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Effects of bioactive extracellular compounds and paralytic shellfish toxins produced by *Alexandrium minutum* on growth and behaviour of juvenile great scallops Pecten maximus

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Abstract :

Dinoflagellates of the genus *Alexandrium* are a major cause of harmful algal blooms (HABs) that have increasingly disrupted coastal ecosystems for the last several decades. Microalgae from the genus *Alexandrium* are known to produce paralytic shellfish toxins (PST) but also bioactive extracellular compounds (BEC) that can display cytotoxic, allelopathic, ichtyotoxic or haemolytic effects upon marine organisms. The objective of this experimental study was to assess the effects of PST and BEC produced by *A. minutum* upon juvenile great scallops *Pecten maximus*. Scallops were exposed for one week to two different strains of *A. minutum*, the first producing both PST and BEC and the second producing only BEC. Escape response to starfish, daily shell growth, histological effects, and accumulation of PST were recorded after one week of exposure, and after two subsequent weeks of recovery. Daily shell growth was delayed three days in scallops exposed to the BEC-producing *A. minutum* strain, probably during the three first days of exposure. An increase of reaction time to predators was observed in scallops exposed to the BEC condition, suggesting that BEC may have altered sensing processes. Scallops exposed to PST displayed a less-efficient escape response and muscular damage which could reflect the effects of paralytic toxins upon the nervous system of scallops. This study demonstrates contrasting effects of the distinct toxic compounds produced by *A. minutum* upon marine bivalves, thus highlighting the importance to better characterize these extracellular, bioactive compounds to better understand responses of other marine

Highlights

► *Alexandrium* sp. can produce paralytic shellfish toxins and other bioactive extracellular compounds with allelopathic and cytotoxic activity. ► Extracellular bioactive compounds cause tissue alteration, thus altering recognition and feeding processes of *P. maximus*. ► Paralytic shellfish toxins reduce the efficiency of scallop *P. maximus* escape response and cause muscular damages. ► It is important to characterize the different compounds and their effects, when assessing the impact of *Alexandrium* on marine organisms

Keywords : Harmful Algal Bloom (HAB), *Alexandrium minutum*, *Pecten maximus*, Paralytic Shellfish Toxins (PST), extracellular compounds, behaviour, histology

Introduction

Harmful Algal Blooms (HABs) develop mainly near coastal waters (Sellner *et al.*, 2003) and have been reported worldwide (Van Egmond *et al.*, 1993; Bricelj & Shumway, 1998; Sellner *et al.*, 2003; Lassus *et al.*, 2006). Dinoflagellates of the genus *Alexandrium* Halim (1960) are among the most prominent HABs in terms of intensity, diversity, and distribution worldwide (Anderson *et al.*, 2012). Species of *Alexandrium,* such as *A. minutum* or *A. catenella,* are known to produce neurotoxins, which include saxitoxin (STX) and its derivatives (21 congeners), also known as paralytic shellfish toxins (PST). STX blocks the passage of sodium ions in nerve cell sodium channels, leading to severe neuromuscular disorders, especially among consumers of intoxicated flesh (Château-Degat, 2003). Thus, PSTs present a very acute toxicity for aquatic organisms (Landsberg, 2002) and human consumers (Lassus *et al.*, 2006; Anderson *et al.*, 2012). PSTs are responsible for the "Paralytic Shellfish Poisoning" (PSP) syndrome that can modify the trophic structure of marine food webs, by causing intoxication or death of many organisms (Anderson *et al.*, 2012). Species of *Alexandrium* are also known to produce others toxic molecules, independently from PST (Tillman and John, 2002), which are excreted extracellularly and can have deleterious effects upon organisms when excreted into surrounding seawater. These compounds can have cytotoxic (Arzul *et al*., 1999, Ford *et al.,* 2008, Tillmann *et al.,* 2008) allelopathic (Lelong *et al.,* 2011), haemolytic (Arzul *et al*. 1999), or growth inhibition impacts on marine organisms, such as neighbouring phytoplanctonic cells, molluscs, or, fishes (Tang *et al.,* 2007, Landsberg, 2002 Haberkorn *et al.,* 2010a,b; Lambert *et al.,* 2013). Extracellular bioactive compounds associated with *Alexandrium* cytotoxicity still remain unidentified chemically. Emura *et al.* (2004) first identified from *A. taylori* protein-like, hemolytic compounds with molecular weights larger than 10,000 Da. Later, Flores *et al.* (2012) also found evidence for protein-like compounds with cytotoxic activity to heterotrophic protists from *A. tamarense* cultures; in this study, toxicity was also linked to Reactive Oxygen Species (ROS). Conversely, Yamasaki *et al.* (2008) were not able to detect a candidate protein, but suggested the possibility that a polysaccharide-based compound produced by *A. tamarense* could be responsible for effects observed. Meanwhile Ma *et al.* (2009) isolated from *A. tamarense* a large, amphiphathic compound with lytic activity. The nature of the compounds inducing cytotoxic activity is

therefore clearly far from being elucidated. The use of a phytoplankton bioassay in this study thus allows detection and semi-quantification of cytotoxic compounds in solution.

The great scallop *P. maximus* is a marine bivalve that lives in coastal waters at the surface of, or slightly buried in, the sediment (Baird, 1958). This scallop appears to have three responses to disturbance that are mostly chemosensory (Thomas & Gruffydd, 1971; Brand, 2006): closing valves, jumping, and vigorous swimming. Great scallops are exposed to HABs in their habitat (Shumway *et al*., 1990; Chauvaud *et al*., 1998; Chauvaud *et al*., 2001; Lorrain *et al.,* 2000). Despite the important commercial value of *P. maximus*, information is scarce regarding the response of this organism to toxic phytoplankton blooms and the potential for these scallops to accumulate toxins. In mussels and oysters, numerous studies have assessed exposure to *A. minutum* and toxin accumulation, filtration and feeding activities, valve activity, as well as other physiological variables, such as immune response, antioxidant response, tissue damages, or reproduction (Bougrier *et al.*, 2003; Fabioux *et al.*, 2015; Haberkorn *et al.*, 2010a,b, 2011; Tran *et al.*, 2010, 2015). A recent study demonstrated a modified escape response of the Peruvian scallop *Argopecten purpuratus* Lamarck (1819) to the predatory starfish *Meyenaster gelatinosus* Meyen (1834), when exposed to *A. catenella* (Whedon & Kofoid) Balech (1985) (Hégaret *et al.*, 2012). The escape response of scallops appears as a biologically relevant physiological indicator as it represents a consistent response (Ortiz *et al.*, 2003), consisting of altered adduction/abduction movements propulsing the individual by expulsing water from the dorsal side (Brokordt *et al.*, 2006). Further, escape response is relatively simple to measure to evaluate the status of scallops (Guderley *et al.*, 2011).

Previous studies have highlighted alteration of the growth of *P. maximus* in the Bay of Brest, in relation to the effects of microalgal extracellular compounds. For instance, a lack of growth has been reported in *P. maximus* following a bloom of the toxic dinoflagellate *Gymnodinium* cf. *nagasakiense* Takayama & Adachi (1985) (= actual *Karenia mikimotoi* (Miyaka & Kominami ex Oda) Hansen & Moeastrup (2000)) (Chauvaud *et al.* 2001). Moreover, growth alteration and inhibition, as well as mortality, have been observed in great scallop juveniles in response to a *Karenia mikimotoi* bloom (Chauvaud *et al*., 2001; Erard-Le-Denn *et al*., 1990; Le Floc'h *et al*., 2008). This algal species produces several extracellular compounds with putative cytotoxic (allelopathic and haemolytic) activities (Gentien & Arzul, 1990; Arzul *et al.*, 1999; Gentien, 2006; Gentien *et al.*, 2007), which are suspected to be responsible for the altered growth patterns observed in scallops.

The present study was undertaken to experimentally assess, under controlled conditions, the possible effects of PSTs and extracellular compounds produced by *A. minutum* upon growth, escape, and histological responses of juvenile *P. maximus*. Individuals were exposed to two strains of *A. minutum,* one producing both PSTs and bioactive extracellular compounds (BEC), and the other producing only BEC, to discriminate possible different effects of these toxins on the physiological responses of juvenile scallops.

Materials and methods

Biological material

Juvenile *Pecten maximus* (7-8 gr; 38-40 mm) were obtained from the Tinduff hatchery (Plougastel-Daoulas, France) and acclimatized to experimental conditions for 45 days before the start of the experiment. In parallel, starfish *Asterias rubens* were sampled in the Bay of Brest by scuba diving three days before the "escape response assays" and maintained in a 120-L tank with running seawater (14°C).

Algal cultures of *Tisochrysis lutea* (clone T-*iso*) Bendif *et al.* (2013) and *Chaetoceros neogracile* VanLandingham (1968) (= *C. muelleri*, Strain CCAP1010/3), two species commonly used as feed in bivalve hatcheries, were produced as the principal diet. T-*iso* was first cultivated in continuous culture and *C. neogracile* in batch culture, with Conway medium (Walne, 1970) in 1-µm-filtered, UV-sterilised seawater. These strains were cultured in 10-L bottles before being inoculated into 150-L cylinders maintained at 18°C. To optimize growth conditions, cylinders were aerated with a mix of air and CO_2 and the cellular density of the mixture (T-*iso* + *C. neogracile)* was in the range of 2.04 x 10^7 cells per day per scallop ± 2.71 x10^5 cells per day per scallop.

Two strains of *A. minutum* were used in the study. The first, AM89BM, hereafter referred to as "PST" for convenience, was isolated from a bloom in the Bay of Morlaix, Brittany, France (Erard-Le-Denn *et al.*, 1990). This strain is known for high PST content (Haberkorn *et al.* 2010a,b), but produces also bioactive extracellular compounds (BEC) (Lelong *et al.*, 2011). The second strain, CCMI1002, hereafter named "BEC", was isolated from a bloom in Ireland (Tillmann & John, 2002). No PST production has been detected in this strain, but it produces a large amount of BEC (more than the AM89BM strain), with known allelopathic and cytotoxic activity (Lambert *et al.* 2013). Both *A. minutum* strains were grown with L1 medium made with autoclaved, 1-µm-filtered seawater (Guillard & Hargraves, 1993). *Alexandrium* strains were cultured in 2-, 6-, and 10-L glass bottles before being inoculated into 300-L cylinders and incubated at 15°C with a 12h/12h photoperiod. Aeration, without

CO_2 supplement, was maintained in the cylinders, and pH was constantly checked to supply favourable conditions for growth. Cultures were grown without antibiotics and were not free of bacteria.

For the preliminary experiment, cultures of *C. neogracile* were grown with F/2-medium with Si made with autoclaved, 1-µm-filtered seawater (Guillard and Ryther 1962, Guillard 1975) at 18°C with a 12h/12h photoperiod.

Preliminary experiment: cytotoxicity assay

To assess the cytotoxicity of both *A. minutum* strains, the production of cytotoxic, allelopathic compounds by the two *A. minutum* strains was tested on *C. neogracile* according to the protocol developed by Lelong *et al.* (2011), which was used as a bioassay. When *A. minutum* cultures reached the end of the exponential growth phase, they were centrifuged and resuspended in clean, new medium in triplicate at 10^4 cell mL^{-1}. Analyses of allelopathic effects were performed 24h after inoculating cultures to standardize the time allowed for production of allelopathic compounds. Before each sampling, cultures were mixed by gentle, manual shaking.

Supernatants from *A. minutum* cultures was separated by centrifugation (10 min, 800×g, 18°C) and filtered to 0.2 µm (acetate cellulose filters, Minisart, Sartorius, Göttingen, Germany). The supernatant was prepared just before the experiment and added to *C. neogracile* cultures directly after filtration. For each condition, 2 mL of the supernatant was added to 3 mL of *C. neogracile* culture, with a final cell density of 10^5 cell mL^{-1} of *C. neogracile* in exponential growth phase. Un-inoculated F/2 medium was used as a control, as performed in Lelong *et al*. (2011).

Bioassays were performed in triplicate in 15-ml, pre-sterilized falcon tubes maintained in culture conditions for 3.5 hours prior to analyses, which consisted in measuring the quantum yield and the chlorophyll fluorescence of *C. neogracile*, which were the first variables affected in the experiment by Lelong *et al*. (2011) prior to cell mortality

Quantum Yield (QY), a measure of the efficiency of photosynthesis, was measured using a PAM AquaPen-C AP-C 100 fluorometer (Photo Systems Instruments, Czech Republic), on cells of *C. neogracile* after 20 min of dark adaptation at 16°C according to the formula:

$$QY = Fv/Fm = (Fm - F0)/Fm,$$

where F0 and Fm are the minimum and maximum fluorescence of cells at 455 nm, respectively.

Chlorophyll fluorescence was assessed using a FACSCalibur (Becton Dickinson) flow cytometer, with an argon blue laser (488 nm) and three fluorescence detectors: FL1 (green, 530 nm), FL2 (orange, 585 nm), FL3 (red, 670 nm). Red fluorescence (FL3) is linearly correlated with the chlorophyll content of cells and was thus used to discriminate living microalgal cells in cytograms.

Experimental design for the exposure experiment

A population of 1620 scallops was distributed haphazardly into six, 120-L tanks (*i.e.* 270 per tank). Two replicate tanks were used per condition (see below for the conditions). Each tank was filled with three replicate trays containing 90 juvenile great scallops each. The tanks were supplied with running seawater, pumped from the bay of Brest, filtered successively to 10 µm and 5 µm at a continuous flow of 200 mL min^{-1}.

Juvenile scallops were fed with a regular diet of *C. neogracile* and T-*iso,* which was mixed at the inlet of seawater (T-iso and *C. neogracile:* 50/50 dry weight (DW) per DW of scallops). During the first week of acclimation, scallops were maintained at 10.5 °C. Then, every three-four days, the temperature and photoperiod were increased respectively by 0.5°C and 30 min day light, to reach 14°C and 14 hours of day light after acclimation, *i.e.* 45 days. The protocol was intended to simulate the gradual increase of the spring photoperiod and temperature, to stimulate scallop growth (Fig. 1A).

After 45 days of acclimation, scallops were exposed for one week to either of the two *A. minutum* strains or to a control diet (as during acclimation). The supply in *A. minutum* in the two HAB treatments was supplemented to the regular diet in four of the tanks and inserted in parallel (25 mL min^{-1} per tank, 4.17 mL min^{-1} per tray at the concentration of 3 x 10^4 cell · mL^{-1}). Three different conditions were thus run in the experiment:

(1) A control condition, in which scallops were fed with the regular food diet only, containing of a mix diet of *T-iso* and *C. neogracile*, hereafter named "CON";

(2) Scallops exposed to "PST" *A. minutum* and

(3) Scallops exposed to "BEC" *A. minutum*.

The daily rations were quantified at 2.33 x 10^6 (± 3.45 x 10^4) of *A. minutum* cells per day per scallops for the PST strain and 2.08 x 10^6 (± 7.14 x 10^4) of *A. minutum* cells per day per scallop for the BEC strain.

At the end of the seven days of exposure to the harmful algae, scallops from the three conditions were fed two extra weeks with the regular food diet to simulate a post-bloom phase.

For the purpose of the study, only 120 scallops distributed among all trays were analysed over the course of the experiment (Fig. 1A). First, 30 non-tagged scallops (10 per condition) were used to assess muscle condition index (MCI), 5 per condition were sampled after the week of exposure, and 5 per condition after the two-week recovery. Additionally, 30 scallops per condition (90 in total) were individually tagged with a RFID chip and weighed and measured once a week over the entire course of the experiment. After the seven days of exposure to the harmful algae, these 30 scallops per condition were challenged with the escape-response test, 15 per condition were sacrificed to perform histological and weight condition index (WCI) measurements, among which 10 per condition were used to measure toxin content in the digestive glands. The 45 remaining, live, tagged scallops (15 per condition) were put back in the tanks for 2 extra weeks of depuration. After the depuration phase, these 45 tagged scallops (15 per treatment) were subjected to the escape response test before being scarified to measure and to perform analyses of toxin content in the digestive glands (on 10 per condition). Shells of all these 120 scallops were collected, after exposure and after depuration, to measure shell growth (Fig. 1B).

Estimation of algal cell consumption

Algal cells (T-*iso*, *C. neogracile,* and *A. minutum*) from the inflow and outflow of the tanks from all conditions were monitored during the exposure phase of the experiment. Cell counts were performed using flow cytometry, as described above. The cytograms allowed us to differentiate the three algal species.

The clearance rate (CR) was evaluated by calculating the consumption of diet algae according to the formula:

$$CR = F \times \frac{Ci - Co}{Co}$$

where F corresponds to the flow rate in L h^{-1},

Ci the cell concentration in the inflow, in cell mL^{-1} ,

and Co the cell concentration in the outflow, in cell mL^{-1}.

During the exposure phase, biodeposits were collected three times per week and observed by fluorescence microscopy to confirm algal ingestion.

Evaluation of escape response

Each tested scallop was placed individually in a 25-L flat, rectangular container filled with filtered seawater at 14°C, following a protocol adapted from Brokordt *et al.* (2006).

The starfish *A. rubens* was selected as the predator, as Guderley et *al.,* (2015) demonstrated that the predatory starfishes evoke the strongest response. The mantle of scallops was thus stimulated with an arm of the starfish. Several indicators of escape response were evaluated (adapted from Brokordt *et al.*, 2006):

1. Reaction time (Rt): the time elapsed between the first contact of the starfish arm with the mantle edge of the scallop and the first clap.
2. Clapping time (Ct): duration of the clapping response.
3. Total time (Tt): total duration of the experiment (=Rt+Ct).
4. Number of claps (Nc): number of claps performed by the scallop to escape the starfish.
5. Clapping rate (Cr): number of claps per second (=Nc/Ct).

After exhaustion, when the scallop stopped clapping, it was allowed to recuperate for 5 minutes before being challenged a second time to test for individual repeatability.

Containers were rinsed after 2-3 scallop assays to eliminate all residues derived from interactions between the starfish and the scallops (flesh residues or molecules). Starfish were changed after 2-3 assays, but the same starfish was used for the two successive stimulations of each scallop.

At the end of the escape response challenge, scallops were dissected for histology and toxin measurements on digestive gland.

Condition index

Condition index was calculated using the wet flesh weight.

The first was the weight condition index (WCI) adapted of Fulton *et al.* (1911) and Ernst *et al.* (1991), based on the individual total weight (Twi (g)) and the shell length (L (mm)):

$$WCI = \frac{Twi \times 10^4}{L^3}$$

The muscular condition index (MCI) was also also assessed, following Fulton *et al.* (1911) and Ernst *et al.* (1991), based on the weight of the adductor muscle (Wam):

$$MCI = \frac{Wam \times 10^4}{L^3}$$

Toxin quantification

After both exposure and recovery phases, PSTs in scallops (n=10) were quantified in the digestive glands of 10 individuals, which is the organ wherein accumulation is maximal (Manfrin *et al.*, 2012). After dissection, digestive glands were preserved in liquid nitrogen before being homogenized and preserved in hydrochloric acid (HCl 0.1 M). Samples were then centrifuged, and hydrolysed by heating at 104°C, and PSTs were quantified using a Saxitoxin (PSP) ELISA kit (Microtiter Plate, Abraxis, Warminster, Pennsylvania, USA), following the manufacturer's protocol.

Histological assessments

Fifteen scallops per condition were examined by eye and microscopically after exposure to search for the eventual occurrence of tissue damage at the end of the exposure period. Individual scallops were prepared for histological analyses as follows: after examination for gross abnormalities, a section of soft-tissues (5 mm thick), including gills, mantle, digestive gland, and adductor muscle, was excised and fixed in Davidson's solution (Shaw and Battle, 1957) for 48h at 4°C before being preserved in 70° ethanol. Tissue sections were dehydrated in ascending ethanol solutions, then cleared with claral, and embedded in paraffin wax. Five-µm thick sections were stained with Harris' hematoxylin and Eosin (Howard *et al.*, 1983), and histopathological lesions were observed under a light microscope. Intensity of each histopathological observation was rated using a three-level, semi-quantitative scale ranging from 0 to 3.

Evaluation of shell growth

Shell growth was assessed in all 120 scallops. Shell growth was measured 20 days after the recovery period, to readjust the curves, for all conditions. External shell growth rings were examined individually at the surface of the external face of the left valve (Clark, 1974; Chauvaud *et al.*, 1998). Scallops (class 0+), spawned in April 2013 (class A), were brought to the laboratory in January 2014 after half a winter in the natural environment in the Bay of Brest. Shells thus included an early winter bulge, characterised by a tightening of growth

rings (Antoine, 1978). Shell growth rate was measured from the outside to the inside (bulge) of the shell, according to a protocol adapted from Chauvaud *et al.* (1998) and Lorrain *et al.* (2002), which preconizes to read the rings along the dorsal-ventral axis only. Growth rings were counted in triplicate along three separate axes (the central axis, as well as two axes 25° right or left from this central axis). To improve the reading, excessively-developed ridges of daily growth rings were removed by a 1 min bath in 10% acetic acid.

The distance between rings was measured under a microscope using image analysis (Visilog 7 software). The distance (D_{AB}) between any two adjacent rings (A and B) was determined using the equation: $D_{AB} = \sqrt{(x_a - x_b)^2 + (y_a - y_b)^2}$,

where X and Y are point coordinates. Pixel measurements were converted into distance (µm) after calibration with known measurement unit (0.4 mm).

Statistical analyses

All statistical analyses were conducted with the R software v. 3.1.2. (Boston, Massachusetts, United States, 2013). An analysis of variance (ANOVA) was performed on data following a normal law (Shapiro test) and showing homogeneity of variances (Bartlett test). A Tukey post-hoc test was further performed to compare the different treatments. Data which did not follow a normal distribution and/or homogeneity were analysed using nonparametric Kruskal-Wallis tests and compared by a multiple comparison test (Wilcoxon test).

Dependent data (same individuals, same condition, different times), were compared with an ANOVA with repeated measures (if normality and/or homogeneity of data were confirmed). If the H1 was rejected ($p<0.05$), a Tukey post-hoc test was used after student test. If normality or homogeneity of data were insufficient for parametric statistics, a Paired Wilcoxon test was applied with function Pairwise Wilcoxon test after false discovery rate (fdr) multiple correction.

For histopathology results, a unilateral Mann–Whitney U test ($\alpha= 0.1$) was used to assess the effects of algal treatments after the exposure phase to *A. minutum*. A Pearson correlation test was used to fit all growth curves between conditions, using shell growth-ring measurements.

Results

Preliminary experiment: Bioassay of cytotoxicity of the two strains of A. minutum

Significant effects of supernatants of both *A. minutum* strains on *C. neogracile* on both chlorophyll pigments (FL3) and efficiency (QY) were observed (Table 1). The BEC *A. minutum* strain was the most allelopathic strain, significantly decreasing quantum yield and chlorophyll fluorescence of *C. neogracile* after only 3.5 h of incubation (Table 1). The PSP *A. minutum* strain also produced some extracellular bioactive compounds with allelopathic effects, but to a lesser extent than the BEC strain (Table 1).

Effects of A. minutum upon scallop physiology

Consumption of food and A. minutum during exposure

Monitoring of algal cells in the inflow and outflow of the tanks showed no significant differences in the amount of T-iso and *C. neogracile* concentrations between the treatments, and no *A. minutum* cells were detected in the CON condition. Microscopic observations of scallop faeces showed that intact cells of *A. minutum* had been ingested but not totally digested (Fig. 2). Again, no *A. minutum* cells were observed in the CON condition.

Clearance rates of scallops exposed to both *A. minutum* strains were significantly lower than those of individuals in the CON condition (Table 2). This decrease in clearance rate was stronger for scallops exposed to the BEC condition, even though it started to recover slightly after five days of exposure.

Effects of A. minutum upon escape response

At the end of the exposure phase, the reaction time of scallops increased significantly in the BEC condition in the second challenge, but not in the first challenge (Fig. 3.A, 3.B, Supplementary data 1). The number of claps and the clapping time were significantly lower in the PST condition for both challenges (Fig. 3.C, 3.D, 3.E, 3.F, Supplementary data 1). Moreover, the clapping time of BEC scallops was significantly higher than control and PST treatments in the second challenge (Fig. 3.F, Supplementary data 1). No significant difference in clapping rate was observed in any of the treatments regardless of the challenge (Fig. 3.G, 3.H, Supplementary data 1).

At the end of the exposure phase, scallops also showed a significantly faster mean reaction time in the second challenge than the first, which corresponded to the first contact with the starfish (Fig. 3.A, 3.B, Table 3) for scallops exposed to both BEC and PST. Scallops exposed to BEC also showed a significantly higher number of claps (Fig. 3.C, 3.D, Supplementary

data 1) and clapping time (Fig. 3.E, 3.F, Supplementary data 1) in the second challenge compared to the first.

After the recovery phase, all differences observed between treatments, as well as between challenges, disappeared (Supplementary data 1).

When comparing the escape response between the exposure and the recovery phase, a few significant differences can be highlighted. After the recovery phase, a significant reduction in the reaction time was observed in the first challenge for the BEC and CON conditions (Supplementary data 1). The number of claps also significantly increased for the BEC and PST conditions in the first challenge (Supplementary data 1). The clapping rate also significantly increased for the first challenge in the BEC condition and the second challenge for the PST condition (Supplementary data 1).

Condition index

No significant differences were found for weight condition index (WCI) and muscular condition index (MCI) between conditions (CON, PST, UEM) or periods (after exposure and recovery).

Paralytic shellfish toxin (PST) accumulation in great scallops exposed to A. minutum

As expected, no PSTs were detected in CON and BEC conditions. In contrast, scallops from the PST condition accumulated toxins in the digestive gland, at a concentration of 4.4 (± 1.87) µg STX.g^{-1}. This concentration appeared to begin to decrease, but not significantly (Kruskal Wallis, p = 0,17) to 1.16 (± 0.16) µg STX.g^{-1} after the two weeks of recovery.

Histological assessment

Histological observations of scallops after the exposure phase showed significant effects of both *A. minutum* strains (Table 3). In particular, the BEC strain caused a significantly higher level of melanisation.

Despite some melanisation and haemocyte infiltration, which occurred regardless of the treatment, no significant effect was found in scallop gills (Table 3). Melanisation and haemocyte infiltrations were, however, significantly higher in the mantle of scallops exposed to both *A. minutum* strains (Table 3, Fig. 4A, B). Overall, the adductor muscles of scallops exposed to both BEC and PST conditions displayed higher levels of haemocyte infiltration, hyalinisation of muscle fibers, and atrophy (Table 3, Fig. 4C, D). In particular, haemocyte infiltration in the adductor muscle were significantly higher in the BEC condition. The presence of brown cells, haemocyte infiltration, and alterations were observed in digestive tubules of all scallops exposed to *A. minutum*, but alterations were significantly higher only in scallops exposed to the PST strain (Table 3, Fig. 4E, F).

Effect of A. minutum upon daily shell growth

Shell growth (number and size of growth rings) appeared equivalent for PST and CON conditions (Fig. 5). In contrast, a mean difference of three daily growth rings was found for scallops exposed to the BEC condition, indicating slower growth. The Pearson correlation coefficient (r) between growth curves of BEC and other conditions was maximal with a three-day shift (Pearson correlation, r= 0.95, p-value= 6.82×10^{-6}). It was thus assumed that these scallops in the BEC treatment had a growth deficit during the first three days of exposure and then expressed the same growth pattern as in the other two treatments after three days (Fig. 5).

Discussion

The present study investigated the effects of two strains of *A. minutum* upon the physiology, growth, and behaviour of juvenile great scallops. To that end, ingestion rate, toxin accumulation, tissue alteration, growth rate, and escape response were investigated after exposure of scallops to each strain. The experiment was also designed to compare the effects of PSTs with those of BEC upon scallop physiology.

Differences between the two tested A. minutum *strains*

The *Chaetoceros* bioassay results presented here show that both strains produce extracellular bioactive compounds with allelopathic effects, but that the BEC *A. minutum* strain has a stronger allelopathic effect, suggesting higher production of BEC or a higher toxicity of this strain. Bioactive extracellular compounds of these two strains have already been shown to affect other cell types, such as haemocytes or spermatozoa (Lambert *et al.* 2013), with the similar intensity as observed in the present study. Indeed, the BEC strain was previously demonstrated to cause responses in oysters *Crassostrea gigas* and clams *Ruditapes philippinarum,* in terms of feeding behaviour (Contreras, 2011) (much more than the PST strain), presumably because of BEC. The cytotoxicity of extracellular compounds released by *Alexandrium* spp. cultures have clearly been shown, with damages to cell membranes inducing immobilization, cell distortion, lysis, and mortality *(*Tillmann *et al*., 2008, Tillmann *et al.,* 2007, Tillmann & John, 2002). Lelong *et al.* (2011) also clearly showed that these mechanisms are associated to damages to the photosynthetic system, which is affected very rapidly after contact with the compounds (less than 30 min). Our results thus highlight in a short amount of time the cytotoxic effect of the extracellular compounds released by *A. minutum.*

Behavioral and physiological effects upon scallops resulting from exposure to BEC

Shell Growth

A 3-day growth delay was observed in the BEC condition. Daily shell growth of scallops *P. maximus* is food-dependent (Antoine 1979; Chauvaud *et al.*, 1998), and a growth delay observed in the wild has been attributed to exposure to the toxic algae *Gymnodinium* cf *nagasakiense* (syn. *Karenia mikimotoi*) (Chauvaud *et al.*, 1998). Congruently, this algal species is known to produce bioactive, extracellular compounds (Arzul *et al.*, 1995; Satake *et al.*, 2002) that may have deleterious effects upon the gills and/or mantle of scallops. As a consequence, scallops may stop feeding in response to BEC, which affects shell growth. Nielsen and Strømgren (1991) have also reported a reduction in shell growth of *Mytilus edulis* after feeding on the toxic alga *Gyrodinium aureolum* (=syn. *K. mikimotoi= G. nagasakiense)*, as well as *Alexandrium ostenfeldii, Chrysochromulina polylepis*, and *Gymnodinium galatheanum.* Exposure to these algae leads to valve closure and reduced filtration, and this may be a response to the bioactive compounds produced by most of these algal species (Smolowitz & Shumway, 1997; Li *et al.*, 2002; Gentien *et al.*, 2006; Krock *et al.,* 2006).

Feeding rate

Reduced filtration rate was observed in great scallops exposed to the BEC strain; scallops were constantly open with tentacles retracted (data not shown). This behaviour was not observed in scallops exposed to the PST strain, which still caused an intermediate, decreased filtration rate. Filtration rate of bivalves has been shown to be relatively unaffected by PST-producing algae (Shumway & Cucci, 1987; Gainey & Shumway, 1988; Li *et al.* 2002; Wilkens, 2006), which suggests that the intermediate response to the PST condition could be induced by the BEC produced by the "PST" AM89BM *A. minutum* strain (as shown by the intermediate allelopathic effect).

Two hypotheses can explain this decrease in filtration rate. First, bioactive compounds excreted by both *A. minutum* strains may have allelopathic effects upon food cells (T-*iso* and *C. neogracile*), either affecting nutritional value or inducing the formation of undigested aggregates. As stated in the Introduction, the allelopathic and deleterious effects of *Alexandrium* spp. have been documented on other algal species (Arzul *et al.* 1999; Tillman *et al.* 2007; Lelong *et al.*, 2011). Additionally, BEC could also induce the production of Transparent Exopolymeric Particles (TEPs) by food algae (Villacorte *et al.*, 2013), causing them to form aggregates, and clogging scallop gills. Thus, BEC could affect sympatric

microalgal taxa and indirectly affect food quality and availability for scallops. To test this hypothesis, growth, physiology and general welfare of T. *Iso* and *C. neogracile* should be assessed in the presence of *A. minutum*. Second, cytotoxicity could affect scallop tissues directly, notably gills. Filtration rate, and thus food intake, could then be hindered. Negative effects of extracellular compounds upon tissues have been hypothesized to explain a stronger decrease of filtration rates in oysters *C. gigas* and clams *Ruditapes philippinarum*, as well as inhibition of the clearance rate in *C. gigas,* during exposure to the BEC strain (Contreras, 2011). Similarly, unknown extracellular polysaccharides have been suspected to affect the feeding activity and clearance rate of *Mytilus edulis* and *Mercenaria mercenaria* exposed to *Aureococcus anophagefferens* (Tracey, 1988; Bricelj & Lonsdale, 1997 Bricelj *et al.*, 2001). This direct contact with brown tide cells was firstly highlighted by Ward & Targett (1989a, b). Feeding rate of scallops exposed to BEC in the present study could be affected in a similar way. Histological observations of the present study reinforced this hypothesis. Overall, melanisation in gills was higher in great scallops exposed to the BEC strain than for both other conditions. Further, there were significant effects of both, BEC and PST strains upon the mantle. Melanisation of the mantle and gills has been described in other scallop species exposed to *A. catenella* (Hégaret *et al.* 2012). This species of algae produces lytic compounds and can induce haemocyte infiltration and melanisation with direct contact with tissues (Basti *et al.* 2015). Histology revealed haemocyte infiltration higher in the BEC condition, with trends for mantle and digestive gland, and significantly, for muscle. All these observations could indicate direct tissue damage by the harmful alga.

Escape behaviour

The higher reaction time of scallops exposed to the BEC strain clearly indicated an effect of bioactive, extracellular compounds. The escape response of BEC-exposed scallops seemed to be less effective following contact with starfish. Chemosensitivity is involved in the escape response of scallops, and the behavioural reflex of escape is dependent upon tactile and chemical stimuli that are perceived by ciliated epithelial cells of the mantle, particularly on tentacles (Wilkens, 2006). Present results suggest that the recognition of a predator may be disrupted by direct tissue damage (such as melanisation) caused by bioactive, extracellular compounds from *Alexandrium*. The decrease in reaction time between the first and second challenge in all conditions suggested a faster recognition of predator after a first contact. The clapping time was also significantly higher in the BEC condition. At the beginning of the escape response, the striated adductor muscle allows for quick shell closures creating a jet-propulsion of water from the mantle cavity (Wilkens, 2006). But, during following swimming

episodes, the smooth adductor muscle is used to gradually reopen the valves (Wilkens, 2006). So, an increase in clapping time, as observed here, might result from the response of the smooth adductor muscle to bioactive compounds, as suggested by the haemocyte infiltrations into the muscle of BEC-exposed scallops. Such haemocyte infiltrations in the adductor muscle have previously been observed in *A. ventricosus* exposed to *Gymnodinium catenatum* (Escobedo-Lozano *et al.* 2012), and this has been interpreted as a defence mechanism to evacuate bioactive extracellular compounds and limit the alteration of the muscle.

Behavioral and physiological effects associated to exposure to PST

Toxin accumulation in scallops exposed to the PST strain indicated that some toxic microalgae were ingested. Quantification of STX was performed in the digestive glands of scallops, the principal organ of PST accumulation (Estrada *et al*., 2010), where most alterations could also be observed. These alterations of digestive gland observed mainly in the PST condition could be linked to inflammatory responses (Habekorn *et al.,* 2010a) of scallops responding to the presence of saxitoxin and its derivates. The saxitoxin and derivates could indeed cause damage in the digestive gland by being directly assimilated and biotransformed, or by being directly released in the digestive gland.

No effect of the PST strain exposure was observed upon scallop growth, but a significant effect upon escape response was found, potentially increasing scallop vulnerability to predators, as previously stated by Brokordt et *al.* (2013) with hypoxia. The number of claps and clapping time of scallops exposed to the PST strain were significantly lower in both challenges. Numerous studies have shown alteration of valve activity after exposure to toxic microalgae. Hence, *A. catenella* significantly degraded the escape response of *Argopecten purpuratus* (Hégaret *et al*. 2012*),* and *A. monilatum* reduced the valve gape in three shellfish species (*M. mercenaria, C. virginica, P. viridis*) (May *et al.,* 2010*)*. Moreover, swimming/clapping activity patterns were shorter during exposure to *Protogonyaulax tamarensis* (=syn. *A. tamarense*) (Shumway & Cucci, 1987), and valve movements of the scallop *N. subnodosus* can be modified by exposure to PST producers (Estrada *et al.*, 2010). Such modifications in valve activity might be attributable to the deleterious effects of PSTs upon the adductor muscle, as suggested by the correlation reported in *C. gigas* between the accumulation of PSTs in the digestive gland and the reduction in valve-opening amplitude (Mat *et al.*, 2013), or the increase in the frequency of opening duration (Haberkorn *et al.*, 2011). Results presented here showed myopathy of the adductor muscle in scallops exposed to both *A. minutum* strains, characterised by myoatrophy and hyaline degeneration of the muscle fibers. These results are consistent with observations in juvenile scallops *A.*

purpuratus exposed to *A. catenella* (Hégaret *et al.*, 2012), or in oysters *C. gigas* exposed to *A. minutum* (PST-producing strain) (Haberkorn *et al.* 2010a). Such myopathy could result from the direct effect of PSTs on muscle fibers, as hypothesized in *C. gigas* (Haberkorn *et al.* 2010a). Bricelj *et al.* (2005) showed that burrowing of *Mya arenaria* was not affected by exposure to non-PST-producing *A. tamarense* strain (CCMP115), but that incapacity to burrow was toxin-induced in clams and resulted from muscle paralysis (Bricelj *et al.*, 1990; 2005). The link between the accumulation of PSTs and the paralysis of the adductor muscle has been observed in several bivalve species, such as *C. gigas, M. edulis* or *A. ventricosus* exposed to PST-producing dinoflagellates (Hégaret *et al.*, 2007; Galimany *et al.*, 2008; Escobedo-Lozano *et al.*, 2012). Moreover, the injection of gonyautoxin (GTX, a saxitoxin-like toxin), produced by *G. catenatum*, into the muscle of the scallop *N. subnodosus* induced paralysis (Estrada *et al.*, 2010). The paralytic effects of PSTs are caused by STX and derivatives, which block sodium channels (Bricelj *et al.*, 2005) and can block nervous transmissions. The locomotory system depends upon the neuromuscular system: nervous information is sent through sodium channels to initiate movement by muscle contractions (Catterall, 1992). Thus, the decrease in the number of claps and clapping time observed in the present study is consistent with the action of STX upon scallop muscle by alteration of the neuro-muscular system, hindering the capacity of the muscle to contract and produce movement.

Conclusion

A *Chaetoceros neogracile* allelopathic assay clearly demonstrated the production of bioactive, extracellular compounds by both *Alexandrium* strains studied. This confirms that *Alexandrium* spp. can produce different types of toxic compounds, such as paralytic shellfish toxins, but also allelopathic compounds, which are not always related to each other.

Physiological and behavioural responses of scallops were clearly different between the two *A. minutum* strains. Scallops exposed to the "PST" strain, showed a decrease in the number of claps and clapping time. However, paralytic shellfish toxins did not seem to affect the recognition processes when exposed to starfish. Conversely, altered recognition processes could be observed in scallops exposed to the non-PST strain, which could be associated to the alteration of tissues responsible for this recognition. These alterations can be caused by the cytotoxic effects of BEC released by *A. minutum*, wich are also likely responsible for the reduction in filtration rate, thus causing cessation of growth by altering the tissues.

These results clearly highlight the importance of working with different strains of *Alexandrium* sp. when assessing their possible interactions with other marine organisms, as a species producing high levels of PST may not be as toxic to other organisms as a strain producing a higher level of bioactive extracellular compounds. This study also demonstrates the importance to better characterize these extracellular bioactive compounds to better understand their interactions with sympatric organisms, such as phytoplankton species via allelopathy, but also other marine organisms via ichtyo- or cytotoxicity.

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Figures

Fig. 1: Experimental design of the experiment **A:** Juvenile scallops fed with a regular diet of *C. neogracile* and T-iso for 45 days of the acclimation phase, before being exposed for one week to the two *A. minutum* strains, one producing BEC (BEC condition), other producing PST (PST condition), and a control diet, similar as during acclimation phase (CON condition). Scallops were then fed with a regular diet of *C. neogracile* and T-iso for an extra two weeks (depuration phase). **B:** Forty (30 tagged and 10 non-tagged) scallops per condition were used for analyses: After the seven days of exposure, 5 scallops per condition were sampled for muscle condition index, 30 tagged scallops per condition were submitted to the escape response test, half of them were then sacrificed in order to perform histological measurements (on 15 individuals per condition), weight condition index (on the same 15 individuals per condition) and toxin analyses in the digestive glands (on 10 individuals per condition). The 20 sacrificed scallops per condition were analysed for shell growth rate. After the depuration phase, 5 scallops per condition were sampled for muscle condition index, and the 15 remaining tagged scallops per condition were submitted to the escape response test, before being sacrificed in order to perform analyses of weight condition index (on 15 individuals per condition) and toxin content in the digestive glands (on 10 scallops per condition).

Fig. 2: Light and epifluorescent pictures of fecal pellets of king scallops *Pecten maximus* containing intact *A. minutum* cells in scallops exposed to PST-*A. minutum* strain (PST) (A) or in scallops exposed to BEC *A. minutum* strain (BEC) (B)

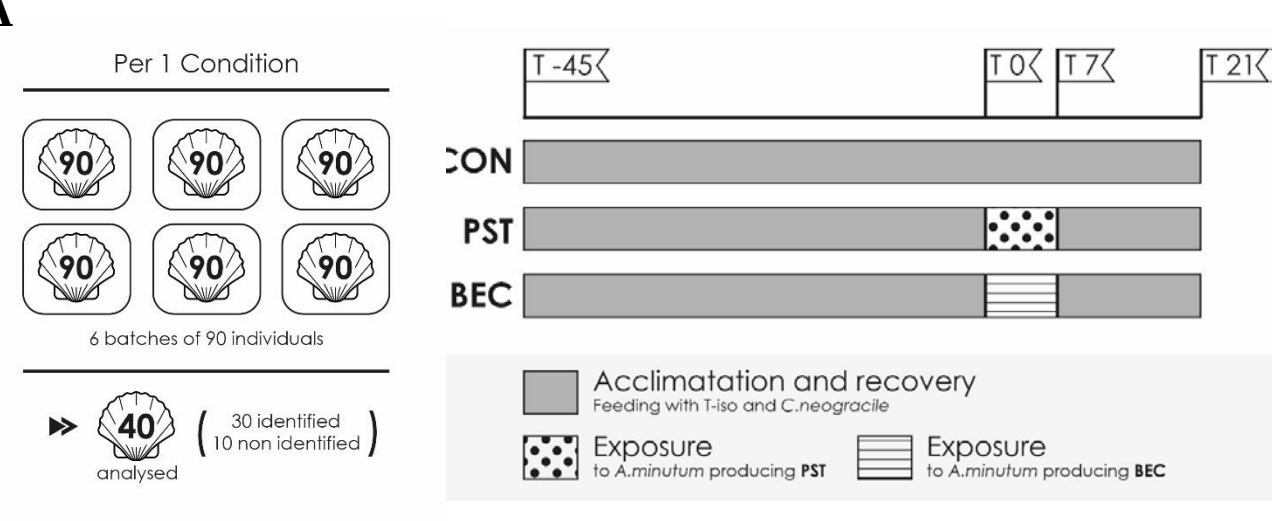
Fig. 3: Escape response of *P. maximus* to predator; fed the control diet (CON), BEC *A. minutum* strain (CCMI1002) (BEC), or PST-*A. minutum* strain (AM89BM) (PST), in terms of reaction time (A, B), number of claps (C, D), clapping time (E, F) and clapping rate (G, H). (Kruskal-Wallis, P>0,05; median +/- quantiles, cross = mean. Letters a, b, c apply to significant differences between treatments for each challenge ($p<0.05$). Fig. A, C, E, G: challenge 1; Fig. B, D, F, H: challenge 2. The second challenge (challenge 2) was performed 5 minutes after the first challenge (challenge 1), in order to test the repeatability of response.

Fig. 4: Histopathological observations in control scallops (A, C, E), in scallops exposed to BEC *A. minutum* strain (BEC) (B) and in scallops exposed to PST-*A. minutum* strain (PST)

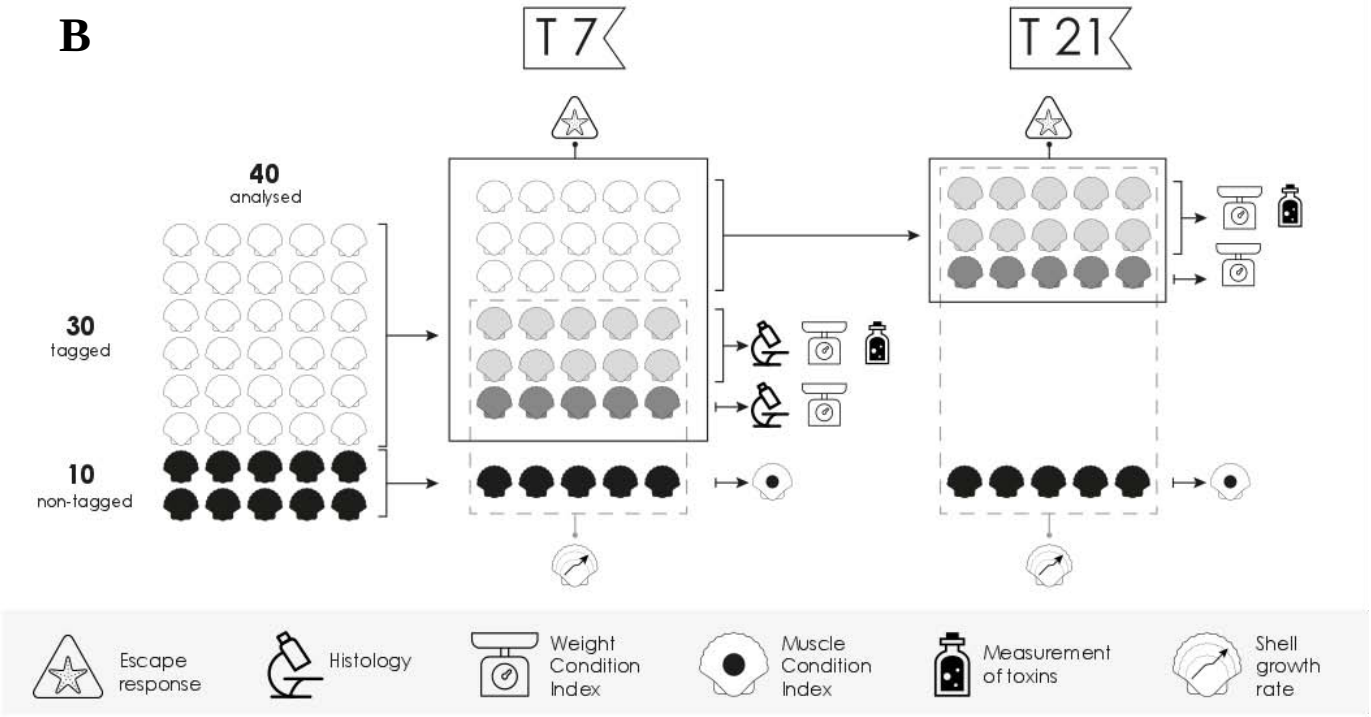
(D, F) on distinct tissues: mantle (A, B), adductor muscle (C, D) and digestive gland (E, F). **M**: Melanisation of mantle tissues in BEC scallops (B). **H**: Hyalinisation of muscle fibers in PST scallops (D). **a**: Digestive tubule alteration of PST scallops (F).

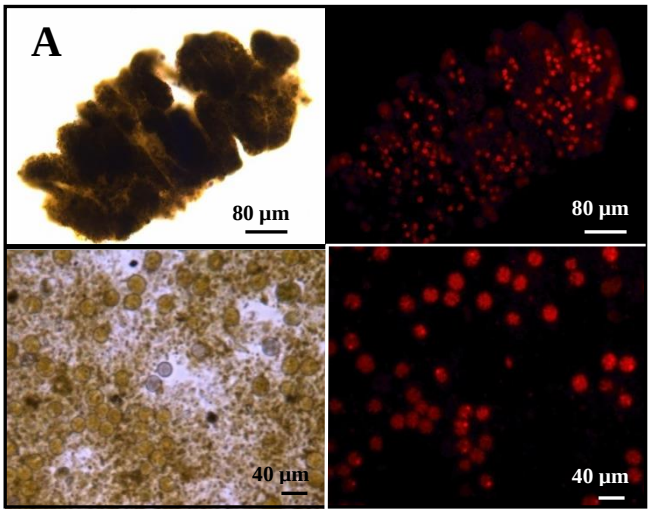
Fig. 5: Reconstituted growth curves of scallops fed the control diet (CON) (continuous line), scallops exposed to PST-*A. minutum* strain (PST) (dashed line) and scallops exposed to BEC *A. minutum* strain (BEC) (dotted line). Acclimation period with T-*iso*/*C. neogracile*, exposure period to *A. minutum* strains and recovery period with T-*iso*/*C. neogracile* are represented. A stop of growth is simulated for the BEC condition from 2 days of exposition.

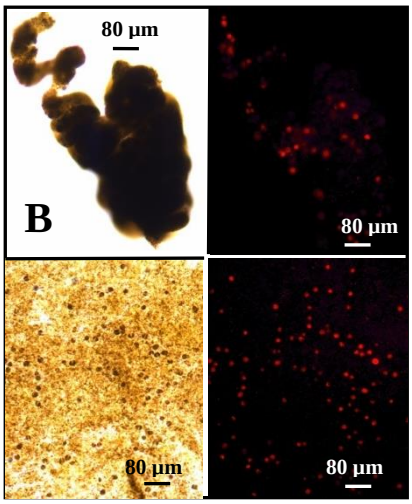
A


Per 1 Condition
90
6 batches of 90 individuals
40
analysed
30 identified
10 non identified
T -45
T 0
T 7
T 21
CON
PST
BEC
Acclimatation and recovery
Feeding with T-iso and C.neogracile
Exposure
to A.minutum producing PST
Exposure
to A.minutum producing BEC

B


T 7
T 21
40
analysed
30
tagged
10
non-tagged
Escape response
Histology
Weight Condition Index
Muscle Condition Index
Measurement of toxins
Shell growth rate


A
80 µm
80 µm
40 µm
40 µm


80 µm
B
80 µm
80 µm
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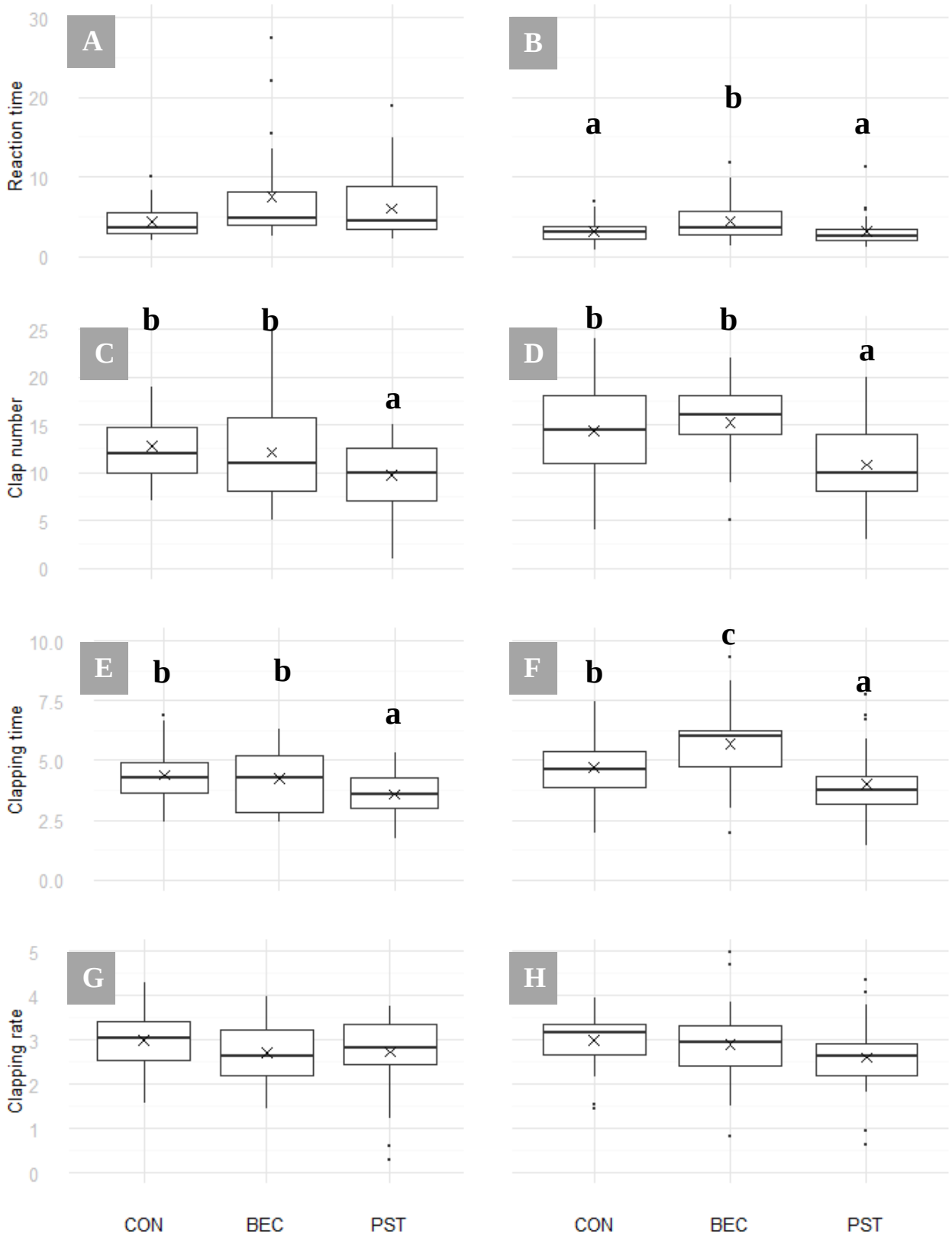
A
B
C
D
E
F
G
H
Reaction time
Clap number
Clapping time
Clapping rate
a
b
b
a
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b
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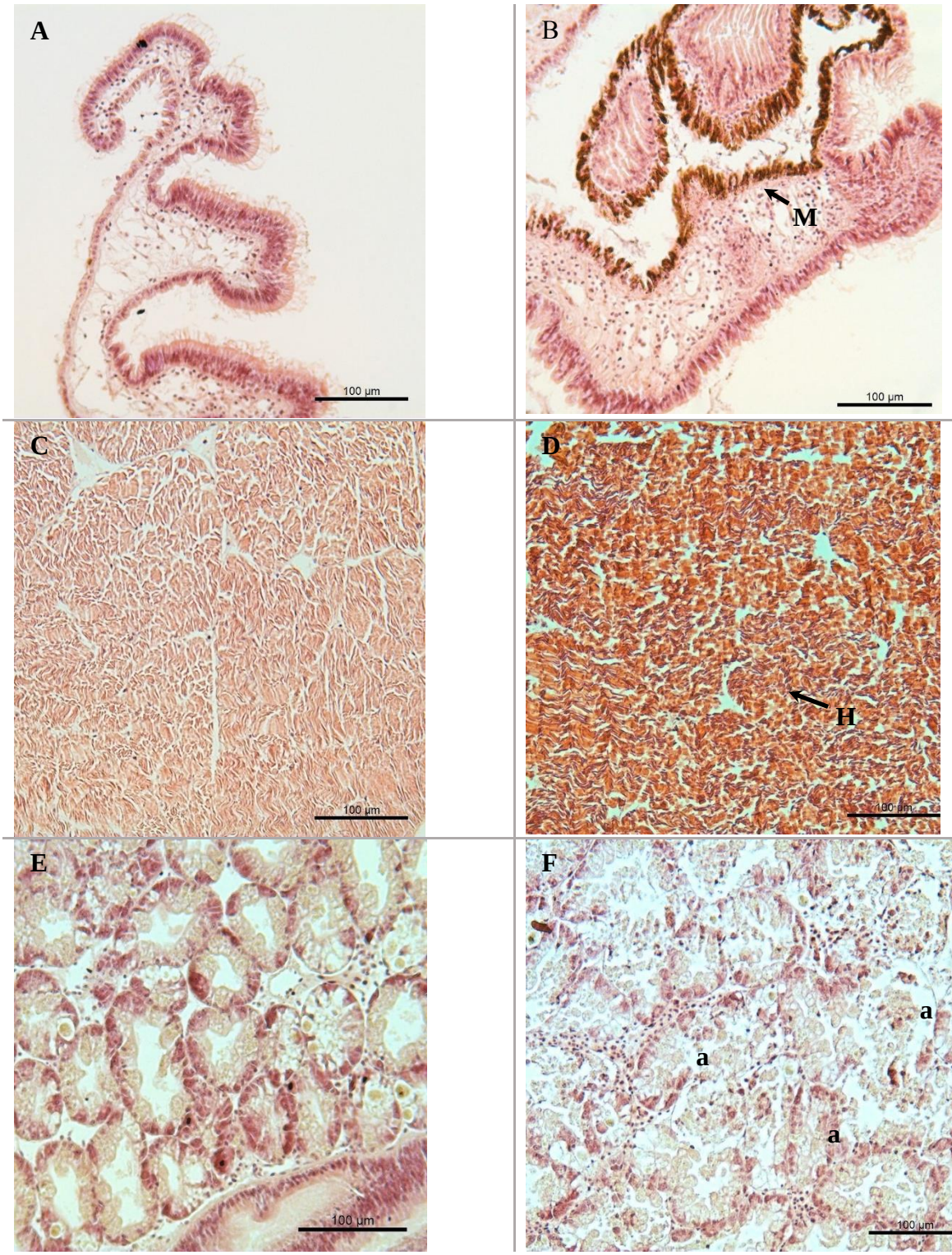
c
a
CON
BEC
PST
CON
BEC
PST


A
100 µm
B
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D
H
100 µm
E
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F
a
a
a
100 µm

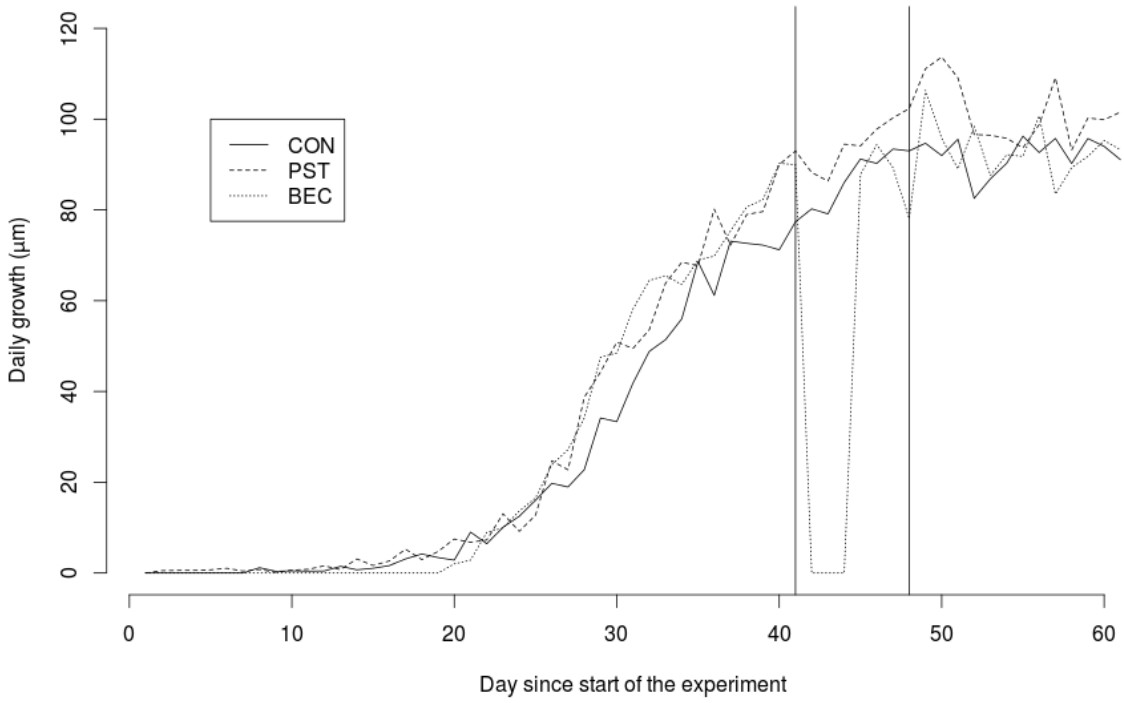
CON
PST
BEC
Daily growth (µm)
0
20
40
60
80
100
120
0
10
20
30
40
50
60
Day since start of the experiment

Table 1: Impact of supernatant of two strains of *A. minutum* (PST and BEC) or a L1-medium control on quantum yield and "FL3" chlorophyll fluorescence of the diatom *C. neogracile* (n=3, ANOVA, Mean ± SE, letters a,b,c apply to significant differences among treatments)

	P-value	**CON**	**BEC**	**PST**
Quantum Yield	P<0,01	0,67±0,010 (a)	0,42±0,005 (c)	0,61±0,033 (b)
FL3	P<0,01	319,0±9,88 (a)	214,1±3,56 (c)	280,6±6,56 (b)

Table 2: Clearance rate ($L \cdot h^{-1} \cdot ind^{-1}$) of scallops *P. maximus* after three and five days of exposure the control diet (CON) or exposed to BEC *A. minutum* strain (CCMI1002) (BEC), or PST-*A. minutum* strain (AM89BM) (PST). (ANOVA, Mean ± SE; letters a, b, c apply to significant differences among treatments).

Days of exposure	**P-value**	**CON**	**BEC**	**PST**
Three days	P<0,05	4,12±0,87 (a)	0,29±0,007 (c)	2,03±0,1 (b)
Five days	P<0,05	3,67±0,17 (a)	1,01±0,1 (bc)	1,94±0,29 (b)

Table 3: Impact of two strains of *A. minutum* (BEC and PST) on tissues of king scallops compare to control (CON). Results are expressed as mean of stage intensity ± SE (n=15 per condition, Kruskal-wallis, *: $p < 0.05$, #: $p < 0.10$; letters a, b, c apply to significant differences among treatments).

Tissues	Histological features	CON	BEC	PST	P-value
Gills	**Melanisation**	0,86 ±0.18	1,38 ±0.13	0,97 ±0.17	NS
	Hemocyte infiltration	2.07 ±0.16	1.93 ±0.19	2 ±0.19	NS
Mantle	**Melanisation**	0,73 (a) ±0.16	1,46 (b) ± 0.19	1,5 (b) ±0.22	*
	Hemocyte infiltration	1.21 (a) ±0.11	2.17 (b) ±0.22	1.93 (b) ±0.24	*
Muscle	**Hyalinisation of muscle fibers**	0.68 ±0.12	1.30 ±0.25	1.13 ±0.26	NS
	Atrophy	0.59 ±0.19	1 ±0.26	1.33 ±0.31	NS
	Hemocyte infiltration	0 (a)	0.34 (b) ±0.16	0 (a)	*
	Hyalinisation + Atrophy + Hemocyte infiltration	1.18 (a) ±0.29	2.65 (b) ±0.41	2.47 (b) ±0.36	*
Digestive gland	**Presence of brown cells**	1.5 ±0.15	1.54 ±0.17	1.5 ±0.17	NS
	Hemocyte infiltration	1.29 ±0.17	1.36 ±012	1.32 ±0.13	NS
	Alterations	0 (a) ±0	0.27 (a) ±0.26	1 (b) ±0.20	#
All tissues	**Mean of all melanisation**	1.58 (a) ±0.24	3 (b) ±0.25	2,5 (a) ±0.30	#
	Mean of all hemocyte infiltration	4.5 ±0.25	5.54 ±0.46	5.31 ±0.45	NS
	Mean of all histological features	6.8 (a) ±0.17	9.14 (b) ±0.70	9.80 (b) ±0.71	#