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The toxic dinoflagellate *Alexandrium minutum* impairs the performance of oyster embryos and larvae

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24 settlement of larvae in response to the PST-producing strain. This work provides evidences that
25 *A. minutum* blooms could hamper settlement of shellfish.

26 **Keywords:** Harmful Algal Bloom (HAB), Paralytic Shellfish Toxin (PST), Bioactive
27 Extracellular Compounds (BEC), *Crassostrea gigas*, embryo, bivalve larvae

28 1. Introduction

29 Harmful algal blooms (HAB) are globally distributed and represent a real concern for marine
30 environment quality (Shumway, 1990; Kudela et al., 2015). Beside their toxicity to humans
31 through consumption of phycotoxin-contaminated shellfish or fishes (Van Dolah, 2000;
32 Berdalet et al., 2015; Grattan et al., 2016), HAB affect biology of marine organisms and
33 functioning of marine coastal ecosystems (Landsberg, 2002; Hallegraeff et al., 2003). The
34 dinoflagellate genus *Alexandrium* is remarkable among HAB-genera, owing to the acute and
35 diverse consequences of its blooms, its ability to colonize a wide variety of habitats, and its
36 persistence over large spatial and temporal scales (Anderson et al., 2012).

37 Some *Alexandrium* species are known to produce paralytic shellfish toxins (PST), a group
38 of more than 50 neurotoxic derivatives of saxitoxin (Wiese et al., 2010). PST can bioaccumulate
39 in filter-feeding bivalves and other marine species, causing paralytic shellfish poisonings (PSP)
40 outbreaks (Bricelj and Shumway, 1998; Cembella et al., 2000) and other harmful effects
41 (Shumway, 1990; Landsberg, 2002). These endogenous water-soluble alkaloids bind with high
42 affinity to the voltage-gated Na⁺ channels, blocking conduction of action potential in excitable
43 cells, eventually leading to paralysis (Cestèle and Catterall, 2000), and interact with Ca²⁺ and
44 K⁺ channels, modifying ionic fluxes into cells (Llewellyn, 2006). Several species of
45 *Alexandrium* also produce, independently from PST (Tillmann and John, 2002), extracellular
46 bioactive compounds (BEC) with allelopathic (Arzul et al., 1999; Tillmann et al., 2008; Lelong
47 et al., 2011; Zheng et al., 2016; Long et al., 2018a, 2018b), ichthyotoxic (Ogata and Kodama,

48 1986; Mardones et al., 2015), cytotoxic (Lush and Hallegraeff, 1996; Borcier et al., 2017;
49 Banno et al., 2018; Castrec et al., 2018), and/or hemolytic activities (Eschbach et al., 2001;
50 Yang et al., 2010; Cho et al., 2017). These compounds, which molecular structures remain
51 largely unknown (Ma et al., 2011), likely contribute to *Alexandrium* bloom toxicity. In
52 particular, distinct negative effects of PST and BEC have been shown on adult *Crassostrea*
53 *gigas* valve behavior and cellular characteristics (Castrec et al., 2018). In addition to
54 extracellular compounds, synergistic effects of superoxide production, free fatty acids, and
55 peroxide products may be responsible for the ichthyotoxic activity of *Alexandrium catenella*
56 strains (Dorantes-Aranda et al., 2015).

57 HAB frequently occur during bivalve reproduction period (Lewis et al., 2018; Gourault et
58 al., 2019a). Along the French coasts, in the English Channel and the North-East Atlantic, the
59 duration of *A. minutum* blooms varies between 2 weeks and 6 months and it can reach high cell
60 densities ($>10^7$ cells L⁻¹) (Guallar et al., 2017). In French coastal waters, *A. minutum* bloom
61 period extends from April to October (Chapelle et al., 2015; Guallar et al., 2017), which is often
62 concomitant with breeding period of bivalves, including the oyster *C. gigas* (Pouvreau et al.,
63 2016; Gourault et al., 2019b). In coastal areas, the recruitment of bivalves is characterized by
64 significant year-to-year variability (Beukema and Dekker, 2005). Inter-annual variability of *C.*
65 *gigas* recruitment has been observed along French coasts, from the north to the south of the
66 Atlantic coast (Auby et al., 2014; Gourault et al., 2019b) and in Mediterranean Sea (Lagarde et
67 al., 2019). Repeated recruitment failures could be detrimental for traditional bivalve culture,
68 which relies on the collection of juveniles from the wild (Rico-Villa et al., 2010). It is not
69 possible, at present, to prove that HAB are directly responsible for bivalve recruitment failures,
70 but observational accounts (Erard-Le Denn et al., 1990; Summerson and Peterson, 1990;
71 Pouvreau et al., 2015) suggest that some blooms may, in part, contribute to lower survival
72 among early life stage bivalves. In addition, laboratory experiments evidenced that

73 *Alexandrium* species, such as *A. catenella*, *A. tamarense*, and *A. minutum*, alter embryo
74 development of *C. gigas* (Mu and Li, 2013), *Chlamys farreri* (Yan et al., 2001), and *Mytilus*
75 *edulis* (De Rijcke et al., 2016). The quality and viability of Japanese pearl oyster *Pinctada*
76 *fucata martensii* and *C. gigas* gametes were affected by *A. catenella* and *A. minutum* (Banno et
77 al., 2018). Direct exposure of early life stages to PST-producing *Alexandrium* strains decreased
78 larval activity, survival, growth, and settlement of several bivalve species, including *C. gigas*,
79 *Pinctada fucata martensii*, *Chlamys farreri*, and *Argopecten irradians* (Matsuyama et al., 2001;
80 Yan et al., 2001, 2003; Mu and Li, 2013; Basti et al., 2015). Moreover, adult oysters *C. gigas*
81 exposed to *A. minutum* produced spermatozoa with decreased motility and larvae of smaller
82 size which exhibited higher mortalities during settlement, demonstrating that larval
83 development could also be affected via exposure of parents to harmful algae (Haberkorn et al.,
84 2010; Castrec et al., 2019).

85 Addressing specific reprotoxic effects of *Alexandrium* attributable to either PST or bioactive
86 extracellular compounds (BEC) is challenging. Most studies, to date, have not been able to
87 experimentally decouple the effects of BEC and PST (Matsuyama et al., 2001; De Rijcke et al.,
88 2016), hence some authors have implicated PST as associated with harmful impacts. Known
89 non-PST-producing *Alexandrium* strains, *i.e.* *A. taylori*, *A. affine*, *A. ostensfeldii*, and *A.*
90 *monilatum*, however, altered cleavage of embryos, hatching into D-larvae, and activity and
91 survival of larvae of bivalves (Matsuyama et al., 2001; May et al., 2010; Basti et al., 2015; De
92 Rijcke et al., 2016). These findings suggest that some bioactive compounds, non-related to PST,
93 could contribute to the detrimental effects of *Alexandrium* upon bivalve early development.

94 To evaluate the respective vulnerabilities of *C. gigas* early life stages to PST and BEC
95 produced by *A. minutum*, the effects of two strains, producing only BEC or both PST and BEC,
96 were analyzed on various developmental stages including: embryos, umbonate, and eyed larvae.
97 Morphological abnormalities, feeding activity, and growth were assessed, and potential

98 consequences on oyster recruitment were investigated by assessing larval survival and
99 settlement after exposure of embryos, umbonate or eyed larvae to *A. minutum*.

100 **2. Materials and methods**

101 *2.1 Algal strains*

102 Two strains of *Alexandrium minutum* (Halim) were selected based upon their different
103 toxicities in terms of both PST and BEC (Castrec et al., 2018). The “PST+BEC” strain
104 (AM89BM strain, isolated from a bloom in the Bay of Morlaix, France; (Erard-Le Denn et al.,
105 1990)) produces both bioactive extracellular compounds (BEC) and paralytic shellfish toxins
106 (PST), and the “BEC” strain (CCMI1002 strain, isolated from a bloom in Gearhies, Bantry Bay,
107 Ireland) produces only BEC. The BEC strain is significantly more cytotoxic than the AM89BM
108 strain according to a bioassay on cells of a diatom species, *Chaetoceros muelleri* (Long et al.,
109 2018a). The PST+BEC strain is known to produce 10.6 fmol PST cell⁻¹ which corresponds to
110 1.3 pg STX eq. cell⁻¹ (Fabioux et al., 2015), whereas no PST were detected in the BEC strain
111 (Borcier et al., 2017). The cultures were grown in 6 L of filtered seawater (0.2 µm)
112 supplemented with L1-Si medium (Guillard and Hargraves, 1993) and maintained at 17 ± 1 °C,
113 under a continuous light intensity of 100-110 µmol photons m⁻² s⁻¹. Cultures of *A. minutum*
114 were harvested in exponential growth phase for the experiment.

115 *Tisochrysis lutea* (formerly *Isochrysis* sp., T. iso strain, CCAP 927/14) and *Chaetoceros* sp.
116 (formerly *Chaetoceros neogracile*, strain CCAP 1010-3) were used as the primary non-toxic
117 food for oyster larvae. The strains were cultured with continuous light (200 µmol photons m⁻²
118 s⁻¹) in separate 300-L transparent vertical cylinders containing filtered seawater (1 µm) enriched
119 with Conway medium (Walne, 1970) at 20 °C, and with silica for *Chaetoceros* sp. Microalgae
120 cultures were harvested at the late-logarithmic phase (after 3–4 days).

121 *2.2 Oysters*

122 Adult *C. gigas* (Bayne et al., 2017a) originated from a cohort of specific-pathogen-free
123 oysters produced according to a standardized protocol (Petton et al., 2015). Oysters were reared
124 in Ifremer experimental facilities (Argenton, France) and transferred as early-juveniles (i.e.
125 spat) to the field in Marennes-Oléron, France. Gravid oysters (18 months old, mean total weight
126 63.5 g) were then transferred to the Ifremer experimental facilities (Argenton, France) in June
127 2017.

128 2.3 Experimental design

129 Three independent exposures were performed on three different oyster developmental stages
130 chosen for their distinct feeding behavior (Fig. 1). Planktonic life-stages of oysters were
131 exposed to *A. minutum* either before the D-larvae stage (Exp1), during umbonate stage (Exp2),
132 or just before settlement (Exp3):

- 133 - Exp1 = embryo exposure: from embryos 1.5 hours post-fertilization (hpf) to D-stage (2-day
134 exposure). The embryo development up to D-larvae involves only endogenous exchanges
135 (Bayne, 2017b), and D-larvae are unable to ingest *Alexandrium* cells due to their relatively
136 large size (23–29 μm) (Raby et al., 1997; Helm et al., 2004);
- 137 - Exp2 = umbonate larvae exposure: from 13 days post-fertilization (dpf) (larvae shell height
138 $163.7 \pm 1.3 \mu\text{m}$) to 15 dpf. These umbonate planktotrophic larvae (Rico-Villa et al., 2006)
139 are not able to feed on *A. minutum* cells at a mean shell length of $150 \pm 22 \mu\text{m}$ (Castrec et
140 al., 2019);
- 141 - Exp3 = eyed larvae exposure: from 20 dpf (larvae shell height $295.2 \pm 2.3 \mu\text{m}$, percentage
142 of eyed larvae 40.9 ± 3.9) to 21 dpf. These eyed competent larvae are also planktotrophic
143 larvae with high phytoplankton consumption (Rico-Villa et al., 2006), and are able to feed
144 on *Alexandrium* cells (Castrec et al., 2019).

145 The PST produced by *A. minutum* are mainly intracellular and released in oyster tissues
146 following ingestion and lysis of toxic dinoflagellate cells in the digestive tract (Bricelj and

147 Shumway, 1998). We expect that, unlike embryos and D-larvae which are not able to ingest *A.*
148 *minutum*, large umbonate and eyed larvae will be exposed to PST through consumption of *A.*
149 *minutum* cells. Control larvae were fed *ad libitum* a mixture of non-toxic algal diets (see above
150 for culture conditions) including *T. lutea* and *Chaetoceros* sp. (Tiso/Chaeto) (Fig. 1). For each
151 exposure (Exp1, Exp2, and Exp3), two different algal treatments (BEC or PST+BEC strain of
152 *A. minutum*) were run during the exposures, conducted for 2 days for Exp1 and Exp2, and 1 day
153 for Exp3 (Fig. 1). When outside the exposure period, larvae were fed *ad libitum* with the
154 Tiso/Chaeto diet used for control treatment. Concentration and duration of *A. minutum*
155 exposures were chosen based on environmentally realistic densities and durations of natural
156 blooms that have occurred in oyster production areas (Chapelle et al., 2015).

157 2.4 Preparation of batches of embryos

158 Gender of mature oysters was determined by examination of 2 μ l gonad samples under a
159 light microscope (Leica MZ 125, $\times 4$ objective). Oocytes and sperm were collected from 15
160 females and 17 males, respectively, by stripping gonads individually (Boulais et al., 2015a,
161 2015b). Briefly, gametes were collected with UV-treated, 1 μ m filtered sea water (FSW; 21 $^{\circ}$ C,
162 pH 8.3, salinity 36). Oocytes and spermatozoa were sieved through 100 μ m and 60 μ m meshes,
163 respectively, to eliminate debris (Steele and Mulcahy, 1999). Gamete concentrations were
164 determined individually by flow cytometry (duplicate, EasyCyte Plus cytometer, Guava
165 Millipore) (Le Goïc et al., 2013, 2014). A pool of oocytes from the 15 females were realized
166 by sampling 2.67×10^6 oocytes per oyster, and 4.17×10^8 spermatozoa per oyster were sample
167 from each male.

168 2.5 Embryo and larval rearing

169 Four batches of 5×10^6 pooled oocytes (see section 2.4) were fertilized with a pool of $5 \times$
170 10^8 spermatozoa (see section 2.4) in beakers containing 5 L FSW at $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$. These batches
171 of embryos were made the same day and used as replicates for the different exposures ($n = 4$

172 replicates) (Fig. S1). Embryos 1.5 hours post-fertilization (hpf) from each beaker were
173 concentrated on a 20- μm mesh, rinsed and transferred into a 5-L cylindrical tank already
174 containing the appropriate algal treatment in FSW at 21 °C (Fig. S1, Fig. 1), at a final
175 concentration of 100 embryos mL^{-1} . During Exp1, embryos were incubated in FSW containing
176 10^3 cells mL^{-1} of the PST+BEC or the BEC strain of *A. minutum* (Fig. 1). For control treatment,
177 embryos were grown in FSW.

178 Each tank was sieved through 40- μm mesh 48 hpf and concentrated into a 100-mL graduated
179 cylinder. D-larva yields were estimated under a light microscope (Olympus BX51), adding one
180 drop of 4% formaldehyde solution into a 10 mL subsample of the cylinder to prevent larval
181 movement. D-larva yield was defined as: number of D-larvae/total number of oocytes used for
182 fertilization. Percentages of abnormal D-larvae were recorded by observing at least 100 larvae
183 per replicate using a light microscope (Zeiss Axio Observer Z1). Larvae presenting shell,
184 mantle and/or hinge malformations, or developmental arrest at the embryonic stage were
185 considered as abnormal (Mottier et al., 2013). D-larvae from each replicate tank were then
186 transferred separately to a 5-L cylindrical tank at the density of 50 larvae mL^{-1} for larval rearing.
187 All larvae were maintained in a flow-through rearing system as described by Gonzalez Araya
188 et al. (2012) (100% seawater renewal h^{-1} , FSW, 21 °C, 35 PSU, pH 8.4), and fed continuously
189 *ad libitum* with *T. lutea* and *Chaetoceros* sp. (Tiso/Chaeto), at $1500 \mu\text{m}^3 \mu\text{L}^{-1}$ (at 1:1 equivalent
190 volume), according to Asmani et al. (2016). During Exp2 (2 days) and Exp3 (1 day), larvae
191 were exposed to 10^3 cells mL^{-1} of the PST+BEC strain or the PST strain and fed *ad libitum* a
192 non-harmful diet (Tiso/Chaeto, see above) (Fig. 1).

193 Once $\geq 50\%$ of larvae reached the eyed larvae stage (morphological competence for
194 metamorphosis), the replicate tanks were drained and larvae were collected on 60 μm mesh and
195 counted as described in Tallec et al. (2018). The exposure duration for Exp3 was only 1 day
196 since $\geq 50\%$ of larvae had reached the eyed larvae stage at 21 dpf. Larval survival was

197 determined as the number of remaining larvae / 2.5×10^5 larvae. A subsample of larvae (300-
198 700 mg) was collected from each tank and stored in liquid nitrogen for subsequent PST
199 quantification (see section 2.9). Morphological characteristics, algal consumption, settlement,
200 and toxin quantification of larvae were measured as described below.

201 2.6 *Morphological measurements of larvae*

202 Larvae were sampled every 1–3 d during the entire experiment and stored in a 0.1%
203 formaldehyde-seawater solution. Larval size was assessed by measuring shell height (greatest
204 dorso-ventral dimension) using image analysis on at least 30 larvae per tank per day of sampling
205 (WinImager 2.0 and ImageJ software for image capture and analysis, respectively).

206 The eyed larvae yield was estimated on at least 30 larvae per tank per day of sampling, and
207 expressed as the percentage of larvae developing an eyespot (morphological competence for
208 metamorphosis). Larval height was statistically compared between control and exposed larvae
209 at the end of the exposure (15 dpf for Exp2; 21 dpf for Exp3) and when larvae reached
210 competence for metamorphosis.

211 2.7 *Algal consumption*

212 Once a day throughout larval rearing, flow rates in larval aquaria were measured and the
213 consumption of phytoplankton cells were quantified by measuring phytoplankton counts (cells
214 mL^{-1}) using an electronic particle counter (Multisizer 3 equipped with a 100- μm aperture tube)
215 in inflow and outflow seawater (20 mL) sampled from each tank. Algal consumption (i.e.
216 Tiso/Chaeto) was expressed in algal cell volume per larvae per day (e. g. $\mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$). A
217 subsample of 1 mL was collected every 2-3 days from each larval tank replicate to determine
218 survival by counting larvae using the light microscope. Algal consumption (i.e. Tiso/Chaeto)
219 was statically compared between algal treatments before and during the exposure (at 13, 14,
220 and 15 dpf for Exp2; at 20 and 21 dpf for Exp3).

221 2.8 *Larval settlement*

222 Once $\geq 50\%$ of larvae from one treatment reached the eyed larvae stage as described
223 previously, larvae from the four replicate cylindrical tanks were transferred for settlement into
224 30-L tank with a 150- μm sieve (5×10^4 larvae tank⁻¹, 4 replicate tanks per exposure and algal
225 treatment), maintained in a flow-through system (9 L h^{-1} ; $30 \% \text{ h}^{-1}$ seawater renewal) and fed
226 with the bi-specific diet (Tiso/Chaeto as described previously). During this transfer, larvae from
227 each cylindrical tank were also sampled for toxin quantification and stored in liquid nitrogen
228 (see section 2.9). After 7 days, the number of swimming larvae, dead larvae (empty shells), and
229 larvae settled on tank walls and sieve were quantified. The survival (%) during the settlement
230 step was evaluated as: $100 - (\text{number of dead larvae} \times 100 / 5 \times 10^4)$. The larval settlement
231 yield (%) was evaluated as: $\text{number of settled larvae} \times 100 / \text{total number of remaining alive}$
232 larvae.

233 2.9 *Toxin quantification*

234 PST extraction was performed on one larvae sample per replicate: larvae were homogenized
235 in HCl 0.1 M (1:1, w:v) using a FastPrep bead-grinder (MP Biomedicals), boiled for 5 min, and
236 subsequently centrifuged (10 min, 4 °C, $3500 \times g$). Toxin quantification of larvae exposed to
237 the PST+BEC or BEC strain and control larvae from Exp1, Exp2 and Exp3 was performed
238 spectrophotometrically using the Abraxis ELISA PSP kit (Novakits, France) according to
239 Lassudrie et al. (2015). Supernatants of the replicates were pooled for control larvae and larvae
240 exposed to the BEC strain, for each exposure. Toxin load was expressed in ng STX g⁻¹ of wet
241 larvae weight.

242 2.10 *Statistical analyses*

243 Statistical analyses and graphical representations were performed using R software (R Core
244 Team, 2012). Differences were considered significant when $p < 0.05$. All values are expressed
245 as mean \pm standard error (SE). For Exp1, D-larvae yield and abnormality percentage were
246 compared between control treatment and embryos exposed to the PST+BEC strain using t-tests

247 since no D-larvae was observed following exposure to the BEC strain. For Exp2 and Exp3,
248 algal consumption was compared among the three algal treatments using one-way ANOVA
249 followed by Tukey's honest significant difference test (Tukey HSD). Comparisons for larval
250 height at 15 and 21 dpf, eyed larvae yield, larval survival and height at competence for
251 metamorphosis, larval settlement yield, and survival during settlement period among the 6
252 treatments (control treatment, Exp1: PST+BEC strain, Exp2: PST+BEC or BEC strain, Exp3:
253 PST+BEC or BEC strain) were assessed using one-way ANOVA followed by Tukey HSD for
254 pairwise comparisons when differences were detected (no data are available for Exp1 with the
255 BEC treatment since hatching was completely inhibited with this treatment). The p-values were
256 adjusted using the False Discovery Rate (FDR) controlling procedure following Benjamini and
257 Hochberg (1995) (Benjamini and Hochberg, 1995) for multiple hypothesis testing. While
258 repeated measurements for algal consumption and shell height were obtained from
259 experimental tanks, statistical comparisons were restricted to across treatments (i.e. not within
260 treatments over time) to avoid non-independent comparisons of culture end-points over time.
261 Normality and homogeneity of variance were verified by the Shapiro-Wilk and Levene's tests,
262 respectively. Algal consumption data from Exp2 at 21 dpf were log transformed to conform to
263 a normal distribution with equal variance.

264 3. Results

265 3.1 Exposure of embryos (Exp1)

266 The 2-day exposure of embryos to the BEC strain totally inhibited hatching into D-larvae
267 due to lysis of embryos. The D-larvae yield of embryos exposed to the PST+BEC strain (47.3
268 ± 1.0 %) was similar (*t-test*, $p > 0.05$) to the control (48.3 ± 5.8 %). The abnormality percentage
269 of D-larvae was significantly higher (*t-test*, $p < 10^{-6}$) in larvae exposed to the PST+BEC strain
270 (30.0 ± 0.8 %) than in control larvae (7.0 ± 0.4 %), with higher occurrences of shell and mantle
271 abnormalities, and membrane swelling (Fig. S1).

272 At 21 days post-fertilization (dpf), larval height (300.4 ± 4.5 μm) and eyed larvae yield (51.8
273 ± 4.1 %) of larvae derived from embryos exposed to the PST+BEC strain were similar (Tukey
274 HSD, all $p > 0.05$) to control larvae (306.8 ± 4.0 μm and 51.4 ± 2.9 %, respectively) (Fig. 2A,
275 Fig. 3A, Exp1). Survival up to competence for metamorphosis of larvae (68.7 ± 5.8 %), survival
276 during settlement (97.5 ± 0.6 %), and settlement yield (18.4 ± 1.0 %) of larvae derived from
277 embryos exposed to the PST+BEC strain were similar (Tukey HSD, all $p > 0.05$) to the control
278 (67.9 ± 7.1 %, 97.9 ± 0.1 %, 16.6 ± 0.9 %, respectively) (Fig. 3B-D, Exp1).

279 3.2 Exposure of umbonate larvae (Exp2)

280 Visual observations during the exposure indicated that the BEC strain modified larval
281 behavior, since most larvae stopped swimming and sank at the bottom of the tank (Fig. S2).
282 Microscopic observation of larvae exposed to the BEC strain and sampled at the bottom of the
283 tank showed that larvae were not closed, but rather shells remained slightly open and the velum
284 was rarely protruded (Fig. 4). On the contrary, control larvae and larvae exposed to the
285 PST+BEC strain remained swimming in the tank (Fig. S2). Umbonate larvae exposed to the
286 BEC strain showed mantle malformations, including abnormal protrusion of the velum,
287 abnormal masses in the velum, and loss of the velum cilia (Fig. 4).

288 Before exposure, algal consumption (Tiso/Chaeto) was similar (ANOVA, $F_{2,9} = 4, p = 0.073$)
289 for all umbonate larvae (Table 1). After 1 day of exposure, algal consumption of umbonate
290 larvae exposed to the BEC strain was significantly lower (Tukey HSD, $p < 10^{-2}$ and $p < 10^{-3}$)
291 than in control larvae and larvae exposed to the PST+BEC strain (Table 1). After 2 days of
292 exposure, larvae exposed to the BEC strain ($196.9 \pm 25.3 \mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$) had a further lower
293 (Tukey HSD, $p < 10^{-6}$) algal consumption than larvae exposed to the PST+BEC strain (753.9
294 $\pm 33.7 \mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$), which was significantly lower (Tukey HSD, $p < 10^{-7}$) than control
295 larvae ($940.9 \pm 21.0 \mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$) (Table 1).

296 Larval height was significantly affected by algal treatment (ANOVA, $F_{5,18} = 56.5, p < 10^{-8}$)
297 after 2 days of exposure to *A. minutum* (Fig. 2B, Table S1). At 15 dpf, larvae exposed to the
298 PST+BEC strain ($197.2 \pm 3.3 \mu\text{m}$) or the BEC strain ($172.3 \pm 1.6 \mu\text{m}$) were smaller (Tukey
299 HSD, $p < 10^{-2}$ and $p < 10^{-5}$) than control larvae ($211.3 \pm 1.1 \mu\text{m}$); larvae exposed to the BEC
300 strain being smaller (Tukey HSD, $p < 10^{-4}$) than those exposed to the PST+BEC strain (Fig.
301 2B).

302 At 21 dpf, eyed larvae yield of umbonate larvae exposed to the PST+BEC strain ($23.7 \pm$
303 7.3%) or to the BEC strain ($4.5 \pm 1.4 \%$) was significantly lower (Tukey HSD, $p < 10^{-2}$ and p
304 $< 10^{-2}$) than that of control larvae ($51.4 \pm 2.9 \%$) (Fig. 3A, Exp2). The yield of eyed larvae at
305 21 dpf was not significantly different (Tukey HSD, $p > 10^{-1}$) between PST+BEC and BEC
306 strains (Fig. 3A, Exp2). Competence in larvae exposed to the PST+BEC strain and the BEC
307 strain was delayed by 2 days (23 dpf) and 3 days (24 dpf), respectively, compared to control
308 larvae (21 dpf). Size at competence for metamorphosis was similar (Tukey HSD, $p > 0.05$) in
309 larvae exposed to the PST+BEC strain ($308.4 \pm 8.1 \mu\text{m}$) and control larvae ($306.8 \pm 4.0 \mu\text{m}$),
310 however, larvae exposed to the BEC strain ($284.3 \pm 4.6 \mu\text{m}$) were significantly smaller than
311 control larvae and larvae exposed to the PST+BEC strain (Tukey HSD, both $p < 0.05$). Larval

312 survival up to metamorphosis was not significantly affected by exposure to either HAB
313 treatment (ANOVA, $F_{5,18} = 1.9$, $p = 0.138$) (Fig. 3B, Table S1).

314 Larval survival during settlement was significantly lower (Tukey HSD, $p < 10^{-4}$) after
315 exposure of umbonate larvae to the BEC strain (88.9 ± 1.6 %) compared to control larvae (97.9
316 ± 0.1 %), whereas survival after exposure to the PST+BEC strain (95.3 ± 0.6 %) was similar to
317 the control (Fig. 3C, Exp2). Settlement yields of larvae exposed to the PST+BEC (8.7 ± 1.7 %)
318 and the BEC (5.4 ± 1.0 %) strains were significantly reduced (Tukey HSD, $p < 10^{-2}$ and $p <$
319 10^{-4}) compared to the control (16.6 ± 0.9 %) (Fig. 3D, Exp2).

320 3.3 Exposure of eyed larvae (Exp3)

321 Visual observations during the exposure indicated that the BEC strain modified the behavior
322 of eyed larvae, as described previously for umbonate larvae. Prior to exposure, at 20 dpf, algal
323 consumption was similar (ANOVA, $F_{2,9} = 2.6$, $p = 0.148$) in the three subgroups, whereas after
324 one day of exposure algal consumption was significantly lower (Tukey HSD; $p < 10^{-6}$ and $p <$
325 10^{-6}) in larvae exposed to the BEC strain ($117.1 \pm 25.5 \mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$) than in larvae exposed
326 to the PST+BEC strain ($3435.8 \pm 310.1 \mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$) and in control larvae (2627.2 ± 217.5
327 $\mu\text{m}^3 \text{larva}^{-1} \text{d}^{-1}$; Table 1). After one day of *A. minutum* exposure (21 dpf), larval height was
328 similar in larvae exposed to the PST+BEC strain ($305.9 \pm 2.9 \mu\text{m}$; Tukey HSD, $p > 0.05$), larvae
329 exposed to the BEC strain ($290 \pm 3.6 \mu\text{m}$; Tukey HSD, $p > 0.05$), and control larvae ($306.8 \pm$
330 $4.0 \mu\text{m}$; Tukey HSD, $p > 0.05$) (Fig. 2C). Eyed larvae yield at 21 dpf and larval survival up to
331 competence for metamorphosis of larvae exposed to both *A. minutum* strains were similar
332 (Tukey HSD, $p > 0.05$) to control larvae (Fig. 3A, B).

333 Exposure of eyed larvae to the BEC strain significantly decreased (Tukey HSD, $p < 10^{-5}$ and
334 $p < 10^{-3}$, respectively) survival during settlement (91.4 ± 1.8 %) and settlement yield (4.7 ± 0.6
335 %), as compared to control larvae (97.9 ± 0.1 %, 16.6 ± 0.9 %) (Fig. 3C, D). Exposure of eyed
336 larvae to the PST+BEC strain had no significant effect (Tukey HSD, $p > 0.05$) on survival

337 during settlement ($97.9 \pm 0.5 \%$) and settlement ($18.9 \pm 1.8 \%$), compared to the control (Fig.
338 3C, D).

339 3.4 Paralytic shellfish toxin content

340 Following exposure to the PST+BEC strain, toxin quantification by the ELISA method
341 indicated that PST content in larvae was 15.6 ± 1.1 ng STX g⁻¹ of wet larvae weight at 23 dpf
342 (8 days after exposure at the umbonate stage (Exp2)), and 38.8 ± 5.0 ng STX g⁻¹ of wet larvae
343 weight at 21 dpf (at the end of the exposure at the eyed stage (Exp3)).

344 No PST was detected in control larvae, in larvae following exposure to the PST+BEC strain
345 at the embryo stage (Exp1), and in larvae following exposure to the BEC strain at the umbonate-
346 (Exp2) and eyed-larvae stage (Exp3).

347 4. Discussion

348 4.1 Oyster embryos are highly vulnerable to the BEC produced by *A. minutum*

349 The most striking effect observed in this study was the lysis of oyster embryos exposed to
350 the *A. minutum* BEC strain (Fig. 3A). Embryos, which are not protected by a shell and do not
351 have complex tissues that could assume a protective role, are particularly sensitive to toxic
352 extracellular compounds (Mu and Li, 2013; Basti et al., 2015). Similarly, non-PST producing
353 strains of *A. affine* have been demonstrated to drastically alter the development of Japanese
354 pearl oyster *Pinctada fucata martensii* embryos, causing destabilization of cell membranes,
355 abnormal swellings and cytoplasmic discharges (Basti et al., 2015). Lysis of *C. gigas* embryos
356 observed in the present study could result from an impairment of cell membrane integrity,
357 caused by direct damage to external membranes, as similar cytotoxic impacts have been
358 observed with non-PST producing strains of *A. affine* (Basti et al., 2015). Results of the present
359 work indicated that responses of oysters differed depending on the *A. minutum* strain they were
360 exposed to, and thus potentially on the type and/or amount of bioactive substances produced by
361 the strains. The BEC strain, demonstrated as more cytotoxic on diatoms than the PST+BEC

362 strain (Long et al., 2018a), also appeared as more toxic for oyster early life stages due to either
363 a higher production of BEC and/or a production of more potent BEC. The PST+BEC strain did
364 not induce mortality of oyster embryos, however, sublethal effects were observed in D-larvae,
365 with increased incidence of mantle anomalies and membrane swelling. These abnormalities
366 caused by the PST+BEC strain might be attributable to the toxicity of BEC and not be caused
367 by PST. Indeed, PST are mainly intracellular and released in oyster tissues following ingestion
368 and lysis of toxic dinoflagellate cells (Bricelj and Shumway, 1998) and oysters at the D-larvae
369 stage are unable to ingest cells as large as *A. minutum* (Castrec et al., 2019).

370 4.2 *Exposure of larvae to A. minutum reduces larval growth, survival and settlement*

371 The effects of *A. minutum* on oyster umbonate and eyed larvae are more complex to interpret
372 than those on embryos and D-larvae, since both PST and BEC could be implicated. Indeed,
373 umbonate larvae (mean shell height 197 μm after 2 days of exposure) and eyed larvae (≈ 300
374 μm) ingested *A. minutum* cells and accumulated PST when exposed to the PST+BEC strain.
375 These results complete previous work demonstrating that *C. gigas* eyed larvae (mean shell
376 length 304 μm) fed on *A. minutum* while smaller umbonate larvae (mean shell length 150 μm)
377 did not (Castrec et al., 2019). This also supports the findings of Baldwin (1995) who
378 demonstrated that *C. virginica* larvae larger than 200 μm fed preferably on large food material
379 (22 to 30 μm), presumably dinoflagellate cells, in the presence of large dinoflagellate bloom.

380 Both *A. minutum* strains provoked growth reduction of larvae exposed at the umbonate
381 stage compared to control. The decrease in feeding activity of umbonate larvae most probably
382 explains their growth reduction and consequently, delays in developmental rates. This altered
383 growth likely results from BEC toxicity since BEC strain induced more severe responses than
384 the PST+BEC strain which produces less BEC or less potent BEC (larval height after 2 days of
385 exposure: - 23 % for the BEC strain vs - 9 % for the PST+BEC strain, as compared to control

386 larvae). These results confirm the role of uncharacterized, non-PST toxins produced by
387 *Alexandrium* spp in the toxicity to oyster larvae presumed by Mu and Li (2013).

388 The reduction in feeding activity was coincident with a radical modification of
389 swimming behavior in umbonate and eyed larvae exposed to the BEC strain. Larvae stopped to
390 swim and sunk, remaining slightly opened, the velum kept inside the shells, and cilia of the
391 velum still in movement. Similarly, behavioral changes were observed among adult oysters
392 exposed to the same strain: increased valve opening duration and micro-closure activity,
393 reduced feeding activity, and inflammation responses in gills (Castrec et al., 2018). Two modes
394 of action can be proposed to explain the effects of BEC on swimming and feeding behavior of
395 larvae, as hypothesized for adult oysters (Castrec et al., 2018): (i) the BEC excreted in the water
396 could activate chemoreceptors modifying normal functioning of swimming and feeding organs
397 or (ii) have a cytotoxic effect, altering larval tissues integrity, characterized in umbonate larvae
398 by cilia exfoliation and abnormal masses in the velum.

399 Exposure of umbonate or eyed larvae to the BEC strain also had deleterious consequences
400 on the subsequent larval settlement and survival during settlement. The lower settlement yield
401 and survival could result from a lower energy state due to lower feeding activity caused by
402 BEC. The amount of food consumed during larval development and resulting energy reserve
403 acquisition are essential for successful settlement and metamorphosis (Holland and Spencer,
404 1973) and have been associated with greater survival during metamorphosis (Gallager and
405 Mann, 1986; Plough, 2018). Larval size at competence for metamorphosis was lower in BEC-
406 strain exposed umbonate larvae than in control larvae. Reduced larval size may increase
407 susceptibility to predation in natural habitats. Organ abnormalities caused by BEC in umbonate
408 larvae that could not have been repaired could also contribute to mortality during settlement.

409 For larvae exposed at umbonate stage to the PST+BEC strain of *A. minutum*, PST (still
410 detected in larvae 8 days post-exposure), could also play a role in the reduction of settlement,

411 as proposed in some previous studies (Yan et al., 2003; Mu and Li, 2013; Basti et al., 2015).
412 Saxitoxin, the parent molecule of PST (Cusick and Sayler, 2013), binds to voltage-gated Na⁺
413 channels with high affinity and interacts with, to a lesser extent, Ca²⁺ and K⁺ channels, changing
414 ionic fluxes into cells (Cestèle and Catterall, 2000; Wang et al., 2003; Llewellyn, 2006). PST
415 could affect settlement by modifying K⁺ fluxes into cells, found as important for settlement of
416 oyster larvae (Wang et al., 2015). Nervous system and more specifically acetylcholine-based
417 pathway that plays a role in larvae settlement (Beiras and Widdows, 1995) could also be altered
418 by PST accumulated in larvae as demonstrated in adult oysters exposed to the same strain of *A.*
419 *minutum* (Mat et al., 2018). Eyed larvae exposed to the PST+BEC also accumulated PST,
420 however, their settlement was similar to control larvae. These results suggest that the exposure
421 of eyed larvae (1 day for eyed larvae vs 2 days for umbonate larvae) was too short, or that eyed
422 larvae were less sensitive to PST than umbonate larvae, and/or PST accumulation in larvae
423 tissues was too low to induce visible effects attributable to PST.

424 **5. Conclusion**

425 The present study evidenced that cytotoxic bioactive extracellular compounds (BEC)
426 produced by *Alexandrium minutum* are detrimental for oyster early life stages, affecting embryo
427 development, larval swimming behavior, feeding activity, growth, survival and settlement, thus
428 confirming a PST-independent toxicity of *A. minutum*. The findings indicated that oyster
429 embryos are more sensitive to *A. minutum* than larvae, BEC having a highly potent lysis activity
430 on embryos. BEC also play a major role in *A. minutum* toxicity upon umbonate and eyed larvae
431 of the Pacific oyster, affecting feeding behavior and decreasing algal consumption, which
432 impaired the subsequent larval growth, settlement and survival. The present study revealed that
433 oyster larvae exposed to *A. minutum* accumulated PST which could affect settlement yield. The
434 potential effects of PST on larval development need to be further investigated. Changes in larval
435 swimming and feeding behavior caused by *A. minutum* would influence larval dispersal and

436 reduce growth, thereby increasing the susceptibility of larvae to predation in the environment.
437 Given the fact that *A. minutum* form blooms overlapping with the spawning and larval
438 development seasons of *C. gigas*, this study provides evidence that *A. minutum* could adversely
439 affect *C. gigas* settlement with potential consequences on the structure of wild and cultivated
440 populations of oysters.

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Tables

Table 1

Effects of *A. minutum* exposures on algal consumption (μm^3 of *T. lutea* and *Chaetoceros* sp. larva⁻¹ d⁻¹) of oyster larvae. Umbonate and eyed larvae were exposed for 2 days (Exp2) and 1 day (Exp3) respectively, to 10^3 cells mL⁻¹ of the PST+BEC or the BEC strain of *A. minutum*, whereas control larvae were only fed with *T. lutea* and *Chaetoceros* sp. (n = 4, letters in superscript denote significant groupings; Tukey HSD after ANOVA). Bold numbers are False Discovery Rate (FDR) adjusted p-values as described by Benjamini & Hochberg (1995).

Exposure	Days post fertilization	Algal consumption (μm^3 larva ⁻¹ d ⁻¹)			ANOVA		
		Control	PST+BEC	BEC	df	F	adjusted <i>p</i>
Exposure of umbonate larvae (Exp2)	13 (before exposure)	577.5 ± 23.8	682.3 ± 31.4	583.2 ± 32.4	2	4.0	7.27×10⁻²
	14 (1 d exposure)	704.9 ± 97.0 ^a	950.8 ± 121.4 ^a	134.4 ± 26.0 ^b	2	21.2	6.11×10⁻⁴
	15 (2 d exposure)	940.9 ± 21 ^a	753.7± 33.7 ^b	196.9 ± 25.3 ^c	2	202.6	1.82×10⁻⁷
Exposure of eyed larvae (Exp3)	20 (before exposure)	2848.8 ±167.1	2674.1 ± 284.8	2160.0 ± 194.8	2	2.6	1.48×10⁻¹
	21 (1 d exposure)	2627.2 ± 217.5 ^a	3435.8 ± 310.1 ^a	117.1 ± 25.5 ^b	2	177.9	1.84×10⁻⁷

Figures

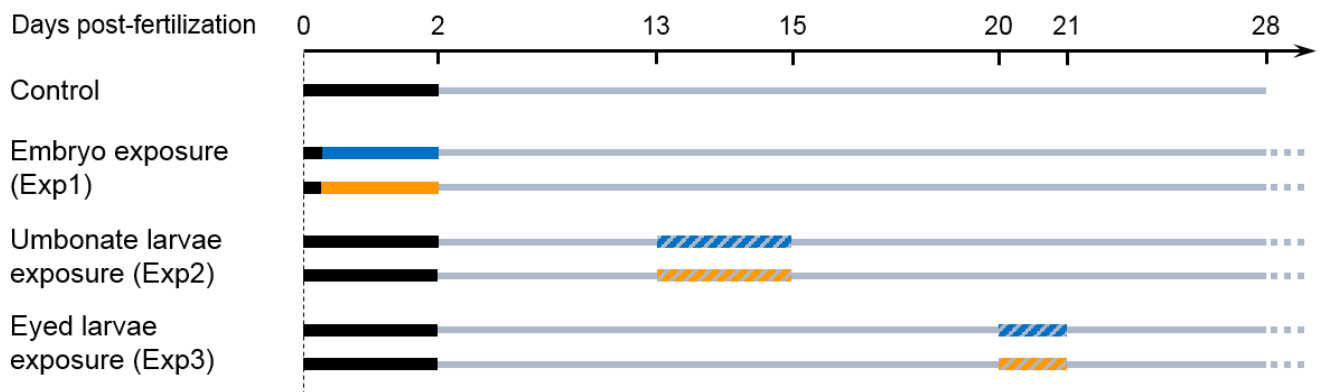


Fig. 1. Flow chart of the experiment. Different early life stages of oysters were exposed to two strains of *A. minutum*: 2-day exposure of embryos (Exp1); 2-day exposure of umbonate larvae (Exp2), and 1-day exposure of eyed larvae (Exp3). In the control treatment, fertilization and embryo development were conducted in filtered sea water (■), and larvae were fed *ad libitum* with non-toxic algae (■, Tiso/Chaeto). During Exp1, embryos were exposed to 10^3 cells mL^{-1} of the PST+BEC strain (■) or the BEC strain (■) of *A. minutum*. During Exp2 and Exp3, larvae were fed *ad libitum* with Tiso/Chaeto, and exposed to 10^3 cells mL^{-1} of the PST+BEC strain (▨) or the BEC strain of *A. minutum* (▨).

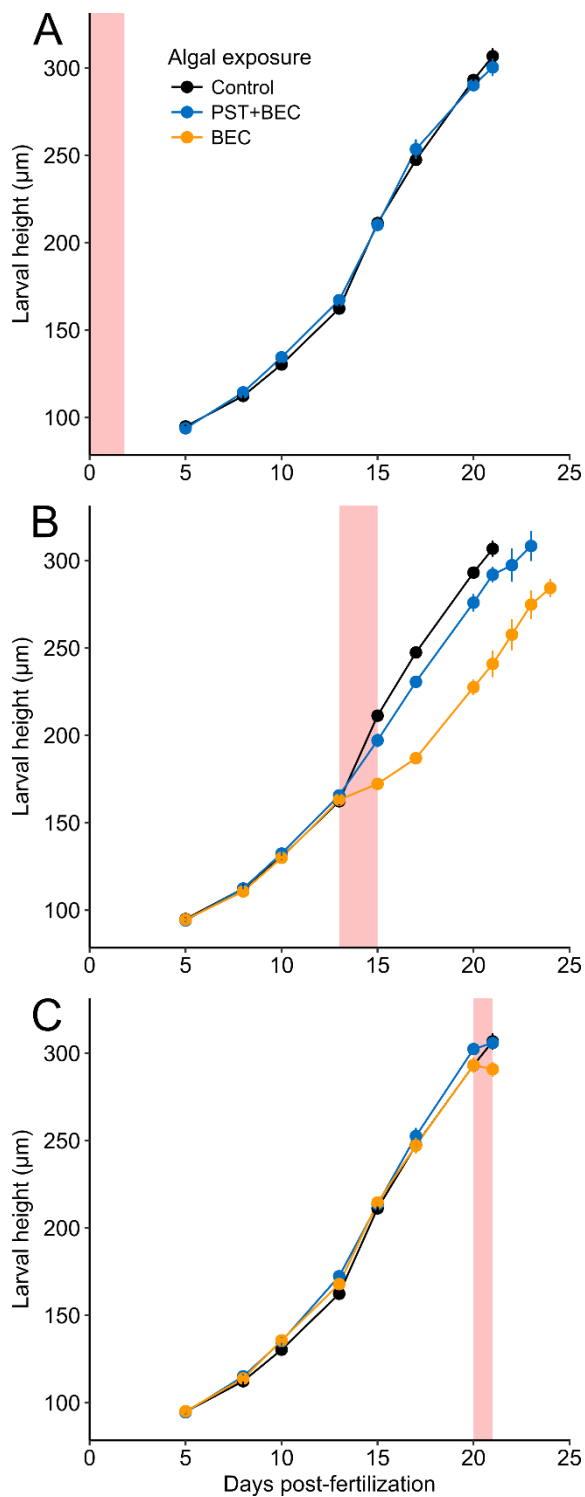


Fig. 2. Effects of *A. minutum* exposure on oyster larval height from 5 days post-fertilization up to competence for metamorphosis ($\geq 50\%$ of eyed larvae). A: Exp1, 2-day exposure of embryos (no data are available for Exp1 with the BEC treatment since hatching was completely inhibited with this treatment); B: Exp2, 2-day exposure of umbonate larvae; C: Exp3, 1-day exposure of eyed larvae. Red bars represent the timing for *A. minutum* exposures. Black, blue, and orange lines represent control larvae only fed with the mix diet of *T. lutea* and *Chaetoceros* sp., larvae exposed to 10^3 cells mL^{-1} of the PST+BEC strain of *A. minutum*, and larvae exposed to 10^3 cells mL^{-1} the BEC strain of *A. minutum*, respectively ($n = 4$, error bars denote \pm standard error). During the experiment, larvae from all treatments were fed *ad libitum* with the mix diet of *T. lutea* and *Chaetoceros* sp.

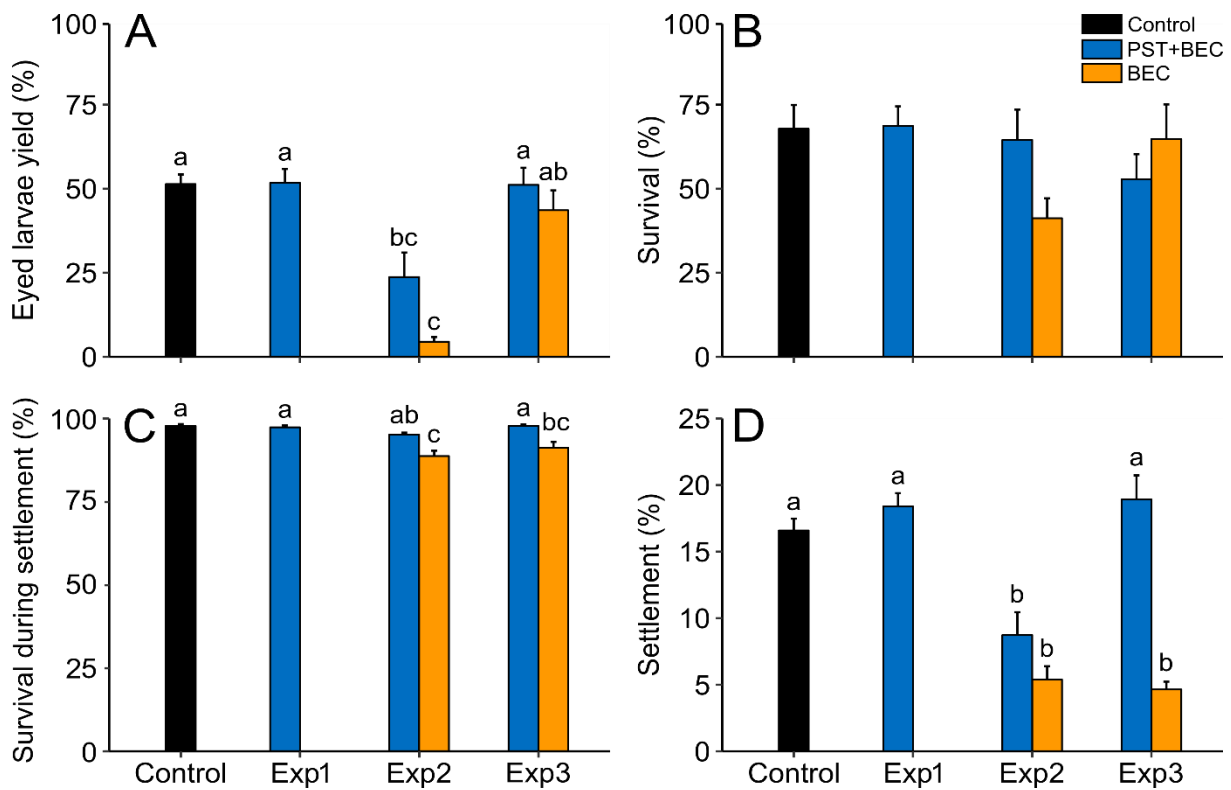


Fig. 3. Effects of *A. minutum* exposure on oyster early life stages: 2-day exposure of embryos (Exp1), 2-day exposure of umbonate larvae (Exp2), and 1-day exposure of eyed larvae (Exp3). Black, blue, and orange bars respectively represent control larvae, larvae exposed to the PST+BEC strain of *A. minutum* (10^3 cells mL^{-1}), and larvae exposed to the BEC strain of *A. minutum* (10^3 cells mL^{-1}). No data are available for Exp1 with the BEC treatment since hatching was completely inhibited with this treatment. A: eyed larvae yield (%) at 21 days post-fertilization (dpf); B: survival (%) of larvae once ≥ 50 % of larvae reached the eyed larvae stage (21-24 dpf depending on the algal treatment); C: larval mortality (%) during the settlement period; D: larval settlement yield ($n = 4$, error bars denote \pm standard error; letters denote significant groupings; $p < 0.05$; Tukey HSD following ANOVA).

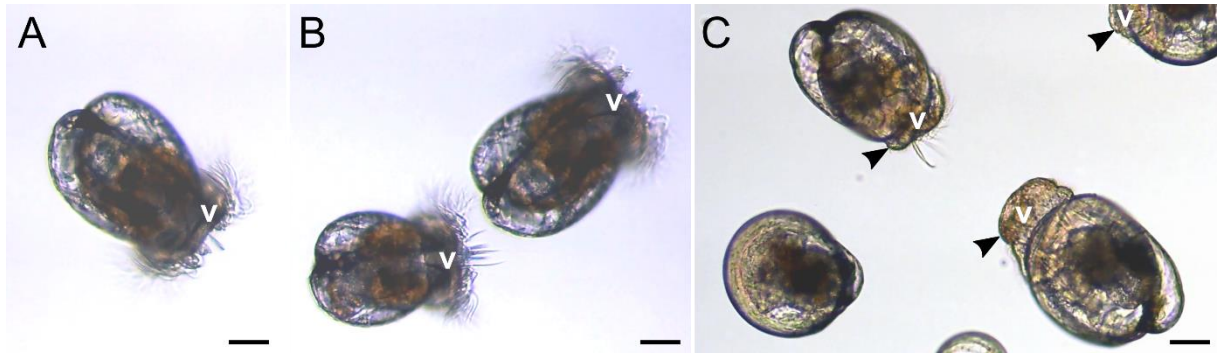


Fig. 4. Light micrographs of oyster umbonate larvae at 15 days post-fertilization, after a 2-day exposure to 10^3 cells mL^{-1} of *A. minutum* (Exp2). Control larvae (A) and larvae exposed to the PST+BEC strain of *A. minutum* (B) were actively swimming with normal velum whereas larvae exposed to the BEC strain of *A. minutum* (C) were mostly immobile and some larvae showed abnormal masses in the velum (indicated by black arrows), and loss of the velum cilia. V: velum. Bar = 50 μm .

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SUPPLEMENTARY MATERIAL FOR:

The toxic dinoflagellate *Alexandrium minutum* impairs the performance of oyster embryos and larvae

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Table S1

Effects of algal treatment on abnormal percentage of D-larvae, D-larvae yield, algal consumption, larval height at 15 and 21 days post-fertilization (dpf), eyed larvae yield at 21 dpf, larval survival and height at competence for metamorphosis, larval settlement yield, and survival during settlement period of *Crassostrea gigas* larvae. dpf: days post-fertilization. (n = 4). No data are available for Exp1 with the BEC treatment since hatching was completely inhibited with this treatment. Bold numbers are False Discovery Rate (FDR) adjusted p-values as described by Benjamini & Hochberg (1995).

Variable	<i>t</i> -test			
	<i>p</i> values		<i>FDR</i> adjusted probabilities	
Abnormality percentage (%)	2.67×10 ⁻⁷		6.23×10⁻⁷	
D-larvae yield (%)	8.67×10 ⁻¹		8.67×10⁻¹	
	One way ANOVA			
	df	F	<i>p</i> values	<i>FDR</i> adjusted probabilities
Algal consumption at 13 dpf (μm ³ of algae larva ⁻¹ d ⁻¹)	2	4.0	5.71×10 ⁻²	7.27×10⁻²
Algal consumption at 14 dpf (μm ³ of algae larva ⁻¹ d ⁻¹)	2	21.2	3.93×10 ⁻⁴	6.11×10⁻⁴
Algal consumption at 15 dpf (μm ³ of algae larva ⁻¹ d ⁻¹)	2	202	3.28×10 ⁻⁸	1.82×10⁻⁷
Algal consumption at 20 dpf (μm ³ of algae larva ⁻¹ d ⁻¹)	2	2.62	1.27×10 ⁻¹	1.48×10⁻¹
Algal consumption at 21 dpf (μm ³ of algae larva ⁻¹ d ⁻¹)	2	178	5.83×10 ⁻⁸	1.84×10⁻⁷
Larval height at 15 dpf (μm)	5	56.5	2.25×10 ⁻¹⁰	3.15×10⁻⁹
Larval height at 21 dpf (μm)	5	30.1	3.89×10 ⁻⁸	1.82×10⁻⁷
Eyed larvae yield at 21 dpf (%)	5	16.2	4.03×10 ⁻⁶	7.05×10⁻⁶
Larval survival at competence for metamorphosis (%)	5	1.9	1.38×10 ⁻¹	1.49×10⁻¹
Larval height at competence for metamorphosis (μm)	5	4.0	1.32×10 ⁻²	1.85×10⁻²
Larval settlement yield (%)	5	28.1	6.56×10 ⁻⁸	1.84×10⁻⁷
Larval survival during settlement period (%)	5	18.9	1.32×10 ⁻⁶	2.64×10⁻⁶

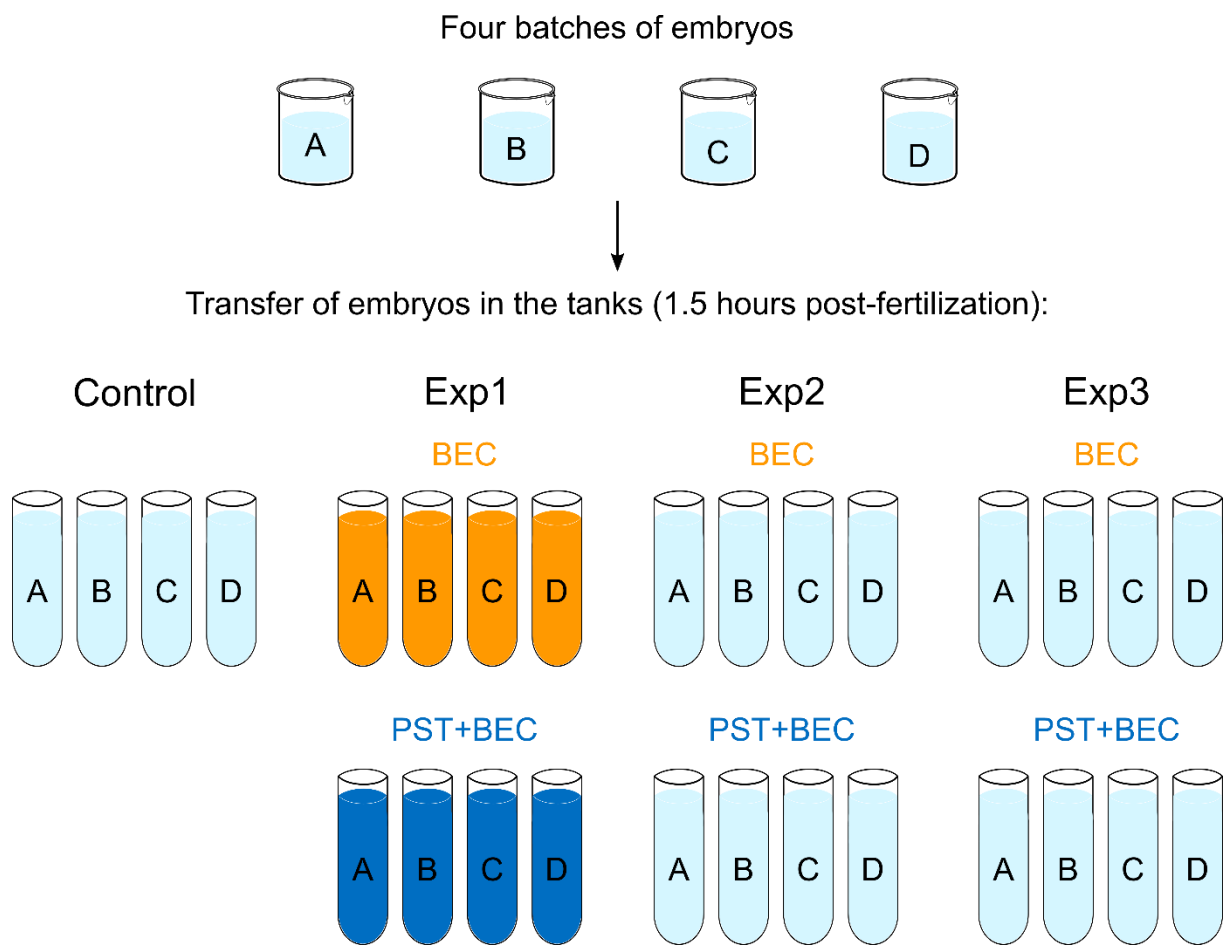


Fig. S1. Flow chart of the experiment representing the transfer of embryos from the four batches (A, B, C and D) to the tanks at 1.5 hours post-fertilization. For Exp1, embryos were transferred in tanks containing 10^3 cells mL^{-1} of the PST+BEC strain (■) or the BEC strain (■) of *A. minutum*. In the control treatment and for Exp2 and Exp3, embryo development was conducted in filtered sea water (■).

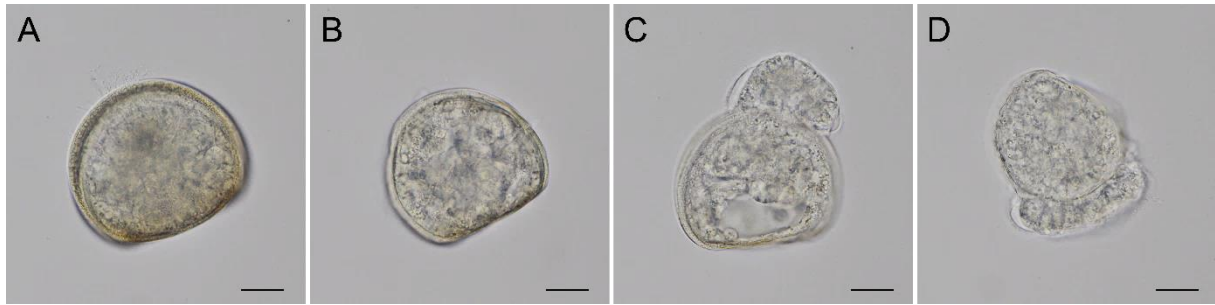


Fig. S2. Light micrographs of normal and abnormal *C. gigas* D-larvae 48 hours post fertilization. Embryos were non exposed (A, control) or exposed to 10^3 cells mL^{-1} of the PST+BEC strain of *A. minutum* (B,C,D) during two days (Exp1). (A) Normal control D-larvae; (B) Shell abnormality; (C) Mantle abnormality; (D) Developmental arrest. No micrographs of the BEC strain effects because it caused lysis and subsequent disintegration of embryos. Bar = 20 μm .

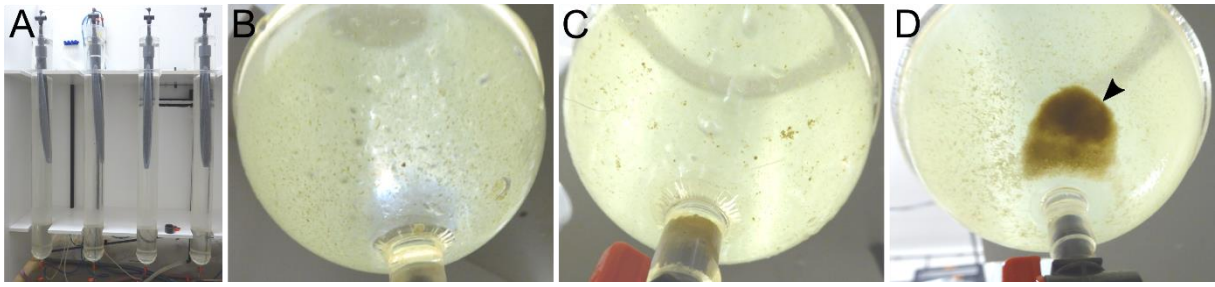


Fig. S3. Photographs illustrating behavior of *C. gigas* umbonate larvae in the experimental cylindrical tanks during Exp2 (A). Control larvae (B) and larvae exposed to the PST+BEC strain (10^3 cells mL⁻¹) (C) were swimming actively, whereas larvae exposed to the BEC strain (10^3 cells mL⁻¹) (D) sank to the bottom of the tanks (indicated by a black arrow) at 16 dpf (one day after the end of the exposure).