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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 2.5×10^4 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 2.5×10^4 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 2.5×10^3 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.

33 1. Introduction

34 Fertilization is essential for reproduction and population sustainability (Marshall et al.,
35 2002). Most marine bivalve molluscs are broadcast spawners depending on external
36 fertilization. Seawater quality is a main factor affecting fertilization and larval development
37 successes in these organisms (Havenhand et al., 2008). In particular, contaminants could
38 negatively affect their reproduction as demonstrated in oysters exposed to anthropogenic
39 pollutants (Akcha et al., 2012; Fitzpatrick et al., 2008; Mai et al., 2013; Vignier et al., 2017,
40 2015). Experimental studies suggest that harmful algal blooms (HAB), often caused by
41 dinoflagellates, can affect marine bivalve reproduction by altering gamete quality and larval
42 development, growth, and survival (Banno et al., 2018; Basti et al., 2013, 2011; Binzer et al.,
43 2018; Bricelj and MacQuarrie, 2007; Castrec et al., 2020, 2019; De Rijcke et al., 2015;
44 Gaillard et al., 2020; Rolton et al., 2018, 2015, 2014; Tang and Gobler, 2012). In coastal
45 areas, HAB are a recurring phenomenon that can co-occur with the reproduction of free
46 spawning marine organisms (Gaillard et al., 2020).

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

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

58 Shumway, 1998) and/or poorly uncharacterized bioactive compounds exerting allelopathic,
59 hemolytic, ichthyotoxic or cytotoxic activities (Arzul et al., 1999; Bianchi et al., 2019;
60 Castrec et al., 2020, 2018; Ford et al., 2008; Lelong et al., 2011; Long et al., 2018a, 2018b;
61 Mardones et al., 2015). These bioactive compounds were proposed to be implicated in the
62 toxicity of *Alexandrium* upon early embryo development of several marine bivalves, such as
63 *Mytilus edulis* (De Rijcke et al., 2016), *C. gigas* (Castrec et al., 2020; Mu and Li, 2013), and
64 *Pinctada fucata martensii* (Basti et al., 2015), independently of the well-known toxins PST,
65 gonyautoxins, and spirolids. However, no clear demonstration of the respective effects of the
66 different toxic compounds has been provided. In addition to the well-known and characterized
67 PST, recent studies demonstrated the toxicity of unidentified bioactive extracellular
68 compounds (BEC) produced by *A. minutum* on other microalgae species (Lelong et al., 2011;
69 Long et al., 2018a, 2018b) and their deleterious effects upon the behavior of the scallop
70 *Pecten maximus* (Borcier et al., 2017) and physiology of the oyster *C. gigas* (Castrec et al.,
71 2018). These BEC were supposed to affect oyster oocytes exposed *in vitro* (Le Goïc et al.,
72 2014) as well as spermatozoa derived from exposed oysters (Castrec et al., 2019; Haberkorn
73 et al., 2010). To our knowledge, the respective involvement of PST and BEC in *A. minutum*
74 toxicity toward *C. gigas* gametes is not yet clearly established.

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

81 **2. Materials and methods**

82 *2.1 Algal strains*

83 Three strains of *Alexandrium minutum* (Halim) were grown in filtered seawater (0.2 μm)
84 supplemented with L1-Si medium (Guillard and Hargraves, 1993) and maintained at 17 ± 1
85 $^{\circ}\text{C}$, under a continuous light intensity of 100-110 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The *A. minutum*
86 strains were (i) the PST+BEC strain (strain AM89BM, isolated from a bloom in the Bay of
87 Morlaix, France) producing 10.6 fmol PST cell⁻¹ (corresponding to 1.3 pg STX eq. cell⁻¹;
88 Fabioux et al., 2015); (ii) the PST strain (strain Da1257, isolated from a bloom in the Bay of
89 Daoulas, France) producing 0.63 fmol PST cell⁻¹ (corresponding to 0.053 pg STX eq. cell⁻¹;
90 Pousse et al., 2018); (iii) the BEC strain (strain CCM11002, isolated from Irish waters), for
91 which no PST were detected (Borcier et al., 2017).

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

101 2.2 Oysters

102 For the experiment assessing cellular responses of gametes exposed to *A. minutum*, adult
103 Pacific oysters (n = 5 females and n = 4 males) *C. gigas* were collected from Daoulas estuary
104 in the Bay of Brest (North Brittany, France). For the fertilization experiment, adult oysters (n
105 = 3 females and n = 3 males) originated from a cohort produced in 2017 according to Petton
106 et al. (2015) and were deployed as early-juveniles (i.e. spat) in the bay of Brest. Gravid
107 oysters were collected during the natural reproduction period (June and July 2018).

108 2.3 Collection of gametes

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

120 2.4 Gamete cellular responses experiment: spermatozoa movement and gamete FCM 121 analyses

122 For each oyster, 2.5 mL of gamete suspension (see section 2.3) were exposed for 2 hours to
123 2.5 mL of the *A. minutum* suspensions (3 *A. minutum* strains \times 4 concentrations) or to 2.5 mL
124 of FSW (control treatment) in 10 mL glass vials for FCM analyses. For spermatozoa motility
125 analyses, 0.5 mL subsample of spermatozoa suspension from each male was transferred in 2
126 mL glass vials containing 0.5 mL of the *A. minutum* suspensions or 0.5 mL of FSW (control
127 treatment). After 2 hours of exposure, spermatozoa were sampled for motility measurements.
128 Final *A. minutum* concentrations were 10, 10^2 , 2.5×10^3 , and 2.5×10^4 cells mL^{-1} . These
129 concentrations were chosen to reflect the range of algal densities observed during natural
130 blooms (Chapelle et al., 2015). In France, the alert threshold triggering weekly concentration
131 monitoring for *A. minutum* blooms is 10 cells mL^{-1} (Guallar et al., 2017).

132 FCM measurements for spermatozoa and oocytes (i.e. relative cell size and complexity,
133 viability (using Sybr-14 and propidium iodide, final concentration 1 μM and 10 $\mu\text{g L}^{-1}$,
134 respectively) and reactive oxygen species production (using DCFH-DA, final concentration
135 10 μM)), were performed using an EasyCyte Plus cytometer (Guava Millipore Luminex)
136 equipped with standard optics and a 488 nm argon laser according to Le Goïc et al. (2013,
137 2014).

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

146 2.5 Fertilization experiment

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

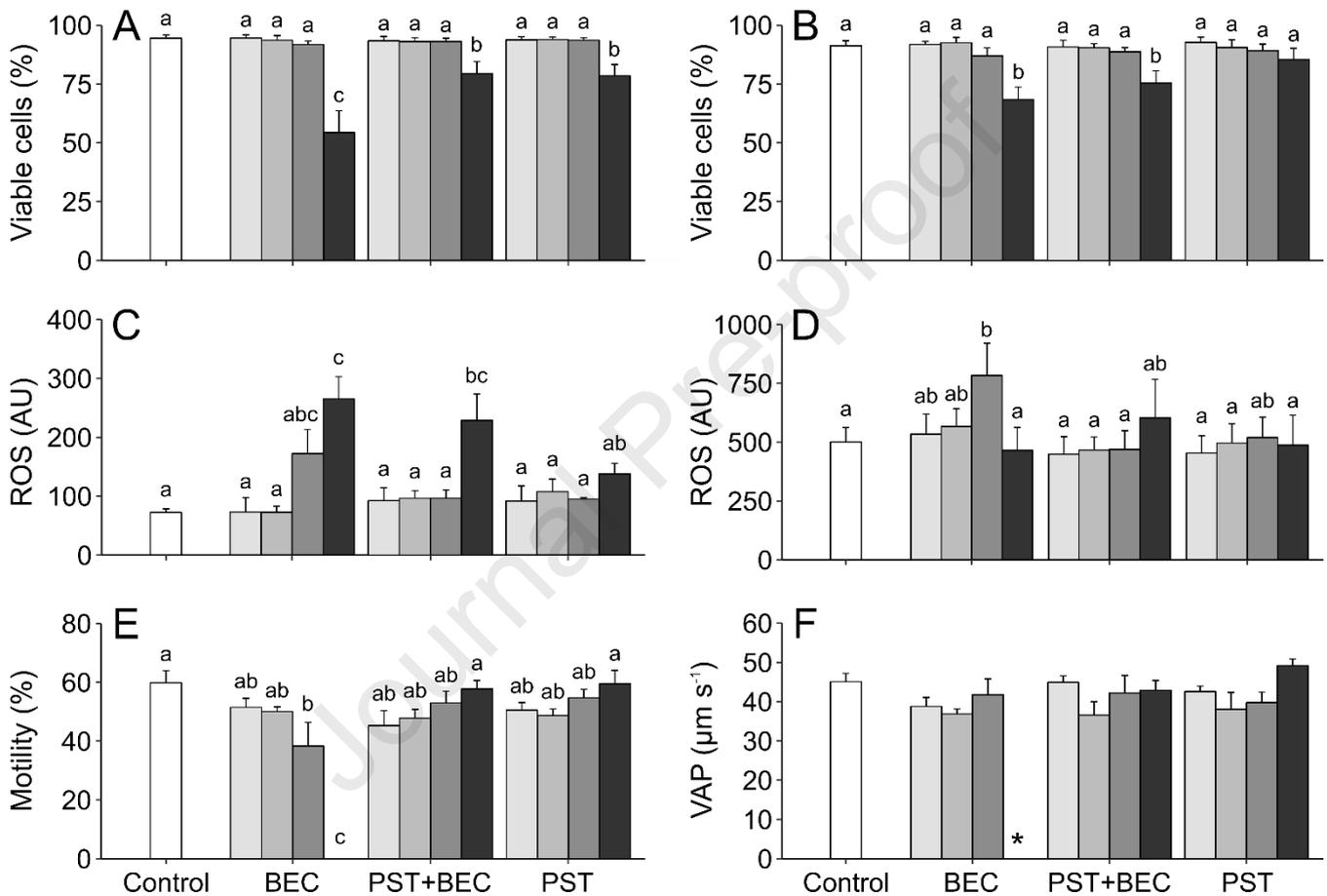
157 were transferred to 24-well microplates (NUNC[®]) filled with 1.4 mL of FSW and 100 μ L of
158 the non-exposed oocytes pool (100:1 spermatozoa:oocyte ratio). After 2 hours of contact
159 between spermatozoa and oocytes, samples were fixed with a formaldehyde-seawater solution
160 (1% final) to estimate the fertilization yield using a light microscope (Leica DM-IRB).
161 Numbers of fertilized (2-cell or 4-cell embryos) and unfertilized (absence of segmentation)
162 oocytes were counted, on at least 100 oocytes per well. Fertilization yield was defined as
163 number of fertilized oocytes \times 100 / number of unfertilized and fertilized oocytes. Each
164 oocytes/spermatozoa fertilization assay was performed in triplicate, the mean of the triplicate
165 fertilizations was used in statistical tests.

166 2.6 Statistical analyses

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

182 **3. Results**183 **3.1 Gamete cellular responses**

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



187 **Fig. 1.** Effects of three *A. minutum* strains producing BEC, PST+BEC, or PST, on cellular features
 188 of *C. gigas* gametes after a 2-hour exposure of spermatozoa ($n = 4$ males) or oocytes ($n = 5$ females) to
 189 10 (\square), 10^2 (\square), 2.5×10^3 (\square), and 2.5×10^4 (\blacksquare) cells mL^{-1} of *A. minutum* or to seawater (\square) (control).
 190 Percentage of viable spermatozoa (A) and oocytes (B), reactive oxygen species (ROS) production of
 191 spermatozoa (C) and oocytes (D), spermatozoa motility (E) and velocity of the average path (VAP)
 192 (F). *: VAP of spermatozoa exposed to 2.5×10^4 cells mL^{-1} of the BEC strain was 0, as movement was
 193 totally inhibited. AU: arbitrary unit. Error bars denote \pm standard error. Letters denote significant
 194 groupings ($p < 0.05$).

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

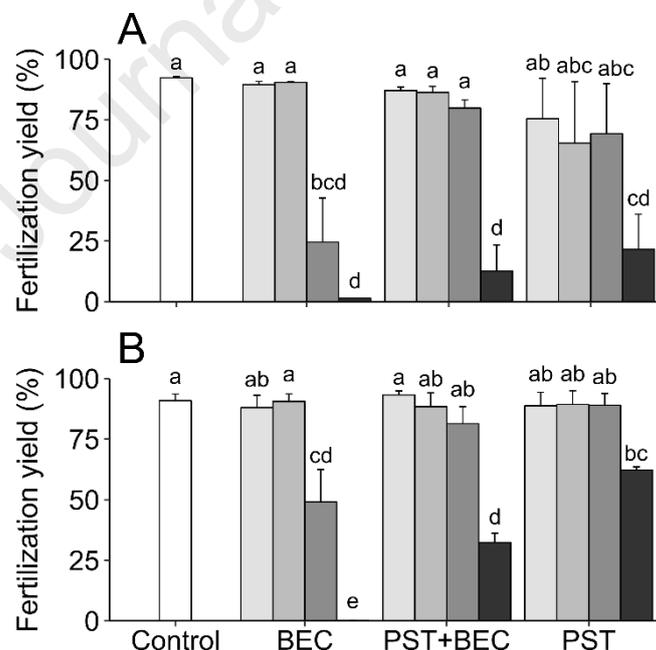
197 the strongest effect (-38%) on spermatozoa viability ($54.3 \pm 9.3\%$) compared to the PST+BEC
198 ($79.4 \pm 5.1\%$, i.e. -14%) and PST strains ($78.5 \pm 4.8\%$, i.e. -15%) (Fig. 1A). The viability of
199 oocytes exposed to 2.5×10^4 cells mL⁻¹ of the BEC strain ($68.4 \pm 5.3\%$) or of the PST+BEC
200 strain ($75.4 \pm 5.2\%$) were lower (-21% and -14%, respectively) than control oocytes ($91.3 \pm$
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 ($38.3 \pm 8.0\%$) was lower (-36%) than in control spermatozoa
215 ($59.8 \pm 4.1\%$) (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

221 Pre-exposure of oocytes or spermatozoa to *A. minutum*, prior to gamete contact for
 222 fertilization test, significantly affected fertilization yields (spermatozoa: $F = 10.78$, $p < 10^{-6}$;
 223 oocytes: $F = 29.60$, $p < 10^{-10}$). The fertilization yields obtained using spermatozoa exposed to
 224 the BEC strain at the two highest concentrations tested (2.5×10^4 and 2.5×10^3 cells mL^{-1})
 225 were significantly lower ($1.4 \pm 1.4\%$ and $24.5 \pm 18.2\%$, i.e. -98 and -73%, respectively) than
 226 fertilization yield obtained using control spermatozoa ($92.3 \pm 0.5\%$). Similarly, spermatozoa
 227 exposure to the highest concentration (2.5×10^4 cells mL^{-1}) of the PST+BEC strain or the
 228 PST strain significantly decreased fertilization yields ($12.6 \pm 10.8\%$ and $21.6 \pm 14.4\%$, i.e. -
 229 86% and -77%, respectively) compared to control (Fig. 2A). Exposure of oocytes also
 230 induced lower fertilization yields compared to control oocytes ($90.9 \pm 2.7\%$) when exposed to
 231 the two highest concentrations tested (2.5×10^4 and 2.5×10^3 cells mL^{-1}) of the BEC strain
 232 ($0.1 \pm 0.1\%$ and $49.0 \pm 13.5\%$, i.e. -99 and -46%, respectively), and to 2.5×10^4 cells mL^{-1} of
 233 the PST+BEC strain ($32.3 \pm 3.9\%$, i.e. -64%) or the PST strain ($62.3 \pm 1.3\%$, i.e. -31%) (Fig.



234 2B). The other concentrations of the *A. minutum* strains induced similar responses than
 235 control condition.

236 **Fig. 2.** Effects of three *A. minutum* strains producing BEC, PST+BEC, or PST, on *C. gigas*
237 fertilization yield after a 2-hour exposure of spermatozoa (n = 3 males) or oocytes (n = 3 females) to
238 10^1 (), 10^2 (), 2.5×10^3 (), and 2.5×10^4 () cells mL⁻¹ of *A. minutum* (Pre-fertilization experiment)
239 or to seawater () (control). Fertilization yields of exposed spermatozoa (A) and exposed oocytes (B).
240 Error bars denote \pm standard error. Letters denote significant groupings ($p < 0.05$).

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241 4. Discussion

242 The present study evidences that concentrations of 2.5×10^3 cells mL^{-1} of *Alexandrium*
243 *minutum* induced sublethal defects on oyster gametes after only two hours of *in vitro*
244 exposure. The extent of the effects depended on the *A. minutum* strain, the toxic compound
245 they produced and exposure concentration. Cellular damages on spermatozoa or oocytes were
246 associated to significant decreases in fertilization yields, the ultimate phenotype translating
247 the quality of gametes. The highest concentrations tested (up to 2.5×10^4 cells mL^{-1}) are in
248 the range of values observed during severe *A. minutum* blooms in France, Spain, Egypt, and
249 South Africa (Chapelle et al., 2015; Garcés et al., 2004; Labib and Halim, 1995; Pitcher et al.,
250 2007) and demonstrate the potentiality of ecological consequences of *A. minutum* blooms for
251 wild and cultivated bivalve populations.

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

259 In addition to a high cellular mortality rate (45.7%), inhibition of spermatozoa motility
260 likely explain the drastic decrease in fertilization yield following spermatozoa exposure to the
261 BEC strain since concentration of motile spermatozoa is a pivotal factor for successful
262 fertilization in free-spawning marine invertebrates (Nice, 2005; Styan, 1998). The motility
263 was significantly reduced after an exposure to 2.5×10^3 *A. minutum* cells mL^{-1} and totally at
264 2.5×10^4 cells mL^{-1} . Alteration of spermatozoa movement could result from membrane
265 alteration or sub-cellular cytotoxic effects. Long et al. (2018b) highlighted the deleterious

266 effects of extracellular bioactive compounds produced by the BEC strain of *A. minutum* on
267 membranes of the diatom *Chaetoceros muelleri*, with modifications of the proportion of lipid
268 classes, pigments, and of the photosynthetic chain suggesting an alteration of membrane
269 integrity, associated with an increase of internal ROS production of the diatom. In human,
270 oxidative stress is largely implicated in reproductive physiopathology. An excessive ROS
271 production was demonstrated to be deleterious for sperm membrane integrity, motility, and
272 fertility (Agarwal et al., 2003), by inducing peroxidative damage to the plasma membrane and
273 DNA fragmentation (Sanocka and Kurpisz, 2004). Consequently, higher ROS production in
274 spermatozoa exposed to 2.5×10^4 cells mL⁻¹ of the BEC strain or the PST+BEC strain could
275 be another element to explain spermatozoa reduced motility and viability. The increase in
276 ROS production in oocytes exposed to 2.5×10^3 cells mL⁻¹ of the BEC strain could be
277 associated with BEC, as ROS generation in cells can be triggered by exposure to xenobiotic
278 or toxins (Manduzio et al., 2005). As proposed by Le Goïc et al. (2014), ROS generation in
279 oocytes exposed to *A. minutum* could lead to cellular damages and subsequently alter
280 fertilization processes. High concentrations of intracellular ROS in human oocytes are
281 responsible for detrimental effects such as disruption of Ca²⁺ homeostasis (Martín-Romero et
282 al., 2008; Takahashi et al., 2003), impaired fertilization, and altered cleavage (Bedaiwy et al.,
283 2002; Hu et al., 2001).

284 Bioactive extracellular compounds produced by different dinoflagellate species generally
285 appear highly toxic for gametes (this study) and early developmental stages of bivalves, not
286 protected by a shell (Banno et al., 2018; Basti et al., 2015; Castrec et al., 2020; Rolton et al.,
287 2015). It is urgent to consider these uncharacterized bioactive compounds in studies assessing
288 the effects of HAB on marine species, to characterize them, and to decipher the mechanisms
289 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 Ca²⁺ channels (Llewellyn, 2006), likely
296 modifies fluxes of these ions in cells. Saxitoxin could therefore alter cellular processes
297 involving Ca²⁺, 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,

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

317 **5. Conclusion**

318 This experiment clearly highlights the toxicity of *A. minutum* on *C. gigas* gametes and their
319 fertilization capacity and suggests that *A. minutum* blooms could have negative consequences
320 on oyster recruitment. The most striking effects were observed following exposure of oyster
321 gametes to the BEC-producing *A. minutum* strain, which decreased spermatozoa motility,
322 increased gamete ROS production, and virtually inhibited the subsequent fertilization, thus
323 confirming a PST-independent toxicity of *A. minutum*. Results illustrate that negative effects
324 of *A. minutum* on oyster gametes, as well as on embryos and larvae, as we demonstrated
325 previously (Castrec et al., 2020), are strain specific and mainly caused by uncharacterized
326 bioactive extracellular compounds, released as exudates and/or associated to algal cell
327 membranes. Further research is necessary to determine the chemical identity of these
328 bioactive compounds produced by *A. minutum* and the mechanism by which *Alexandrium*
329 spp. toxins affect bivalve gametes and embryos. These results also participate to illustrate that
330 toxicity vary substantially among clones of the same *Alexandrium* species (Castrec et al.,
331 2018; Long et al., 2018a; Touzet et al., 2007). As suggested for *A. ostensfeldii* (Brandenburg et
332 al., 2018), this intraspecific trait variation could be an advantage for development and
333 resilience of *A. minutum* blooms.

334 **CRedit authorship contribution statement**

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

339 - Review & Editing. **Philippe Soudant:** Supervision, Writing - Review & Editing. **Hélène**
340 **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:

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