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# Bioactive extracellular compounds produced by the dinoflagellate *Alexandrium minutum* are highly detrimental for oysters

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**Abstract :**

Blooms of the dinoflagellate *Alexandrium* spp., known as producers of paralytic shellfish toxins (PSTs), are regularly detected on the French coastline. PSTs accumulate into harvested shellfish species, such as the Pacific oyster *Crassostrea gigas*, and can cause strong disorders to consumers at high doses. The impacts of *Alexandrium minutum* on *C. gigas* have often been attributed to its production of PSTs without testing separately the effects of the bioactive extracellular compounds (BECs) with allelopathic, hemolytic, cytotoxic or ichthyotoxic properties, which can also be produced by these algae. The BECs, still uncharacterized, are excreted within the environment thereby impacting not only phytoplankton, zooplankton but also marine invertebrates and fishes, without implicating any PST. The aim of this work was to compare the effects of three strains of *A. minutum* producing either only PSTs, only BECs, or both PSTs and BECs, on the oyster *C. gigas*. Behavioral and physiological responses of oysters exposed during 4 days were monitored and showed contrasted behavioral and physiological responses in oysters supposedly depending on produced bioactive substances. The non-PST extracellular-compound-producing strain primarily strongly modified valve-activity behavior of *C. gigas* and induced hemocyte mobilization within the gills, whereas the PST-producing strain caused inflammatory responses within the digestive gland and disrupted the daily biological rhythm of valve activity behavior. BECs may therefore have a significant harmful effect on the gills, which is one of the first organ in contact with the extracellular substances released in the water by *A. minutum.* Conversely, the PSTs impact the digestive gland, where they are released and mainly accumulated, after degradation of algal cells during digestion process of bivalves. This study provides a better understanding of the toxicity of *A. minutum* on oyster and highlights the significant role of BECs in this toxicity calling for further chemical characterization of these substances.

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**Highlights**

► Paralytic shellfish toxins (PSTs) and bioactive extracellular compounds (BECs) of *Alexandrium minutum* have contrasted effects upon *Crassostrea gigas* oysters. ► BECs affect nutrition and valve-activity behavior. ► BECs induce hemocyte mobilization in gills. ► PSTs disrupt biological rhythm. ► PSTs provoke inflammation in the digestive gland.

**Keywords** : Harmful Algal Bloom (HAB), *Crassostrea gigas*, Paralytic Shellfish Toxin (PST), Bioactive Extracellular Compounds, Histology, Behavior

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## 1. Introduction

Harmful algal blooms (HABs) are present in freshwater, brackish and marine environments, as naturally occurring phenomena. For the last decades, there is clear evidence confirming the geographical extension of HABs worldwide (Anderson et al., 2012a). Factors contributing to this phenomenon are various: local hydrological events, global change through the selection of species according to their thermal preferences, N:P ratio (possible role of eutrophication), accidental transfer of marine species, and appearance of toxin-producing strains caused by genetic modification of harmless strains (Lassus et al., 2016). Those factors sometimes act in a complex interplay, and more importantly, vary among geographical areas. Effects of HABs are expected to intensify even more as the use of coastal waters for aquaculture and shellfish farming is globally increasing (Lassus et al., 2016).

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Among the microalgae responsible for HABs, the genus *Alexandrium* (Halim 1960) is one of the most important, in terms of diversity, distribution, and intensity (Anderson et al., 2012b). Blooms of *Alexandrium* are globally distributed, with species present in tropical, temperate and subarctic regions (Tillmann et al., 2016). Species of *Alexandrium* are known to produce Paralytic Shellfish Toxins (PSTs), composed of saxitoxin (STX) and approximately 50 derivatives of STX (Wiese et al., 2010). This group of toxins have a particularly acute toxicity (Kodama, 2010), causing neurotoxic syndromes. PSTs block the conduction of action potential by binding to the voltage-gated sodium channels that play an essential role in membrane excitability in nerve and muscle fibers cells (Cestèle and Catterall, 2000). In human seafood consumers, paralytic shellfish poisoning (PSP) causes dizziness, tingling sensation of the mouth, headache, nausea, vomiting, diarrhea and in severe cases death by asphyxiation (Etheridge, 2010). Blooms of *Alexandrium* can also have ecological consequences by alteration of marine trophic structure, and death of marine fish, seabirds, and mammals, but also have dramatic economic effects, causing impairment of tourism and recreational activities, fishery closure and sell prohibition of shellfish (Anderson et al., 2012b). Filter-feeder bivalves including several commercial species, such as the Pacific oyster *Crassostrea gigas*, are known to accumulate PSTs by feeding on toxic algae (Bricelj and Shumway, 1998).

Many studies have also highlighted that *A. minutum* impacts oyster behavior and physiology. Laboratory experiments demonstrated that exposure to *A. minutum* deeply modified valve behavior (Tran et al., 2010; Haberkorn et al., 2011) and disrupted biological rhythms of oysters (Tran et al., 2015; Payton et al., 2017a). Functioning and structure of digestive organs (Lassus et al., 1999; Haberkorn et al., 2010a), and oyster defense responses were negatively affected (Haberkorn et al., 2010b; Hégaret et al., 2011; Mat et al., 2013; Mello et al., 2013).

From the above studies, relationships between *A. minutum* effects and PST microalgae content or PST bioaccumulation by oysters could not be established. *Alexandrium* species, however, were also shown to produce bioactive extracellular compounds (BECs) excreted in the surrounding seawater, independently from PSTs. The BECs produced by *Alexandrium* species can be allelopathic (Fistarol et al., 2004; Lelong et al., 2011; Zheng et al., 2016), cytotoxic (Ford et al., 2008; Flores et al., 2012), haemolytic (Arzul et al., 1999; Yang et al., 2010), or ichthyotoxic (Ogata and Kodama, 1986; Mardones et al., 2015). To date, knowledge on the chemical nature and the mode of action of the BECs produced by *Alexandrium* spp. is still scarce. Cytotoxic extracellular compounds produced by *Alexandrium* spp were suggested to act primarily on cell membranes, with a high lysis potential (Emura et al., 2004; Tillmann et al., 2007, 2008). The lytic allelochemical activity of different strains of *Alexandrium* spp.

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studied on various protists appeared not related to the cellular PST content or composition (Tillmann and John, 2002; Tillmann et al., 2009).

An exposure of *Pecten maximus* juveniles to a non-PST BEC-producing strain of *A. minutum* (strain CCMI1002) resulted in a decrease of daily shell growth, tissues alterations and an increased reaction time when exposed to predators (Borcier et al., 2017). Similarly, this *A. minutum* non-PST strain induced dramatic effects on oyster hemocytes and spermatozoa, and strongly affected feeding behavior of oyster *C. gigas* and clam *Ruditapes philippinarum*, presumably because of BECs (Contreras, 2011; Lambert et al., 2013). These harmful effects of non-PST producing strains on marine organisms raise the question of the respective involvement of PSTs and BECs in the toxicity of *A. minutum*.

To discriminate the respective effects of PSTs and BECs produced by *A. minutum*, we analyzed, under controlled conditions, the effects of different strains of *A. minutum* on the oyster *C. gigas*. To address this question, we selected three *A. minutum* strains with very different toxicity in terms of both PSTs and BECs: the "PST" strain (Daoulas1257 strain, isolated from the Bay of Brest in France; (Boullot et al., 2017) producing only PSTs; the "PST+BEC" strain (AM89BM strain, isolated from the Bay of Morlaix in France; (Erard-Le Denn et al., 1990) producing both PSTs and BECs; and the "BEC" strain (CCMI1002 strain, isolated from Irish waters; (Tillmann and John, 2002) producing only BECs. This work clarifies the respective effects of PSTs and BECs produced by *A. minutum* on valve behavior and physiology of the oyster.

## 2. Materials and methods

### 2.1 *Oyster characteristics and general conditions*

Diploid pacific oysters, *Crassostrea gigas* (*Magallana* gen. nov.; Salvi and Mariottini, 2017), used in this study came from a single cohort produced in March 2014 in the Argenton Ifremer facilities (France). At the time the experiment began (January 2015), oysters were 10 months old, measured 56 ± 1 mm length (mean ± SE), and weighed 25.1 ± 0.9 g (total wet weight; mean ± SE). Three hundred and sixty oysters were randomly distributed into nine 30-liter tanks, including 2 or 3 oysters equipped with valvometric electrodes per tank (n = 6-7 per condition) for valve activity measurements. Each tank was continuously supplied with 1-µm filtered UV-treated seawater and algae at a total flow of 19 ml $min^{-1}$, allowing a renewal of the volume of the tank every day. Every tank was equipped with a circulation pump to homogenize microalgae and seawater. Experiments were carried out with a photoperiod of 10 h light (8 am to 6 pm) and 14 h dark (6 pm to 8 am) and seawater was maintained at a temperature of 16 ± 1

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°C. Oysters were maintained in the same tanks during the experiment, in a blind room to minimize any external influences and disturbance due to handling on animal behavior.

### 2.2 *Algal culture*

The three strains of the dinoflagellate *Alexandrium minutum* (Daoulas1257, AM89BM and CCMI1002 strains) were cultured in 6-L batch culture using seawater filtered to 0.2 µm supplemented with L1 medium. Cultures were maintained at 17 ± 1 °C with a dark:light cycle of 12:12 h. According to the bioassay developed by Long et al. (personal communication), the BEC strain is more cytotoxic than the PST+BEC strain. The PST+BEC strain is known to produced more PSTs (10.6 fmol cell$^{-1}$ which corresponds to 1.3 pg STX eq. cell$^{-1}$ (Haberkorn et al., 2010a)) than the PST strain (0.63 fmol cell$^{-1}$ which corresponds to 0.053 pg STX eq. cell$^{-1}$ (Pousse et al., 2018)), whereas no PSTs were detected in the BEC strain (Borcier et al., 2017). The dinoflagellate *Heterocapsa triquetra* (Ehrenberg, 1840; strain HT99PZ) was grown in 150-L cylinders containing 1-µm filtered, UV-treated seawater enriched with L1 nutrients (Guillard and Hargraves, 1993) and cultures were maintained at 19 ± 1 °C with continuous light. Cultures of *A. minutum* and *H. triquetra* were not axenic, grown without antibiotics, and harvested in exponential growth phase for the experiment.

### 2.3 *Experimental design of* A. minutum *exposure*

Oysters were acclimated for 7 days with increasing *H. triquetra* densities from 1.65 x 10$^3$ to 3.3 x 10$^3$ cells mL$^{-1}$. The non-toxic *H. triquetra* was chosen for acclimation because of its similarity to *A. minutum* in terms of size and shape: *H. triquetra* cell size (19-28 µm) is similar to *A. minutum* cell size (23-29 µm). After the 7-day acclimation, oysters were fed continuously with the *A. minutum* strains for 4 days at 19 mL min$^{-1}$. Each *A. minutum* strain was distributed to three replicate tanks at a density of 3.1 x 10$^3$ cells mL$^{-1}$. This algal density and exposure time were environmentally realistic (Chapelle et al., 2015).

### 2.4 *Measurement of* A. minutum *concentrations by flow cytometry*

During the exposure phase, *A. minutum* cell concentration in the seawater was monitored from the inflow and outflow of the tanks from all conditions. Cell counts were performed using a FACScalibur (BD Sciences, San Jose, CA, USA) flow cytometer with a 488 nm argon blue laser. Threshold was set to FL3 (red fluorescence 670 nm Long pass filter, i.e. > 670 nm) to detect only chlorophyll-containing cells. Concentrations of *A. minutum* were estimated using cell counts during 30 seconds of flow-cytometer acquisition and flow rate, measured according to Marie et al. (1999).

### 2.5 *Oyster behavioral analysis*

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### *2.5.1 Valve activity measurement*

Valve activity of *C. gigas* was studied using High Frequency Non Invasive valvometry (n = 2-3 oysters per tank). Lightweight electromagnets were glued on each valve of each animal and connected to a lab valvometer by flexible wires (Tran et al., 2003; Chambon et al., 2007). The sampling frequency for each individual was 0.2 Hz. Data were processed using LabView 8.0 software (National Instruments, Austin, TX, USA). The record of valve activity started 4 days before exposure to define oyster behavior under reference conditions (non-toxic *H. triquetra* feeding). Following initiation of experiments, valve activity was measured continuously during the 4 days of exposure to the *A. minutum* strains.

Several parameters of valve activity were analyzed. Hourly opening duration of each individual was expressed as the percentage of time oysters spent with their valves opened, and ranged from 100% (valves open for the entire hour) to 0 % (valves closed throughout the hour). Additionally, hourly valve-opening amplitude and hourly number of valve micro-closures, which corresponds to the number of partial and fast valve closures, were measured for each individual. For each valve activity parameter, mean hourly values for the condition were calculated as the mean of hourly individual values of each condition.

### *2.5.2 Chronobiological analysis*

Chronobiological analyses were made with the software Time Series Analysis Seriel Cosinor 6.3 (http://www.euroestech.net/mainuk.php). Different steps were performed to validate a biological rhythm in oysters (see Tran et al. (2011) and Mat et al. (2012)). Briefly, the quality of the dataset was checked by controlling the absence of randomness using the autocorrelation diagram and the absence of any stationary character by a partial autocorrelation function calculation. The periodicities in the recorded data were tested with the spectral method of the Lomb and Scargle periodogram. These methods give a threshold of probability ($p$ = 0.95) defining the limit below which the signal can be regarded as "noise". The confidence interval of the period was calculated with the method of Halberg (1969). The rhythmicity was then modeled with the Cosinor model, which uses a cosine function calculated by regression (Bingham et al., 1982). To validate the model two tests must be validated: the elliptic test must be rejected and the probability (p-value) for the null amplitude hypothesis must be lower than 0.05. Finally, the percent rhythm (PR), i.e., percentage of the cyclic behavior explained by the model, was calculated.

## 2.6 *Oyster sampling*

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At the end of the exposure period, 12 oysters per tank were sampled and used as follows: for each tank, 7 oysters including oysters equipped with electrodes were analyzed for individual hemocyte variable measurements and condition index, and 5 oysters for histology. A 3-mm cross-section of soft tissues including digestive gland, gills, mantle, gonad, and a section of adductor muscle were taken for each of the 45 oysters sampled for histopathology. Hemolymph was withdrawn from the adductor muscle of each oyster (400 µL minimum) with a syringe before oysters were shucked. Total and soft tissues weights were measured for condition index measurement. Digestive glands were dissected, weighted individually, and were ground with a "MM 400" mixer mill (Retsch) in liquid nitrogen and stored at -80 °C for further toxin analyses. Condition index (CI) was determined for 62 individuals as follows (Bodoy et al., 1986): CI = (wet flesh weight / total weight) x 100.

### 2.7 *Histopathology*

Dissected tissues were fixed immediately in modified Davidson's fixative (Latendresse et al., 2002) for 24 h at 4°C. Tissues were processed and observed as described by Hermabessiere et al. (2016). The histopathological figures observed per organ were classified as follows:

- Gills: (i) presence of mucus, (ii) hemocyte infiltration, (iii) hemocytes outside the gills;
- Digestive gland (stomach, intestine, digestive ducts and tubules): (i) hemocyte infiltration, (ii) hemocytes in diapedesis in the digestive epithelium, (iii) presence of *A. minutum* cells in the lumen.

Intensity of each histopathological observation was rated using a three-level semi-quantitative scale and the sum calculated for each organ. Each pathological condition was graded as stage 0 (absence or very light), stage 1 (light-moderate), and stage 2 (heavy). Based upon this scale, mean intensity of each histopathological figure were calculated for each specific tissue as follows:

$$\sum(\text{stage}_{\text{individual 1}} + \text{stage}_{\text{individual 2}} + \ldots + \text{stage}_{\text{individual } n})/n$$

(where $n$ is the number of diagnosed animals per condition).

### 2.8 *Analyses of hemolymph variables*

The quality of hemolymph sample (absence of contamination) was checked using microscope and then stored temporarily on ice before flow-cytometric analysis. Mortality (percentage of dead hemocytes) and characteristics determined in live circulating hemocytes, i.e. total and differential hemocyte counts (granulocytes, hyalinocytes and small agranulocytes) (in cell $mL^{-1}$), size, and internal complexity (in arbitrary units a.u.), were assessed following Haberkorn et al. (2010b) with an Easy-Cyte-Plus flow-cytometer (Guava-Millipore).

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Functional variables, i.e. production of Reactive Oxygen Species (ROS) (specifically $H_2O_2$ and $O_2^-$) by unstimulated hemocytes, mitochondrial membrane potential (MMP) and phagocytosis were determined as described in Lambert et al. (2003), Donaghy et al. (2012) and Haberkorn et al. (2010b), respectively. Hemocyte functional analyses were performed with a FacsVerse flow-cytometer (BD Biosciences, San Jose, C.A., USA) for ROS production and a FACScalibur (BD Biosciences) for MMP and phagocytosis.

### 2.9 *Toxin quantification by liquid chromatography/fluorescence detection*

The digestive glands of 14 oysters exposed to the PST strain and of 14 oysters exposed to the PST+BEC strain (2-6 per tank) were sampled for toxin content measurement. Toxins were also assayed in pooled digestive glands of 6 oysters (2 oysters per tank) exposed to the BEC strain to verify that the BEC strain did not produce PSTs. Acetic acid (1 ml of 0.1 N) was added to 100 mg of ground digestive gland and the sample was frozen at -20 °C until extraction and analysis were performed. Toxin quantification were performed in triplicate for each sample using the liquid chromatography with fluorescence detection (LC/FD), as described by Boullot et al. (2017). The molar concentration (µmol $L^{-1}$) was converted to µg STX equivalent 100 $g^{-1}$ of digestive gland wet weight using the conversion factors of EFSA (EFSA Journal, 2009).

### 2.10 *Statistical analyses*

Differences between microalgal treatments were investigated using One-Way ANOVA followed by Tukey HSD post hoc test, after checking assumptions (normality of data and equal variance tests). When assumptions were not validated, the non-parametric Kruskal-Wallis test (non-nested – comparison of three conditions categorized by algal treatment) was applied, followed by the Nemenyi–Damico–Wolfe–Dunn (NDWD) post hoc test. For each condition and each valve activity parameter, Mann Whitney tests were used to compare the mean hourly values of the condition between the last 4 days of acclimation (n = 96 hours) and the 4-day *A. minutum* exposure period (n = 96 hours). Intensity of each pathological condition was compared statistically using the Kruskal-Wallis test. For the sum of binary pathological data (presence/absence), One-Way ANOVA was used. Differences were considered significant when $p < 0.05$. Statistical analyses were performed using R version 3.2.2 (R Core Team, 2012). All values are expressed as mean ± standard error (SE).

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## 3. Results

### 3.1 *Paralytic shellfish toxin content in the digestive gland of* Crassostrea gigas

The mean PST toxicity in the digestive glands of oysters after a 4-day exposure to *A. minutum* was 3.3-fold higher ($p < 0.01$, t test) in oysters exposed to the PST strain (174.2 ± 40.3 µg STX eq. 100 $g^{-1}$ wet digestive gland, n = 14) compared to oysters exposed to the PST+BEC strain (52.2 ± 17.2 µg STX eq. 100 $g^{-1}$ wet digestive gland, n = 14). The mean total PST load in the digestive glands of oysters from the PST condition (39.3 ± 8.8 µmol $kg^{-1}$ wet digestive gland, n = 13) was significantly higher (4.9-fold; $p < 0.001$, t test) compared to oysters from the PST+BEC condition (8.1 ± 2.4 µmol $kg^{-1}$ wet digestive gland, n = 14). No PSTs were detected in the digestive glands of oysters exposed to the BEC strain.

Toxin composition of oyster digestive glands were similar in oysters exposed to the PST or the PST+BEC strains: carbamates C2 and C1, N-sulfocarbamoyls GTX3 and GTX2, plus a minor quantity of decarbamoyls dc-GTX3 and dc-GTX2 (Fig. 1). Very low levels of saxitoxin (STX) and dc-STX were also detected in tissues of 4 oysters exposed to the PST strain (≈ 0.2%) and only in one oyster exposed to the PST+BEC strain (≈ 0.5%). The mean proportions of C1, GTX3, GTX2, dc-GTX3 and dc-GTX2 were significantly higher ($p < 0.01$, Mann-Whitney) and the proportion of C2 was significantly lower ($p < 0.001$, Mann-Whitney) in oysters exposed to the PST+BEC strain compared to oysters exposed to the PST strain (Fig. 1). The proportions of the other STX derivatives in digestive gland of oysters were not significantly different between the PST+BEC and PST conditions.

### 3.2 *Oyster valve-activity behavior*

Behavioral analyses were conducted on oysters during the 4 last days of acclimation and during the 4-day exposure to *A. minutum*. After acclimation period, valve behavior of oysters varied depending on the *A. minutum* strain they were exposed to (Table 1). During exposure to the PST strain, oysters showed no significant modifications of valve-activity behavior, in terms of hourly valve-opening duration, hourly valve-opening amplitude and hourly micro-closure activity (i.e. number of micro-closures per hour) as compared to the acclimation period (Table 1). On the contrary, oysters fed the PST+BEC or the BEC strains during the 4 days of *A. minutum* exposure were opened hourly for a significantly longer time ($p < 0.001$ in both conditions, Table 1) and with a higher amplitude ($p < 0.05$ and $p < 0.001$, respectively; Table 1) than during the acclimation period. Additionally, oysters from the PST+BEC and the BEC conditions exhibited significantly more valve micro-closures than during acclimation (1.84 and 3.46 fold higher, respectively; $p < 0.001$ in both conditions).

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### 3.3 *Daily rhythm of oyster behavior*

Chronobiological analyses were performed on the mean valve hourly opening duration in each condition before and during the *A. minutum* exposure. Spectral analysis (Lomb and Scargle periodogram) allows the determination of significant rhythmic periodicity in valve behavior. The results show a daily valve activity with a circadian period (range: 20-28 h) during the acclimation period with *H. triquetra* in the three groups (Fig. 2). All the significant periods were tested in the chronobiological model (Cosinor model), which validated the existence of a circadian rhythm. During the *A. minutum* exposure, valve activity of oysters exposed to the BEC strain still showed a significant circadian period ($p < 0.05$), whereas no significant period were found in oysters exposed to the PST+BEC or PST strains (Fig. 2).

### 3.4 Alexandrium minutum *consumption*

During the exposure, the percentage of *A. minutum* cells filtered by oysters from the PST condition was 93.6% the first day and then reached 100% during the 3 following days of exposure (Table 2). The percentage of *A. minutum* cells filtered by oysters ranged from 61.3% to 95.6% in the PST+BEC condition, while for the BEC condition this percentage increased from 14.8% to 53.2% at the end of the exposure (Table 2).

### 3.5 *Condition index*

At the end of the exposure, wet weights of oyster digestive glands were not statistically different between the three conditions. Condition index in oysters exposed to the BEC strain (14.9 ± 0.5, n = 21) was, however, significantly lower ($p < 0.05$ and $p < 0.001$, Tukey HSD test) compared to oysters of the two groups, exposed to the PST+BEC strain (17.1 ± 0.7, n = 21) and to the PST strain (18.2 ± 0.6, n = 21).

### 3.6 *Histopathology*

Histology revealed hemorrhages between the gills filaments in 53% and 27% of oysters exposed to the BEC (Fig. 3A) and PST+BEC (Fig. 3C) strains respectively (Table 3). Hemocytes migrated outside gill tissue via diapedesis (Fig. 3A) and aggregated around *A. minutum* cells (Fig. 3B). In the gills, hemocyte infiltration, diapedesis and hemorrhage were significantly more intense in oysters exposed to the BEC strain compared to oysters exposed to the PST strain (Table 3). Although gill tissue was not altered, mucus production was significantly higher in oysters from the BEC condition compared to oysters from the PST condition (Fig. 3C, Table 3). Intensity of histopathological observations in the gills of oysters from the PST+BEC condition was not significantly different from the other conditions, except

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for the intensity of hemocyte diapedesis which was intermediate between BEC and PST conditions (Table 3).

All oysters from the three conditions were actively feeding, as indicated by star-shaped digestive tubules (Fig. 3E). A relatively large number of intact *A. minutum* cells were observed in stomach and intestine lumen in oysters of the 3 conditions (Table 3). No *A. minutum* cell was detected in digestive ducts or tubules, except in the tubules of one oyster of the PST condition. Pathological alterations observed in the digestive gland consisted of inflammatory responses characterized by intense hemocyte diapedesis, mainly through the epithelium of the stomach and digestive tubules, and hemocyte infiltrations detected mostly in digestive ducts and tubules (Fig. 3D, E). In the stomach, hemocyte diapedesis was significantly more intense ($p < 0.05$, Kruskal-Wallis test) in oysters exposed to the PST strain (1.7 ± 0.1) compared to oysters exposed to the BEC strain (1.1 ± 0.2, Table 3). More rarely, hemocytes were observed in the lumen of the stomach, intestine and digestive ducts, in oysters exposed to the PST or the PST+BEC strain, but never in oysters exposed to the BEC strain.

The sum of mean intensities of pathological figures observed in the gills revealed that hemocyte responses were more intense in oysters exposed to the BEC strain (3.8) compared to oysters exposed to the PST strain (2.3), oysters from the PST+BEC condition being intermediate (1.1, Fig. 4A).

### 3.7 *Hemocyte characteristics and functioning analyzed by flow cytometry*

Total hemocyte count of oysters at the end of the experiment was significantly affected by algal exposure ($p < 0.05$, one-way ANOVA) (Fig. 5A). This effect was mainly attributable to variation in small agranulocyte counts between the conditions ($p < 0.01$, Kruskal Wallis): small agranulocyte count of oysters from the PST condition ($1.1 \pm 0.1 \times 10^5$) was significantly lower compared to oysters exposed to the PST+BEC ($2.5 \pm 0.6 \times 10^5$) and BEC strains ($2.4 \pm 0.5 \times 10^5$). The composition of hemocyte subpopulations also appeared impacted: the percentage of small agranulocytes and granulocytes was significantly different in the three conditions ($p < 0.01$, Kruskal-Wallis, and $p < 0.01$, ANOVA) (Fig. 5B, C). Hemolymph from oysters exposed to the PST strain contained a significantly lower ($p < 0.01$, Kruskal-Wallis) percentage of small agranulocytes (24.6 ± 1.4%) compared to oysters from PST+BEC (35.2 ± 2.3%) and BEC condition (34.0 ± 2.8%), and a significantly higher ($p < 0.01$, ANOVA) percentage of granulocytes (22.2 ± 2.0%) compared to oysters from the PST+BEC condition (14.1 ± 1.4%) (Fig. 5B, C). Lastly, mortality of hemocytes (percentage of dead hemocytes) in oysters exposed to the PST strain was significantly higher (9.6 ± 2.5%) compared to oysters exposed the

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PST+BEC strain (2.3 ± 0.3%) and the BEC strain (2.8 ± 0.6%) (Fig. 5D). No significant difference in MMP, phagocytosis, ROS production, and morphological parameters was observed between the three conditions.

## 4. Discussion

Although the effects of the toxic dinoflagellate *A. minutum* on bivalves have been well described, addressing specific oyster response to either paralytic shellfish toxins (PSTs) or bioactive extracellular compounds (BECs) is still challenging, since most studies either did not assess the production of BECs (Bricelj et al., 1993; Bricelj and Shumway, 1998) or often used strains producing both types of toxins (Haberkorn et al., 2010a, 2010b, 2011; Tran et al., 2010, 2015; Mat et al., 2013; Payton et al., 2017a). The present work investigated the effects of the different types of toxic compounds produced by the dinoflagellate *A. minutum*, the PSTs and the BECs, which chemical nature is still unknown, on the behavior and physiology of adult *C. gigas*. The most striking responses in this experiment were observed in oysters exposed to the BEC strain of *A. minutum*, on feeding activity, valve activity behavior, and on tissue and cellular gill parameters.

### 4.1 *BECs reduce algal consumption of oyster and thus PST accumulation*

In this study, the PST+BEC *A. minutum* strain appeared more toxic than the PST strain regarding cellular PST content and toxicity. However, oysters exposed to the PST+BEC strain accumulated less PSTs in their digestive glands than oysters exposed to the PST strain. This could be the result of a reduced feeding activity. Reduced feeding activity is supported by the reduction of algal consumption observed for oysters of the PST+BEC condition compared to the PST condition, this reduction being even more drastic in the BEC condition. The BECs might be responsible for the reduction of feeding activity, as this response was only observed in oysters exposed to the BEC producing strains and more marked with the BEC strain, which is the most cytotoxic strain tested. Similarly, the BEC strain elicited a stronger decrease in clearance and ingestion rates, and a lower absorption rate than the PST+BEC strain in *C. gigas* and *R. philippinarum* (Contreras, 2011). Feces production also seemed to be lower in the BEC condition than in the PST and PST+BEC conditions, and a noticeable production of pseudofeces was observed in the BEC condition (data not shown). Pseudofeces has been described as a secondary regulatory mechanism of ingestion rate in marine bivalves and also a mechanism for pre-ingestive selection (Shumway and Cucci, 1987; Navarro and Widdows, 1997; Wildish et al., 1998). Previous studies showed that *C. gigas* reduced filtration, ingestion

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and biodeposition rates when exposed to different *Alexandrium* species (Bardouil et al., 1993; Lassus et al., 1999, 1996; Bougrier et al., 2003). Lassus et al. (2004) and Laabir et al. (2007) reported partial inhibition of filtration rate in oysters exposed to the PST+BEC *A. minutum* strain compared to non-toxic *Skeletonema costatum*, related to a decreased feeding time activity and a lower biodeposit production rate. The decreased filtration rate coupled with pseudofeces production would be at the origin of the significantly-reduced condition index of oysters of the BEC condition, compared to oysters of the PST and PST+BEC conditions. This reduced feeding activity might consist in an avoidance response after direct contact between *Alexandrium* cells and oyster external organs.

### 4.2 *BECs disturb valve-activity behavior*

Valve activity behavior analysis showed increased hourly valve-opening duration and hourly micro-closure activity in oysters exposed to the BEC strain and, to a lesser extent in oysters exposed to the PST+BEC strain. Concerning the PST+BEC strain, similar results were already obtained by Tran et al. (2010, 2015), but without defining the respective effects of PSTs and BECs produced by the strain. These behavioral responses may be mostly related to the production of BECs by *A. minutum*. Indeed, the non-PST producing BEC strain caused more intense behavioral changes in oysters than the PST+BEC strain, whereas the PST strain did not induce any significant changes in oyster behavioral parameters. The frequency of micro-closures during a 2-day exposure to the PST+BEC strain of *A. minutum* was positively correlated to the *A. minutum* concentration in the ambient water and not to the PST content in the digestive gland of oysters (Haberkorn et al., 2011). This supports our hypothesis, as a higher *A. minutum* concentration would imply a greater quantity of bioactive extracellular compounds in the water resulting in a higher impact on oyster micro-closure activity.

The BECs excreted in the water could activate chemoreceptors of the mantel edge or directly affect oyster gills when *A. minutum* cells come into contact with the tissues in the pallial cavity, responsible for the quick increase in frequency of valve micro-closures. Conversely, intracellular PSTs are mainly released in the digestive gland, once dinoflagellate cells have been digested by oysters. During the toxification phase, the digestive gland is considered as the initial repository of toxic cells following ingestion and is the organ where PST are initially accumulated in bivalves (Bricelj and Shumway, 1998). The hypothesis that behavioral changes might be caused by internal contamination by PSTs released after *A. minutum* cell digestion is not supported by our results, as behavioral changes were mostly observed in response to the BEC strain.

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The increase in frequency of valve micro-closures is probably a protective behavior to increase intervalvar water renewal and minimize contact with BECs, potentially irritant to gill tissues. Increased closures and expulsions observed in *Pecten maximus* exposed to the PST+BEC strain was also hypothesized to serve as a reaction to expulse *A. minutum* cells from the mantle cavity (Coquereau et al., 2016). Exposing juvenile *Pecten maximus* to the BEC strain of *A. minutum* led to a less effective escape response after a contact with a predator supposedly due to direct tissue damage caused by BECs (Borcier et al., 2017). Given the noteworthy oyster behavioral changes in response to BEC-producing strains, *C. gigas* gills integrity could be impaired.

### 4.3 *BECs induce hemorrhage and inflammation in gills*

The BECs induced hemorrhage in gills and aggregation of hemocytes around *A. minutum* cells. These symptoms are indeed stronger with the BEC strain, whereas these pathologies were not observed in gills of oysters exposed to the PST strain, which had no detectable cytotoxic activity. Aggregation of hemocytes surrounding toxic algal cells had already been observed in *Argopecten irradians irradians* and *Mercenaria mercenaria* respectively exposed to *Prorocentrum minimum* which also produce uncharacterized cytotoxic compounds (Wikfors and Smolowitz, 1993; Hégaret et al., 2011).

Besides, the intensity of hemocyte infiltration, diapedesis, and mucus production in the gills was significantly higher for oysters in the BEC condition than in the PST condition. The pathologies could be interpreted as defensive inflammation responses against *A. minutum* cells and bioactive compounds released by those algal cells. Haberkorn et al. (2010a) previously reported an increased mucus production in *C. gigas* exposed to the PST+BEC strain of *A.* minutum, probably reflecting tissue irritation and/or defense reaction to BECs. Indeed, mucus contains lytic enzymes that play an important role in defense against a wide range of stressors (Fisher, 1992). Similarly, contact with the non-PST producing *Alexandrium ostenfeldii* led to edema in the mantle, associated with hemocyte infiltration in the gills of Manila clams (Lassudrie et al., 2014). This inflammatory response in external organs was hypothesized to be partly induced by uncharacterized extracellular compounds produced by *A. ostenfeldii* shown to be lytic to protists (Tillmann et al., 2007).

In the present study, the higher counts of hemocytes in circulating hemolymph may reflect hemocyte *de novo* production or mobilization from connective tissues to hemolymph to supply responses in specific tissues. Particularly, hemocyte participation in wound repair has been suggested based upon the observation of tissue lesions caused by toxic dinoflagellate exposure

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associated with hemocyte aggregates or infiltrations, a typical response to *Alexandrium* spp. exposure in bivalves.

### 4.4 *PSTs disrupt biological rhythm*

The two PST-producing strains disrupted the daily rhythm of oyster valve activity. Biological rhythms allow synchronization and anticipation of environmental cycles, allowing the temporal organization of biological processes and maximizing the fitness of organisms with their biotope (Yerushalmi and Green, 2009). Oyster behavior is known to follow circadian, ultradian, and infradian rhythms (Tran et al., 2011; Bernard et al., 2016). Their daily rhythm is driven by a circadian clock (Mat et al., 2012, 2013; Payton et al., 2017b), which genetic components, i.e. clock genes, have been recently identified (Perrigault and Tran, 2017). The clock genes are involved in the generation and/or synchronization of circadian rhythmic activities (Chaves et al., 2011). Additionally, *A. minutum* exposure (PST+BEC strain) can deeply modify temporal organization of gills transcriptome and induce a loss of daily cycle in oyster valve behavior, crystalline style length, and expression of genes involved in detoxification and oxidative stress (Tran et al., 2015; Payton et al., 2017a). These disruptions have been linked to the repression of the transcription of most of the clock genes in oysters exposed to *A. minutum* (Mat et al., 2013; Payton et al., 2017a).

PSTs are presumably responsible for the disappearance of the valve cyclic activity observed in the present study via down-regulation of clock gene expression, as showed in Payton et al. (2017a) . Indeed, saxitoxin and its derivatives have a mechanism of action close to tetrodotoxin, targeting the same voltage-gated sodium channels (Heggeness and Starkus, 1986); and tetrodotoxin is known to repress clock gene expression (van den Pol and Obrietan, 2002).

### 4.5 *PSTs provoke inflammation in the digestive gland*

Toxin accumulation measured in the digestive gland, the main storage organ for PSTs (Lassus et al., 2007; Guéguen et al., 2008), indicated that the PST-producing strains were ingested. Oysters exposed to the PST strain had higher PST burden in their digestive glands, where the strongest inflammatory response was also observed. Hemocyte infiltration and diapedesis across digestive epithelium were more intense in the PST condition than in the BEC condition, and intermediate in the PST+BEC condition. These typical hemocyte responses in bivalves exposed to *Alexandrium* spp. (Galimany et al., 2008; Lassudrie et al., 2014; Borcier et al., 2017) have been linked to the presence of PSTs (Haberkorn et al., 2010a). Saxitoxin and its derivatives could be directly released within the organ by lysis of *Alexandrium* cells, or assimilated and biotransformed within the tissues. Hemocytes could participate to toxin

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depuration by eliminating toxins through the lumen of the intestine and/or isolating *Alexandrium* cells.

Indeed, the percentage of dead circulating hemocytes was higher in the PST condition. Mortality of oyster hemocytes was already noticed *in vitro* in response to toxic *Alexandrium fundyense* and *A. minutum* (PST+BEC strain) (Hégaret et al., 2011). In the lion-paw scallop *Nodipecten subnodosus*, a lower count of hemocytes was attributed to the elicited caspase-dependent apoptosis of hemocytes (Estrada et al., 2010, 2014). Similarly, *in vitro* exposure of oyster hemocytes to different saxitoxin analogs (STX and C1/C2) caused apoptosis in hemocytes in a dose dependent manner, a process dependent on caspase activation (Abi-Khalil et al., 2017). Since these PST derivatives were detected in oysters of the PST condition (data not shown), we suggest that the large amount of accumulated PSTs induced hemocyte apoptosis responsible of the higher mortality of circulating hemocytes.

## 5. Conclusions

In the present study, behavioral and physiological responses of oysters greatly differed depending on the *A. minutum* strain they were exposed to, and thus potentially on the type of bioactive substances produced by the strains. The results showed for the first time that the cytotoxic BECs produced by *A. minutum* could impair oyster feeding, by altering valve activity and gill tissues. These findings highlight the urgent need to characterize the bioactive extracellular compounds in toxic algae, as *Alexandrium* spp. The PSTs produced by *A. minutum* have disrupted the biological rhythm of oyster behavior, with significant impacts on the digestive gland. The potential genotoxic impacts of PSTs on clock genes, potentially leading to the loss of rhythmicity is to further investigate.

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## Figures

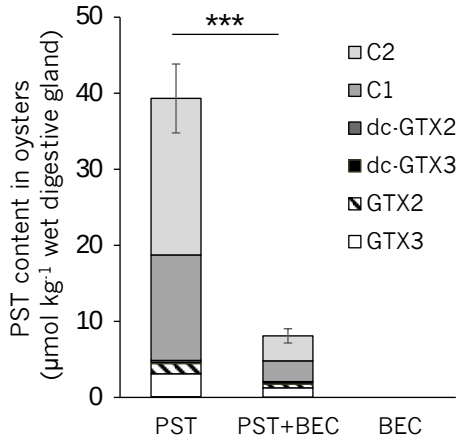


**Fig. 1.** Paralytic shellfish toxin accumulation (in µmol $kg^{-1}$ of wet digestive gland) in the digestive glands of *C. gigas* exposed for 4 days to the PST strain (n = 14 oysters), the PST+BEC strain (n = 14 oysters), or the BEC strain (n = 1 pool of 6 oysters) of *A. minutum*. Mean ± SE. *** Significant difference is indicated by $p < 0.001$ (Mann-Whitney test). No PSTs were detected in the pool of 6 oysters from the BEC condition. The values are given as the mean contribution (in percentage molar concentration) of each saxitoxin derivate to the global toxicity. STX and dc-STX were detected in digestive glands of oysters in both conditions but are not presented because they represented less than 1% of total toxicity. Saxitoxin (STX) and gonyautoxins (GTX2 and GTX3) belong to carbamate toxins. C-toxins (C1 and C2) belong to N-sulfocarbamoyl toxins. dc-STX, dc-GTX2 and dc-GTX3 are decarbamoyl toxins.

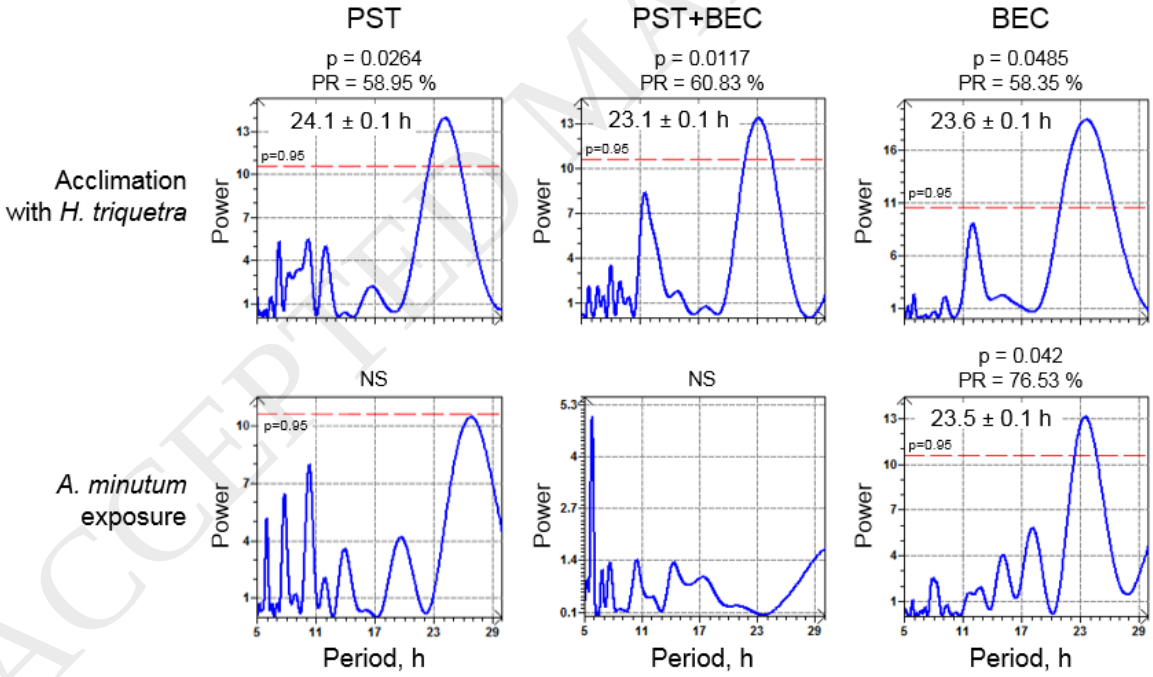


**Fig. 2.** Effect of three *A. minutum* strains producing PST, PST+BEC, or BEC, on the behavioral rhythm of *C. gigas*. Chronobiological analysis was made for each condition, during the 4 last days of the acclimation with non-toxic *H. triquetra* (top graphs), and during the 4-day exposure to *A. minutum* (bottom graphs). To reveal significant rhythm, spectral analysis by Lomb and Scargle periodogram was done first, the red dotted line corresponds to the significant threshold ($p$ = 0.95), and the rhythmic activity is characterized by the period of the condition. Then, the rhythm is validated by the p-value and the percent rhythm (PR) of the Cosinor model. n = 4-7 oysters per condition. NS: Non-significant at p-value = 0.05, i.e. no significant period.

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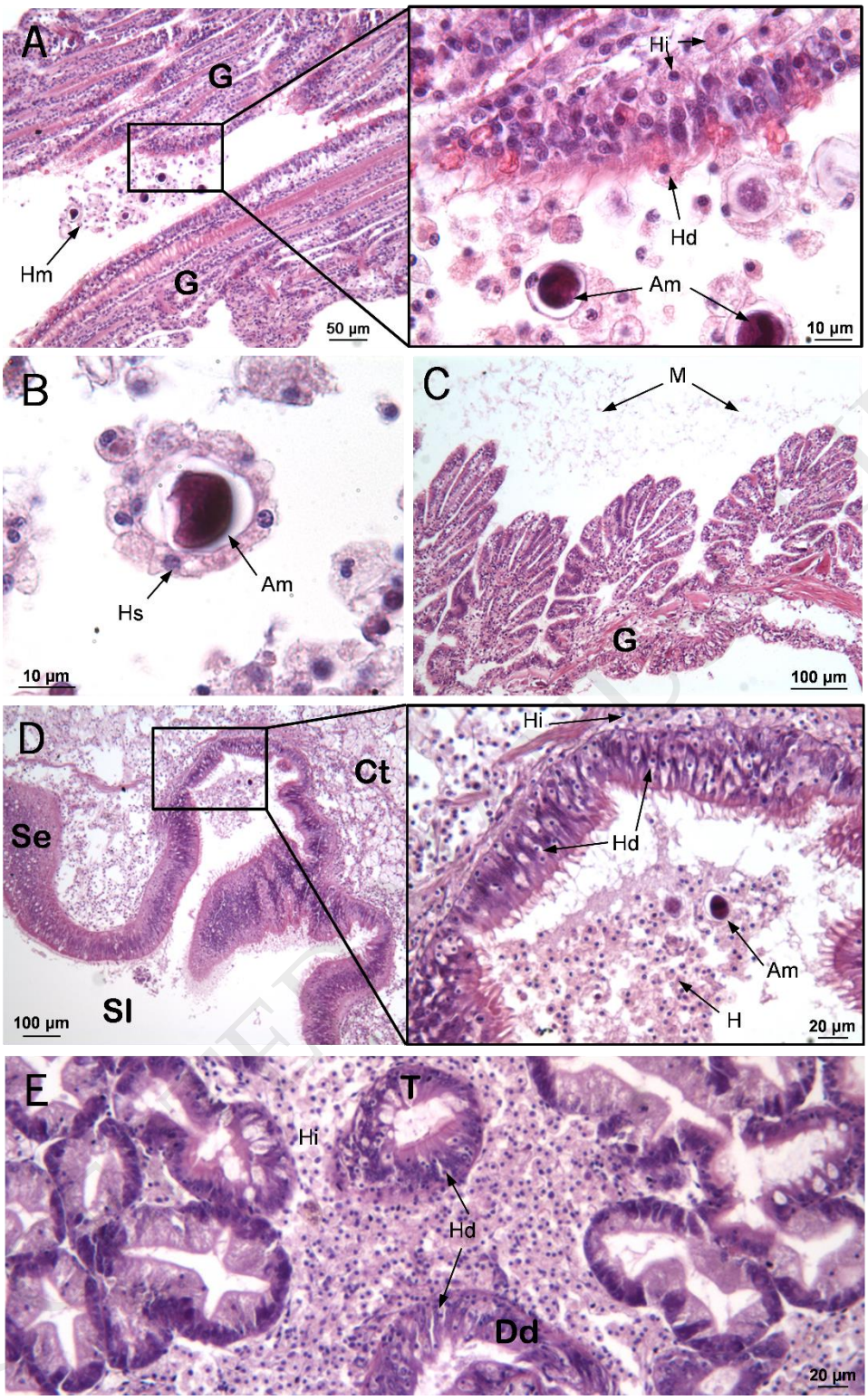


**Fig. 3.** Histological analysis (Hematoxylin-eosin stained paraffin sections) of gills of *C. gigas* exposed to the BEC strain of *A. minutum* (A, B) or to the PST+BEC strain (C) and digestive gland of oysters exposed to the PST strain (D, E). (A) Hemorrhage (Hm) between gill filaments (G), hemocyte diapedesis (Hd) and infiltration (Hi) in gill filament, and *A. minutum* cells (Am) outside the gills; (B) Detail of hemocytes surrounding (Hs) an *A. minutum* cell (Am); (C) Mucus (M) produced by gills (G); (D) Moderate inflammatory responses in the stomach characterized by infiltration of hemocytes (Hi) in the connective tissue (Ct) and intense hemocyte diapedesis (Hd) through stomach epithelium (Se), with an *A. minutum* cell and hemocytes in the stomach lumen (Sl); (E) Diapedesis through the epithelium of digestive ducts (Dd) and tubules (T), and hemocyte infiltration (Hi) in the connective tissue.

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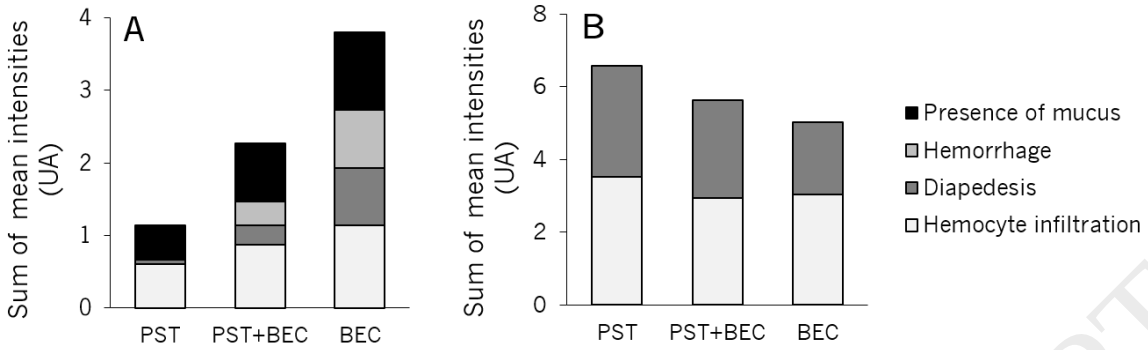


**Fig. 4.** Sum of mean intensities of each histopathological observations in the gills (A) and in the digestive gland (B) of oysters *C. gigas* after a 4-day exposure to the PST, PST+BEC or BEC strain of *A. minutum* (n = 15 in each condition).

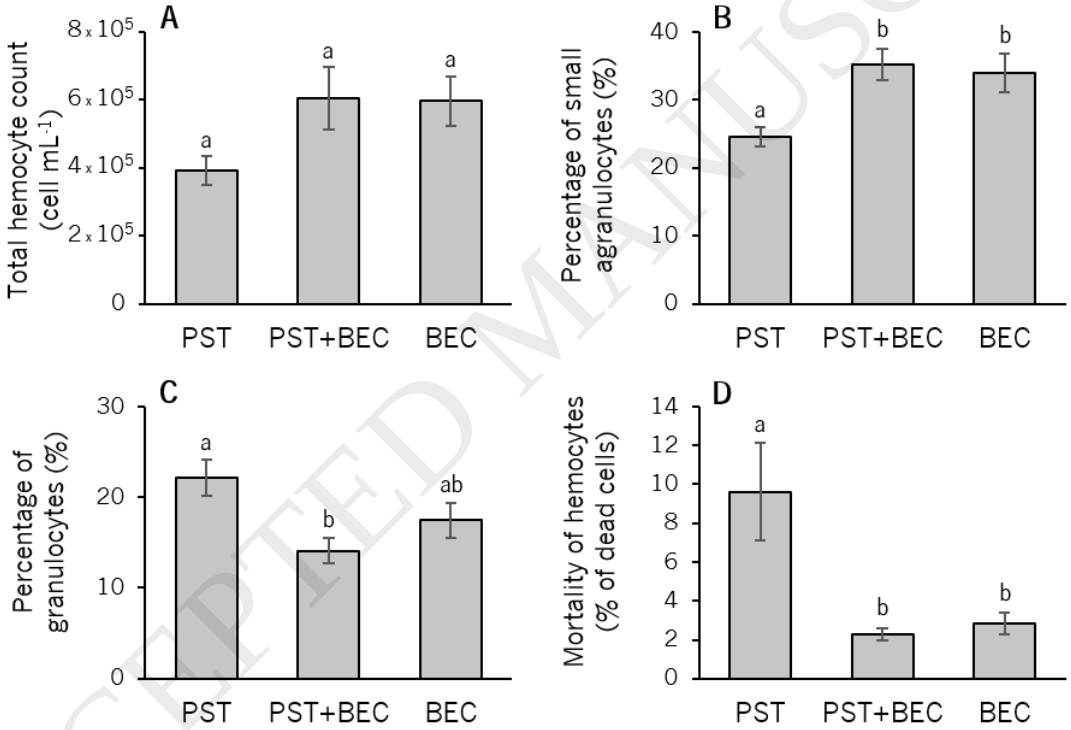


**Fig. 5.** Hemocyte variables (mean ± SE) in the circulating hemolymph of oysters *C. gigas* after a 4-day exposure to different strains of *A. minutum*: the PST strain (n = 18 oysters), the PST+BEC strain (n = 18 oysters) or the BEC strain (n = 19 oysters). (A) Total hemocyte count (cells $mL^{-1}$); (B) Percentage of small agranulocytes; (C) Percentage of granulocytes; (D) Hemocyte mortality (percentage of dead hemocytes). Letters indicate significant differences between conditions (ANOVA followed by post-hoc Tukey HSD test).

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## Tables

**Table 1**
Valve activity behavior modifications of *C. gigas* induced by exposure to different *A. minutum* strains: PST, PST+BEC and BEC strains. For each condition, Mann Whitney tests were used to compare the mean hourly values of valve activity parameter between acclimation (n = 96 hours, i.e. 4 last days of acclimation) and *A. minutum* exposure (n = 96 hours). For each condition, fold change of each valve activity parameter was calculated by dividing the mean hourly value of the parameter for the condition during *A. minutum* exposure by the mean hourly value during acclimation.

| Condition | Valve-activity modifications between *A. minutum* exposure and acclimation | | | | | |
|---|---|---|---|---|---|---|
| | Opening duration | | Opening amplitude | | Micro-closure | |
| | Test | Fold change | Test | Fold change | Test | Fold change |
| PST | NS | 1.06 | NS | 1.03 | NS | 0.88 |
| PST+BEC | *** | 1.25 | * | 1.13 | *** | 1.84 |
| BEC | *** | 1.55 | *** | 1.23 | *** | 3.46 |

NS = non-significant
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

**Table 2**
Percentage (%) of *A. minutum* cells filtered by oysters during the 4-day exposure period to the PST, PST+BEC and BEC strains. Mean ± SE, n = 3 tanks per condition.

| Condition | Day 1 | Day 2 | Day 3 | Day 4 |
|---|---|---|---|---|
| PST | 93.6 ± 3.7 | 100.0 ± 0 | 100.0 ± 0 | 100.0 ± 0 |
| PST+BEC | 61.3 ± 2.0 | 62.5 ± 2.3 | 85.0 ± 0.5 | 95.6 ± 2.6 |
| BEC | 50.3 ± 1.7 | 14.8 ± 2.4 | 41.8 ± 2.7 | 53.2 ± 4.6 |

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**Table 3**
Mean intensities of histological figures in *C. gigas* digestive gland and gills, after a 4-day exposure to the PST, PST+BEC or BEC strain of *A. minutum*. Results are expressed as mean of stage intensity ± SE (n = 15), with stage 0 = absence or very light, stage 1 = light-moderate, and stage 2 = heavy. Letters indicate significant differences between conditions (Kruskal-Wallis test followed by NDWD post-hoc test).

| Organs | Pathologies | Conditions | | | Kruskal-Wallis test |
|---|---|---|---|---|---|
| | | PST | PST+BEC | BEC | |
| Digestive gland | | | | | |
| Stomach | Hemocyte infiltration | 0.6 ± 0.1 | 0.5 ± 0.2 | 0.5± 0.2 | NS |
| Intestine | | 0.9 ± 0.2 | 0.6 ± 0.2 | 0.6 ± 0.1 | NS |
| Digestive ducts | | 0.9 ± 0.2 | 0.7 ± 0.2 | 0.9 ± 0.1 | NS |
| Digestive tubules | | 1.2 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.1 | NS |
| Stomach | Diapedesis | 1.7 ± 0.1[a] | 1.2 ± 0.2[ab] | 1.1 ± 0.2[b] | * |
| Intestine | | 0.9 ± 0.2 | 0.9 ± 0.1 | 0.5 ± 0.2 | NS |
| Digestive ducts | | 0.3 ± 0.2 | 0.6 ± 0.2 | 0.4 ± 0.1 | NS |
| Digestive tubules | | 0.1 ± 0.1 | 0 ± 0 | 0 ± 0 | NS |
| Stomach | Presence of *A. minutum* cells in the lumen | 1.1 ± 0.2 | 0.7 ± 0.2 | 0.5 ± 0.2 | NS |
| Intestine | | 1.6 ± 0.1 | 1.2 ± 0.2 | 1.0 ± 0.2 | NS |
| Gills | Hemocyte infiltration | 0.6 ± 0.2[a] | 0.9 ± 0.2[ab] | 1.1 ± 0.1[b] | * |
| | Diapedesis | 0.1 ± 0.1[a] | 0.3 ± 0.1[b] | 0.8 ± 0.1[c] | *** |
| | Hemorrhage | 0 ± 0[a] | 0.3 ± 0.2[ab] | 0.8 ± 0.2[b] | ** |
| | Mucus production | 0.5 ± 0.1[a] | 0.8 ± 0.1[ab] | 1.1 ± 0.2[b] | * |

NS = non-significant
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.