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

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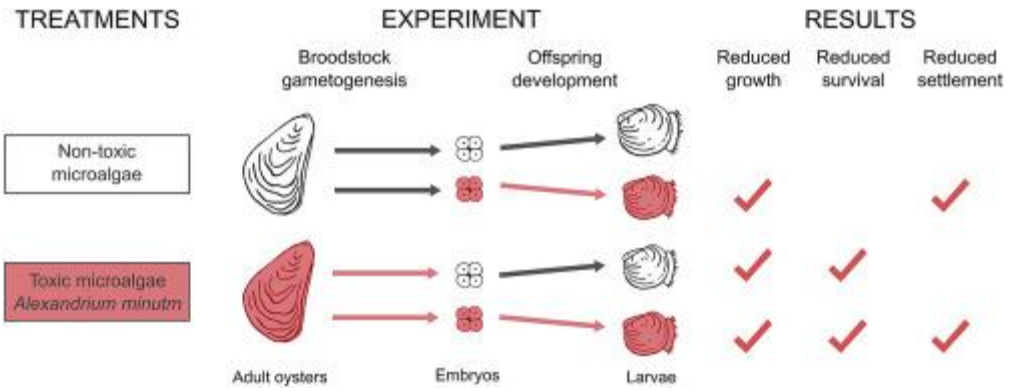
Abstract :

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^{-1}) 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^{-1}. 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

Introduction

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

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

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

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

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

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

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

1. Materials and methods

1.1 *Biological materials*

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

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

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

The flow chart of experimental procedure is presented in Fig. 1. After acclimation, the oysters were placed in six experimental 50-L tanks (46 oysters per tank) supplied with filtered running seawater (1-µm filtered and UV-treated (FSW); 17.0 ± 0.1 °C, pH 8.3 ± 0.1, and 35.1

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

1.3 *Algal consumption of adult oysters*

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

1.4 *Sampling*

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

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

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

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

1.5 *Histology*

A 3-mm cross section of the visceral mass was excised in front of the pericardial region and immediately fixed in modified Davidson's solution (Latendresse et al., 2002) for 24 h at 4 °C. Tissues were processed, stained with Harris' hematoxylin–eosin, and observed as described by Hermabessiere et al. (2016). Tissue sections were examined under a light microscope (Leica DMIRB) equipped with a digital camera (Imaging RETIGA 2000R) to determine the sex and gametogenesis stage of each oyster according to the reproductive scale reported by Steele and Mulcahy (1999). In addition, measurement of reproductive effort was determined by image software (Adobe® Photoshop®): gonadal occupation index is the percentage of whole gonadal 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 5×10^4 oocytes mL^{-1} and 1×10^7 spermatozoa mL^{-1}, 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^{-1} 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).

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

1.7 *Quantification of total RNA in oocytes*

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

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

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

1.9 *Toxin quantification*

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

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

1.10 *Larval rearing*

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

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

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

A small complementary experiment was carried out on oyster larvae raised on the same conditions as the control larvae to test the capacity of larvae to ingest *A. minutum* cells. Umbonate larvae (mean shell length 150 ± 22 µm) and eyed larvae (mean shell length 304 ± 15 µm) were exposed to *A. minutum* (AM89BM strain) and the presence of *A. minutum* cells in larvae digestive tract and feces was checked under light microscope.

1.11 *Larval survival and settlement*

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

1.12 *Statistical analyses*

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

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

2. Results

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

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

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

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

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

2.2 *Gamete quality*

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

2.3 *Paralytic shellfish toxin content*

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

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

2.4 *Larval length, survival and settlement*

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

326 **3**A). At 22 dpf, non-exposed larvae derived from exposed parents (248.3 ± 11.3 µm) were
327 significantly smaller (-15 %, $p < 0.01$; Tukey HSD) compared to non-exposed larvae derived
328 from control parents (293.2 ± 3.7 µm).

329 Exposed larvae derived from control parents (223.9 ± 4.4 µm) 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 ± 5.1 µm) 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 %,
334 $p < 0.01$; 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 ± 10.8 % and 32.0 ± 7.1 % for non-exposed and
338 exposed larvae, respectively) compared to larvae derived from control parents (67.3 ± 4.2 %
339 and 41.2 ± 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 % ± 0.3 and 5.1
342 % ± 1.8 for exposed larvae, and 11.9 % ± 1.1 and 30.0 % ± 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* (23–29 µm) (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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In the present study, growth of larvae derived from exposed parents was less affected by *A. minutum* exposure than growth of larvae derived from non-exposed oysters. This result suggests that parental exposure to *A. minutum* may have led to an improved capacity to cope with the stress caused by *A. minutum* exposure. Similarly, exposure of adult Sydney rock oysters to elevated pCO_2 improved the capacity of their offspring to regulate extracellular pH at elevated pCO_2 (Parker et al., 2012). Boullot et al. (2018) revealed that the sensitivity of *C. gigas* nerves to saxitoxin was decreased when oysters had been previously exposed to PST-producing *A. minutum*. It can be hypothesized that larvae derived from PST-containing gametes produced by exposed parents may be less sensitive to PST during larval development.

4. Conclusions

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

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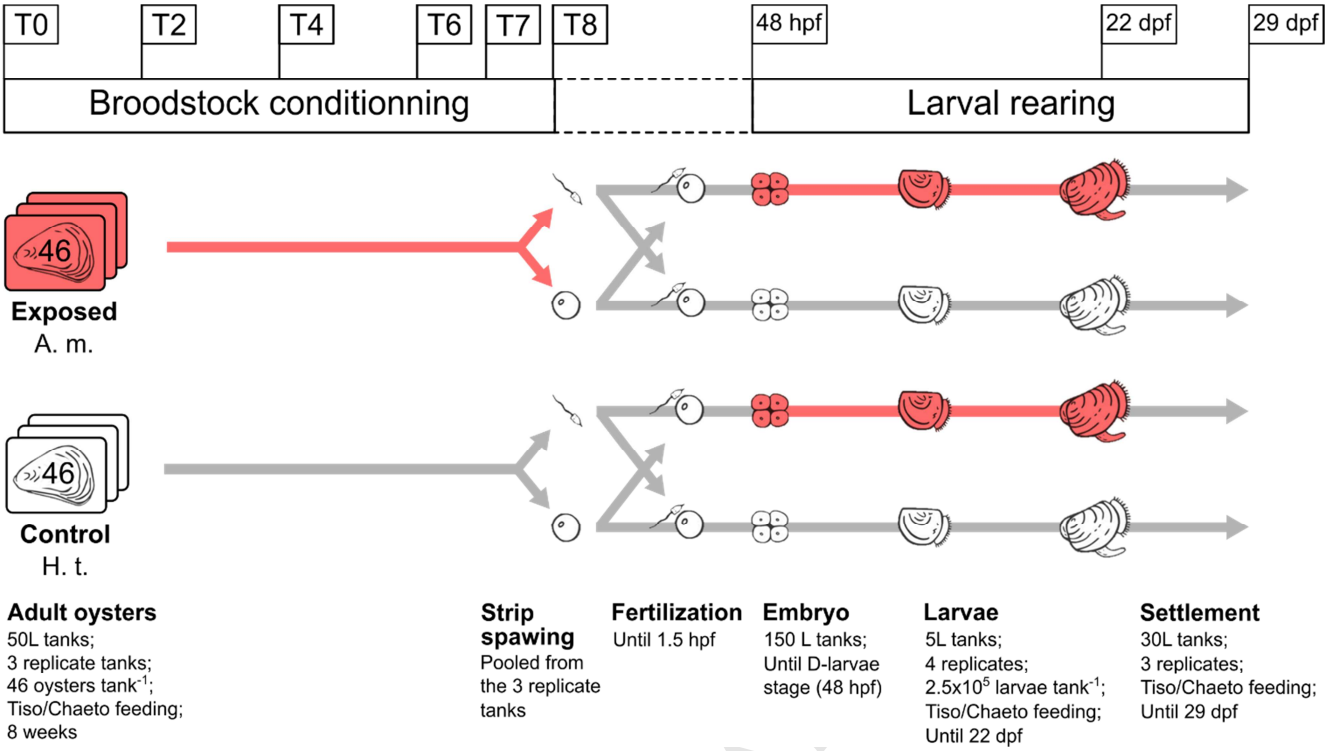


Figures

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

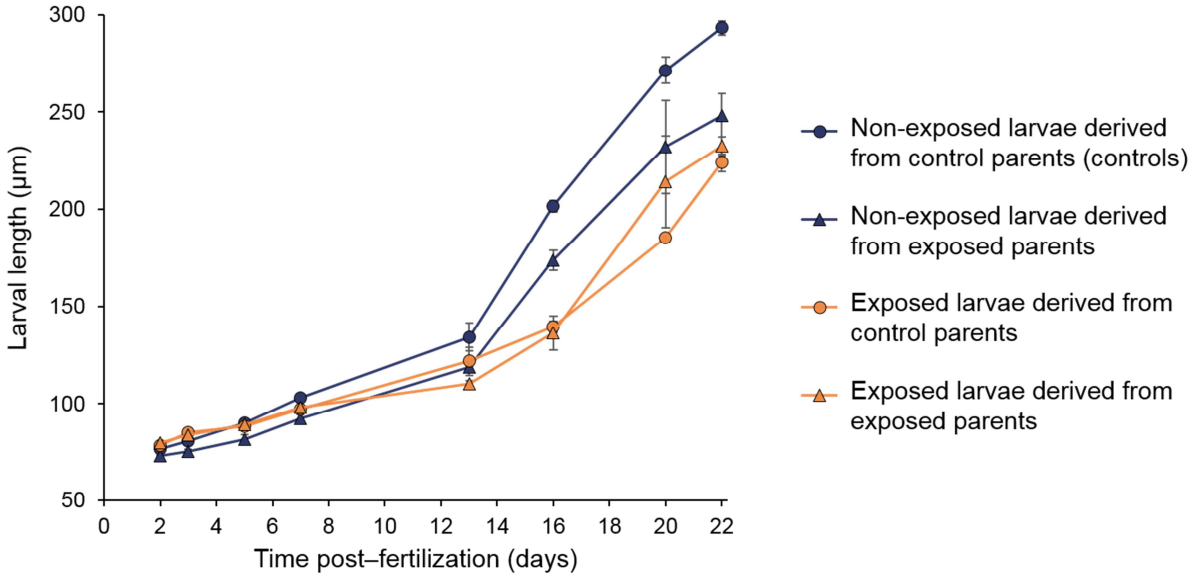
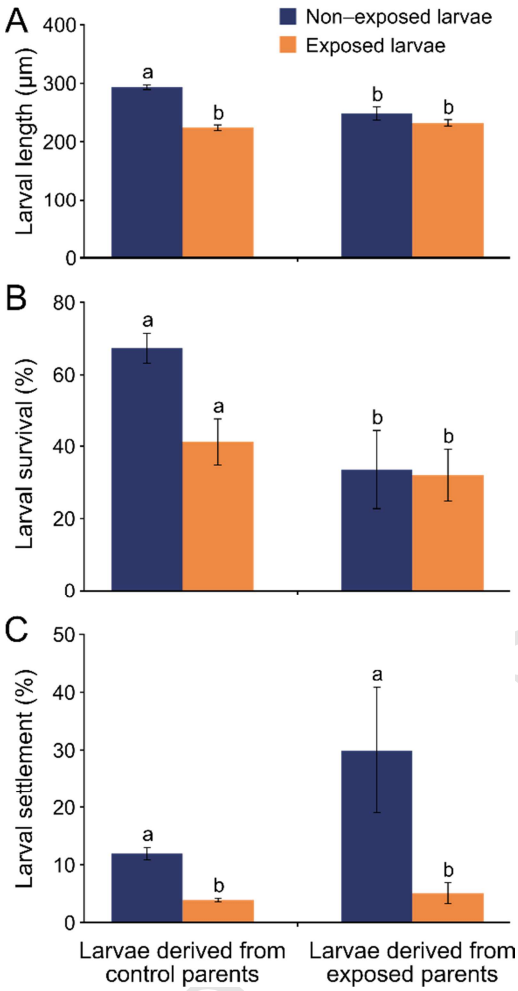
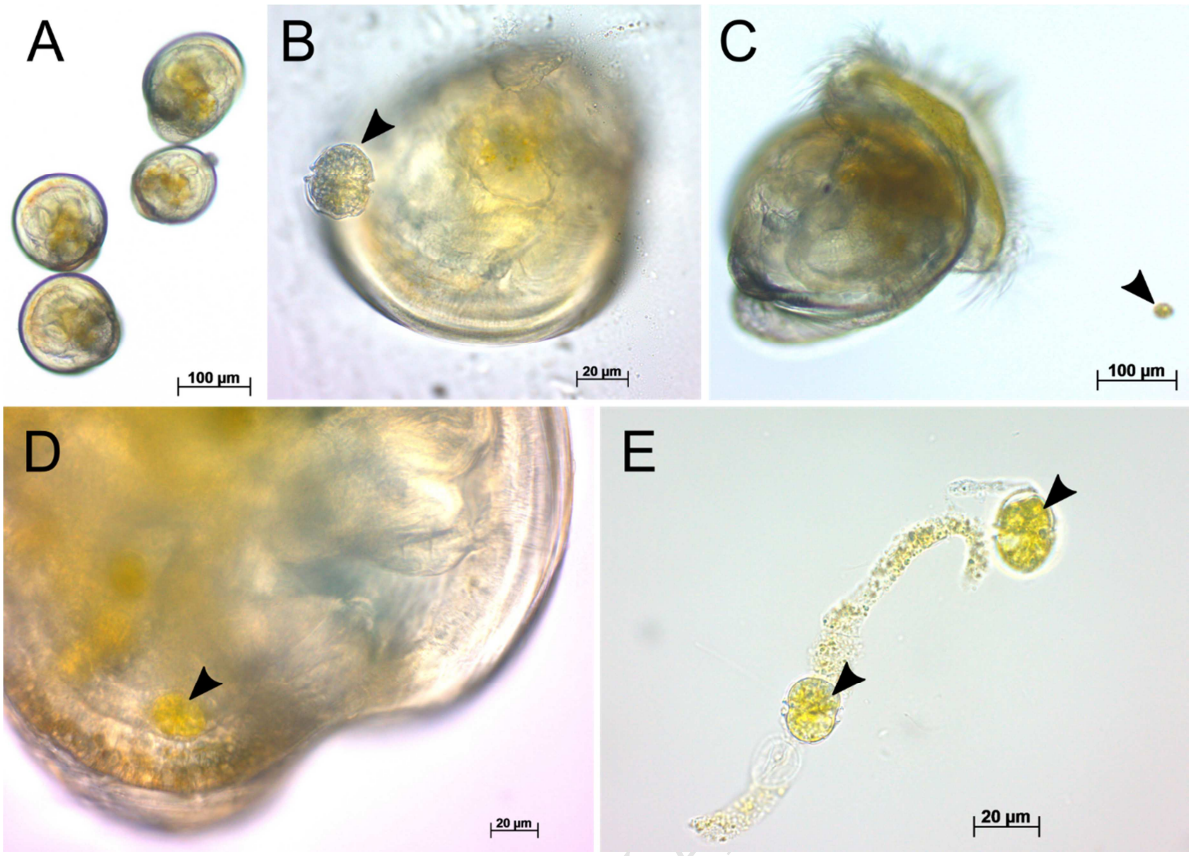


Fig. 2. Larval length from D-larvae up to metamorphosis of the controls (non-exposed larvae derived from control parents) and the three other combination of parental and larval exposures. Larval groups were obtained by crossing gametes collected from adult *Crassostrea gigas* exposed to the non-toxic *Heterocapsa triquetra* (control parents) and from oysters exposed to *Alexandrium minutum* (exposed parents), and then exposing the offspring continuously to *A. minutum* (exposed larvae) or not (non-exposed larvae). Mean ± 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 ± 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 ± 22 µm) were unable to ingest *A. minutum* cells due to their relative
508 large size (23–29 µm), whereas eyed larvae (C-E; mean shell length 304 ± 15 µm) 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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