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Abstract

The dinoflagellate genus *Alexandrium* comprises species that produce highly potent neurotoxins known as paralytic shellfish toxins (PST), and bioactive extracellular compounds (BEC) of unknown structure and ecological significance. The toxic bloom-forming species, *Alexandrium minutum*, is distributed worldwide and adversely affects many bivalves including the commercially and ecologically important Pacific oyster, *Crassostrea gigas*. In France, recurrent *A. minutum* blooms can co-occur with *C. gigas* spawning and larval development, and may endanger recruitment and population renewal. The present study explores how *A. minutum* affects early-oyster development by exposing embryos and larvae, under controlled laboratory conditions, to two strains of *A. minutum*, producing only BEC or both PST and BEC. Results highlight the major role of BEC in *A. minutum* toxicity upon oyster development. The BEC strain caused lysis of embryos, the most sensitive stage to *A. minutum* toxicity among planktonic life-stages. In addition, the non-PST-producing *A. minutum* strain inhibited hatching, disrupted larval swimming behavior, feeding, growth, and induced drastic decreases in survival and settlement of umbonate and eyed larvae (9 and 68 %, respectively). The findings indicated PST accumulation in oyster larvae (*e.g.* umbonate stages), possibly impairing development and

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

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

1. Introduction

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

Some *Alexandrium* species are known to produce paralytic shellfish toxins (PST), a group of more than 50 neurotoxic derivatives of saxitoxin (Wiese et al., 2010). PST can bioaccumulate in filter-feeding bivalves and other marine species, causing paralytic shellfish poisonings (PSP) outbreaks (Bricelj and Shumway, 1998; Cembella et al., 2000) and other harmful effects (Shumway, 1990; Landsberg, 2002). These endogenous water-soluble alkaloids bind with high affinity to the voltage-gated Na^+ channels, blocking conduction of action potential in excitable cells, eventually leading to paralysis (Cestèle and Catterall, 2000), and interact with Ca^{2+} and K^+ channels, modifying ionic fluxes into cells (Llewellyn, 2006). Several species of *Alexandrium* also produce, independently from PST (Tillmann and John, 2002), extracellular bioactive compounds (BEC) with allelopathic (Arzul et al., 1999; Tillmann et al., 2008; Lelong et al., 2011; Zheng et al., 2016; Long et al., 2018a, 2018b), ichthyotoxic (Ogata and Kodama,

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

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

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

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

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

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

2. Materials and methods

2.1 Algal strains

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

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

2.2 Oysters

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

2.3 Experimental design

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

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

The PST produced by *A. minutum* are mainly intracellular and released in oyster tissues 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 µl gonad samples under a
159 light microscope (Leica MZ 125, ×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 µm filtered sea water (FSW; 21 °C,
162 pH 8.3, salinity 36). Oocytes and spermatozoa were sieved through 100 µm and 60 µm 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°C ± 1°C. These batches
171 of embryos were made the same day and used as replicates for the different exposures (n = 4

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

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

Once ≥ 50% of larvae reached the eyed larvae stage (morphological competence for metamorphosis), the replicate tanks were drained and larvae were collected on 60 µm mesh and counted as described in Tallec et al. (2018). The exposure duration for Exp3 was only 1 day since ≥ 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. μm^3 $larva^{-1}$ 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*

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

2.9 Toxin quantification

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

2.10 Statistical analyses

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

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

3. Results

3.1 Exposure of embryos (Exp1)

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

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

3.2 Exposure of umbonate larvae (Exp2)

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

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

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

At 21 dpf, eyed larvae yield of umbonate larvae exposed to the PST+BEC strain (23.7 ± 7.3 %) or to the BEC strain (4.5 ± 1.4 %) was significantly lower (Tukey HSD, $p < 10^{-2}$ and $p < 10^{-2}$) than that of control larvae (51.4 ± 2.9 %) (Fig. 3A, Exp2). The yield of eyed larvae at 21 dpf was not significantly different (Tukey HSD, $p > 10^{-1}$) between PST+BEC and BEC strains (Fig. 3A, Exp2). Competence in larvae exposed to the PST+BEC strain and the BEC strain was delayed by 2 days (23 dpf) and 3 days (24 dpf), respectively, compared to control larvae (21 dpf). Size at competence for metamorphosis was similar (Tukey HSD, $p > 0.05$) in larvae exposed to the PST+BEC strain (308.4 ± 8.1 µm) and control larvae (306.8 ± 4.0 µm), however, larvae exposed to the BEC strain (284.3 ± 4.6 µm) were significantly smaller than 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 ± 25.5 μm^3 $larva^{-1}$ d^{-1}) than in larvae exposed
326 to the PST+BEC strain (3435.8 ± 310.1 μm^3 $larva^{-1}$ d^{-1}) and in control larvae (2627.2 ± 217.5
327 μm^3 $larva^{-1}$ 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 ± 2.9 µm; Tukey HSD, $p > 0.05$), larvae
329 exposed to the BEC strain (290 ± 3.6 µm; Tukey HSD, $p > 0.05$), and control larvae (306.8 ±
330 4.0 µm; 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 ± 0.5 %) and settlement (18.9 ± 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

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

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

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

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

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

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

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

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

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

5. Conclusion

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

reduce growth, thereby increasing the susceptibility of larvae to predation in the environment. Given the fact that *A. minutum* form blooms overlapping with the spawning and larval development seasons of *C. gigas,* this study provides evidence that *A. minutum* could adversely affect *C. gigas* settlement with potential consequences on the structure of wild and cultivated 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})					
		Control	PST+BEC	BEC	ANOVA		
					df	F	adjusted *p*
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

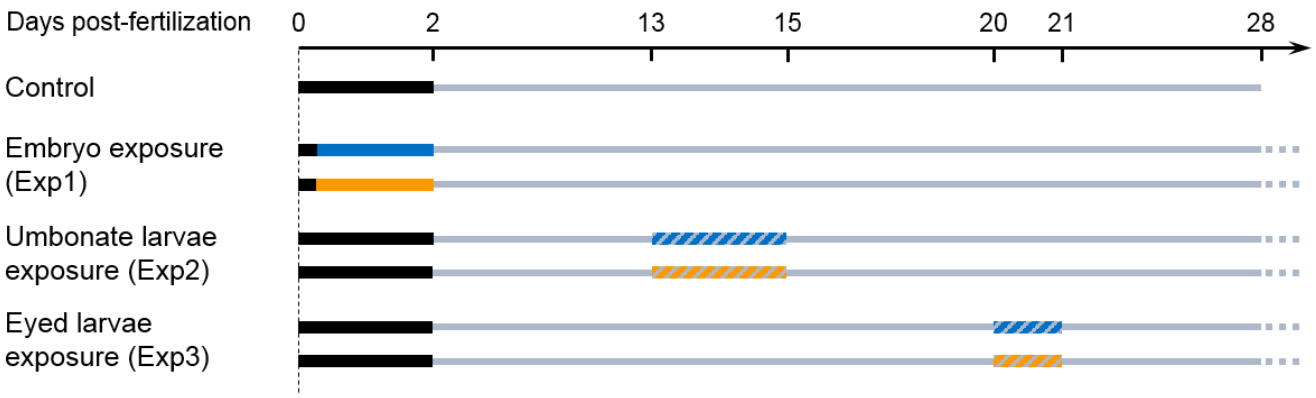

Fig. 1. Flow chart of the experiment. Different early life stages of oysters were exposed to two strains of *A. minutum*: 2-day exposure of embryos (Exp1); 2-day exposure of umbonate larvae (Exp2), and 1-day exposure of eyed larvae (Exp3). In the control treatment, fertilization and embryo development 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* (▬).

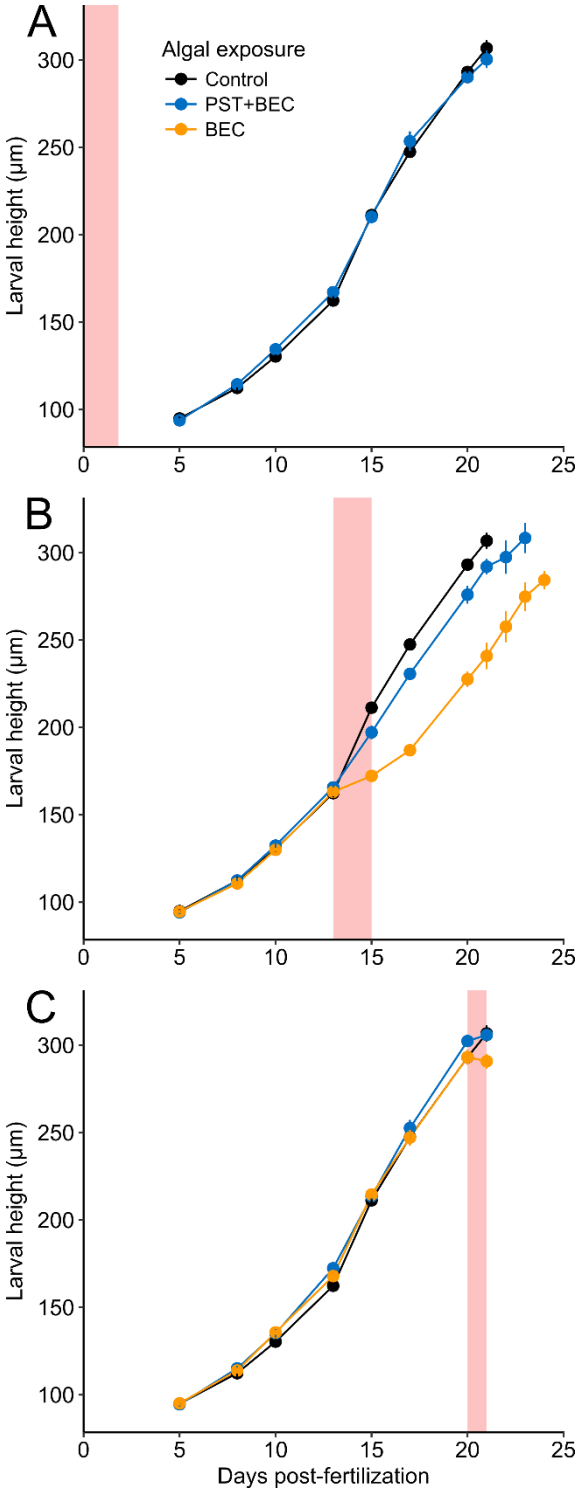

Fig. 2. Effects of *A. minutum* exposure on oyster larval height from 5 days post-fertilization up to competence for metamorphosis (≥ 50 % of eyed larvae). A: Exp1, 2-day exposure of embryos (no data are available for Exp1 with the BEC treatment since hatching was completely inhibited with this treatment); 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 ± standard error). During the experiment, larvae from all treatments were fed *ad libitum* with the mix diet of *T. lutea* and *Chaetoceros* sp.

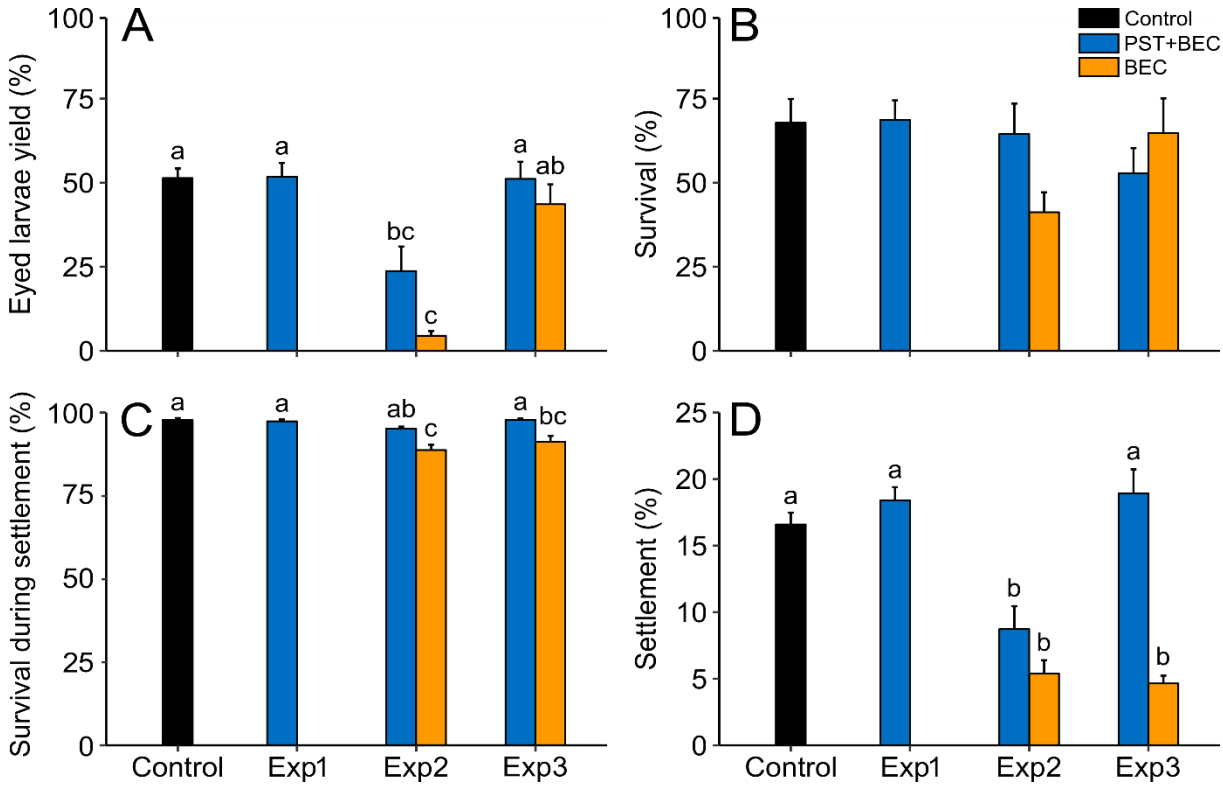

Fig. 3. Effects of *A. minutum* exposure on oyster early life stages: 2-day exposure of embryos (Exp1), 2-day exposure of umbonate larvae (Exp2), and 1-day exposure of eyed larvae (Exp3). Black, blue, and orange bars respectively represent control larvae, larvae exposed to the PST+BEC strain of *A. minutum* (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 ± standard error; letters denote significant groupings; $p < 0.05$; Tukey HSD following ANOVA).

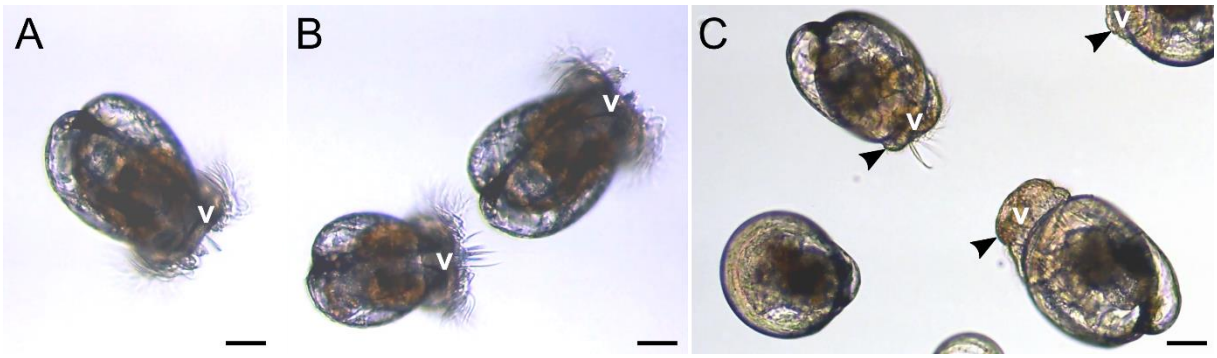

Fig. 4. Light micrographs of oyster umbonate larvae at 15 days post-fertilization, after a 2-day exposure to 10^3 cells mL^{-1} of *A. minutum* (Exp2). Control larvae (A) and larvae exposed to the PST+BEC strain of *A. minutum* (B) were actively swimming with normal velum whereas larvae exposed to the 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	***t*-test**		
	p values		*FDR adjusted probabilities*
Abnormality percentage (%)	2.67×10^{-7}		$\mathbf{6.23\times10^{-7}}$
D-larvae yield (%)	8.67×10^{-1}		$\mathbf{8.67\times10^{-1}}$
	One way ANOVA		
	df	F	*p values*	*FDR adjusted probabilities*
Algal consumption at 13 dpf (μm^3 of algae $larva^{-1}$ d^{-1})	2	4.0	5.71×10^{-2}	$\mathbf{7.27\times10^{-2}}$
Algal consumption at 14 dpf (μm^3 of algae $larva^{-1}$ d^{-1})	2	21.2	3.93×10^{-4}	$\mathbf{6.11\times10^{-4}}$
Algal consumption at 15 dpf (μm^3 of algae $larva^{-1}$ d^{-1})	2	202	3.28×10^{-8}	$\mathbf{1.82\times10^{-7}}$
Algal consumption at 20 dpf (μm^3 of algae $larva^{-1}$ d^{-1})	2	2.62	1.27×10^{-1}	$\mathbf{1.48\times10^{-1}}$
Algal consumption at 21 dpf (μm^3 of algae $larva^{-1}$ d^{-1})	2	178	5.83×10^{-8}	$\mathbf{1.84\times10^{-7}}$
Larval height at 15 dpf (µm)	5	56.5	2.25×10^{-10}	$\mathbf{3.15\times10^{-9}}$
Larval height at 21 dpf (µm)	5	30.1	3.89×10^{-8}	$\mathbf{1.82\times10^{-7}}$
Eyed larvae yield at 21 dpf (%)	5	16.2	4.03×10^{-6}	$\mathbf{7.05\times10^{-6}}$
Larval survival at competence for metamorphosis (%)	5	1.9	1.38×10^{-1}	$\mathbf{1.49\times10^{-1}}$
Larval height at competence for metamorphosis (µm)	5	4.0	1.32×10^{-2}	$\mathbf{1.85\times10^{-2}}$
Larval settlement yield (%)	5	28.1	6.56×10^{-8}	$\mathbf{1.84\times10^{-7}}$
Larval survival during settlement period (%)	5	18.9	1.32×10^{-6}	$\mathbf{2.64\times10^{-6}}$

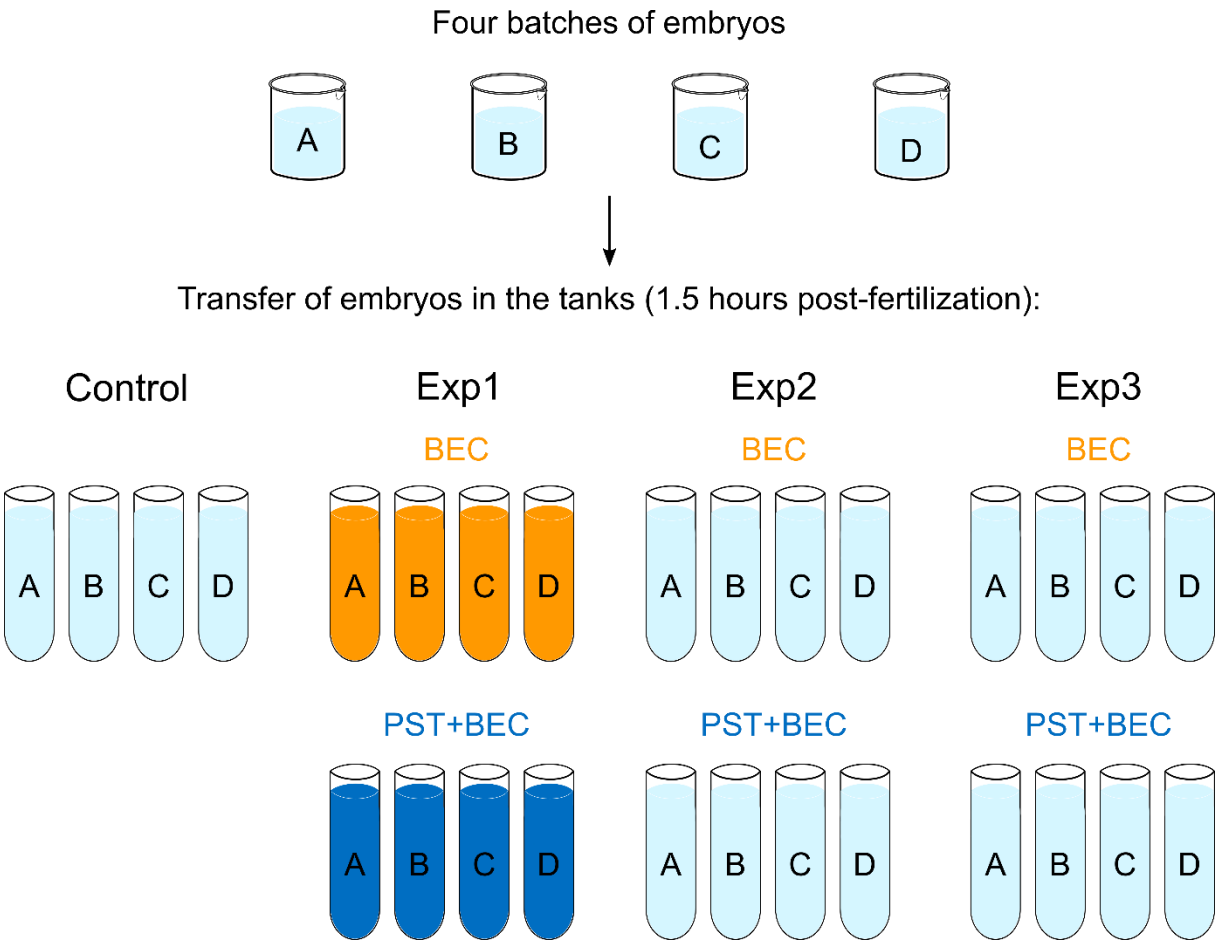

Fig. S1. Flow chart of the experiment representing the transfer of embryos from the four batches (A, B, C and D) to the tanks at 1.5 hours post-fertilization. For Exp1, embryos were transferred in tanks containing 10^3 cells mL^{-1} of the PST+BEC strain ( ) or the BEC strain ( ) of *A. minutum*. In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

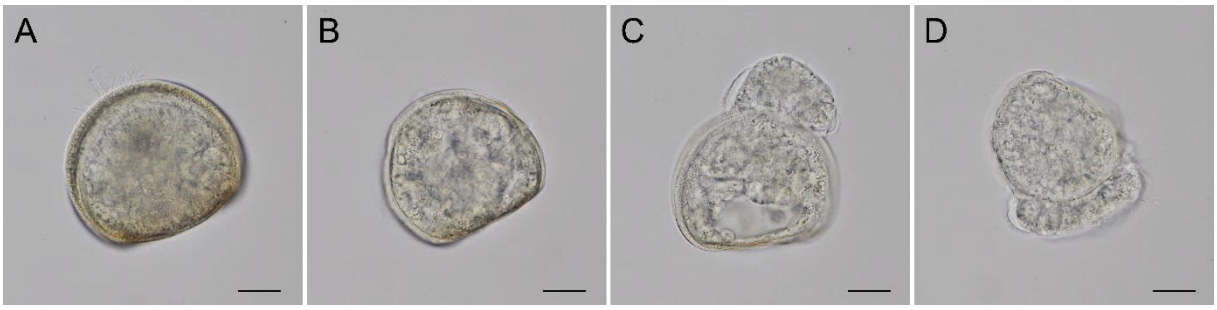

Fig. S2. Light micrographs of normal and abnormal *C. gigas* D-larvae 48 hours post fertilization. Embryos were non exposed (A, control) or exposed to 10^3 cells mL^{-1} of the PST+BEC strain of *A. minutum* (B,C,D) during two days (Exp1). (A) Normal control D-larvae; (B) Shell abnormality; (C) Mantle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

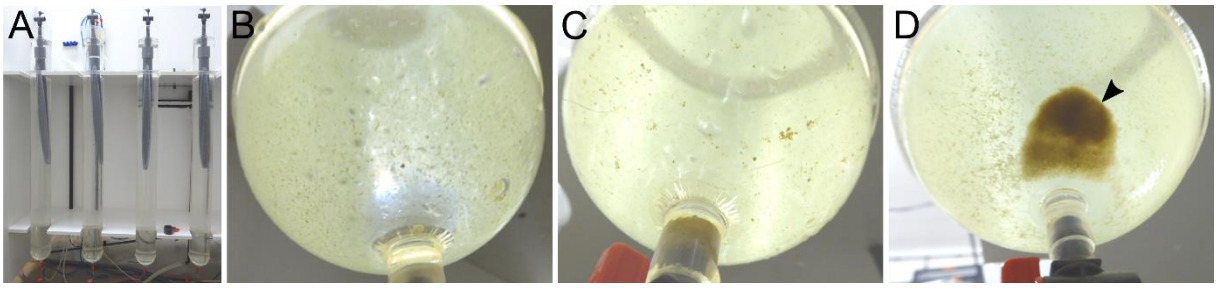

Fig. S3. Photographs illustrating behavior of *C. gigas* umbonate larvae in the experimental cylindrical tanks during Exp2 (A). Control larvae (B) and larvae exposed to the PST+BEC strain (10^3 cells mL^{-1}) (C) were swimming actively, whereas larvae exposed to the BEC strain (10^3 cells mL^{-1}) (D)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