Lambert et al. [53]. Hemocyte analyses were performed with a FACScalibur flow-cytometer (BD), and data were processed using WinMDI 2.8 software. Additionally, failure to withdraw a sufficient volume of hemolymph to perform these analyses from individual oysters (i.e. <100µL) was recorded.

2.10 Statistical analyses

Differences in total *Vibrio* DNA content between experimental conditions were tested at each sampling time with Mann-Whitney or Kruskal-Wallis test followed by Nemenyi-Damico-Wolfe-Dunn (NDWD) post-hoc test.

In *A. catenella*-exposed oysters, differences in measurements of PST accumulation regarding challenge condition, field-exposure and sampling time were estimated with Kruskal-Wallis test followed by NDWD post-hoc test.

Hemocyte variables first were analyzed with 3-way ANOVA to test effects of “time”, “algal exposure” and “challenge condition” and interactions. Then, when “time” was not significant, this factor was removed from the analysis, thus data were analyzed over the entire experiment with 2-way ANOVA followed by the LSD post-hoc test. When the factor “time” was significant, 2-way ANOVA testing the effects of “algal exposure”, “challenge condition” and the interaction was performed at each sampling time, followed by the LSD post-hoc test. When needed, data were transformed as log(X+1) or 1/X to meet normality of residuals and homoscedasticity. Percentage data were transformed as Asin(sqrt(X/100)). Multiple correlations were tested between PST accumulation, total *Vibrio* burden and hemocyte

variables using Spearman rank correlations. In addition, differences between the percentage of oysters in which hemolymph could not be withdrawn from the adductor muscle between the four conditions defined by algal exposures and challenge conditions (i.e. four conditions: "unchallenged – *A. catenella*"; "unchallenged – *H. triquetra*"; "challenged – *A. catenella*"; "challenged – *H. triquetra*") were tested at T_4 and at T_9 with a Chi-square test with Holm-Bonferroni correction for multiple comparisons.

Differences were considered significant when $p<0.05$ for all statistical tests. Statistical analyses were performed using Statgraphics Plus statistical software (Manugistics, Inc., Rockville, MD, USA) and R version 2.15.1 [54]. All values were expressed as mean ± standard error.

3 Results

3.1 OsHV-1 DNA and *Vibrio aesturianus* DNA quantification

Neither OsHV-1 nor *V. aesturianus* DNA were detected in any of the mantle samples analyzed, regardless of the sampling time (T_0, T_4, T_9) or the experimental condition (field-exposed, challenged or unchallenged oysters; exposed to *Alexandrium catenella* or *Heterocapsa triquetra*).

3.2 Total *Vibrio* quantification

Over the entire experiment, bacteria related to the *Vibrio* Genus were detected in the mantle of 93 ± 3% of the oysters.

At T_0, a significantly ($p<0.05$) higher mean amount of total *Vibrio* DNA was detected in mantle of field-exposed oysters ($2.5 \pm 0.8 \times 10^3$ DNA copies mg^{-1} wet mantle, n=10) compared to SPF oysters ($2.6 \pm 1.0 \times 10^2$ DNA copies mg^{-1} wet mantle, n=24) (Figure 2).

After 4 days, no significant difference in total *Vibrio* DNA quantification was detected between unchallenged and challenged oysters, exposed to *H. triquetra* or *A. catenella* ($4.3 \pm 0.7 \times 10^2$ DNA copies mg^{-1} wet mantle, n=36) (Figure 2).

After 9 days, no significant difference in mean, total *Vibrio* quantification was detected between algal exposure, challenge condition, or field-exposure, although total *Vibrio* load tended to remain higher in field-exposed oysters ($1.3 \pm 0.7 \times 10^3$ DNA copies mg^{-1} wet mantle, n=18) compared to SPF oysters (both unchallenged and challenged) ($4.8 \pm 1.2 \times 10^1$ DNA copies mg^{-1} wet mantle, n=36) (p=0.068) (Figure 2).

3.3 Parasite detection by histology

No parasite infestation was observed in histological slides.

3.4 PST accumulation

No PST was detected in the digestive glands of oysters exposed to the non-toxic dinoflagellate *H. triquetra*, challenged or unchallenged, at both T_4 and T_9.

After 4 days, PSTs were detected in the digestive glands of *A. catenella*-exposed oysters, and no significant difference was detected between challenged and unchallenged oysters ($1.3 \pm 0.1 \times 10^3$ µg STX kg^{-1} wet digestive gland; n=24 per condition). After 9 days, however, challenged oysters ($9.5 \pm 1.7 \times 10^2$ µg STX kg^{-1} wet digestive gland; n=24) accumulated significantly less (2.6-fold) PSTs than unchallenged oysters ($2.5 \pm 0.4 \times 10^3$ µg STX kg^{-1} wet digestive gland; n=24) (Figure 3).

3.5 Hemocyte variables

Statistical effects of algal exposure (*A. catenella* or control *H. triquetra*) and challenge condition upon hemocyte variables of juvenile *Crassostrea gigas* are reported in Table 2 and represented in Figure 4. When a significant effect of time of sampling (T_4 and T_9) was detected, results were examined at T_4 and T_9, otherwise, results were considered over the entire course of the experiment.

The 2-way ANOVA revealed that total hemocyte count (THC) and agranular hemocyte count (Figure 4A) were significantly impacted by the interaction of both algal exposure and challenge condition, and granulocyte count was impacted by both factors “algal exposure”

and "challenge condition". Post-hoc test tests indicated that THC, granulocyte and agranular hemocyte count were significantly higher in *A. catenella*-exposed oysters compared to oysters exposed to the control dinoflagellate *H. triquetra*. Additionally, these variables were higher in challenged oysters compared to unchallenged oysters only when exposed to the control dinoflagellate *H. triquetra*. In *A. catenella*-exposed oysters, however, measurements of these variables were not higher in response to the challenge.

Exposure to *A. catenella* and the challenge condition led to significantly higher ROS production in hemocytes (Figure 4B) and to higher granulocyte internal complexity (Figure 4C) during the entire experiment; and to higher granulocyte size (Figure 4D) after 4 days of experimental treatment. After 9 days, however, only the effect of challenge condition persisted upon granulocyte size (Figure 4D), which was significantly higher in challenged oysters compared to unchallenged oysters.

Interaction of both algal exposure and challenge condition significantly impacted agranular hemocyte internal complexity (Figure 4E) and size (Figure 4F) over the entire experiment. *A. catenella* exposure led to higher agranular hemocyte complexity and size. Additionally, in oysters exposed to the control dinoflagellate *H. triquetra*, the challenge condition increased these variables. In *A. catenella*-exposed oysters, however, measurements of these variables were not higher in response to challenge.

Finally, after 4 days of experimental treatment, a significant synergistic effect was detected with the interaction of both *A. catenella* exposure and challenge by cohabitation with field-exposed oysters, causing 5-fold increase in mortality of hemocytes (Figure 4G) (both granulocytes and agranular hemocytes, with respectively 23 ± 6 % and 8 ± 2 % mortality in granulocytes and agranular hemocytes of challenged oysters exposed to *A. catenella* vs. respectively 6 ± 2 % and 1.2 ± 0.3 % mortality in granulocytes and agranular hemocytes of

oysters in other experimental conditions). After 9 days of experimental treatment, however, no significant differences between experimental treatments in hemocyte mortality was detected.

Correlations between PST accumulation, total *Vibrio* burden, and hemocyte variables over the entire course of the experiment (T_4 and T_9 together), according to experimental conditions, are presented in Table 3. In unchallenged oysters exposed to *A. catenella*, PST accumulation was negatively correlated with complexity and size of agranular hemocytes ($p<0.05$). In challenged - *A. catenella*-exposed oysters, PST accumulation was positively correlated with granulocyte complexity ($p<0.05$).

At T_4, hemolymph was successfully withdrawn from 66% of the experimental oysters, without any significant experimental differences. At T_9, a significant difference ($p<0.05$) in the ability to withdraw hemolymph was noted between conditions. Indeed, although hemolymph was successfully withdrawn from 83% of the unchallenged oysters exposed to *H. triquetra*, only 43% of the other oysters were successfully bled.

4 Discussion

This study assessed hemocyte responses of juvenile oysters *Crassostrea gigas* exposed to two different microbiotic conditions: a toxic dinoflagellate *Alexandrium catenella* and a cohabitation challenge with field-exposed oysters, presumably carrying environmental micro-organisms, as well as the combination of these two conditions.

4.1 Hemocyte response upon *A. catenella* exposure

Exposure to *A. catenella* for 4 and 9 days induced clear hemocyte responses in juvenile oysters. These results contrasted with those reported on adult oysters *C. gigas* after exposure to *A. catenella*, which did not induce strong modifications of hemocyte variables [37]. The young age and possibly life history of oysters used in the present study (i.e. grown exclusively in a hatchery) possibly increased their sensitivity to *A. catenella*. In Manila clams, higher vulnerability of juveniles to *Alexandrium tamarens*e exposure compared to adults was also described, with observation of high mortality and burrowing impairment [32].

In the present study, *A. catenella* exposure in unchallenged oysters led to higher agranular hemocyte size and complexity, which were however negatively correlated with paralytic shellfish toxin (PST) content. These results suggest a specific functional differentiation, putatively associated with PST detoxication. Based upon functional and morphological studies, many authors suggested that the different hemocyte sub-populations (granulocytes and agranular hemocytes) would represent different stages of the same cell, reflecting differentiation in response to environmental challenges [55–57]. Hemocytes could, thus, develop into different functional types by shifting from one morphologically-based sub-

371 population to another, but also change characteristics within the same sub-population. A role
372 of hemocytes in detoxication of *Alexandrium* spp. toxins in bivalves has been suggested also
373 by other authors [29–31,36], considering hemocyte infiltration in the digestive gland (the
374 main organ accumulating toxins in bivalves, [46,58,59]) and the associated observation of
375 hemocyte diapedesis across the digestive epithelium. A hypothesis that hemocyte diapedesis
376 eliminated toxins from the tissues towards the lumen of the stomach and intestine to be
377 expelled within the faeces has been proposed [30,60].

378 Exposure to *A. catenella* also led to higher hemocyte counts, ROS production, and size and
379 complexity of granulocytes in the circulating hemolymph which were not related to PST
380 accumulation (no significant correlation). These results highlight that effects of *A. catenella*
381 exposure are not necessarily associated with PSTs alone, suggesting effects of other
382 compounds, possibly extracellular compounds produced by *A. catenella*. Although chemical
383 compositions of extracellular compounds from *Alexandrium* spp. remain unclear, allelopathic,
384 hemolytic, ichtyotoxic, and oxidative properties have been reported for different unicellular
385 organisms [61–63]. Algal extracellular compounds could thus be responsible for deleterious
386 effects upon bivalves independently from PSTs. Indeed, inflammatory reactions associated
387 with tissue lesions were reported in gills and mantle, *i.e.* organs in contact with the intact algal
388 cells before digestive processes break the algal walls and release intracellular toxins in the
389 digestive tract [29,64]. In the present study, the higher counts of hemocytes in circulating
390 hemolymph may reflect hemocyte *de novo* production or mobilization from connective tissues
391 to hemolymph to supply responses in specific tissues. Particularly, hemocyte participation in
392 wound repair has been suggested based upon the observation of tissue lesions caused by toxic
393 dinoflagellate exposure associated with hemocyte aggregates or infiltrations, a typical
394 response to *Alexandrium* spp. exposure in bivalves [29–31,36,65].

Increases of both granulocyte size and internal complexity (ie. granulation) was observed in this study; whereas, other studies reported an opposite pattern, with hemocyte degranulation and size decrease upon *Alexandrium fundyense* and *Alexandrium minutum* exposure in mussels and adult *C. gigas*, respectively [30,36]. Those discrepancies may be attributed to the use of different algal strains with different toxicity (from PSTs or extracellular compounds), bivalve species, or bivalve age. These morphological changes may be associated with immune functions, as granulocytes are the main hemocyte sub-population involved in immune defense [57,66,67]. In fact, Ford et al. [68] demonstrated deleterious effects of algal extracellular compounds upon immune functions of clam hemocytes (phagocytosis and adherence) by *in vitro* exposure to non-PST-producing *A. tamarense* strain.

Hemocyte ROS production increased upon *A. catenella* exposure, suggesting stimulation of hemocyte metabolic activity and possible associated oxidative stress. Haberkorn et al. [36] also reported an increase in hemocyte ROS production in *C. gigas* exposed to *A. minutum*. In the previous study, this response was correlated positively with PST content; whereas, in the present study hemocyte ROS production may have increased in response to *A. catenella* extracellular compounds. Indeed, in other strains of *Alexandrium* spp., oxidative properties have been reported to be involved in toxicity of extracellular compounds to protists [63].

4.2 Hemocyte responses induced by cohabitation challenge

Challenge of specific pathogen-free (SPF) oysters by cohabitation with oysters previously held in the field also led to marked hemocyte responses in the naïve, SPF oysters. Higher total hemocyte count (THC) in hemolymph of challenged oysters suggests stimulation of hematopoïesis and/or mobilization of hemocytes from tissues towards hemolymph. Furthermore, cohabitation challenge increased hemocyte size and complexity of both

agranular hemocytes and granulocytes, as well as hemocyte ROS production. These hemocyte morphological and metabolic differentiations may reflect functional changes, suggesting an immune response to exposure to a new microbial community. Indeed, field-exposed oysters likely carried microorganisms that colonized them during field exposure, as suggested by the higher load of total *Vibrio* compared to SPF oysters at the beginning of the experiment. No specific pathogens known to be involved in juvenile oyster massive mortality events (OsHV-1 and *V. aesturianus*), however, were detected in these field-exposed oysters. Moreover, cohabitation with field-exposed oysters did not modify total *Vibrio* load in the mantle of challenged oysters compared to unchallenged individuals. Nevertheless, the presence of pathogens, either at low concentration or colonizing other organs, may have rendered them undetectable, despite the initiation of an early immune response. Indeed, Labreuche et al. [69] reported enhancement of hemocyte ROS production in *C. gigas* after injection with the pathogenic *V. aesturianus* strain 01/32, despite very low infection intensity in hemolymph, which suggested pathogen colonization of other compartments. The authors further identified a metalloprotease as a major virulence factor contained in extracellular products of *V. aesturianus* strain 01/32 as responsible for this effect upon hemocytes [70,71]. Similarly, in the present study, the effects on hemocytes may have been induced by non-septicemic pathogen colonization and strong effects of a putative virulence factor. Additionally, exposure of juvenile oysters *C. gigas* to herpesvirus OsHV-1µVar through cohabitation challenge can induce physiological responses (perturbation of cardiac activity and ingestion rates) in oysters without inducing detectable OsHV-1 infection (B. Petton, pers. com.). Finally, some bacteria considered to be non-pathogenic for their host have been reported to affect immune response variables. For example, morphology and lysosomal membrane stability of hemocytes were altered in *Mytilus galloprovincialis* challenged with *Vibrio splendidus*, a bacterium not considered to be pathogenic to *Mytilus* spp. [72]. Also, humoral immune components, such as

444 phenoloxidase (PO) and superoxide dismutase (SOD) activities in mantle and extrapalleal
445 fluid, were reported to increase in Manila clam *Venerupis philippinarum* after injection of a
446 *Vibrio tapetis* strain considered to be non-pathogenic [73].

447

448 4.3 Interaction between cohabitation challenge and *A. catenella* exposure

449 The increases in THC and in agranular hemocyte size and complexity induced by either
450 cohabitation challenge or *A. catenella*-exposure were not exacerbated when both factors were
451 combined. It could be argued that the absence of additional effects on these hemocyte
452 characteristics would be directly associated with the lower PST accumulation in challenged
453 oysters after 9 days of experimental treatment. Nevertheless, granulocyte complexity was
454 positively correlated with PST accumulation as a consequence of the interaction between
455 challenge and *A. catenella* exposure. Such relationships between PSTs and granulocytes may
456 suggest an alternative strategy of hemocyte response to avoid excessive responses when
457 oysters are responding to two or more environmental stimuli. This absence of exacerbated
458 hemocyte response also could be associated with the effect of *A. catenella* exposure either on
459 the introduced new microorganisms and/or bacterial virulence factor production believed to
460 cause the cellular immune response, or on the nature of the immune response itself.

461 The first hypothesis is that extracellular compounds and/or PSTs produced by *A. catenella*
462 would have partially eliminated the factor(s) triggering the immune response through
463 modification of microbial composition or interference with production of the microbial
464 virulence factors putatively involved in the response. As mentioned above, *Alexandrium* spp.
465 can produce non-PST-extracellular compounds that can have deleterious effects upon
466 different target cells (e.g., unicellular algae, bivalve hemocytes) [61–63,68]. In addition,
467 *Alexandrium* cells can release extracellular PSTs, especially during encystment that widely

468 occurs when grazed by bivalves [74–77]. Possible toxic effects of PSTs upon prokaryotes,
469 including Gram-negative bacteria such as *Vibrio* spp., have been reported, with alteration of
470 sodium and potassium fluxes [78]. Previous studies already reported modification of host-
471 pathogen interactions in bivalves exposed to harmful algal blooms (HABs) [34,65,79,80],
472 some being caused by direct, negative effects of HABs upon bivalve pathogens [65,80].

473 Another hypothesis is that simultaneous cohabitation challenge and *A. catenella* exposure
474 could have modified hemocyte responses. A strong synergistic effect of both cohabitation
475 challenge and *A. catenella* exposure upon hemocyte mortality observed at T_4 may reflect
476 apoptosis of hemocytes that would explain the absence of exacerbated immune responses
477 upon exposure to combined stimuli. PSTs or extracellular compounds produced by *A.*
478 *catenella* could have interfered with recognition or signaling pathways that trigger the cellular
479 immune responses. Apoptosis could thus represent an alternative immune strategy. Indeed,
480 apoptosis is an important mechanism of immune defense against intra-cellular pathogens [81]
481 that was also activated by *A. catenella* exposure in *C. gigas* hemocytes [82]. Alternately,
482 mortality of hemocytes may reflect cytotoxic effects, resulting in necrosis of hemocytes and
483 thus altering the cellular immune responses. In another study, exposure to the PST-producer
484 *A. fundyense* inhibited *Crassostrea virginica* oyster cellular immune responses (i.e.
485 infiltrations in tissues) to trematode infestation, concomitantly with a higher parasitic load
486 compared to oysters fed a non-toxic dinoflagellate [33].

487 Finally, the lower PST accumulation in challenged oysters compared to unchallenged
488 individuals may be the consequence of perturbations of feeding processes that would be
489 associated with the newly introduced microorganisms. Challenge by cohabitation with OsHV-
490 1 infected oysters was observed to induce physiological responses, including abnormal
491 ingestion rates, in individual juvenile oysters, although no OsHV1 DNA was detected (B.
492 Petton, pers. com.). In the present study, the lower PST accumulation in challenged oysters

493 could thus be revealing some alterations in feeding processes (filtration, ingestion or
494 digestion) as a consequence of first stage disease development. In a previous study [34],
495 challenge by cohabitation with oysters infected with herpesvirus OsHV-1µVar and possible
496 other pathogens also lowered PST accumulation in challenged oyster spat, putatively because
497 of disease induced by these pathogens. Negative effects of perkinsosis (caused by the
498 protozoan parasite *Perkinsus olseni* syn. *atlanticus*) and Brown Ring Disease (BRD, caused
499 by the bacterium *Vibrio tapetis*) were reported, respectively, upon clearance rate of Manila
500 clams [83,84] Moreover, alterations of digestive diverticula similar to starvation symptoms
501 was reported in BRD-diseased clams [85]. Measurement of feeding processes in oysters
502 subjected to the same conditions as in the present experiment would be useful to further
503 explore this hypothesis.

504

505 Overall, oyster health status seemed to be affected after 9 days of both cohabitation challenge
506 and *A. catenella*-exposure, as also suggested by the difficulty to withdraw hemolymph from
507 57 % of these oyster groups (as opposed to 17 % in the control group). In different
508 laboratories, such difficulties to withdraw hemolymph were recurrently noticed from bivalves
509 infected with pathogenic agents or exposed to toxic dinoflagellates and were assumed to be
510 associated with compromised health status (G. H. Wikfors, C. Lambert, P. Soudant, H.
511 Hégaret, pers. com.). The mechanisms underlying this reaction, however, remain unclear but
512 could be associated with compromised muscle drainage by hemolymph.

513

5 Conclusions

Exposure of *Crassostrea gigas* spat to *A. catenella* induced strong hemocyte responses related to PSTs and/or extracellular bioactive compounds. Additionally, challenge by cohabitation with field-exposed oysters also led to hemocyte responses, with increased concentration of circulating hemocytes changing morphology, suggesting an immune reaction probably associated with a change in microorganism community or virulence factor exposure. This response may have restrained potential opportunistic infection, as suggested by the absence of specific pathogen detection and low *Vibrio* load. These hemocyte responses to cohabitation challenge were modulated by *A. catenella* exposure, suggesting either the implementation of alternative defense mechanisms, or cytotoxic effects (of PSTs and/or extracellular compounds) upon hemocytes. Such physiological impairment also may have resulted in the alteration of feeding-related processes, as suggested by the lower PST accumulation in challenged oysters.

This study highlights the sensitivity of juvenile oysters, in terms of cellular immune responses, to changes in environmental biotic conditions. Such response should be taken into account when transferring young bivalve livestock, as this could increase susceptibility to diseases by weakening spat immunological and physiological status.

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Figure captions

Fig. 1 Scheme of the experimental design. All oysters used (5 months-old) were produced in the hatchery following a standardized process to obtain specific pathogen-free (SPF) *Crassostrea gigas* oysters. SPF and field-exposed oysters were sampled prior to exposure and cohabitation (T_0); challenged and unchallenged oysters were sampled after 4 days and 9 days of algal exposure and cohabitation with field-exposed oysters (T_4 and T_9)

Fig. 2 Quantification of total *Vibrio* in mantle of experimental oysters at the beginning of the experiment (T_0), and after 4 and 9 days of experimental treatment (T_4 and T_9) in unchallenged oysters, challenged oysters, and field-exposed oysters (not sampled at T_4) exposed to the non-toxic *Heterocapsa triquetra* (*Ht*) or the toxic *Alexandrium catenella* (*Ac*).*: significant difference between conditions, $p<0.05$ (Mann-Whitney and Kruskal-Wallis tests). Mean ± SE. N_{T0}=24 unchallenged oysters and 10 field-exposed oysters; $N_{T4 \text{ and } T9}$ = 9 oysters per condition

Fig. 3 Paralytic shellfish toxin (PST) accumulation after 4 (T_4) and 9 (T_9) days of exposure to *Alexandrium catenella* in the digestive glands of unchallenged, challenged, and field-exposed juvenile oysters (analyzed at T_9 only). Letters indicate significant differences between conditions (Kruskal-Wallis test followed by NDWD post-hoc test; $p<0.05$). Mean ± SE. N=24 oysters per condition

Fig. 4 Hemocyte variables in the circulating hemolymph of juvenile oysters *Crassostrea gigas* according to "challenge condition" (unchallenged or challenged by cohabitation with field-exposed oysters) and "algal treatment" (*Heterocapsa triquetra* or *Alexandrium catenella*), either over the entire experiment (T_4 + T_9) when no statistical effect of the time of sampling was detected, or at T_4 and T_9 separately, as specified, when effect of the time of sampling was significant (3-way ANOVA, with "time", "challenge condition" and "algal treatment" as main factors and their interactions). (A) Total and differential hemocyte count; (B) ROS production; (C) Granulocyte complexity; (D) Granulocyte size; (E) Agranular hemocyte

847 complexity; (F) Agranular hemocyte size; (G) Hemocyte mortality (percentage of dead
848 hemocytes). NS: No significant difference. Letters indicate significant differences between
849 groups (ANOVA followed by LSD post-hoc test): greek letters for total hemocytes, capital
850 letters for agranular hemocytes, small letters for granulocytes. Mean ± SE. N_{T4}= 12-18
851 oysters per group, N_{T9}= 10-20 oysters per group, N_{T4+T9} =23-36 oysters per group

Table 1 Method reference, chemistry and primers used for detection and quantification of herpesvirus OsHV-1, total *Vibrio* and *Vibrio aesturianus* DNA by qPCR.

Analysis	**Method reference**	**Chemistry**	**Primers**
OsHV-1	Standard protocol of the European Union Reference Laboratory for mollusc diseases [43,81]	SYBR Green	Forward HVDP-F : 5' ATTGATGATGTGGATAATCTGTG 3' Reverse HVDP-R : 5' GGTAAATACCATTGGTCTTGTTCC 3'
Total *Vibrio*	Thompson et al. 2004	SYBR Green	Forward 567F: 5' GGCGTAAAGCGCATGCAGGT 3' Reverse 680R: 5' GAAATTCTACCCCCCTCTACAG 3'
Vibrio aesturianus	Standard protocol of the European Union Reference Laboratory for mollusc diseases [83]	TaqMan (*dnaJ*-probe: 5' Texas Red TGGTAGCGCAGACTTCG GCGAC BHQ-2 3')	Forward *dnaJ*-F: 5' GTATGAAATTTTAACTGACCCACAA 3' Reverse *dnaJ*-R: 5' TCAATTTCTTTCGAACAACCAC 3'

Table 2 Statistical effects of "time" (4 or 9 days of experiment), "algal exposure" (toxic *Alexandrium catenella* or control *Heterocapsa triquetra*), "challenge condition" (challenged by cohabitation with field-exposed oysters or unchallenged) upon hemocyte variables of juvenile oysters *Crassostrea gigas*, either over the entire experiment ("$T_4 + T_9$") when no significant effect of "time" was detected, or, when significant effect of "time" was detected, after 4 days of experiment ("T_4") and 9 days of experiment ("T_9"). NS: No significant difference; Significant difference indicated by *: $p<0.05$; **: $p<0.01$; **: $p<0.001$ (3-way ANOVA including factor "time"; 2-way ANOVA excluding factor "time"); "-": no analysis. N in each group is indicated

Hemocyte variables	Over the entire experiment ($T_4 + T_9$)				T_4			T_9		
	Time	Algal exposure	Challenge	Interaction Algal exposure × Challenge	Algal exposure	Challenge	Interaction Algal exposure × Challenge	Algal exposure	Challenge	Interaction Algal exposure × Challenge
	N=51-63	N=50-64	N=51-63	N=23-36	N=29-34	N=30-33	N=12-18	N=21-30	N=21-30	N=10-20
THC	NS	***	NS	*	-	-	-	-	-	-
Gran. count	NS	***	*	NS	-	-	-	-	-	-
Agran. count	NS	***	NS	*	-	-	-	-	-	-
ROS production	NS	***	***	NS	-	-	-	-	-	-
Gran. complexity	NS	**	**	NS	-	-	-	-	-	-
Gran. size	*	-	-	-	**	*	NS	NS	*	NS
Agran. complexity	NS	***	*	*	-	-	-	-	-	-
Agran. size	NS	***	NS	*	-	-	-	-	-	-
Mortality	**	-	-	-	***	***	**	NS	NS	NS

THC: total hemocyte count; Gran.: granulocytes; Agran.: agranular hemocytes; ROS: reactive oxygen species

Table 3 Spearman rank correlations between hemocyte variables, PST accumulation in the digestive gland (for *Alexandrium catenella* exposed oysters only), and total *Vibrio* burden in the mantle of experimental juvenile oysters *Crassostrea gigas* according to experimental conditions. Data from both T_4 and T_9 sampling times were used. Spearman rank correlation coefficient, (N) and *p*-value are presented. Bold indicates significant correlations (α=0.05)

Experimental condition	Variables	THC	Gran. count	Agran. count	ROS	Gran. complexity	Gran. size	Agran. complexity	Agran. size	Hem. mortality	PSTs
Unchallenged	**Total**	0,42	0,29	0,50	-0,07	-0,44	-0,29	0,14	0,33	0,08	NA
H. triquetra	***Vibrio***	(15)	(15)	(15)	(15)	(15)	(15)	(15)	(15)	(15)	NA
		0,111	0,273	0,056	0,806	0,100	0,279	0,581	0,211	0,765	NA
Unchallenged	**PST**	-0,10	-0,19	-0,09	0,02	0,13	0,11	**-0,47**	**-0,44**	0,32	NA
A. catenella		(26)	(26)	(26)	(26)	(26)	(26)	**(26)**	**(26)**	(26)	NA
		0,594	0,340	0,635	0,914	0,489	0,559	**0,019**	**0,028**	0,096	NA
	Total	0,08	-0,21	0,10	-0,32	0,06	0,01	0,17	0,23	-0,09	-0,01
	Vibrio	(12)	(12)	(12)	(12)	(12)	(12)	(12)	(12)	(12)	(18)
		0,786	0,479	0,723	0,288	0,841	0,962	0,558	0,429	0,728	0,948
Challenged	**Total**	0,09	0,07	0,11	-0,41	-0,25	-0,12	0,19	0,24	-0,27	0,09
H. triquetra	***Vibrio***	(13)	(13)	(13)	(13)	(13)	(13)	(13)	(13)	(13)	(13)
		0,739	0,784	0,695	0,153	0,383	0,667	0,498	0,399	0,321	0,739
Challenged	**PST**	0,15	0,33	0,08	0,08	**0,45**	-0,10	0,42	0,16	0,21	NA
A. catenella		(21)	(21)	(21)	(21)	**(21)**	(21)	(21)	(21)	(21)	NA
		0,489	0,140	0,688	0,710	**0,046**	0,629	0,060	0,450	0,334	NA
	Total	0,01	-0,13	0,03	-0,17	-0,06	0,00	-0,43	-0,24	0,01	-0,02
	Vibrio	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(11)	(19)
		0,950	0,672	0,912	0,583	0,838	0,987	0,170	0,442	0,659	0,9310

THC: total hemocyte count; Gran.: granulocytes; Agran.: agranular hemocytes; ROS: reactive oxygen species; Hem.: hemocyte; PSTs: paralytic shellfish toxins; NA: not available

Spearman rank correlation coefficient
(N)
p-value