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The toxic dinoflagellate *Alexandrium minutum* affects oyster gamete health and fertilization potential

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Abstract :

Dinoflagellates from the globally distributed genus Alexandrium are known to produce both paralytic shellfish toxins (PST) and uncharacterized bioactive extracellular compounds (BEC) with allelopathic, ichthyotoxic, hemolytic and cytotoxic activities. In France, blooms of Alexandrium minutum appear generally during the spawning period of most bivalves. These blooms could therefore alter gametes and/or larval development of bivalves, causing severe issues for ecologically and economically important species, such as the Pacific oyster Crassostrea (=Magallana) gigas. The aim of this work was to test the effects of three strains of A. minutum producing either only PST, only BEC, or both PST and BEC upon oyster gametes, and potential consequences on fertilization success. Oocytes and spermatozoa were exposed in vitro for 2 hours to a range of environmentally realistic A. minutum concentrations (10 to 2.5 × 104 cells mL-1). Following exposure, gamete viability and reactive oxygen species (ROS) production were assessed by flow cytometry, spermatozoa motility and fertilization capacities of both spermatozoa and oocytes were analysed by microscopy. Viability and fertilization capacity of spermatozoa and oocytes were drastically reduced following exposure to 2.5 × 104 cells mL-1 of A. minutum. The BEC-producing strain was the most potent strain decreasing spermatozoa motility, increasing ROS production of oocytes, and decreasing fertilization, from the concentration of 2.5 × 103 cells mL-1. This study highlights the significant cellular toxicity of the BEC produced by A. minutum on oyster gametes. Physical contact between gametes and motile thecate A. minutum cells may also contribute to alter oyster gamete integrity. These results suggest that oyster gametes exposure to A. minutum blooms could affect oyster fertility and reproduction success.

Highlights

► Oyster spermatozoa and oocytes were exposed *in vitro* to *Alexandrium minutum* strains. ► Viability and fertilization capacity of gametes were drastically reduced. ► The toxicity of *Alexandrium minutum* upon oyster gametes is strain-specific. ► A non-PST-producing strain increased reactive oxygen species production in oocytes. ► This strain also decreased spermatozoa motility and gamete fertilization.

Keywords : Algal blooms, Algal toxins, Fertilization, Paralytic Shellfish Toxins (PST), Bioactive Extracellular Compounds (BEC), Spermatozoa, Oocyte, Flow cytometry, Crassostrea (=Magallana) gigas, Bivalves.

1. Introduction

Fertilization is essential for reproduction and population sustainability (Marshall et al., 2002). Most marine bivalve molluscs are broadcast spawners depending on external fertilization. Seawater quality is a main factor affecting fertilization and larval development successes in these organisms (Havenhand et al., 2008). In particular, contaminants could negatively affect their reproduction as demonstrated in oysters exposed to anthropogenic pollutants (Akcha et al., 2012; Fitzpatrick et al., 2008; Mai et al., 2013; Vignier et al., 2017, 2015). Experimental studies suggest that harmful algal blooms (HAB), often caused by dinoflagellates, can affect marine bivalve reproduction by altering gamete quality and larval development, growth, and survival (Banno et al., 2018; Basti et al., 2013, 2011; Binzer et al., 2018; Bricelj and MacQuarrie, 2007; Castrec et al., 2020, 2019; De Rijcke et al., 2015; Gaillard et al., 2020; Rolton et al., 2018, 2015, 2014; Tang and Gobler, 2012). In coastal areas, HAB are a recurring phenomenon that can co-occur with the reproduction of free spawning marine organisms (Gaillard et al., 2020).

In France, blooms of *Alexandrium minutum* usually occur from April to November (Pouvreau et al., 2016) overlapping with Pacific oyster breeding and spawning periods (Fig. S1). An understanding of the effects of *A. minutum* cells and its toxic compounds on gametes and early life stages of *Crassostrea* (=*Magallana*) *gigas* (Bayne et al., 2017) is therefore critically important, considering the economic and ecological importance of this bivalve species. The known consequences of *Alexandrium* blooms are both ecological (e.g., alteration of marine trophic structure, large-scale mortality of fish and shellfish) and economic (e.g., impairment of tourism and recreational activities, fishery closure and sell prohibition of shellfish) (Anderson et al., 2012b).

HAB species from the genus *Alexandrium* are known to produce paralytic shellfish toxins (PST) that can cause human poisoning through shellfish-mediated intoxications (Bricelj and

Shumway, 1998) and/or poorly uncharacterized bioactive compounds exerting allelopathic, hemolytic, ichthyotoxic or cytotoxic activities (Arzul et al., 1999; Bianchi et al., 2019; Castrec et al., 2020, 2018; Ford et al., 2008; Lelong et al., 2011; Long et al., 2018a, 2018b; Mardones et al., 2015). These bioactive compounds were proposed to be implicated in the toxicity of *Alexandrium* upon early embryo development of several marine bivalves, such as *Mytilus edulis* (De Rijcke et al., 2016), *C. gigas* (Castrec et al., 2020; Mu and Li, 2013), and *Pinctada fucata martensii* (Basti et al., 2015), independently of the well-known toxins PST, gonyautoxins, and spirolids. However, no clear demonstration of the respective effects of the different toxic compounds has been provided. In addition to the well-known and characterized PST, recent studies demonstrated the toxicity of unidentified bioactive extracellular compounds (BEC) produced by *A. minutum* on other microalgae species (Lelong et al., 2011; Long et al., 2018a, 2018b) and their deleterious effects upon the behavior of the scallop *Pecten maximus* (Borcier et al., 2017) and physiology of the oyster *C. gigas* (Castrec et al., 2018). These BEC were supposed to affect oyster oocytes exposed *in vitro* (Le Goïc et al., 2014) as well as spermatozoa derived from exposed oysters (Castrec et al., 2019; Haberkorn et al., 2010). To our knowledge, the respective involvement of PST and BEC in *A. minutum* toxicity toward *C. gigas* gametes is not yet clearly established.

In the present study, we investigated the toxicity of *A. minutum* on *C. gigas* gametes by measuring their cellular characteristics by flow cytometry and the fertilization success of spermatozoa or oocytes by microscopy. Spermatozoa motility and swimming velocity were analysed by microscopy and image analysis. To determine the respective toxicity of BEC and PST, three *A. minutum* strains were tested, either producing only PST, only BEC, or both PST and BEC.

2. Materials and methods

2.1 Algal strains

Three strains of *Alexandrium minutum* (Halim) were grown in filtered seawater (0.2 µm) supplemented with L1-Si medium (Guillard and Hargraves, 1993) and maintained at 17 ± 1 °C, under a continuous light intensity of 100-110 µmol photons m^{-2} s^{-1}. The *A. minutum* strains were (i) the PST+BEC strain (strain AM89BM, isolated from a bloom in the Bay of Morlaix, France) producing 10.6 fmol PST $cell^{-1}$ (corresponding to 1.3 pg STX eq. $cell^{-1}$; Fabioux et al., 2015); (ii) the PST strain (strain Da1257, isolated from a bloom in the Bay of Daoulas, France) producing 0.63 fmol PST $cell^{-1}$ (corresponding to 0.053 pg STX eq. $cell^{-1}$; Pousse et al., 2018); (iii) the BEC strain (strain CCMI1002, isolated from Irish waters), for which no PST were detected (Borcier et al., 2017).

According to the bioassay developed by Long et al. (2018a), the BEC strain appears four times more cytotoxic than the PST+BEC strain, whereas the PST strain has a negligible cytotoxic potency. Cultures were not axenic, grown without antibiotics, and harvested in exponential growth phase for the experiments. Algal concentrations (cells mL^{-1}) were determined by counts using a FACScalibur (BD Sciences, San Jose, CA, USA) flow cytometer equipped with a blue laser (excitation 488 nm) according to Marie et al. (1999). For gamete cellular responses and fertilization experiments, cultures were re-suspended in filtered seawater 24 h prior to the exposures to reach cell concentrations of 2×10^1, 2×10^2, 5×10^3, and 5×10^4 cells mL^{-1}.

2.2 Oysters

For the experiment assessing cellular responses of gametes exposed to *A. minutum*, adult Pacific oysters (n = 5 females and n = 4 males) *C. gigas* were collected from Daoulas estuary in the Bay of Brest (North Brittany, France). For the fertilization experiment, adult oysters (n = 3 females and n = 3 males) originated from a cohort produced in 2017 according to Petton et al. (2015) and were deployed as early-juveniles (i.e. spat) in the bay of Brest. Gravid oysters were collected during the natural reproduction period (June and July 2018).

2.3 Collection of gametes

Oyster gender determination and gamete quality check, i.e. round shape of oocytes and spermatozoa motility, were performed by examination of 2 µL gonad samples under a light microscope (Leica MZ 125, ×10 objective) before each experiment. Oocytes and spermatozoa were then collected by stripping gonads individually (Boulais et al., 2015a, b). Briefly, gametes were collected in 0.2-µm filtered seawater (FSW; pH 8.12, salinity 34.7, 21 °C) and sieved through 100-µm mesh to eliminate cellular debris. Cell concentrations were determined individually by flow cytometry (FCM) (duplicate, EasyCyte Plus cytometer, Guava Millipore Luminex) according to Le Goïc et al. (2014, 2013). The concentration of oocyte suspensions were adjusted to 1×10^5 cells mL^{-1} with FSW. Spermatozoa suspensions were diluted to 1×10^8 cells mL^{-1} with FSW for movement measurements, and to 2×10^6 cells mL^{-1} for gamete cellular responses and fertilization experiments.

2.4 Gamete cellular responses experiment: spermatozoa movement and gamete FCM analyses

For each oyster, 2.5 mL of gamete suspension (see section 2.3) were exposed for 2 hours to 2.5 mL of the *A. minutum* suspensions (3 *A. minutum* strains × 4 concentrations) or to 2.5 mL of FSW (control treatment) in 10 mL glass vials for FCM analyses. For spermatozoa motility analyses, 0.5 mL subsample of spermatozoa suspension from each male was transferred in 2 mL glass vials containing 0.5 mL of the *A. minutum* suspensions or 0.5 mL of FSW (control treatment). After 2 hours of exposure, spermatozoa were sampled for motility measurements. Final *A. minutum* concentrations were 10, 10^2, 2.5×10^3, and 2.5×10^4 cells mL^{-1}. These concentrations were chosen to reflect the range of algal densities observed during natural blooms (Chapelle et al., 2015). In France, the alert threshold triggering weekly concentration monitoring for *A. minutum* blooms is 10 cells mL^{-1} (Guallar et al., 2017).

FCM measurements for spermatozoa and oocytes (i.e. relative cell size and complexity, viability (using Sybr-14 and propidium iodide, final concentration 1 µM and 10 µg L^{-1}, respectively) and reactive oxygen species production (using DCFH-DA, final concentration 10 µM)), were performed using an EasyCyte Plus cytometer (Guava Millipore Luminex) equipped with standard optics and a 488 nm argon laser according to Le Goïc et al. (2013, 2014).

Spermatozoa movement was analyzed under phase contrast microscope coupled to a video camera (Qicam Fast 1394) according to (Boulais et al., 2018). Briefly, each spermatozoa suspension was diluted 2 times in FSW containing pluronic acid (final concentration of 1 g L^{-1}) to avoid spermatozoa sticking to the cell surface. Then, 12 µL of diluted spermatozoa suspension was immediately transferred to a Fast-Read® cell 102 (Fisher Scientific) and the percentage of motile spermatozoa and their velocity (VAP: Velocity of the Average Path) were assessed on a minimum of 30 spermatozoa for each sample, using the ImageJ software and a CASA plug-in calibrated to Pacific oyster (Boulais et al., 2015b).

2.5 Fertilization experiment

For each oyster, 2.5 mL of gamete suspension (oocytes or spermatozoa, see section 2.3) were exposed for 2 hours to 2.5 mL of the *A. minutum* culture (3 *A. minutum* strains × 4 concentrations) or to 2.5 mL of FSW (control treatment) in 10 mL glass vials. Fertilization of exposed oocytes was performed using a pool of control spermatozoa, at 5×10^7 cells mL^{-1} prepared from the initial spermatozoa suspensions of 3 males (see section 2.3). Fertilization of exposed spermatozoa was performed using a pool of control oocytes, at 5×10^4 cells mL^{-1}, prepared from initial oocyte suspensions of 3 female oysters (see section 2.3). After the exposure to *A. minutum*, exposed oocytes (5×10^3 oocytes) were transferred to 24-well microplates (NUNC©) filled with 2 mL of FSW and 10 µL of the non-exposed spermatozoa pool (100:1 spermatozoa:oocyte ratio). Similarly, exposed spermatozoa (5×10^5 spermatozoa)

were transferred to 24-well microplates (NUNC©) filled with 1.4 mL of FSW and 100 µL of the non-exposed oocytes pool (100:1 spermatozoa:oocyte ratio). After 2 hours of contact between spermatozoa and oocytes, samples were fixed with a formaldehyde-seawater solution (1% final) to estimate the fertilization yield using a light microscope (Leica DM-IRB). Numbers of fertilized (2-cell or 4-cell embryos) and unfertilized (absence of segmentation) oocytes were counted, on at least 100 oocytes per well. Fertilization yield was defined as number of fertilized oocytes × 100 / number of unfertilized and fertilized oocytes. Each oocytes/spermatozoa fertilization assay was performed in triplicate, the mean of the triplicate fertilizations was used in statistical tests.

2.6 Statistical analyses

Statistical analyses and graphical representations were performed using R software (R Core Team, 2012). Differences were considered significant when $p < 0.05$. All values are expressed as mean ± standard error (SE). Normality and homogeneity of variance were verified by the Shapiro-Wilk and Levene methods, respectively. Cellular characteristics measured by flow cytometry and fertilization yields were compared between the 13 treatments (3 *A. minutum strains* × 4 concentrations, and the control) for oocytes and spermatozoa using repeated measures ANOVA (‘nlme’ package), with ‘treatment’ as a fixed factor and ‘oyster’ as a random factor. For all models, we checked the assumption of homogeneity of variance and normality of residuals graphically using normal quantile-quantile plots and scale-location plots of sqrt(|residuals|). The viability variable was transformed as arcsin(squareroot) before statistical analysis to pass normality, but were presented as non-transformed data in figures. A repeated measures ANOVA was used to test the effect of *A. minutum* exposure on spermatozoa motility and velocity, accounting for oyster as a random factor. If the effect of treatment was significant, post-hoc analyses were performed using emmeans function with Tukey’s correction.

3. Results

3.1 Gamete cellular responses

The viability of spermatozoa ($F = 23.47$, $p < 10^{-12}$) and oocytes ($F = 12.76$, $p < 10^{-10}$) were only affected at the highest *A. minutum* concentration tested, i.e. 2.5×10^4 cells mL^{-1} (Fig. 1A, B).

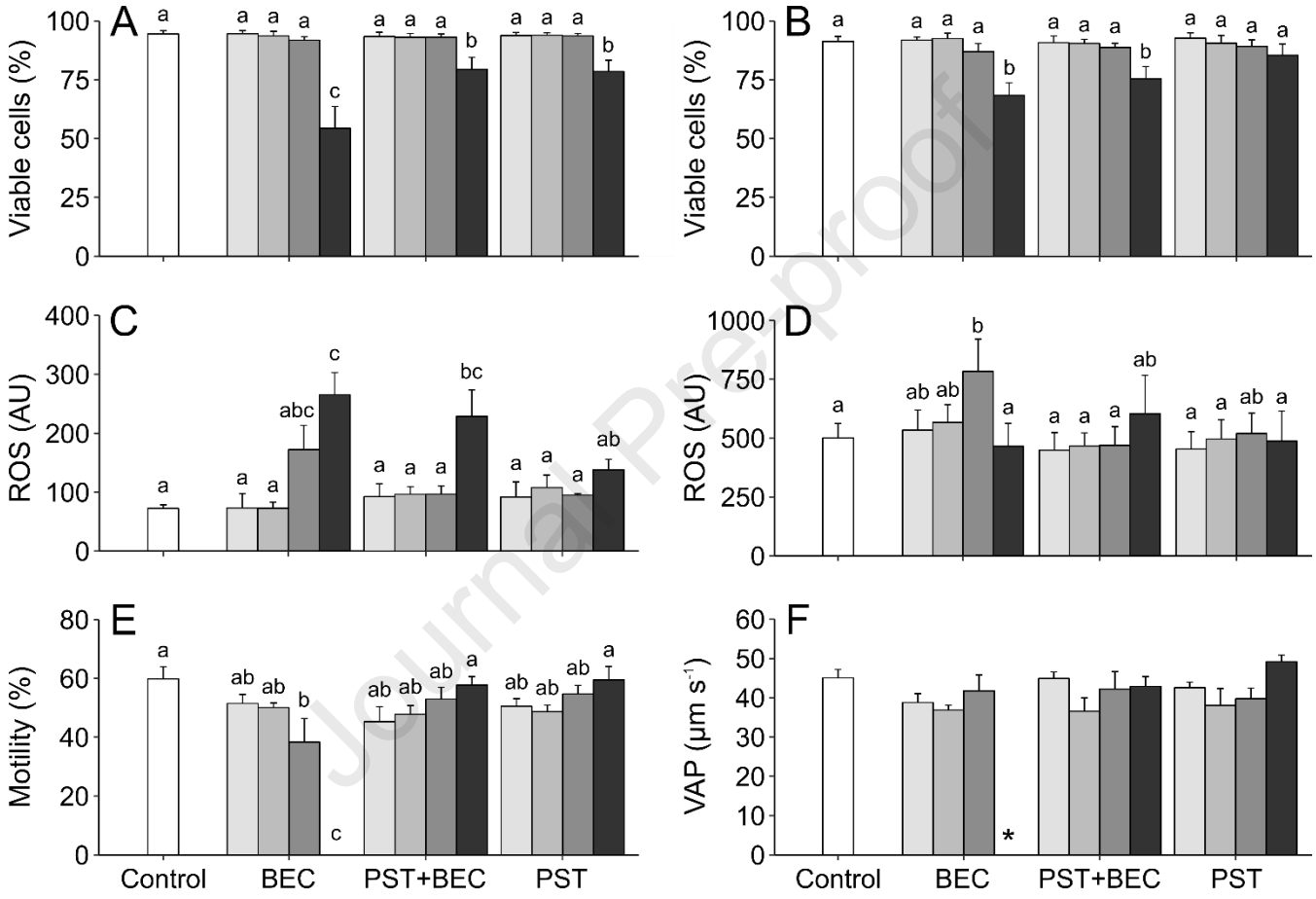

Fig. 1. Effects of three *A. minutum* strains producing BEC, PST+BEC, or PST, on cellular features of *C. gigas* gametes after a 2-hour exposure of spermatozoa (n = 4 males) or oocytes (n = 5 females) to 10 (□), 10^2 (▫), 2.5×10^3 (▪), and 2.5×10^4 (■) cells mL^{-1} of *A. minutum* or to seawater (□) (control). Percentage of viable spermatozoa (A) and oocytes (B), reactive oxygen species (ROS) production of spermatozoa (C) and oocytes (D), spermatozoa motility (E) and velocity of the average path (VAP) (F). *: VAP of spermatozoa exposed to 2.5×10^4 cells mL^{-1} of the BEC strain was 0, as movement was totally inhibited. AU: arbitrary unit. Error bars denote ± standard error. Letters denote significant groupings ($p < 0.05$).

At this concentration, the percentages of viable spermatozoa exposed to the three *A. minutum* strains were lower than control spermatozoa (94.4 ± 1.4%), the BEC strain having

197 the strongest effect (-38%) on spermatozoa viability (54.3 ± 9.3%) compared to the PST+BEC
198 (79.4 ± 5.1%, i.e. -14%) and PST strains (78.5 ± 4.8%, i.e. -15%) (Fig. 1A). The viability of
199 oocytes exposed to 2.5×10^4 cells mL^{-1} of the BEC strain (68.4 ± 5.3%) or of the PST+BEC
200 strain (75.4 ± 5.2%) were lower (-21% and -14%, respectively) than control oocytes (91.3 ±
201 2.1%) (Fig. 1B). The PST strain did not significantly affect oocyte viability compared to
202 control (Fig. 1B).

203 The production of reactive oxygen species (ROS) of spermatozoa ($F = 8.29$, $p < 10^{-6}$) and
204 oocytes ($F = 2.70$, $p < 10^{-2}$) were modified by exposure to *A. minutum*. The ROS production
205 of spermatozoa exposed to 2.5×10^4 cells mL^{-1} of the BEC strain (265.5 ± 37.5 AU) or the
206 PST+BEC strain (228.6 ± 45.0 AU) were higher (+140% and +113%, respectively) than
207 control spermatozoa (72.4 ± 6.0 AU) (Fig. 1C). The ROS production of oocytes exposed to
208 2.5×10^3 cells mL^{-1} of the BEC strain (783.9 ± 136.8 AU) was higher (+56%) than control
209 oocytes (500.8 ± 61.4 AU) (Fig. 1D). In the other conditions, ROS production of exposed
210 gametes were similar to control (Fig. 1C, D).

211 Spermatozoa motility was significantly affected by *A. minutum* exposure ($F = 19.66$,
212 $p < 10^{-11}$). Exposure to 2.5×10^4 cells mL^{-1} of the BEC strain totally inhibited spermatozoa
213 movement (Fig. 1E). The percentage of motile spermatozoa in spermatozoa exposed to $2.5 \times$
214 10^3 cells mL^{-1} of the BEC strain (38.3 ± 8.0%) was lower (-36%) than in control spermatozoa
215 (59.8 ± 4.1%) (Fig. 1E). The motility of spermatozoa in the other conditions were similar to
216 control (Fig. 1E). The velocity of motile spermatozoa (VAP: Velocity of the Average Path)
217 were not significantly affected by *A. minutum* exposure (Fig. 1F), except for the VAP of
218 spermatozoa exposed to 2.5×10^4 cells mL^{-1} of the BEC strain, which could not be measured
219 since spermatozoa movement was totally inhibited (Fig. 1E).

220 3.2 *Fertilization of exposed oocytes or spermatozoa*

Pre-exposure of oocytes or spermatozoa to *A. minutum*, prior to gamete contact for fertilization test, significantly affected fertilization yields (spermatozoa: $F = 10.78$, $p < 10^{-6}$; oocytes: $F = 29.60$, $p < 10^{-10}$). The fertilization yields obtained using spermatozoa exposed to the BEC strain at the two highest concentrations tested (2.5×10^4 and 2.5×10^3 cells mL^{-1}) were significantly lower (1.4 ± 1.4% and 24.5 ± 18.2%, i.e. -98 and -73%, respectively) than fertilization yield obtained using control spermatozoa (92.3 ± 0.5%). Similarly, spermatozoa exposure to the highest concentration (2.5×10^4 cells mL^{-1}) of the PST+BEC strain or the PST strain significantly decreased fertilization yields (12.6 ± 10.8% and 21.6 ± 14.4%, i.e. -86% and -77%, respectively) compared to control (Fig. 2A). Exposure of oocytes also induced lower fertilization yields compared to control oocytes (90.9 ± 2.7%) when exposed to the two highest concentrations tested (2.5×10^4 and 2.5×10^3 cells mL^{-1}) of the BEC strain (0.1 ± 0.1% and 49.0 ± 13.5%, i.e. -99 and -46%, respectively), and to 2.5×10^4 cells mL^{-1} of the PST+BEC strain (32.3 ± 3.9%, i.e. -64%) or the PST strain (62.3 ± 1.3%, i.e. -31%) (Fig. 2B). The other concentrations of the *A. minutum* strains induced similar responses than control condition.

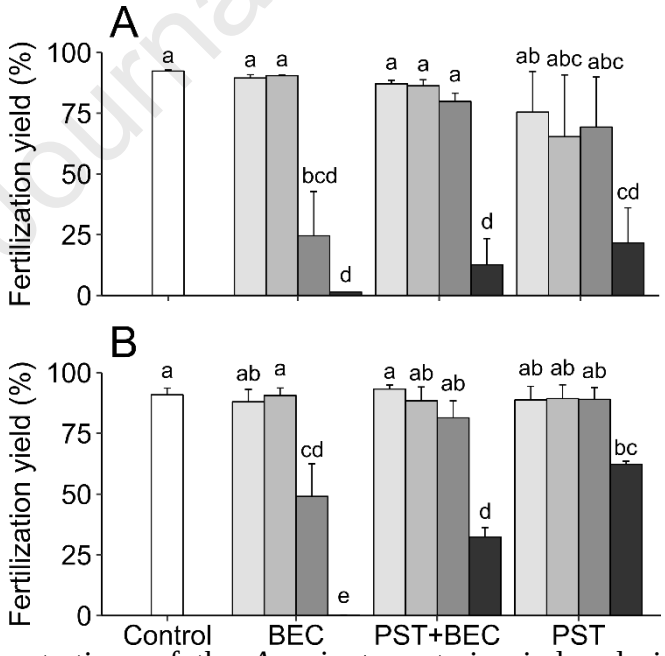

Fig. 2. Effects of three *A. minutum* strains producing BEC, PST+BEC, or PST, on *C. gigas* fertilization yield after a 2-hour exposure of spermatozoa (n = 3 males) or oocytes (n = 3 females) to 10 ( ), 10^2 ( ), 2.5×10^3 ( ), and 2.5×10^4 ( ) cells mL^{-1} of *A. minutum* (Pre-fertilization experiment) or to seawater ( ) (control). Fertilization yields of exposed spermatozoa (A) and exposed oocytes (B). Error bars denote ± standard error. Letters denote significant groupings ($p < 0.05$).

4. Discussion

The present study evidences that concentrations of 2.5×10^3 cells mL^{-1} of *Alexandrium minutum* induced sublethal defects on oyster gametes after only two hours of *in vitro* exposure. The extent of the effects depended on the *A. minutum* strain, the toxic compound they produced and exposure concentration. Cellular damages on spermatozoa or oocytes were associated to significant decreases in fertilization yields, the ultimate phenotype translating the quality of gametes. The highest concentrations tested (up to 2.5×10^4 cells mL^{-1}) are in the range of values observed during severe *A. minutum* blooms in France, Spain, Egypt, and South Africa (Chapelle et al., 2015; Garcés et al., 2004; Labib and Halim, 1995; Pitcher et al., 2007) and demonstrate the potentiality of ecological consequences of *A. minutum* blooms for wild and cultivated bivalve populations.

Sublethal cellular damages and consequences on fertilization were most severe with the BEC-producing *A. minutum* strain, illustrating the high toxicity of bioactive extracellular compounds to oyster gametes. This strain significantly decreased fertilization when spermatozoa (-73%) or oocytes (-49%) were exposed at 2.5×10^3 cells mL^{-1} and almost totally inhibited it for gamete exposure at 2.5×10^4 cells mL^{-1}. This strain was also characterized as the most allelopathic, i.e. cytotoxic, for diatoms (Long et al., 2018a) and oyster embryos (Castrec et al., 2020).

In addition to a high cellular mortality rate (45.7%), inhibition of spermatozoa motility likely explain the drastic decrease in fertilization yield following spermatozoa exposure to the BEC strain since concentration of motile spermatozoa is a pivotal factor for successful fertilization in free-spawning marine invertebrates (Nice, 2005; Styan, 1998). The motility was significantly reduced after an exposure to 2.5×10^3 *A. minutum* cells mL^{-1} and totally at 2.5×10^4 cells mL^{-1}. Alteration of spermatozoa movement could result from membrane alteration or sub-cellular cytotoxic effects. Long et al. (2018b) highlighted the deleterious

effects of extracellular bioactive compounds produced by the BEC strain of *A. minutum* on membranes of the diatom *Chaetoceros muelleri*, with modifications of the proportion of lipid classes, pigments, and of the photosynthetic chain suggesting an alteration of membrane integrity, associated with an increase of internal ROS production of the diatom. In human, oxidative stress is largely implicated in reproductive physiopathology. An excessive ROS production was demonstrated to be deleterious for sperm membrane integrity, motility, and fertility (Agarwal et al., 2003), by inducing peroxidative damage to the plasma membrane and DNA fragmentation (Sanocka and Kurpisz, 2004). Consequently, higher ROS production in spermatozoa exposed to 2.5×10^4 cells mL^{-1} of the BEC strain or the PST+BEC strain could be another element to explain spermatozoa reduced motility and viability. The increase in ROS production in oocytes exposed to 2.5×10^3 cells mL^{-1} of the BEC strain could be associated with BEC, as ROS generation in cells can be triggered by exposure to xenobiotic or toxins (Manduzio et al., 2005). As proposed by Le Goïc et al. (2014), ROS generation in oocytes exposed to *A. minutum* could lead to cellular damages and subsequently alter fertilization processes. High concentrations of intracellular ROS in human oocytes are responsible for detrimental effects such as disruption of Ca^{2+} homeostasis (Martín-Romero et al., 2008; Takahashi et al., 2003), impaired fertilization, and altered cleavage (Bedaiwy et al., 2002; Hu et al., 2001).

Bioactive extracellular compounds produced by different dinoflagellate species generally appear highly toxic for gametes (this study) and early developmental stages of bivalves, not protected by a shell (Banno et al., 2018; Basti et al., 2015; Castrec et al., 2020; Rolton et al., 2015). It is urgent to consider these uncharacterized bioactive compounds in studies assessing the effects of HAB on marine species, to characterize them, and to decipher the mechanisms underlying their toxicity.

290 Exposure of spermatozoa or oocytes to the PST-producing *A. minutum* strain at 2.5×10^4
291 cells mL^{-1} also altered fertilization and induced mortality in spermatozoa. Although PST is
292 mainly intracellular in dinoflagellates, low levels of extracellular PST have been measured in
293 the culture media of some *Alexandrium* spp. isolates (Lefebvre et al., 2008). Extracellular PST
294 might have been present in the PST strain culture media and could be involved in the toxic
295 effects observed. Saxitoxin, binding to Na^+ and Ca^{2+} channels (Llewellyn, 2006), likely
296 modifies fluxes of these ions in cells. Saxitoxin could therefore alter cellular processes
297 involving Ca^{2+}, such as spermatozoa motility (Boulais et al., 2017), oocyte maturation
298 (Leclerc et al., 2000) and, fertilization (Togo and Morisawa, 1999) in *C. gigas*. Further studies
299 should investigate the effects of oyster gamete exposure to purified PST, assessing cellular
300 integrity using electronic microscopy, cellular physiology including spermatozoa motility or
301 oocyte maturation, and the consequences on fertilization processes.

302 In addition to the toxicity of BEC and PST, repeated contacts between oyster gametes and
303 motile thecate dinoflagellates at high concentration (2.5×10^4 cells mL^{-1}) also likely
304 contribute to alter oocytes and spermatozoa cellular integrity, reducing their fertilization
305 capacity. Physical contact with algal cells seems to be an important mechanism involved in
306 the toxicity of HAB species to bivalve gametes and embryos, and has been suggested for
307 *Alexandrium* species such as *A. tamarense, A. taylori* and *A. affine* (Basti et al., 2015;
308 Matsuyama et al., 2001; Yan et al., 2001).

309 In our experiments, *A. minutum* cells and their toxins were still present during fertilization
310 since oocytes and spermatozoa were not filtered following exposure to remove algal cells.
311 Therefore, algal concentrations during fertilization ranged from 2.5 to 6.25×10^3 cells mL^{-1}
312 when performed with previously exposed spermatozoa and from 0.5 to 1.25×10^3 cells mL^{-1}
313 with previously exposed oocytes. Thus, *A. minutum* toxins could have directly impaired
314 fertilization process or prevent first cleavage of embryos. Indeed, some *Alexandrium* strains,

i.e. *A. catenella* and *A. affine*, affected the first and second cleavages of the Japanese pearl oyster *P. fucata martensii* embryos (Basti et al., 2015).

5. Conclusion

This experiment clearly highlights the toxicity of *A. minutum* on *C. gigas* gametes and their fertilization capacity and suggests that *A. minutum* blooms could have negative consequences on oyster recruitment. The most striking effects were observed following exposure of oyster gametes to the BEC-producing *A. minutum* strain, which decreased spermatozoa motility, increased gamete ROS production, and virtually inhibited the subsequent fertilization, thus confirming a PST-independent toxicity of *A. minutum*. Results illustrate that negative effects of *A. minutum* on oyster gametes, as well as on embryos and larvae, as we demonstrated previously (Castrec et al., 2020), are strain specific and mainly caused by uncharacterized bioactive extracellular compounds, released as exudates and/or associated to algal cell membranes. Further research is necessary to determine the chemical identity of these bioactive compounds produced by *A. minutum* and the mechanism by which *Alexandrium* spp. toxins affect bivalve gametes and embryos. These results also participate to illustrate that toxicity vary substantially among clones of the same *Alexandrium* species (Castrec et al., 2018; Long et al., 2018a; Touzet et al., 2007). As suggested for *A. ostenfeldii* (Brandenburg et al., 2018), this intraspecific trait variation could be an advantage for development and resilience of *A. minutum* blooms.

CRediT authorship contribution statement

Justine Castrec: Conceptualization, Methodology, Investigation, Writing - Original Draft, Formal analysis, Visualization. **Caroline Fabioux:** Conceptualization, Investigation, Supervision, Writing - Review & Editing. **Nelly Le Goïc:** Investigation, Resources, Writing - Review & Editing. **Myrina Boulais:** Conceptualization, Methodology, Investigation, Writing

- Review & Editing. **Philippe Soudant:** Supervision, Writing - Review & Editing. **Hélène Hégaret:** Conceptualization, Investigation, Writing - Review & Editing, Supervision.

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Highlights

- Oyster spermatozoa and oocytes were exposed *in vitro* to *Alexandrium minutum* strains.
- Viability and fertilization capacity of gametes were drastically reduced.
- The toxicity of *Alexandrium minutum* upon oyster gametes is strain-specific.
- A non-PST-producing strain increased reactive oxygen species production in oocytes.
- This strain also decreased spermatozoa motility and gamete fertilization.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: