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## The dinoflagellate *Alexandrium minutum* affects development of the oyster *Crassostrea gigas*, through parental or direct exposure

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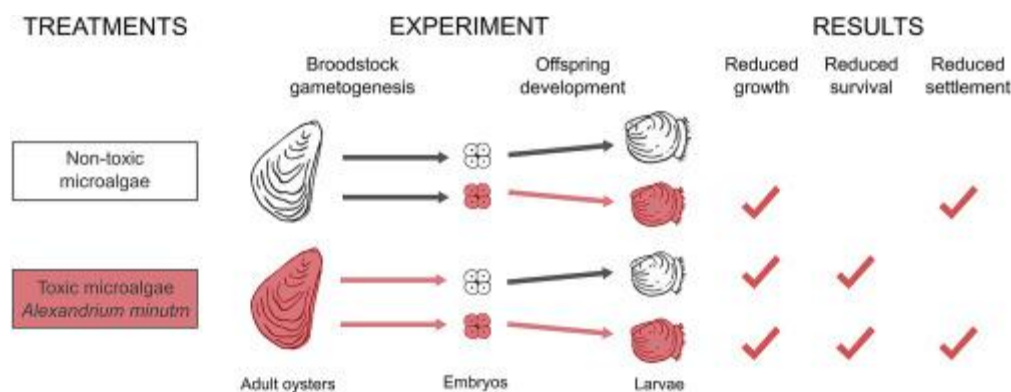
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### Abstract :

Harmful algal blooms are a threat to aquatic organisms and coastal ecosystems. Among harmful species, the widespread distributed genus *Alexandrium* is of global importance. This genus is well-known for the synthesis of paralytic shellfish toxins which are toxic for humans through the consumption of contaminated shellfish. While the effects of *Alexandrium* species upon the physiology of bivalves are now well documented, consequences on reproduction remain poorly studied. In France, *Alexandrium minutum* blooms have been recurrent for the last decades, generally appearing during the reproduction season of most bivalves including the oyster *Crassostrea gigas*. These blooms could not only affect gametogenesis but also spawning, larval development or juvenile recruitment. This study assesses the effect of toxic *A. minutum* blooms on *C. gigas* reproduction. Adult oysters were experimentally exposed to *A. minutum*, at environmentally realistic concentrations ( $10^2$  to  $10^3$  cells mL<sup>-1</sup>) for two months during their gametogenesis and a control group, not exposed to *A. minutum* was fed with a non-toxic dinoflagellate. To determine both consequences to next generation and direct effects of *A. minutum* exposure on larvae, the embryo-larval development of subsequent offspring was conducted with and without *A. minutum* exposure at  $10^2$  cells mL<sup>-1</sup>. Effects at each stage of the reproduction were investigated on ecophysiological parameters, cellular responses, and offspring development. Broodstock exposed to *A. minutum* produced spermatozoa with decreased motility and larvae of smaller size which showed higher mortalities during settlement. Embryo-larval exposure to *A. minutum* significantly reduced growth and settlement of larvae compared to non-exposed offspring. This detrimental consequence on larval growth was stronger in larvae derived from control parents compared to offspring from exposed parents. This study provides evidence that *A. minutum* blooms, whether they occur during gametogenesis, spawning or larval development, can either affect gamete quality and/or larval development of *C. gigas*, thus potentially impacting oyster recruitment.

## Graphical abstract



## Highlights

► Two-month exposure of adult oysters to *Alexandrium minutum* decreased sperm motility. ► Oocytes and spermatozoa of exposed oysters contained paralytic shellfish toxins. ► Larvae derived from these gametes showed reduced growth and survival. ► Exposure of oyster larvae to *A. minutum* altered larval growth and settlement. ► Adult exposure influenced offspring response to *A. minutum* exposure.

**Keywords :** Harmful algal bloom (HAB), Paralytic shellfish toxin (PST), *Crassostrea gigas*, Gametes, Larvae

## 44 **Introduction**

45 Harmful algal blooms (HABs) have increasingly disrupted coastal ecosystems for the last  
46 few decades (Kudela et al., 2015). Among toxic species, blooms of microalgae producing  
47 paralytic shellfish toxins (PST) represent an important threat for marine ecosystems, human  
48 health and the economy in coastal areas. Blooms of *Alexandrium* affect marine ecosystems by  
49 disrupting community and food web structures (Hallegraeff, 2010), and causing death of  
50 seabirds and mammals (Hattenrath-Lehmann et al., 2017). The resulting PST can affect  
51 humans via shellfish poisoning events (Anderson et al., 2012a), as filter-feeding bivalves  
52 accumulate PST in their flesh by feeding on PST-producing microalgae (Bricelj and

53 Shumway, 1998). Prohibition of shellfish harvesting, impairment of tourism and recreational  
54 activities are direct socio-economic consequences of PST-producing microalgae blooms in  
55 coastal regions (Anderson et al., 2012b). Detrimental effects of those phenomena are expected  
56 to intensify as the geographical distribution of HABs and the use of coastal waters for  
57 aquaculture are increasing worldwide (Lassus et al., 2016).

58 In marine ecosystems, outbreaks of Paralytic Shellfish Poisonings (PSPs) are mainly  
59 caused by the globally distributed dinoflagellate genus *Alexandrium* (Rossini, 2014). Blooms  
60 of *Alexandrium* spp. affect the physiology of bivalves including exploited species such as  
61 *Mytilus edulis*, *Pecten maximus*, *Venerupis philippinarum*, and *Crassostrea gigas* (Borcier et  
62 al., 2017; Bricelj et al., 1993; Haberkorn et al., 2010a; Lassudrie et al., 2014). *Alexandrium*  
63 *minutum* modifies valve behavior, disrupts biological rhythms, affects defense responses and  
64 digestion of *C. gigas* (Haberkorn et al., 2010b, 2011; Mat et al., 2013; Mello et al., 2013;  
65 Payton et al., 2017; Tran et al., 2010).

66 In France, *A. minutum* blooms usually occur from April to November and can reach high  
67 concentrations ( $> 10^7$  cells L<sup>-1</sup>) (Guallar et al., 2017, Chapelle et al., 2015). In French coastal  
68 waters, *A. minutum* blooms may last several months (Guallar et al., 2017) and are often  
69 concomitant with breeding period of bivalves, including the oyster *C. gigas* (Pouvreau et al.,  
70 2016; Suquet et al., 2016; Ubertini et al., 2017). Marine bivalves are ecosystem engineers and  
71 a key resource in coastal areas (Ekstrom et al., 2015). In France, 64,200 tons of Pacific oysters  
72 were produced in 2016, representing a value of 360,400 USD (FAO, 2018). In France, the  
73 natural recruitment of oyster spat constitutes a large part of cultivated stocks and repeated  
74 recruitment failures could have negative impacts on local oyster aquaculture. Given the  
75 economic importance of oysters, an understanding of the effects of *Alexandrium* blooms on  
76 the reproduction of *C. gigas* is critically important. Adult Pacific oysters which reproduce  
77 during the summer are frequently exposed to the harmful algae during gametogenesis.

78 Gametes, embryos and larvae, the free-living stages of oysters, also directly experience the  
79 toxicity of *Alexandrium* with potential consequences on recruitment.

80 Short-term exposure of mature Pacific oysters to *Alexandrium* spp. affected spermatozoa  
81 quality, and *in vitro* exposure of oocytes to *A. minutum* increased reactive oxygen species  
82 production in oocytes (Haberhorn et al., 2010b; Le Goïc et al., 2013). Knowledge regarding  
83 potential effects of adult oyster exposure to ecological concentrations of HAB species on the  
84 next generation remains scarce (Rolton et al., 2018, 2016; Vasconcelos et al., 2010). Adult  
85 eastern oysters *Crassostrea virginica* exposed to a natural bloom of the brevetoxin producer,  
86 *Karenia brevis*, produced smaller larvae with higher mortality than non-exposed oysters  
87 (Rolton et al., 2016). Northern quahogs *Mercenaria mercenaria* exposed to *K. brevis* showed  
88 reduced gonadal allocation and fertilization success, and the early development of the  
89 subsequent offspring was also affected (Rolton et al., 2018). Concerning direct exposure of  
90 larvae, acute short-term exposures of *Alexandrium* spp. negatively affected survival, growth,  
91 activity, and settlement of bivalve larvae, including *C. gigas*, *Pinctada fucata martensii*,  
92 *Mercenaria mercenaria*, *Chlamys farreri*, and *Argopecten irradians concentricus* (Basti et al.,  
93 2015; Matsuyama et al., 2001; Mu and Li, 2013; Tang and Gobler, 2012; Yan et al., 2003,  
94 2001). However, the effects of *A. minutum* blooms on oyster larvae remain unknown.

95 The present study investigated the effects of a 3-month exposure to toxic *A. minutum* at  
96 concentrations similar to natural bloom events on the gametogenesis of adult *C. gigas* and on  
97 their offspring. We assessed the weight (total, shell, and flesh), feeding, sex and  
98 gametogenesis stage of adult oysters, the quality and cellular, molecular and biochemical  
99 characteristics of gametes, and the growth, survival and settlement of larvae. The hypotheses  
100 tested were: (1) Gamete quality will be negatively affected by a long-term exposure to *A.*  
101 *minutum*, (2) Parental exposure may adversely affect subsequently produced offspring, and  
102 (3) Direct exposure of larvae to *A. minutum* will alter larval development.

## 103 1. Materials and methods

### 104 1.1 *Biological materials*

105 *Oysters*. Adult oysters *Crassostrea gigas* (*Magallana* gen. nov.; Salvi and Mariottini,  
106 2017) were produced and cultured in controlled conditions according to a standardized  
107 protocol (Petton et al., 2015), in Ifremer experimental facilities (Argenton and Bouin, France).  
108 Oysters were never exposed to any harmful algal bloom. Histological inspection at the start of  
109 the experiment in April 2016 showed that oysters (12 months old, mean total weight  $18.0 \pm$   
110  $0.4$  g) were in early gametogenesis (stage 1), according to Steele and Mulcahy (1999).

111 *Algal cultures*. *Tisochrysis lutea* (formerly *Isochrysis* sp., T. iso strain, CCAP 927/14) and  
112 *Chaetoceros* sp. (formerly *Chaetoceros neogracile*, strain CCAP 1010-3) were used as non-  
113 toxic food for oysters. They were cultured with continuous light ( $200 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ ) in  
114 separated 300-L cylinders enriched with Conway medium (Walne, 1970), and with silicium  
115 for *Chaetoceros* sp. The dinoflagellates *Alexandrium minutum* (Halim, AM89BM strain) and  
116 *Heterocapsa triquetra* (Ehrenberg, 1840; HT99PZ strain) were grown in filtered seawater ( $0.2$   
117  $\mu\text{m}$ ) supplemented with L1 medium (Guillard and Hargraves, 1993) and kept in exponential  
118 growth phase. Cultures were maintained at  $17 \pm 1$  °C. This *A. minutum* strain produced a  
119 quantity of Paralytic Shellfish Toxins (PST) equivalent to  $1.3 \pm 0.1$  pg eq. STX per cell in the  
120 exponential growth phase (Haberkorn et al., 2010 a, b). This strain also produces bioactive  
121 extracellular compounds (BECs), which have allelopathic and cytotoxic activities (Borcier et  
122 al., 2017; Castrec et al., 2018; Lelong et al., 2011). The non-toxic *H. triquetra* was chosen as  
123 a control because of its similarity to *A. minutum* in terms of size and shape (Tran et al., 2015).

### 124 1.2 *Experimental exposure of adult oysters to A. minutum*

125 The flow chart of experimental procedure is presented in Fig. 1. After acclimation, the  
126 oysters were placed in six experimental 50-L tanks (46 oysters per tank) supplied with filtered  
127 running seawater (1- $\mu\text{m}$  filtered and UV-treated (FSW);  $17.0 \pm 0.1$  °C, pH  $8.3 \pm 0.1$ , and 35.1

128  $\pm 0.1$  PSU;  $12.5 \text{ L h}^{-1}$ ), and fed continuously on an algal mixture (1:1 equivalent volume) of *T.*  
129 *lutea* and *Chaetoceros* sp. (Tiso/Chaeto) at a daily ratio equal to 6 % dry mass algae/dry mass  
130 oyster (Petton et al., 2015). The toxic *A. minutum* was continuously added to Tiso/Chaeto in 3  
131 replicate tanks (exposed treatment) at a concentration of  $10^2$  cells  $\text{mL}^{-1}$ , except during the  
132 seventh week of the experiment where the concentration was gradually increased to  $10^3$  cells  
133  $\text{mL}^{-1}$  for 4 days, mimicking an *A. minutum* bloom event (Chapelle et al., 2015). The 3 other  
134 tanks (control treatment) were exposed to the same concentrations of the non-toxic  
135 dinoflagellate *H. triquetra*. Algal concentrations and exposure time correspond to  
136 environmentally realistic conditions (Chapelle et al., 2015). To prevent algal sinking, the  
137 water inflow was pressurized to create recirculating flow in the tank and air bubbling was  
138 used. The oysters were conditioned for 8 weeks to reach ripeness.

### 139 1.3 Algal consumption of adult oysters

140 For each algal treatment, a fourth tank was deployed without oysters to evaluate algal  
141 sinking. Once a day, inflow and outflow seawater was sampled from each tank (20 mL).  
142 Phytoplankton counts were made using an electronic particle counter (Multisizer 3 equipped  
143 with a 100- $\mu\text{m}$  aperture tube) to provide 56 days of continuous data. Algal sinking (S) was  
144 evaluated in percentage of microalgae retained in the tank without oysters:  $S = [(V_o - V_i) / V_i]$   
145  $\times 100$ ;  $V_i$  being the number of algae at the inlet of the control tank, and  $V_o$  the number of  
146 algae at the outlet. Algal sinking was low ( $< 4$  %) and similar in the two treatments. Total  
147 algal consumption, i.e. dinoflagellate and Tiso/Chaeto, was expressed in algal cell volume per  
148 oyster per hour ( $\mu\text{m}^3 \text{ oyster}^{-1} \text{ h}^{-1}$ ), as in Sussarellu et al. (2016).

### 149 1.4 Sampling

150 At 2, 4, 6, and 8 weeks after the beginning of exposure (corresponding to T2, T4, T6 and  
151 T8, respectively), five animals per tank were sampled for flesh mass, and a transversal section  
152 of the gonadic area for histological examination (Fig. 1). The remainder of the digestive gland



153 was flash frozen in liquid nitrogen and stored at -20 °C for subsequent PST quantification. At  
154 the beginning (T0) and at the end (T8) of the experiment, 15 oysters per algal treatment were  
155 sampled to measure biometric parameters (total wet mass (TWM), wet shell mass (WSM),  
156 and dry flesh mass (DFM)). Condition index was calculated as:  $DFM \times 100 / (TWM - WSM)$ .

157 Six control and 6 exposed oysters of each sex were collected after 7 weeks of broodstock  
158 conditioning (T7) for gamete quality measurements (see section 1.6).

159 Gametes were collected at T8 in the remaining control and exposed animals (45 oysters per  
160 treatment, 15 oysters per tank) by stripping the gonad, and pooling gametes of oysters from  
161 the same tank, for each sex. Oocytes were suspended in FSW, filtered in a 100- $\mu$ m sieve, and  
162 oocyte concentrations were determined by flow cytometry (FCM) according to Le Goïc et al.  
163 (2014). Oocytes were transferred in 2 mL tubes for PST quantification and biochemical  
164 composition measurements ( $2 \times 10^6$  oocytes for each analysis, stored at -20 °C), and for RNA  
165 analyses ( $4 \times 10^6$  oocytes, stored in liquid nitrogen). Sperm samples for PST content and  
166 biochemical composition were obtained by direct pipetting the incised male gonad. For larval  
167 rearing, gametes were pooled for each treatment and each sex (Fig. 1).

### 168 1.5 *Histology*

169 A 3-mm cross section of the visceral mass was excised in front of the pericardial region and  
170 immediately fixed in modified Davidson's solution (Latendresse et al., 2002) for 24 h at 4 °C.  
171 Tissues were processed, stained with Harris' hematoxylin–eosin, and observed as described by  
172 Hermabessiere et al. (2016). Tissue sections were examined under a light microscope (Leica  
173 DMIRB) equipped with a digital camera (Imaging RETIGA 2000R) to determine the sex and  
174 gametogenesis stage of each oyster according to the reproductive scale reported by Steele and  
175 Mulcahy (1999). In addition, measurement of reproductive effort was determined by image  
176 software (Adobe® Photoshop®): gonadal occupation index is the percentage of whole gonadal  
177 area in relation to the total transverse section area (Fabioux et al., 2005).

178 1.6 *Gamete cellular analyses*

179 *Gamete collection.* Oocytes and spermatozoa were collected at T7 for each oyster by  
180 stripping entirely the gonad, according to Boulais et al. (2017) for spermatozoa and Le Goïc et  
181 al. (2014) for oocytes. Spermatozoa were collected in 10 mL of FSW at 19 °C and sieved  
182 through 60 µm mesh. Oocytes were suspended in FSW, sieved through 100 µm to remove  
183 pieces of gonad tissue and concentrated on a 20-µm sieve. Oocyte and spermatozoa  
184 concentrations were determined by FCM according to Le Goïc et al. (2013, 2014) and  
185 adjusted to  $5 \times 10^4$  oocytes mL<sup>-1</sup> and  $1 \times 10^7$  spermatozoa mL<sup>-1</sup>, for FCM and cellular  
186 analyses.

187 *Cellular parameters by flow cytometry.* FCM measurements were performed using an  
188 EasyCyte Plus cytometer (Guava Millipore) equipped with standard optics and a 488 nm  
189 argon laser. Analyses of gamete relative size and complexity, viability, mitochondrial  
190 membrane potential (MMP), and reactive oxygen species (ROS) production were performed  
191 according to Le Goïc et al. (2013, 2014) for spermatozoa and oocytes.

192 *Oocyte morphological measurements.* Oocyte circularity (ranging from 0 to 1, where a  
193 value of 1 indicates a perfect circle) and Feret's diameter (Ferreira and Rasband, 2012) were  
194 measured under microscope, using ImageJ software (n = 30 oocytes for each female)  
195 according to Boulais et al. (2015a).

196 *Characterization of spermatozoa movement.* Spermatozoa movement was triggered using a  
197 two-step dilution in an activating solution (FSW, 5 g L<sup>-1</sup> bovine serum albumin, Tris 20 mM,  
198 pH 8.1, dilution rate 1:30) and analyzed under microscope using a CASA plug-in for ImageJ  
199 software. The percentage of motile spermatozoa and their velocity (VAP: Velocity of the  
200 Average Path) were assessed on a minimum of 30 spermatozoa for each male, according to  
201 Boulais et al. (2015b).

202 *Quantification of ATP content in spermatozoa.* Intracellular ATP content of spermatozoa  
203 was estimated in triplicates using  $5 \times 10^6$  spermatozoa for each male as described in Boulais  
204 et al. (2015b), by bioluminescence (kit ATPlite, Perkin Elmer) using a plate reader  
205 (EnSpire™ 2300 Multilabel Reader, PerkinElmer).

#### 206 1.7 *Quantification of total RNA in oocytes*

207 Total RNA was isolated using Tri-reagent (Sigma), treated with rDNase (Macherey-  
208 Nagel), purified using affinity chromatography (Nucleospin RNA kit, Macherey-Nagel)  
209 according to the manufacturer's instructions, and assayed for concentration using a ND-1000  
210 spectrophotometer (Nanodrop Technologies).

#### 211 1.8 *Protein, lipid, and carbohydrate compositions of gametes*

212 Total lipids and carbohydrates were analyzed as described by Bligh and Dyer (1959), and  
213 Dubois et al. (1956), respectively. Total protein content was assayed as described by Da Costa  
214 et al. (2016). Dry weights were determined with a 400  $\mu$ L aliquot of the first fraction  
215 distributed in pre-weighed capsules and dried at 80 °C for 48 h. Content of each constituent  
216 was expressed as: biochemical content of each constituent in  $\text{mg} \times 100 / \text{dry mass of gonad in}$   
217  $\text{mg}$ .

#### 218 1.9 *Toxin quantification*

219 PST extraction was performed individually following manufacturer instructions: digestive  
220 gland tissue was homogenized in HCl 0.1 M (1:1, w:v) using a Precellys®24 beads-grinder,  
221 then boiled for 5 min. For the sampling times T2, T4, and T8, individual homogenates were  
222 pooled for each tank ( $n = 3$  pools of 5 oysters for each sampling time). The PST digestive  
223 gland content was analyzed individually just before (T6,  $n = 15$ ) and after (T7,  $n = 12$ ) the  
224 high dinoflagellate bloom simulated in this experiment. Toxin quantification was performed  
225 by spectrophotometry using the Abraxis ELISA PSP kit (Novakits, France; see methods in

226 Lassudrie et al. (2015)). Toxin load was expressed in  $\mu\text{g STX } 100 \text{ g}^{-1}$  of wet digestive gland  
227 weight (DG) or gonad weight.

#### 228 1.10 Larval rearing

229 To test the influence of parental exposure on offspring, fertilization was performed for  
230 each treatment; a pool of  $15 \times 10^6$  oocytes (from 33 control females or 29 exposed females)  
231 were fertilized separately in a beaker using a pool of sperm (from 12 control males or 16  
232 exposed males) using a non-limiting sperm to oocyte ratio (100:1) (Fig. 1). Embryos were  
233 transferred 1.5 hours post-fertilization (hpf) in 150-L tanks in UV-treated FSW at a  
234 concentration of  $50 \text{ embryos mL}^{-1}$  and maintained 48 h at  $21 \text{ }^\circ\text{C}$ . D-larvae were then  
235 transferred to 5-L cylindrical triplicate tanks at the density of  $50 \text{ larvae mL}^{-1}$ , and maintained  
236 in a flow-through rearing system (100% seawater renewal  $\text{h}^{-1}$ ,  $21 \text{ }^\circ\text{C}$ , 35 PSU) (Fig. 1). The  
237 larvae were fed continuously with Tiso/Chaeto as described by Asmani et al. (2016).

238 To test for influence of offspring exposure, fertilization and larval rearing were performed  
239 for each algal treatment as described above, but the subsequent offspring were continuously  
240 exposed to *A. minutum* ( $10^2 \text{ cells mL}^{-1}$ ), from 4-cell embryos (2.5 hpf) to veliger larvae (22  
241 days post-fertilization, dpf) (Fig. 1).

242 Larvae were sampled every 2–3 days and fixed in a 0.1% formaldehyde-seawater solution  
243 until image analysis for size monitoring. Larval size was assessed by measuring shell length  
244 using image analysis on at least 30 larvae per tank per day of sampling (WinImager 2.0 and  
245 ImageJ software for image capture and analysis, respectively).

246 A small complementary experiment was carried out on oyster larvae raised on the same  
247 conditions as the control larvae to test the capacity of larvae to ingest *A. minutum* cells.  
248 Umbonate larvae (mean shell length  $150 \pm 22 \mu\text{m}$ ) and eyed larvae (mean shell length  $304 \pm$   
249  $15 \mu\text{m}$ ) were exposed to *A. minutum* (AM89BM strain) and the presence of *A. minutum* cells  
250 in larvae digestive tract and feces was checked under light microscope.

251 1.11 *Larval survival and settlement*

252 All the tanks were drained and the total number of larvae was determined at 22 dpf (Fig.  
253 1), when  $\geq 50\%$  of larvae reached the eyed larvae stage (morphological competence for  
254 metamorphosis) in the control tanks (non-exposed oysters derived from control parents). Each  
255 larval population was transferred to a PVC container with a 125- $\mu\text{m}$  nylon mesh base (in  
256 triplicate per treatment), maintained in a flow-through system (9 L h<sup>-1</sup>, 30% h<sup>-1</sup> seawater  
257 renewal, 21 °C, 35 PSU) and fed the Tiso/Chaeto diet as described previously. After 7 days  
258 (29 dpf), the number of remaining swimming larvae, dead larvae, and larvae settled on tank  
259 walls and sieve were counted (Fig. 1). The percentage of survival during the settlement step  
260 was evaluated as: total number of alive larvae at 29 dpf  $\times$  100 / total number of larvae initially  
261 stocked at 22 dpf. The larval settlement was evaluated as: number of settled larvae at 29 dpf  $\times$   
262 100 / total number of alive larvae at 29 dpf.

263 1.12 *Statistical analyses*

264 Statistical analyses were performed using R version 3.2.2 (R Core Team, 2012). All values  
265 are expressed as mean  $\pm$  standard error (SE). Differences were considered significant when  $p$   
266  $< 0.05$ . Differences in oyster algal consumption were evaluated using repeated measures  
267 ANOVA, where ‘algal exposure’ and ‘day’ were fixed factors (Huvet et al., 2015).  
268 Comparison for oyster gonadal maturation and occupation index between the two algal  
269 treatments were investigated for each sampling time using Mann-Whitney U test and  $t$ -test,  
270 respectively. Results of oyster condition index, gonadal maturation and occupation were  
271 pooled for the 3 tanks of each algal treatment, after verifying there were no statistical  
272 differences between tanks using one-way ANOVA, Fisher’s exact test, and  $t$ -test,  
273 respectively. Number of oocytes, and gamete parameters were compared using  $t$ -test.  
274 Comparison for PST content in oyster digestive glands between sampling times was assessed  
275 using a non-parametric Kruskal-Wallis test with Mann-Whitney pairwise as post hoc test, as

276 homoscedasticity assumption was not met. To determine any significant differences between  
277 'parental exposure', and 'larval exposure' on larvae, larval length, survival, and settlement  
278 were analyzed using a two-way ANOVA, where 'parental exposure' and 'larval exposure'  
279 were fixed and orthogonal factors. Levene's test was used to determine any heterogeneity of  
280 variances and data were transformed if significant. In case of a significant interaction between  
281 the two factors, a Tukey HSD was used to detect differences among means.

## 282 2. Results

### 283 2.1 *Oyster algal consumption, gonad development and biochemical composition*

284 No mortality was observed in adult oysters during the experiment. A significantly higher  
285 algal consumption (+10 %,  $F = 66.93$ ,  $df = 1$ ,  $p < 0.01$ ; two-way ANOVA) was observed over  
286 the whole experiment for oysters exposed to *A. minutum* ( $3.32 \times 10^9 \pm 5.72 \times 10^7 \mu\text{m}^3$  of  
287 algae oyster<sup>-1</sup> h<sup>-1</sup>) compared to control oysters ( $3.02 \times 10^9 \pm 5.87 \times 10^7 \mu\text{m}^3$  of algae oyster<sup>-1</sup>  
288 h<sup>-1</sup>) when averaged over the whole experiment. There was a significant effect of date ( $F =$   
289  $114.88$ ,  $df = 47$ ,  $p < 0.001$ ; two-way ANOVA), and a date-exposure interaction on algal  
290 consumption ( $F = 4.92$ ,  $df = 47$ ,  $p < 0.001$ ; two-way ANOVA). No difference ( $t = 0.39$ ,  $df =$   
291  $28$ ;  $t$ -test) in condition index was observed between exposed and control oysters at the end of  
292 the conditioning ( $10.8 \pm 0.5$  and  $10.6 \pm 0.4$ , respectively).

293 Gonadal maturation was not different between exposed and control oysters during  
294 broodstock conditioning (Table S1). At T6, all oysters were ripe (stage 3) in both treatments  
295 and gonadal occupation index was not significantly different between exposed ( $61.5 \pm 3.0$  %)  
296 and control ( $55.1 \pm 3.1$  %) oysters (Table S1). The total number of oocytes collected by  
297 stripping at the time of breeding was not significantly different ( $t = -1.06$ ,  $df = 4$ ;  $t$ -test) in  
298 exposed ( $5.4 \times 10^6 \pm 0.4$ ) and control ( $6.5 \times 10^6 \pm 0.9$ ) females.

299 Gonad lipid content was higher (+55 %,  $t = 3.98$ ,  $df = 4$ ,  $p < 0.05$ ;  $t$ -test) in exposed males  
300 ( $17.0 \pm 1.5$  %) than in controls ( $10.9 \pm 0.4$  %), but protein and carbohydrate contents were not

301 different (Table S2). No significant difference in oocyte biochemical composition was  
302 observed between both treatments (Table S2).

### 303 2.2 *Gamete quality*

304 Significantly lower percentage of motile spermatozoa ( $-36\%$ ,  $t = -2.94$ ,  $df = 9$ ,  $p < 0.05$ ;  $t$ -  
305 test) was observed in exposed oysters ( $30 \pm 5\%$ ) compared to control ( $48 \pm 3\%$ ) oysters  
306 (Table S3). Spermatozoa velocity and ATP content were similar between the two treatments  
307 (Table S3). Oocyte diameter and circularity did not differ between treatments (Table S3).  
308 Oocyte and spermatozoa cellular characteristics measured by flow cytometry were similar in  
309 exposed and control oysters (Table S3).

### 310 2.3 *Paralytic shellfish toxin content*

311 The PST content in the digestive glands of exposed oysters was  $10.9 \pm 0.5$ ,  $26.7 \pm 1.6$ , and  
312  $19.3 \pm 3.5 \mu\text{g STX } 100 \text{ g}^{-1}$  of DG after 2 (T2), 4 (T4), and 6 weeks of conditioning (T6),  
313 respectively. The PST content in the digestive glands of exposed oysters was higher ( $p < 0.05$ ;  
314 Kruskal-Wallis) at T7 ( $131.3 \pm 22.4 \mu\text{g STX } 100 \text{ g}^{-1}$  of DG) and T8 ( $52.5 \pm 5.0 \mu\text{g STX } 100$   
315  $\text{g}^{-1}$  of DG) (i.e. after the mimicked *A. minutum* bloom at  $10^3$  cells per mL) than at T2, T4 and  
316 T6.

317 The PST content in exposed oyster gonads measured at T8 was  $1.8 \pm 0.1 \mu\text{g STX } 100 \text{ g}^{-1}$   
318 wet gonad weight ( $n = 3$  pools of 4-6 oysters) for exposed males, and  $0.3 \pm 0.1 \mu\text{g STX } 100$   
319  $\text{g}^{-1}$  wet oocytes weight ( $n = 3$  pools of 9-10 oysters) for exposed females, corresponding to 3.3  
320 % and 0.6 % of the PST content measured in the digestive glands, respectively.

### 321 2.4 *Larval length, survival and settlement*

322 Larval length was significantly affected by both parental and larval exposures to *A.*  
323 *minutum* (Fig. 2). Larval and parental exposures had significant independent ( $F = 39.04$ ,  $df =$   
324  $1$ ,  $p < 0.001$ , and  $F = 7.134$ ,  $df = 1$ ,  $p < 0.05$ , respectively; two-way ANOVA) and interactive  
325 effects ( $F = 15.22$ ,  $df = 1$ ,  $p < 0.01$ ; two-way ANOVA) upon larval length at 22 dpf (Fig. 3Fig.

326 3A). At 22 dpf, non-exposed larvae derived from exposed parents ( $248.3 \pm 11.3 \mu\text{m}$ ) were  
327 significantly smaller (-15 %,  $p < 0.01$ ; Tukey HSD) compared to non-exposed larvae derived  
328 from control parents ( $293.2 \pm 3.7 \mu\text{m}$ ).

329 Exposed larvae derived from control parents ( $223.9 \pm 4.4 \mu\text{m}$ ) were smaller (-24 %,  $p <$   
330  $0.001$ ; Tukey HSD) compared to non-exposed larvae derived from control parents, whereas  
331 the length of exposed larvae derived from exposed parents ( $232.3 \pm 5.1 \mu\text{m}$ ) was not  
332 significantly different (-6 %) from non-exposed larvae derived from exposed parents (Fig.  
333 3A). The length of exposed larvae derived from exposed parents was, however, lower (-20 %,  $p <$   
334  $0.01$ ; Tukey HSD) than non-exposed larvae derived from control parents (Fig. 3A).

335 Parental exposure, but not larval exposure, significantly affected larval survival during the  
336 settlement step (Fig. 3B,  $F = 8.21$ ,  $df = 1$ ,  $p < 0.05$ ; two-way ANOVA). Larvae derived from  
337 exposed parents had reduced survival ( $33.6 \pm 10.8 \%$  and  $32.0 \pm 7.1 \%$  for non-exposed and  
338 exposed larvae, respectively) compared to larvae derived from control parents ( $67.3 \pm 4.2 \%$   
339 and  $41.2 \pm 6.3 \%$  for non-exposed and exposed larvae, respectively; Fig. 3B).

340 Larval exposure, but not parental exposure, significantly reduced larval settlement ( $F =$   
341  $8.77$ ,  $df = 1$ ,  $p < 0.05$ ; two-way ANOVA, Fig. 3C). Settlement rates were  $3.9 \% \pm 0.3$  and  $5.1$   
342  $\% \pm 1.8$  for exposed larvae, and  $11.9 \% \pm 1.1$  and  $30.0 \% \pm 10.9$  for non-exposed larvae  
343 derived from control and exposed parents, respectively (Fig. 3C).

344 Ingestion tests on control larvae indicated that eyed larvae fed on *A. minutum*, as these  
345 algal cells were observed in the intestine and feces of eyed larvae (Fig. 4C-E). Conversely,  
346 ingestion was not observed with early umbonate larvae due to the relative large cell size of *A.*  
347 *minutum* ( $23\text{--}29 \mu\text{m}$ ) (Fig. 4A, B).

### 348 3. Discussion

349 Coastal areas regularly experience few days to several weeks of HAB during bivalve  
350 reproductive season (Guallar et al., 2017). This experiment was designed to assess the



351 consequences of the toxic dinoflagellate *Alexandrium minutum* upon reproduction, early  
352 development, and settlement of *Crassostrea gigas* oysters, an environmentally and economic  
353 important species.

### 354 3.1 *Presence of A. minutum modifies feeding of maturing oysters*

355 Algal consumption was significantly higher for oysters exposed to *A. minutum* than for  
356 control oysters over the whole experiment. Pousse et al. (2018) applied a mechanistic model  
357 based on Dynamic Energy Budget theory to the data of the present study, coupling the  
358 kinetics of PST accumulation and bioenergetics in *C. gigas*. They evidenced that toxicant  
359 stress provoked by *A. minutum* affected the energy balance of oysters, more energy being  
360 needed for tissue damage repair and detoxification of toxic substances produced by *A.*  
361 *minutum*. Exposed adult oysters would therefore increase their food consumption to adjust  
362 energy intake. Such modification of feeding activity was observed in oysters exposed to  
363 polystyrene microspheres to compensate digestive interference caused by plastic particles  
364 (Sussarellu et al., 2016) and has been proposed for female copepods *Acartia (Acartiura)*  
365 *clausi* exposed to *A. minutum* (previously *A. lusitanicum*) (Dutz, 1998).

366 Under our experimental conditions of dual feeding with *A. minutum* and non-toxic  
367 Tiso/Chaeto algae, the higher feeding rate seemed to partly counterbalance the higher energy  
368 demand due to *A. minutum*. Coupled with active detoxification of toxins (Fabioux et al.,  
369 2015), this could explain the absence of major visible effects on gonadal maturation and  
370 reproductive effort. However, the lower percentage of motile spermatozoa in oysters exposed  
371 to *A. minutum* still suggests that this response might not be sufficient to overcome PST  
372 toxicity on broodstock.

### 373 3.2 *Broodstock exposure to A. minutum affected quality of gametes*

374 In male oysters, exposure to *A. minutum* decreased the percent of motile spermatozoa.  
375 Haberkorn et al. (2010b) evidenced that a short acute exposure of mature oysters to *A.*

376 *minutum* reduced spermatozoa motility and ATP content, and altered structural and reserve  
377 lipids of the digestive gland. In the present study, neither decreased ATP content nor sperm  
378 mortality can explain this reduced motility. Total lipid content in male gonads of exposed  
379 oysters increased maybe reflecting modifications in lipid metabolism. Lipids are key  
380 component of cellular membranes of spermatozoa and modifications in lipid metabolism  
381 could be associated with changes in gamete features. PST bind to voltage-gated Na<sup>+</sup> channels  
382 with high affinity and interact, to a lesser extent, with Ca<sup>2+</sup> and K<sup>+</sup> channels, modifying ionic  
383 fluxes into cells (Llewellyn, 2006) and associated metabolic pathways (Mat et al., 2018).  
384 Spermatozoa motility in Pacific oysters is a key factor for reproduction and notably depends  
385 on concentrations of ions including K<sup>+</sup>, Ca<sup>2+</sup> and Na<sup>+</sup> (Alavi et al., 2014). Indeed, the  
386 percentage of motile sperm is drastically reduced in Na<sup>+</sup>-free seawater (Boulais et al., 2018).  
387 In the present study, PST could be responsible for the decreased motility observed in  
388 spermatozoa through membrane alterations or ionic fluxes changes. In the field, the  
389 fertilization rate of oysters could be impaired by fewer motile spermatozoa with negative  
390 consequences on recruitment, as proposed for another free-spawning invertebrate, the sea  
391 urchin exposed to high doses of cadmium (Au et al., 2001). The present experimental  
392 conditions probably hide this negative effect, as spermatozoa are put in excess compared to  
393 oocytes for *in vitro* fertilization, in a small limited volume, increasing pairing probability  
394 compared to natural conditions.

### 395 3.3 Broodstock exposure to *A. minutum* affected offspring growth and survival

396 Broodstock exposure to *A. minutum* decreased offspring growth and induced higher  
397 mortality in larvae during the settlement period. Intracellular PST initially accumulate in the  
398 digestive gland of bivalves following algal cell lysis and are then transferred into other  
399 organs, including gonads (Bricelj and Shumway, 1998). In this study, PST was detected in  
400 oocytes ( $0.3 \pm 0.1 \mu\text{g STX } 100 \text{ g}^{-1}$  wet oocytes weight) and male gonad ( $1.8 \pm 0.1 \mu\text{g STX}$

401 100 g<sup>-1</sup> wet gonad weight), which is consistent with PST content observed in oocytes of  
402 mature oysters exposed to a natural *A. minutum* bloom (Hermabessiere et al., 2016).  
403 *Crassostrea virginica* larvae derived from oysters naturally exposed to *Karenia brevis* showed  
404 significantly higher mortalities and smaller length than larvae derived from non-exposed  
405 oysters, suggesting that these negative effects on larval development may be due to the  
406 presence of brevetoxins in oocytes (Rolton et al., 2018, 2016). In the present study, toxic  
407 effects on the next generation could thus originate from deleterious effect of PST transferred  
408 to offspring via gametes. Maternal effects in eggs influence embryogenesis and larval  
409 development (Bayne, 2017). PST may also have resulted in functional cell damage during the  
410 process of gametogenesis with consequences on development of offspring. Larval physiology  
411 could be affected even later during development and settlement like in the present study.

#### 412 3.4 Larval growth and settlement are affected by direct exposure to *A. minutum*

413 A substantial alteration of larval growth was observed for larvae exposed to *A. minutum*,  
414 both derived from control and exposed adult oysters. *Alexandrium* toxicity comes from  
415 intracellular PST but also from bioactive extracellular compounds (BECs) produced and  
416 excreted in the surrounding water by some *Alexandrium* strains, such as the *A. minutum* strain  
417 tested in this study (Borcier et al., 2017; Castrec et al., 2018; Long et al., 2018). These BECs  
418 can be allelopathic, cytotoxic, haemolytic, or ichthyotoxic (Arzul et al., 1999; Ford et al.,  
419 2008; Lelong et al., 2011; Mardones et al., 2015), however, their molecular structures remain  
420 largely unknown (Ma et al., 2011). This detrimental effect of *A. minutum* on larvae could be  
421 attributed sequentially to BEC and PST during development.

422 Toxic effects on small *C. gigas* larvae (D-larvae to larvae < 150 µm) which are unable to  
423 feed on *A. minutum* could not be related to PST toxicity arising from algal cell consumption,  
424 but rather arise from BECs. These bioactive substances produced by *Alexandrium minutum*  
425 mainly exert their action by direct contact with external tissues, e.g. the gills (Borcier et al.,

426 2017; Castrec et al., 2018) or cells, e.g. gametes (Le Goïc et al., 2014). These BECs could be  
427 cytotoxic to the velum, the feeding and swimming organ of the larvae, thereby reducing  
428 energy uptake and subsequent larval growth. Similarly, the toxic effects of *A. minutum* and *A.*  
429 *ostenfeldii* on *M. edulis* larvae observed by De Rijcke et al. (2016) could mainly result from  
430 extracellular bioactive substances, as suggested by the authors. This hypothesis was also  
431 suggested by Banno et al. (2018) who identified some unknown bioactive compounds as  
432 responsible for the decrease of sperm mobility and egg viability of oysters *P. fucata martensii*  
433 exposed to two *Alexandrium* species.

434 The PST probably become harmful from the moment larvae are able to ingest *A. minutum*  
435 cells. In this study, *C. gigas* eyed larvae (mean length  $\pm$  SD:  $304 \pm 15 \mu\text{m}$ ) ingested *A.*  
436 *minutum* cells. Veliger larvae ( $> 200 \mu\text{m}$ ) of the oyster *C. virginica* fed preferably on large  
437 food material (22 to  $30 \mu\text{m}$ ) in the presence of large cell dinoflagellate bloom (Baldwin,  
438 1995), suggesting that oyster larvae ( $> 200 \mu\text{m}$ ) likely fed on *A. minutum* in the present study.  
439 Thus, both the PST accumulated through *A. minutum* consumption and the BECs could have  
440 contributed to the adverse effects on growth at the end of the larval development. This  
441 hypothesis supports the findings of Mu and Li (2013) who suggested that the reduced growth  
442 of early umbonate larvae of *C. gigas* following a 4-day exposure to  $3 \times 10^2$  cells  $\text{mL}^{-1}$  of *A.*  
443 *catenella* might relate to both PST and unknown toxins produced by *A. catenella*.

444 Larval exposure to *A. minutum* also altered the settlement of oyster larvae. Larval mortality  
445 does not explain the reduced settlement in exposed larvae. The decreased settlement could  
446 either result from the lagged growth observed after 22 days of exposure, as most exposed  
447 larvae did not reach the competence for settlement and metamorphosis, and/or from altered  
448 physiology caused by *A. minutum* toxins. Similarly, the activity of Japanese pearl oyster (*P.*  
449 *fucata martensii*) pre-settling larvae was decreased when exposed to *Alexandrium affine* and  
450 *A. catenella*, at  $2.5 \times 10^2$  cells  $\text{mL}^{-1}$  and 10 cells  $\text{mL}^{-1}$ , respectively (Basti et al., 2015). This

451 effect was attributed to non-PST metabolites with potent lytic activity produced by the non-  
452 PST *A. affine* or to the PST produced by *A. catenella*, following ingestion of algal cells,  
453 leading to paralysis and/or altered cellular homeostasis (Basti et al., 2015).

### 454 3.5 Broodstock conditioning influenced larval response to *A. minutum* exposure

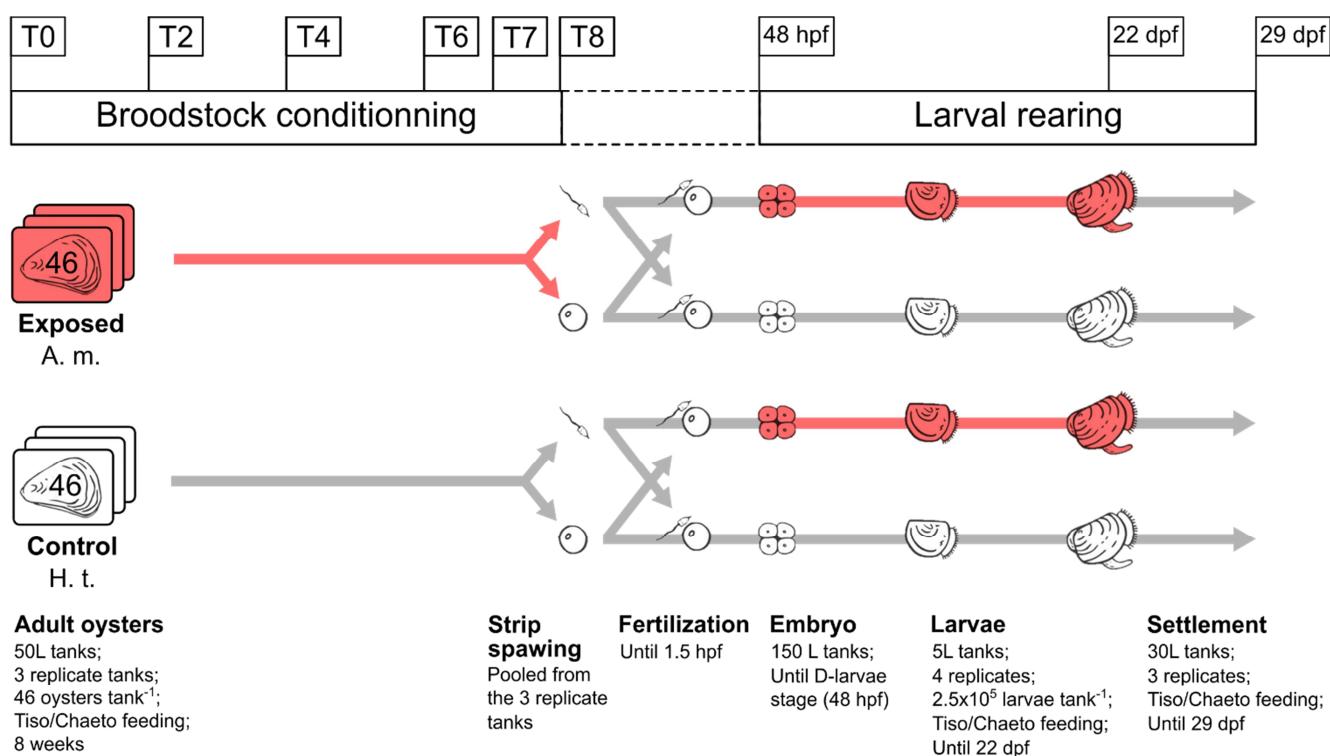
455 In the present study, growth of larvae derived from exposed parents was less affected by *A.*  
456 *minutum* exposure than growth of larvae derived from non-exposed oysters. This result  
457 suggests that parental exposure to *A. minutum* may have led to an improved capacity to cope  
458 with the stress caused by *A. minutum* exposure. Similarly, exposure of adult Sydney rock  
459 oysters to elevated pCO<sub>2</sub> improved the capacity of their offspring to regulate extracellular pH  
460 at elevated pCO<sub>2</sub> (Parker et al., 2012). Boullot et al. (2018) revealed that the sensitivity of *C.*  
461 *gigas* nerves to saxitoxin was decreased when oysters had been previously exposed to PST-  
462 producing *A. minutum*. It can be hypothesized that larvae derived from PST-containing  
463 gametes produced by exposed parents may be less sensitive to PST during larval  
464 development.

## 465 4. Conclusions

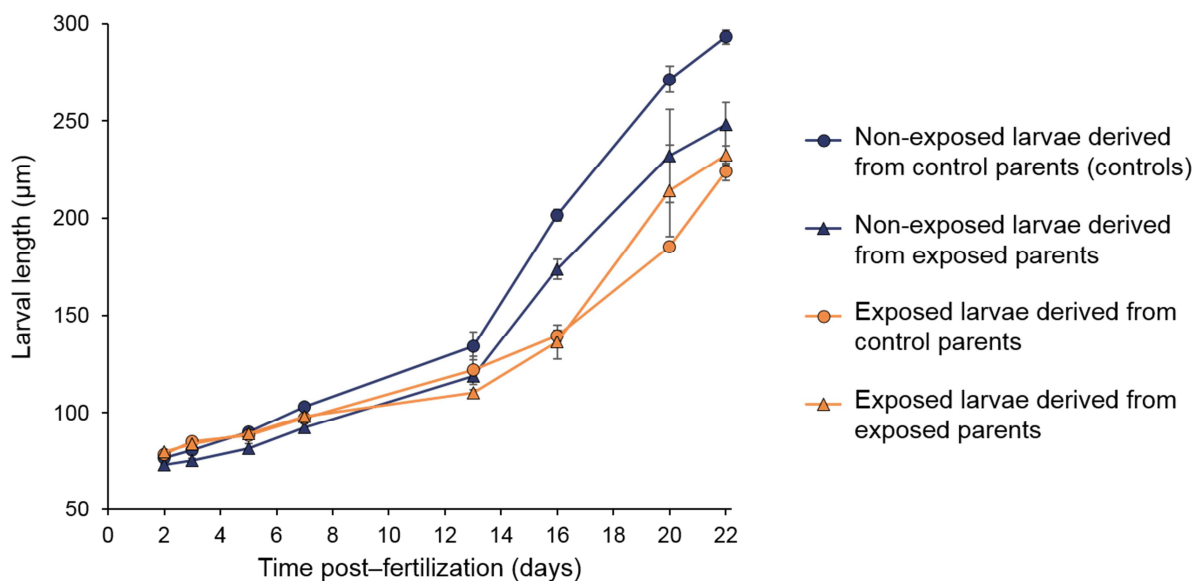
466 Successful reproduction is essential for the sustainability of marine populations. This study  
467 demonstrates that long term exposure of adult oysters to *A. minutum* during gametogenesis  
468 affected spermatozoa motility, and reduced growth and survival of the subsequent offspring.  
469 The present laboratory experiment also evidenced that direct *A. minutum* exposure during  
470 oyster embryo-larval development significantly altered growth and settlement of larvae. These  
471 effects of *A. minutum* blooms on oyster reproduction are likely to compromise recruitment of  
472 benthic post-larvae of *C. gigas* by slowing down growth, prolonging the time larvae remain in  
473 the seawater column, thus making them more vulnerable to predation. Further research is  
474 needed to investigate potential long term effects on marine bivalve populations by studying  
475 the consequences of recurrent *Alexandrium* blooms over multiple generations.

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483 Quéré.

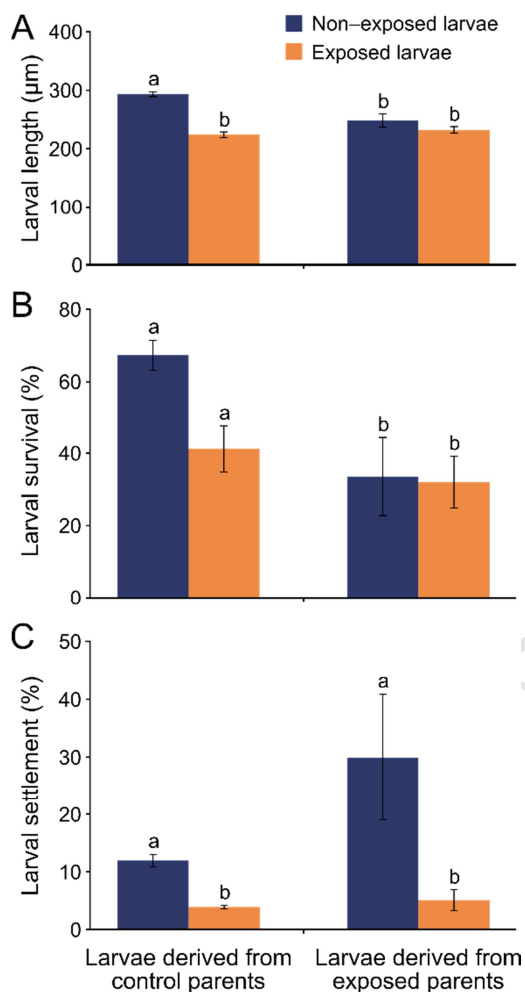
484 **Figures**

485 **Fig. 1.** Flow chart of the experiment. For broodstock conditioning, oysters in red are exposed to the  
 486 toxic *Alexandrium minutum* (A. m.), whereas oysters in white are fed with non-toxic *Heterocapsa*  
 487 *triquetra* (H. t. = control treatment). During adult oyster exposure, oysters were sampled every two  
 488 weeks (T0, T2, T4, T6 and T8) for PST accumulation and histological analyses to study  
 489 gametogenesis. Gamete cellular analyses were conducted on oysters sampled after 7 weeks of  
 490 exposure (T7). For larval rearing, embryos and larvae in red are exposed to the toxic *A. minutum*,  
 491 whereas stages in white are non-exposed. Tiso/Chaeto feeding: *Tisochrysis lutea* and *Chaetoceros* sp.  
 492 feeding *ad libitum*; hpf: hours post-fertilization; dpf: days post-fertilization. (For interpretation of the  
 493 references to color in this figure legend, the reader is referred to the web version of this article.)

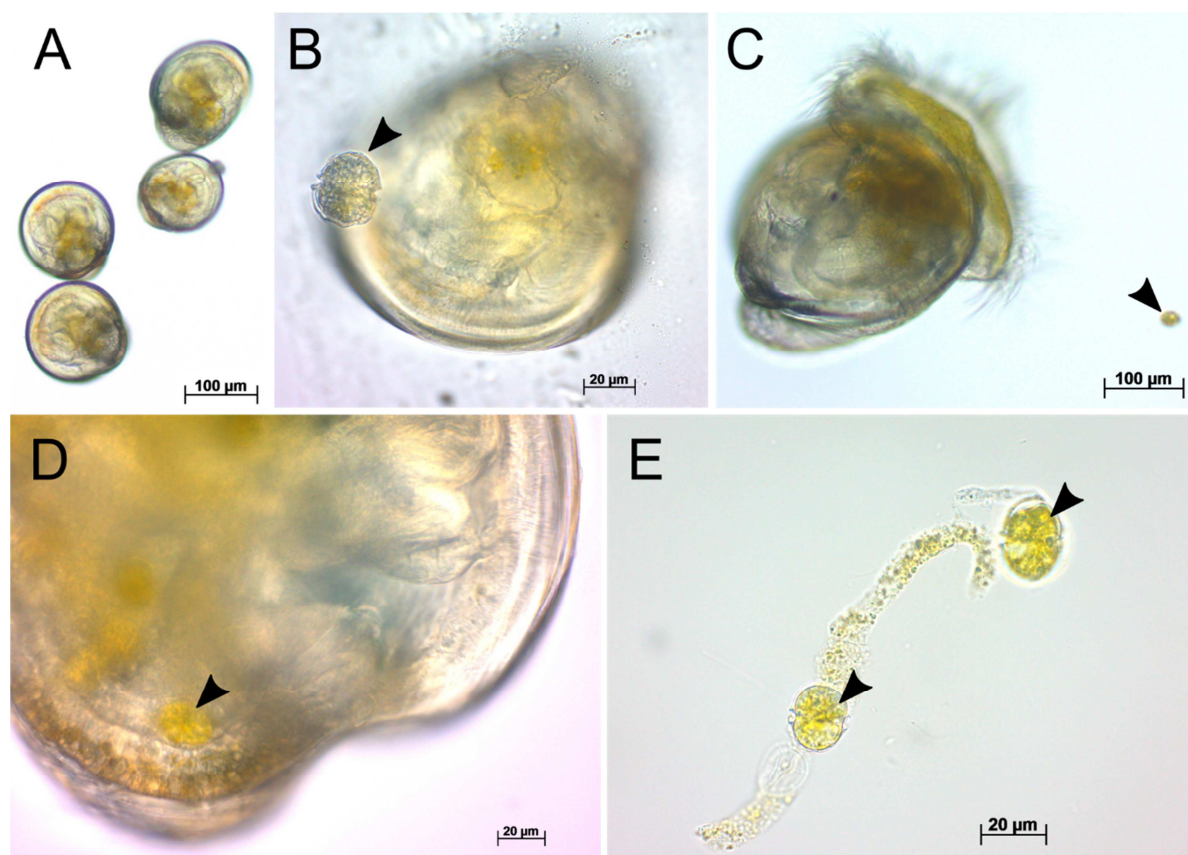


494 **Fig. 2.** Larval length from D-larvae up to metamorphosis of the controls (non-exposed larvae derived  
 495 from control parents) and the three other combination of parental and larval exposures. Larval groups  
 496 were obtained by crossing gametes collected from adult *Crassostrea gigas* exposed to the non-toxic  
 497 *Heterocapsa triquetra* (control parents) and from oysters exposed to *Alexandrium minutum* (exposed  
 498 parents), and then exposing the offspring continuously to *A. minutum* (exposed larvae) or not (non-  
 499 exposed larvae). Mean  $\pm$  SE, n = 3.





500 **Fig. 3.** Larval length 22 days post-fertilization (dpf) (A), survival (B) and settlement (C) of *C. gigas*  
 501 larvae, non-exposed or exposed to *A. minutum*, derived from *A. minutum* exposed parents or control  
 502 parents. Survival is estimated as the total number of alive larvae 29 dpf divided by number of larvae  
 503 initially stocked at 22 dpf. Settlement is calculated as the number of settled larvae at 29 dpf divided by  
 504 the total number of live larvae at 29 dpf. Mean  $\pm$  SE,  $n = 3$ . Letters denote significant groupings ( $p <$   
 505  $0.05$ ; two-way ANOVA and Tukey HSD).



506 **Fig. 4.** Capacity of control *C. gigas* larvae to ingest *A. minutum*. Light micrographs. Umbonate larvae  
507 (A, B; mean shell length  $150 \pm 22 \mu\text{m}$ ) were unable to ingest *A. minutum* cells due to their relative  
508 large size (23–29  $\mu\text{m}$ ), whereas eyed larvae (C-E; mean shell length  $304 \pm 15 \mu\text{m}$ ) fed on *A. minutum*:  
509 algal cells were observed in the intestine (D) and fecal pellets (E). Black arrows indicate *A. minutum*  
510 cells.

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