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Cultures of *Dinophysis sacculus*, *D. acuminata* and pectenotoxin 2 affect gametes and fertilization success of the Pacific oyster, *Crassostrea gigas*

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1 **Title**

2 Cultures of *Dinophysis sacculus*, *D. acuminata* and pectenotoxin 2 affect gametes and
3 fertilization success of the Pacific oyster, *Crassostrea gigas*

4

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20 **Highlights**

21 *Dinophysis sacculus* and *D. acuminata* increased mortality of *Crassostrea gigas* oocytes

22 Exposure of oocytes and spermatozoa to 0.5 cells mL⁻¹ of *D. sacculus* decreased subsequent
23 fertilization success

24 Oyster gametes were negatively affected by exposure to whole culture or resuspended cells of
25 *Dinophysis* spp.

26 5 nM of PTX2 decreased fertilization success of oocytes and 500 nM of PTX2 increased ROS
27 production; OA showed no effect

28 Observed effects may be due to either cell to cell contact or PTX2 or other bioactive compounds
29 or a combination

30

31 **Graphical abstract**

32 End

33

34 **Abstract**

35 Harmful algal blooms (HABs) of toxic species of the dinoflagellate genus *Dinophysis* are a threat
36 to human health as they are mainly responsible for diarrhetic shellfish poisoning (DSP) in the
37 consumers of contaminated shellfish. Such contamination leads to shellfish farm closures causing
38 major economic and social issues. The direct effects of numerous HAB species have been
39 demonstrated on adult bivalves, whereas the effects on critical early life stages remain relatively

40 unexplored. The present study aimed to determine the *in vitro* effects of either cultivated strains
41 of *D. sacculus* and *D. acuminata* isolated from France or their associated toxins (i.e. okadaic acid
42 (OA) and pectenotoxin 2 (PTX2)) on the quality of the gametes of the Pacific oyster *Crassostrea*
43 *gigas*. This was performed by assessing the ROS production and viability of the gametes using
44 flow cytometry, and fertilization success using microscopic counts. Oocytes were more affected
45 than spermatozoa and their mortality and ROS production increased in the presence of *D.*
46 *sacculus* and PTX2, respectively. A decrease in fertilization success was observed at
47 concentrations as low as 0.5 cell mL⁻¹ of *Dinophysis* spp. and 5 nM of PTX2, whereas no effect
48 of OA could be observed. The effect on fertilization success was higher when both gamete types
49 were concomitantly exposed compared to separate exposures, suggesting a synergistic effect. Our
50 results also suggest that the effects could be due to cell-to-cell contact. These results highlight a
51 potential effect of *Dinophysis* spp. and PTX2 on reproduction and recruitment of the Pacific
52 oyster.

53

54 **Capsule**

55 *Dinophysis sacculus*, *D. acuminata* and pectenotoxin 2 increase oocyte mortality and ROS
56 production, and decrease fertilization success of the Pacific oyster, *Crassostrea* (= *Magallana*)
57 *gigas*.

58

59 **Keywords**

60 *Dinophysis* spp.; okadaic acid; pectenotoxins; oyster gametes; fertilization success

61

62 **Abbreviations**

63 DSP, diarrheic shellfish poisoning; DSTs, diarrheic shellfish toxins; DCFH-DA, 2',7'-
64 dichlorofluorescein diacetate; DTX1, dinophysistoxin 1; DTX2, dinophysistoxin 2; DTXs,
65 dinophysistoxins; Extra, extracellular; FCM, flow cytometry; FSC, forward scatter; HABs,
66 harmful algal blooms; Intra, intracellular; OA, okadaic acid; PTX2, pectenotoxin 2; PTX2eq,
67 pectenotoxin 2 equivalent; PTXs, pectenotoxins; PI, propidium iodide; ROS, reactive oxygen
68 species; SSC, side scatter; FSSW, filter-sterilized sea water; FSW, filtered sea water; UHPLC-
69 LRMS/MS, ultra-high performance liquid chromatography coupled to low resolution tandem
70 mass spectrometry

72 **Introduction**

73 Harmful algal blooms (HABs) of toxic microalgae are increasing in terms of frequency,
74 intensity and duration due, in part, to climate change and eutrophication (Gobler et al., 2017;
75 Hallegraeff, 1993; Wells et al., 2019). Toxins associated with HABs can accumulate in marine
76 bivalves (Landsberg, 2002; Shumway, 1990; Simões et al., 2015), causing a threat to human
77 health through direct contact with toxins or consumption of contaminated organisms (Hallegraeff,
78 2010, 1993; Van Dolah, 2000). Consequently, national surveillance programs monitoring
79 phytoplankton and phycotoxin concentrations in water and bivalves have been implemented (e.g.
80 REPHY in France). The European Council has set a maximum limit of 160 µg OA eq. per kg of
81 fresh whole bivalve meat (EU Commission, 2011), above which shellfish harvesting (farming
82 and recreational) is forbidden in order to protect human consumers (Nielsen et al. 2012).

83 Shellfish farming is an important economic sector worldwide. In France, the Pacific
84 oyster, *Crassostrea gigas* (= *Magallana gigas*; Thunberg, 1793) represents the majority of annual
85 shellfish sales (ca. 118,000 tons; France Agrimer, 2018). Shellfish farmers in France annually
86 suffer economic losses due to the presence of several toxic species of the genus *Dinophysis*
87 (Ehrenberg, 1841; Marcaillou et al., 2005; Trainer et al., 2020). Indeed, *D. acuminata* and *D.*
88 *sacculus* are the main responsible of shellfish farm closures, that can last for several weeks per
89 year (Belin and Soudant, 2018; Marchand et al., 2009). Along French coasts, *Dinophysis* spp. are
90 regularly observed at a concentration of 10^2 cells L⁻¹ (Figure S2, REPHY, 2019), which is similar
91 to concentrations typically reported in the literature (Reguera et al., 2012). However, blooms of
92 *Dinophysis* spp. can occasionally reach cell densities up to $10^3 - 10^7$ cells L⁻¹ (reviewed in
93 Reguera et al., 2012), including one instance of 8×10^5 cells L⁻¹ reported in France (REPHY,
94 2019).

95 These dinoflagellates can produce two types of lipophilic toxins, okadaic acid (OA) and
96 its analogs dinophysistoxins (DTXs), and pectenotoxins (PTXs; Marcaillou et al. 2005, Reguera
97 et al. 2014). Okadaic acid and DTXs are responsible for diarrhetic shellfish poisoning (DSP) in
98 humans following shellfish consumption (Lawrence et al., 2000; Reguera and Pizarro, 2008),
99 with symptoms that include diarrhea, nausea, vomiting and abdominal pain (Yasumoto et al.,
100 1978). In contrast, PTXs are not considered diarrhetic shellfish toxins (DSTs) as they do not
101 cause diarrhea in humans (Matsushima et al., 2015). However, PTXs are lethal to mice by
102 intraperitoneal injection (Miles et al., 2004).

103 While HABs have mostly been studied in relation to public health, another fundamental
104 issue is the direct effect they have on filter-feeding bivalves (Landsberg, 2002; Matsuyama et al.,
105 2001; Shumway and Cucci, 1987; Sandra E. Shumway, 1990). National monitoring programs

106 along the French Atlantic coast indicate that spawning, development and recruitment of larvae
107 may co-occur with *Dinophysis* spp. (Figure S2; Pouvreau et al., 2016; REPHY, 2019). While
108 adults and juvenile bivalves can mechanically escape toxic microalgae by cessation of filtration
109 and closing their shells (Hégaret et al., 2007), the planktonic early life stages such as gametes and
110 embryos are directly exposed to HABs and their toxins in the water column and appear more
111 sensitive than adults (Castrec et al., 2019; Glibert et al., 2007; Stoecker et al., 2008; Wang et al.,
112 2006; Yan et al., 2001).

113 Many studies have focused on the effects of toxic dinoflagellate species on oyster
114 gametes, embryos and larvae, e.g. for the genera *Alexandrium* (Banno et al., 2018; Basti et al.,
115 2015a; Castrec et al., 2019, 2020; Matsuyama et al., 2001; Mu and Li, 2013), *Karenia* (Leverone
116 et al. 2006, Rolton et al. 2014, 2015, 2016, Basti et al. 2015a), *Heterocapsa* (Basti et al., 2013,
117 2011), *Gymnodinium* (Matsuyama et al., 2001), *Karlodinium* and *Prorocentrum* (Glibert et al.,
118 2007; Stoecker et al., 2008). Nevertheless, due to the mixotrophy of toxic species of the genus
119 *Dinophysis* and the resulting difficulty in their cultivation until recently (Park et al., 2006), few
120 studies have investigated the effects of *Dinophysis* spp., their toxins or combinations of both on
121 bivalves, such as oysters. The few available studies indicate that *Dinophysis* spp. producing PTXs
122 induce hypersecretion of mucus and pseudofeces, paralysis, alteration of the tissues within the
123 digestive gland and reduced escape response in adult scallops (Basti et al., 2015b). McCarthy et
124 al., (2014) demonstrated that exposure of adult Pacific oysters and blue mussels to OA increased
125 DNA fragmentation. Further studies also highlighted modified hemocyte functions in both
126 Mediterranean mussels (Malagoli et al., 2008; Prado-Alvarez et al., 2012) and carpet shell clams
127 (Prado-Alvarez et al., 2013) exposed to *Dinophysis* spp. and their toxins.

128 The present study investigated the *in vitro* effects of whole culture, resuspended cells and
129 culture filtrate of *Dinophysis sacculus* (Stein, 1883), whole culture of *D. acuminata* (Claparède
130 and Lachmann, 1859) and certified standards of OA and PTX2 on (i) gamete cellular
131 characteristics (i.e., ROS production, mortality, and morphology), and (ii) fertilization success of
132 oocytes and spermatozoa of the Pacific oyster.

133

134 **Materials and methods**

135 *Microalgal cultures*

136 Monoclonal cultures of *D. sacculus* (Stein, 1883) (strain IFR-DSA-01Lt) and *D. acuminata*
137 (Claparède and Lachmann, 1859) (strain IFR-DAU-02Ar) were isolated in Arcachon, France, in
138 2015 and 2018, respectively. These mixotrophic species were cultivated in 0.2 µm filter-sterilized
139 natural seawater (FSSW) for *D. sacculus* and L1/20-Si + K/2-Si (Hernández-Urcera et al., 2018)
140 for *D. acuminata* at salinity 35 and fed every two days with ciliate prey *Mesodinium rubrum*
141 (Lohmann, 1908) (strain MBL-DK2009) at a ratio of 1: 1 (predator: prey) according to Park et al.
142 (2006). The ciliate *M. rubrum* was fed three times a week with the cryptophyte *Teleaulax*
143 *amphioxeia* (Conrad) (Hill, 1992) (strain AND-0710). Both *M. rubrum* and *T. amphioxeia* were
144 cultivated in flasks respectively in L1/20-Si and L1-Si (Guillard and Hargraves, 1993) and
145 diluted every two days for the ciliates and every week for the cryptophyte. All cultures were
146 maintained at 17.8 ± 0.6 °C, at a light intensity of ~ 100 µmol photons m⁻² s⁻¹ provided by cool-
147 white and pink fluorescent tubes (fluora and cool-white fluorescent light, Osram, Munich,
148 Germany) and a 12: 12 (L: D) cycle (Table S1). To increase the biomass of *Dinophysis* spp.,
149 cultures were fed at a ratio of 1: 10 (predator: prey) for 4 months before the experiment. One

150 week before the experiment, cultures of *Dinophysis* spp. were filtered on a nylon sieve (mesh
151 11 μ m) and gently rinsed with 75 mL of FSSW to remove any cryptophyte and ciliate. Cultures
152 were then resuspended in 20 mL of FSSW and starved for one week before the experiment to
153 obtain cells from the mid exponential growth phase on the day of the experiment.

154

155 *Experimental design*

156 All experiments are summarized in Figure S1 and performed with 3 to 11 replicates. A replicate
157 is either a pool of several females or males, or one male or female, as detailed below.

158 Experiment 1 (Exp. 1) – Effect of the whole culture of *D. sacculus* upon gametes

159 The aim of Exp. 1 was to determine the effect of whole cultures of *D. sacculus* on gamete cellular
160 characteristics and fertilization success. In total, four fertilization experiments were performed by
161 crossing exposed or non-exposed oocytes and/or spermatozoa to *D. sacculus*.

162 - (i) Oocytes exposed to *D. sacculus* (n = 8 pools of gametes, each from 3 different
163 females) were crossed with non-exposed spermatozoa (a pool of spermatozoa from 5
164 males)

165 -or (ii) Spermatozoa exposed to *D. sacculus* (n = 8 pools of gametes, each from 3
166 different males) were crossed with non-exposed oocytes (a pool of oocytes from 5
167 females).

168 - (iii) In addition, fertilization success was determined after exposure of both oocytes and
169 spermatozoa to *D. sacculus* (n = 4 pools of gametes, each from 3 different organisms).

170 - (iv) The effects of whole cultures of *D. sacculus* on fertilization success was also
171 investigated by exposing gametes only during fertilization. Oocytes, spermatozoa and *D.*
172 *sacculus* (at final concentrations of 0 (control), 0.5, 5, 50 and 500 cells mL⁻¹) were put in
173 contact simultaneously in FSSW (n = 11 pools of gametes, each from 3 different
174 organisms).

175 Briefly, for (i), (ii) and (iii); oocytes or spermatozoa were exposed for 2 h in glass vials at 20 ± 1
176 °C to the whole culture of *D. sacculus* at a final concentration of 0 (control), 0.5, 5, 50 and 500
177 cells mL⁻¹ in FSSW. These *D. sacculus* cell concentrations were selected to mimic the cell
178 densities in natural blooms occurring in France, from typical (1 x 10² cells L⁻¹) to exceptionally
179 dense blooms (8 x 10⁵ cells L⁻¹, Figure S2, REPHY, 2019).

180

181 Experiment 2 (Exp. 2) – Effect of resuspended cells and culture filtrate of *D. sacculus* upon
182 gametes

183 Experiment 2 was designed to determine the respective effect of resuspended cells and
184 extracellular medium (culture filtrate) of *D. sacculus* on gametes. (i) Oocytes or (ii) spermatozoa
185 (n = 5 individual females or males) were exposed for 2 h in glass vials either to the whole *D.*
186 *sacculus* culture at 500 cells mL⁻¹ (similar to Exp. 1), or to *D. sacculus* cells only, obtained by
187 filtration (11 µm-mesh nylon sieve) of a culture at 500 cells mL⁻¹ and resuspended in FSSW (to
188 remove the extracellular metabolites) or to culture filtrate obtained by filtration (0.2 µm-mesh
189 nylon sieve) of the whole culture (500 cells mL⁻¹) to measure the effect of only the extracellular
190 metabolites of living cultures. After exposure, gamete cellular characteristics and fertilization
191 success were determined using gametes exposed to either whole culture or to resuspended cells of

192 *D. sacculus* or to its culture filtrate and a pool of unexposed spermatozoa or oocytes from 5
193 oysters.

194

195 Experiment 3 (Exp. 3) – Effect the whole culture of *D. acuminata* upon gametes

196 The aim of Exp. 3 was to determine the effect of a 2 h exposure of whole cultures of *D.*
197 *acuminata* (at a final concentration of 0 (control), 0.5, 5, 50 and 500 cells mL⁻¹) on gamete
198 cellular characteristics and fertilization success of both exposed oocytes and spermatozoa (n = 4
199 pools of gametes, each from 3 different organisms), as described in Exp. 1 (iii).

200

201 Experiment 4 (Exp. 4) – Effect of okadaic acid (OA) and pectenotoxin 2 (PTX2) standards on
202 gametes

203 Experiment 4 investigated the effect of 0 (control), 5, 10, 20 and 50 nM solutions of okadaic acid
204 (OA) and pectenotoxin 2 (PTX2) in FSSW and to the corresponding methanol (MeOH) control
205 (2.3 % final) on cellular characteristics and fertilization success of (i) oocytes or (ii) spermatozoa,
206 (iii) both oocytes and spermatozoa exposed to toxins and (iv) gametes exposed to toxins only
207 during fertilization (n = 3 to 4 pools of gametes from 3 different organisms). These
208 concentrations of toxins approximately corresponded to the minimum concentration of OA (i.e. 5
209 nM) found in the studied strains of *D. sacculus* and *D. acuminata* (sum of intra and extracellular
210 toxins of 500 cells) to ca. the maximum concentration of PTX2 (i.e. 50 nM; Table 1). All
211 measurements were performed as detailed in Exp. 1.

212

213 *Toxin analyses of Dinophysis spp. strains*

214 Toxin analysis was adapted from Sibat et al. (2018) and García-Portela et al. (2018) and
215 performed on 1 mL (n=3) sub samples of *Dinophysis* spp. cultures collected in exponential
216 growth phase. After centrifugation (3500 g, 4 °C, 15 min), both cells and culture filtrates
217 (supernatants) were extracted. The pellet (intracellular toxins) was extracted with 0.5 mL
218 methanol and sonicated at 25 kHz for 15 min. Extracellular toxins were recovered from the
219 supernatant after liquid-liquid extraction with dichloromethane, which was evaporated under
220 nitrogen and resuspended in 0.5 mL of methanol. Subsequently, samples were filtered (0.2 µm,
221 Nanosep, MF, Pall, Northborough, MA, USA). Analyses were performed using ultra high
222 performance liquid chromatography coupled to low resolution tandem mass spectrometry
223 (UHPLC-LRMS/MS) with a UHPLC system (UFLC XR Nexera, Shimadzu, Tokyo, Japan)
224 coupled to a triple quadrupole/ion-trap mass spectrometer (API 4000 QTrap, ABSciex, Redwood
225 City, CA, USA), equipped with a turboV[®] ESI source (see details in García-Portela et al. 2018).
226 Certified calibration solutions of PTX2, OA, dinophysistoxin 1 and 2 (DTX1 and DTX2) were
227 obtained from the National Research Council Canada (NRCC, Halifax, NS, Canada). Intracellular
228 (intra) and total (sum of intracellular and extracellular) toxin contents were expressed on a per
229 cell basis (pg cell⁻¹) while extracellular (extra) as equivalent (eq) pg cell⁻¹. Pectenotoxin 2 eq
230 (PTX2eq) was the sum of pectenotoxin 2, pectenotoxin 2b, pectenotoxin 2 seco-acid and 7-*epi*-
231 pectenotoxin 2 seco-acid, all quantified with PTX2 standard by assuming similar molar
232 responses.

233

234 *Sampling and maintenance of oysters*

235 Sexually mature *C. gigas* were collected at La Pointe du Chateau-Baie de Daoulas, France
236 (48°20'01.8"N 4°19'02.6"W) in summer 2018 and 2019 or obtained from Ifremer experimental
237 facilities as described in Castrec et al. (2019). Individuals from the field were cleaned with
238 filtered sea water (FSW) to remove sessile organisms. All oysters were maintained in an aerated
239 tank at 16 ± 1 °C with a continuous flow of FSW for one to three days before the experiments.

240

241 *Collection of gametes*

242 Gonads were dissected and placed in individual Petri dishes to collect gametes according to Song
243 et al., (2009) for oocytes and Boulais et al., (2015) for spermatozoa. Briefly, for each oyster,
244 gametes were collected in 10 mL FSSW and sieved through 100 µm mesh to isolate gametes
245 from gonad debris. Only motile spermatozoa and rounded oocytes were selected for the
246 experiments (Rolton et al., 2015). Spermatozoa and oocyte concentrations were determined by
247 flow cytometry (FCM) according to Le Goïc et al. (2013, 2014) and diluted in FSSW at 10^7 cells
248 and 10^5 mL⁻¹, respectively.

249

250 *Flow-cytometry analysis – morphology, viability and ROS production*

251 Analyses of morphology, viability, and ROS production (i.e. cellular characteristics) of oyster
252 gametes by FCM were adapted from Le Goïc et al. (2013, 2014) and carried out with an
253 EasyCyte Plus cytometer (Guava Technologies, Millipore, Luminex Billerica, USA) equipped
254 with a 488-nm argon laser and three fluorescence detectors: green (525 ± 30 nm), yellow ($583 \pm$
255 26 nm) and red (680 ± 30 nm).

256 For cell morphology measurements, values of the forward scatter (FSC) and side scatter (SSC),
257 respectively proxies of cell size and complexity, were used to estimate cell morphology of
258 spermatozoa and oocytes. Spermatozoa viability was assessed using double staining spermatozoa
259 solution with 2 μL of propidium iodide (PI) and 2 μL of SYBR-14 (Live/Dead® Sperm Viability
260 Kit, Molecular Probes, Eugene, USA) at final concentrations of 2 $\mu\text{g mL}^{-1}$ and 1 μM during 10
261 min in the dark (Le Goïc et al., 2013). For oocyte viability, PI and SYBR-Green-1 (1/10,000 of
262 the commercial solution; Molecular Probes, Eugene, USA) were used (Le Goïc et al., 2014).
263 ROS production of gametes was measured by staining 200 μL of oocytes or spermatozoa solution
264 with 2 μL (final concentration of 10 μM) of dye 2',7',7'-dichlorofluorescein diacetate DCFH-DA
265 (Sigma, St Quentin Fallavier, France) for 1 h in the dark (Le Goïc et al., 2014; Vignier et al.,
266 2017).
267 ROS production was expressed as percentage of control (a.u.). For both viability (expressed as
268 mortality) and ROS production measurements, oocytes and spermatozoa concentrations were 5 x
269 10^4 and 5 x 10^6 cells mL^{-1} , respectively.

270

271 *Microscopy analysis – fertilization success assessment*

272 To assess fertilization success, oocytes and spermatozoa were inoculated in FSSW (20 ± 1 °C) at
273 a ratio of 1: 100 (5 x 10^3 oocytes: 5 x 10^5 spermatozoa) in 12-well plates in a final volume of 4
274 mL FSSW (Boulais et al., 2017). When fertilized oocytes in the control (i.e. non-exposed oocytes
275 and spermatozoa) reached 80 % or, after 2 h of incubation, samples were fixed with 1 %
276 formaldehyde (final concentration). Fertilization success (%) was assessed under an inverted light
277 microscope (Axio observer.Z1, Zeiss, Oberkochen, Germany) by counting fertilized and

278 unfertilized oocytes (polar body extrusion from 2 to 8-cell stages vs. no polar body). For the
279 fertilization success measurements in all experiments except conditions (iv), the concentration of
280 *Dinophysis* spp. or toxins were minimum 30-fold lower due to dilution in FSSW and their
281 potential contribution to the observed effect were considered not significant.

282

283 *Statistical analyses*

284 Statistical analyses were performed on RStudio v 1.1.463. After checking the assumptions of
285 independence (Durbin-Watson test), homoscedasticity (Bartlett test) and normality (Shapiro-Wilk
286 test) of the residuals, t-test or one-way ANOVA followed by a Tukey post hoc test were
287 computed. Otherwise, Mann-Whitney U or Kruskal-Wallis tests were used, followed by a
288 Conover test. Differences were considered statistically significant when $P < 0.05$, for a
289 significance level of $\alpha = 0.05$. Values were expressed as mean \pm SD.

290

291 **Results**

292 *Toxin contents of Dinophysis spp. cultures*

293 The *D. sacculus* strain synthesized OA and three PTX2 derivatives (pectenotoxin 2,
294 pectenotoxin 2b, pectenotoxin 2 seco-acid, 7-*epi*-pectenotoxin 2 seco-acid) whereas the *D.*
295 *acuminata* strain synthesized only OA. Neither of the two algal species produced DTX1 or DTX2
296 (Table 1).

297 For Exp. 1 and Exp. 2, *D. sacculus* produced similar amounts of total OA (4.5 ± 1.4 and $6.0 \pm$
298 0.46 pg cell⁻¹) and total PTX2eq, i.e. sum of concentrations (95 ± 36 and 76 ± 19 pg cell⁻¹; Table
299 1). The majority of OA was in the extracellular compartment in contrast to PTX2 which was

300 mainly intracellular. For Exp. 3 with *D. acuminata*, the total OA content per cell was 16-fold
301 higher (87 ± 11 pg cell⁻¹) than *D. sacculus* and >90 % was intracellular ($P < 0.001$; Table 1).

302

303 *Exp. 1 – Effect of the whole culture of D. sacculus on gametes*

304 None of the tested concentrations affected spermatozoa cellular characteristics. However,
305 mortality of oocytes was 2.9-fold-higher when exposed to *D. sacculus* at a concentration of
306 500 cells mL⁻¹ compared to the control ($P < 0.05$), whereas no effect was observed on ROS
307 production. Moreover, a significant increase in FSC was observed for the same exposure
308 condition compared to control ($P < 0.05$; Table 2).

309 Fertilization success decreased significantly when (i) oocytes or (ii) spermatozoa were exposed to
310 50 and 500 cells mL⁻¹ of *D. sacculus* compared to their respective controls (89 vs. 56 and 2 %
311 and 83 vs. 58 and 33 %, respectively; $P < 0.001$; Figure 1 A-B). Interestingly, a 17-fold
312 difference was noted between spermatozoa and oocytes when exposed to 500 cells mL⁻¹ ($P <$
313 0.001 ; Figure 1 A-B) with oocytes being more sensitive. When (iii) both gametes were exposed
314 to *D. sacculus*, fertilization success compared to control was significantly reduced by 25, 44 and
315 93 % at 5, 50 and 500 cells mL⁻¹, respectively ($P < 0.001$; Figure 1 C). The fertilization success
316 in presence of *D. sacculus* during the fertilization (iv) was significantly reduced by 18, 39 and 57
317 % when exposed to 0.5, 5 and 50 cells mL⁻¹, respectively ($P < 0.001$). Fertilization was however,
318 totally impeded at 500 cells mL⁻¹ (Figure 1 D).

319

320 *Exp. 2 – Effect of resuspended cells and culture filtrate of D. sacculus on gametes*

321 The negative effect of gametes exposed to whole cultures (500 cells mL⁻¹) was confirmed ($P <$
322 0.001), with fertilization success 10 times lower following exposure of oocytes vs. spermatozoa
323 ($P < 0.001$; Figure 2 A-B). While similar results were obtained using resuspended *D. sacculus*
324 cells ($P < 0.05$), no significant difference was noted between the controls and gametes exposed to
325 culture filtrates (Figure 2 A-B).

326

327 *Exp. 3 – Effect the whole culture of D. acuminata on gametes*

328 Only oocytes exposed to 500 cells mL⁻¹ of *D. acuminata* were significantly affected, with a 2.7-
329 fold higher mortality ($P < 0.001$) and a 16 % increase in FSC ($P < 0.001$; Table 2). Again,
330 spermatozoa were not affected. A significant decrease in fertilization success after exposure of
331 both gametes was observed from as few as 5 cells mL⁻¹ (1.8-fold; $P < 0.05$), while fertilization
332 was almost completely inhibited at 500 cells mL⁻¹ of *D. acuminata* ($P < 0.001$; Figure 3).

333

334 *Exp. 4 – Effect of okadaic acid (OA) and pectenotoxin 2 (PTX2) standards on gametes*

335 Production of ROS was around twice higher in oocytes exposed for 2 h to 50 nM of PTX2 ($P <$
336 0.05) compared to the control while spermatozoa were not affected by exposure to OA and PTX2
337 standards (Table 2).

338 A decrease in fertilization success was observed with (i) oocytes exposed for 2 h to 20 and 50 nM
339 of PTX2 compared to control (16 and 0 % vs. 77 %, respectively; $P < 0.001$; Figure 4 A).

340 Similarly, (ii) spermatozoa exposed to 20 and 50 nM of PTX2 decreased fertilization success
341 compared to control (35 and 25 % vs. 81 %; $P < 0.001$; Figure 4 B). However, oocytes exposed
342 to 50 nM, exhibited a more pronounced effect than exposed spermatozoa on fertilization ($P <$

343 0.05; Figure 4 A-B). Exposure of (iii) both oocytes and spermatozoa to 5, 10, 20 and 50 nM of
344 PTX2 reduced the fertilization success by 25, 37, 78 and 97 % compared to control ($P < 0.01$;
345 Figure 4 C). The fertilization in presence of toxins (iv) was significantly diminished only at a
346 concentration of 50 nM of PTX2 (32 %) compared to control (76 %; $P < 0.001$; Figure 4 D).
347 Neither OA (at any of the concentrations tested) nor MeOH in the control affected the
348 fertilization success.

349

350 **Discussion**

351 The present work demonstrated that *Dinophysis sacculus* and *D. acuminata* as well as one of
352 their toxins PTX2 impaired cellular characteristics and fertilization success in gametes of the
353 Pacific oyster, *Crassostrea gigas*, a species of commercial interest.

354 The concentrations of *Dinophysis* spp. used in this exposure study were selected because of their
355 environmental relevance, since concentrations of > 1000 cells L^{-1} were frequently observed in the
356 four regions studied on the French Atlantic coast, whereas concentrations of $> 10,000$ cells L^{-1}
357 were occasionally observed in two of the regions, including major oyster production sites, i.e. the
358 Bay of Arcachon and the Bay of Brest (Figure S2). These concentrations, observed in France
359 over a ten-year period, are moderate compared to other areas affected by *Dinophysis* spp. blooms,
360 e.g. India or Norway, where concentrations of up 1.5×10^6 and 2.3×10^7 cells L^{-1} have been
361 observed (Reguera et al., 2012). e

362 The toxin exposure concentrations (i.e. from 5 to 50 nM) corresponded to the maximum amounts
363 of OA and PTX2, respectively, produced by 500 cells of *D. sacculus* in our experiment.

364 Noteworthy, PTX2 caused an increase in ROS production of oocytes exposed to 50 nM, which

365 could reflect a stimulation of oocyte metabolism or cellular stress. Indeed, the production of ROS
366 is a key mechanism involved in stress responses (Kadomura et al., 2006), but an excess of ROS
367 could lead to cellular toxic effects such as destruction of membrane integrity by lipid
368 peroxidation, DNA damage and associated alteration of cell functioning and ultimately cell death
369 (Cavallo et al., 2003; Landsberg, 2002; Lesser, 2006). This may explain the observed reduced
370 fertilization success of oocytes exposed to PTX2. Similarly, Le Goïc et al. (2014) observed an
371 increased ROS production of oocytes in the presence of the toxic dinoflagellate *Alexandrium*
372 *minutum*, and suggested this increase production may have reduced oocyte quality.

373 Pectenotoxin 2 at concentrations as low as 20 nM reduced fertilization success when either
374 oocytes or spermatozoa were pre-exposed. This is true also at concentration as low as 5 nM of
375 PTX2 when oocytes and spermatozoa were both pre-exposed. This suggests that both oocytes and
376 spermatozoa were negatively affected by PTX2 and that the effect of PTX2 on oyster gametes is
377 cumulative. Secondly, this toxicity is likely mediated by a different mechanism than ROS
378 production since no increase in ROS production was observed below 50 nM concentration of
379 PTX2.

380 Using mammalian and finfish cell lines (e.g. human, rat, rabbit, salmon), PTXs have been shown
381 to interfere with actin assembly/disassembly, thereby affecting cell cytoskeletal functions and
382 leading to cell death, at concentrations ranging from nM to μ M (Ares et al., 2005; Dominguez et
383 al., 2010; Spector et al., 1999). It has been shown that PTX2 causes actin depolymerization
384 (Dominguez et al., 2010), sequestration of monomeric actin (at a concentration of 20 nM; Spector
385 et al., 1999), disrupted F-actin (Hori et al., 1999), and inhibited actin polymerization by a capping
386 process at the barbed-end of F- and G-actin (Allingham et al., 2007). The reduced fertilization
387 success observed in this study could be associated with impairment of the oocyte and

388 spermatozoan cytoskeleton by PTX2, as well as fertilization itself since actin polymerization is a
389 crucial mechanism in oysters, involved in spermatozoan motility and the penetration of the
390 oocyte (Ledu and McCombie, 2003). In the literature, the indirect evidence of the involvement of
391 *Dinophysis* spp. in mortalities observed in the natural environment (reviewed in Landsberg 2002,
392 Basti et al., 2015b) were almost exclusively related to *D. caudata*, a producer of high cellular
393 contents of PTX2 (Basti et al., 2015b, 2015c; Fernández et al., 2006; Marasigan et al., 2001). The
394 action of PTX2 on actin in oyster gametes would be worthy of investigation in further studies.

395
396 Okadaic acid, in contrast to PTX2, affected neither the gametes nor fertilization success, at
397 concentrations up to 50 nM. Okadaic acid is reported to be an inhibitor of serine/threonine protein
398 phosphatase 1 and 2 activities (Bialojan and Takai, 1988; McCarthy et al., 2014) and is also
399 believed to be a tumor promoter in humans (Lago et al., 2005). This toxin induced chromosome
400 loss, apoptosis and DNA damages in mammalian cell lines (see references in Prado-Alvarez et
401 al., 2013). Okadaic acid has been shown to induce an increase in DNA fragmentation in adult
402 Pacific oyster and blue mussel (McCarthy et al., 2014) and modified hemocyte functions in
403 several bivalve species (Malagoli et al., 2008; Prado-Alvarez et al., 2013, 2012) at concentrations
404 between 1.2 to 50 and 10 to 500 nM, respectively. The absence of effects of OA on oyster
405 gametes in this study could be due to the relatively short exposure time and low OA
406 concentrations, reaching respectively 2 h and a maximum of 50 nM concentration of certified
407 standard and 4.5 nM concentration of extracellular OA produced by 500 cells of *D. acuminata* in
408 our experiment.

409 The main structural difference between OA and PTX2 is that PTX2 is a macrocyclic lactone (i.e.
410 cyclic ester). Pectenotoxin 2 biological activity on cytoskeletal dynamics is clearly associated

411 with the macrocyclic ester as the activity disappeared when the esters were hydrolysed and the
412 macrocycle was opened (Allingham et al., 2007; Ares et al., 2007; Miles et al., 2006). The
413 resulting analogue, pectenotoxin 2 seco-acid, is structurally very similar to OA, and is not active
414 on the cytoskeleton (neither is OA). Interestingly, PTX2 has a high structural similarity with
415 goniiodomin A, another algal macrocyclic lactone, which has also been reported to affect the
416 cytoskeleton via F-actin (Espiña et al., 2016). In addition, it should be noted that among the three
417 species of *Alexandrium* that produce goniiodomins (Harris et al., 2020), *A. monilatum* has been
418 clearly associated with fish kills since the middle of the last century (Howell, 1953) and more
419 recently also with shellfish mortalities (Harding et al., 2009; May et al., 2010).

420 Our study also revealed that PTX2 is likely not to be the only bioactive compound responsible for
421 the toxicity of *Dinophysis* spp. on oyster gametes and fertilization success. Firstly, fertilization
422 success was decreased in the presence of 0.5 cell mL^{-1} of *D. sacculus* during fertilization, which
423 corresponded to a non-detectable amount of PTX2, while 50 nM of PTX2 were needed to obtain
424 similar effects. Secondly, our strain of *D. acuminata* did not produce PTX2 but also caused a
425 decrease in fertilization success, when both gametes were pre-exposed to only 5 cells mL^{-1} , and,
426 as described above, these effects could also not be attributed to OA.

427 Additionally, the present study indicated that the decreased fertilization success, specifically for
428 *D. sacculus*, was derived from cells and not from the extracellular compartment, as filtrate had no
429 activity, unlike resuspended cells. This observation could be explained by cell-to-cell contact and
430 the effect of (a) mechanical damages and/or (b) surface-bound toxins and/or (c) quick release of
431 intracellular bioactive compounds (Landsberg, 2002).

432 Contact with *Dinophysis* spp. cells, or by the mean of feeding peduncle (Ojamäe et al., 2016),
433 may have resulted in (a) mechanical damage to the membranes of oyster gametes, as suggested

434 by the increase in FSC morphological parameter of oocytes. This proxy of cell size could indicate
435 a swelling of the cells, when exposed to 500 cells mL⁻¹ of *D. sacculus* associated to an increase in
436 mortality.

437 Furthermore, it has been hypothesized that (b) the presence of toxins on the cell surface of
438 another HAB species, *H. circularisquama* can affect pearl oyster larvae after contact (Basti et al.,
439 2011). In addition, Mu and Li (2013) suggested that the release by *A. catenella* of surface-located
440 toxins may affect Pacific oyster egg hatching success. Surface-bound toxins from *Dinophysis*
441 spp., *Alexandrium* spp. and *Heterocapsa* spp. have not been reported yet but could potentially
442 explain the observed effect on gametes and fertilization success.

443 Another explanation could be (c) a rapid release of intracellular compounds, different from the
444 already known and characterized toxins, with activity towards oyster gametes. While most
445 attention has been paid to toxins affecting humans (i.e. DSTs in a sanitary context), other
446 bioactive compounds from *Dinophysis* spp. have been overlooked despite some interesting
447 observations. Basti et al. (2015b) observed mortality in adult mollusks fed *D. caudata*
448 independently of PTX2 content, thus they hypothesized the presence of unknown toxins and/or
449 other bioactive compounds. Similarly, Mafra et al. (2016) hypothesized that the mechanism of
450 prey capture of several toxic species of *Dinophysis* spp. involved uncharacterized allelochemical
451 compounds, other than the known DSTs, which debilitates *M. rubrum*. The existence of such
452 allelochemicals for *Karenia brevis* affecting *C. virginica* larvae (Rolton et al., 2014) as well as
453 for *A. minutum* affecting early life stages and adults of *C. gigas* has also been observed (Castrec
454 et al., 2020, 2018). Bioguided-fractionation approaches combining suitable bioassays (Long et
455 al., 2018) as well as chromatography coupled to e.g. high resolution mass spectrometry (Nothias
456 et al., 2018) may be useful to identify these molecules. However, the difficulties inherent to the

457 highly challenging culture of *Dinophysis* spp. requiring prey organisms may be a limitation in
458 these kinds of studies.

459 Oyster spermatozoa are motile and small (2 μm) cells, which may thus have limited contact with
460 *Dinophysis* spp. cells or their toxins, as opposed to the immotile and large (75 μm) oocytes,
461 which are more likely to make physical contact with the dinoflagellate or its toxins. Moreover,
462 spermatozoa and oocytes are different in term of composition (e.g. biochemical content),
463 metabolism (e.g. mobility and embryonic development, respectively) and plasma membrane
464 (Boulais et al., 2017, 2015). These observations may explain the different sensitivity between
465 gametes exposed to *Dinophysis* spp. and PTX2 and the absence of effects observed by flow
466 cytometry for spermatozoa.

467 In addition, the decrease in fertilization success when spermatozoa were exposed to *D. sacculus*
468 and *D. acuminata* and the synergistic effect observed when both gametes were exposed,
469 suggested that spermatozoa were indeed impacted by *Dinophysis* spp.. Further measurements on
470 spermatozoa should be focused on motility and velocity, as well as energetic metabolism and
471 mitochondrial membrane potential, which can affect flagellar movements and ultimately
472 fertilization capacity (Boulais et al., 2017, 2015; Le Goïc et al., 2013).

473 The inhibition of fertilization success was higher with gametes exposed to *D. acuminata* than to
474 *D. sacculus* for the same concentration, however whether this species is more toxic or has a
475 different mechanism of action is still unknown.

476 Some preliminary results also indicate that when gametes were exposed to *D. sacculus*, abnormal
477 development of D-shaped larvae could be observed (personal communications), leading to the
478 question of the effects on early life stages of *C. gigas* and ultimately, recruitment. Similarly,

479 when *C. gigas* larvae were exposed to *A. minutum*, anomalies in swimming behavior, feeding and
480 growth were observed which led to a decrease in survival and settlement of older larvae stages
481 (Castrec et al., 2020).

482 Analysis of the data collected in the REPHY and VELYGER monitoring programs clearly
483 demonstrated that the concentrations of *Dinophysis* spp. used in this study are environmentally
484 relevant and can occur during spawning of *C. gigas* (Figure S2). If shifts in climate lead to
485 increased co-occurrence of *Dinophysis* spp. and oyster spawning periods, effects on reproduction
486 could potentially increase. Economic impact assessment of *Dinophysis* spp. blooms in French
487 coasts is underway as part of the CoCliME project. Additionally, any significant increase of
488 *Dinophysis* spp. concentrations, e.g. through increased eutrophication is likely to also amplify
489 such effects. Indeed, *Dinophysis* spp. blooms have been related to nutrient pollution in France
490 (Souchu et al., 2013) and globally (Hattenrath-Lehmann and Gobler, 2015; Hattenrath-Lehmann
491 et al., 2015).

492

493 **Conclusion**

494 This study highlighted for the first time that low cellular concentrations (i.e. 5×10^2 to 5×10^3
495 cells L^{-1}) of toxic species of the genus *Dinophysis*, i.e. *D. sacculus* and *D. acuminata*, and low
496 PTX2 concentration (5 nM) can interfere with fertilization success of *C. gigas* and can potentially
497 affect reproduction of this species.

498 The adverse effects observed on oyster fertilization success and gamete cellular characteristics
499 were similar for both *Dinophysis* species. Whether this activity is a general trait of the genus
500 *Dinophysis* and results from similar mechanisms requires further investigation. Future studies

501 should also include other *Dinophysis* spp., as this is a very diverse genus with species showing
502 different toxin profiles, including some that do not produce toxins. It is important to explore the
503 intraspecific and interspecific diversity of this activity and its broader impacts on shellfish
504 reproduction.

505 Therefore, studies focusing on the effects of *Dinophysis* spp., especially PTX-producers, and
506 their associated allelopathic or bioactive compounds appear fundamental to better assess their
507 effects on marine organisms (i.e. bivalve, fish, zooplankton and phytoplankton).

508

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812 **Tables and figures**

813 Table 1: Mean intracellular (intra, pg cell⁻¹), extracellular (extra, eq pg cell⁻¹), total (sum of intracellular and extracellular, pg cell⁻¹ and
814 nM corresponding to 500 cells mL⁻¹) of okadaic acid (OA) and pectenotoxin 2 eq (PTX2eq) for *Dinophysis sacculus* (Exp. 1 and Exp.
815 2) and *D. acuminata* (Exp. 3). Values are expressed as mean \pm SD (n = 4 for Exp.1 and Exp. 2 and n = 1 for Exp. 3). Treatments with
816 different superscript letter were significantly different and absence of superscript letter means NS difference.

Exp.	OA				PTX2eq				
	Intra (pg cell ⁻¹)	Extra (eq pg cell ⁻¹)	Total (pg cell ⁻¹)	Total (nM in 500 cells mL ⁻¹)	Intra (pg cell ⁻¹)	Extra (eq pg cell ⁻¹)	Total (pg cell ⁻¹)	Total (nM in 500 cells mL ⁻¹)	
<i>D. sacculus</i>	1	2.2 \pm 0.52 ^a	2.3 \pm 1.1 ^a	4.5 \pm 1.4 ^a	2.8 \pm 0.9 ^a	73 \pm 26	22 \pm 9.0	95 \pm 34	55 \pm 20
	2	2.1 \pm 0.17 ^a	3.9 \pm 0.29 ^a	6.0 \pm 0.46 ^a	3.7 \pm 0.3 ^a	64 \pm 16	12 \pm 2.9	76 \pm 19	44 \pm 11
<i>D. acuminata</i>	3	80 \pm 9.0 ^b	7.5 \pm 1.9 ^b	87 \pm 11 ^b	54 \pm 6.8 ^b	< LD	< LD	< LD	< LD

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822 Table 2: Forward scatter (FSC), side scatter (SSC), ROS production (a.u., % of control) and mortality of oocytes and spermatozoa after
823 exposure to a gradient of concentration from 0.5 to 500 cells mL⁻¹ of *D. sacculus* (Exp. 1) or *D. acuminata* (Exp. 3) and to a gradient
824 of concentration from 5 to 50 nM of okadaic acid (OA) or pectenotoxin 2 (PTX2) (Exp. 4) and to sea water and methanol (MeOH)
825 controls. Values are expressed as mean ± SD (n = 4 - 5 for Exp 1, n = 3 - 4 for Exp. 3 and n = 5 for Exp. 4). Treatments with different
826 superscript letter were significantly different. *n.a.* data not available

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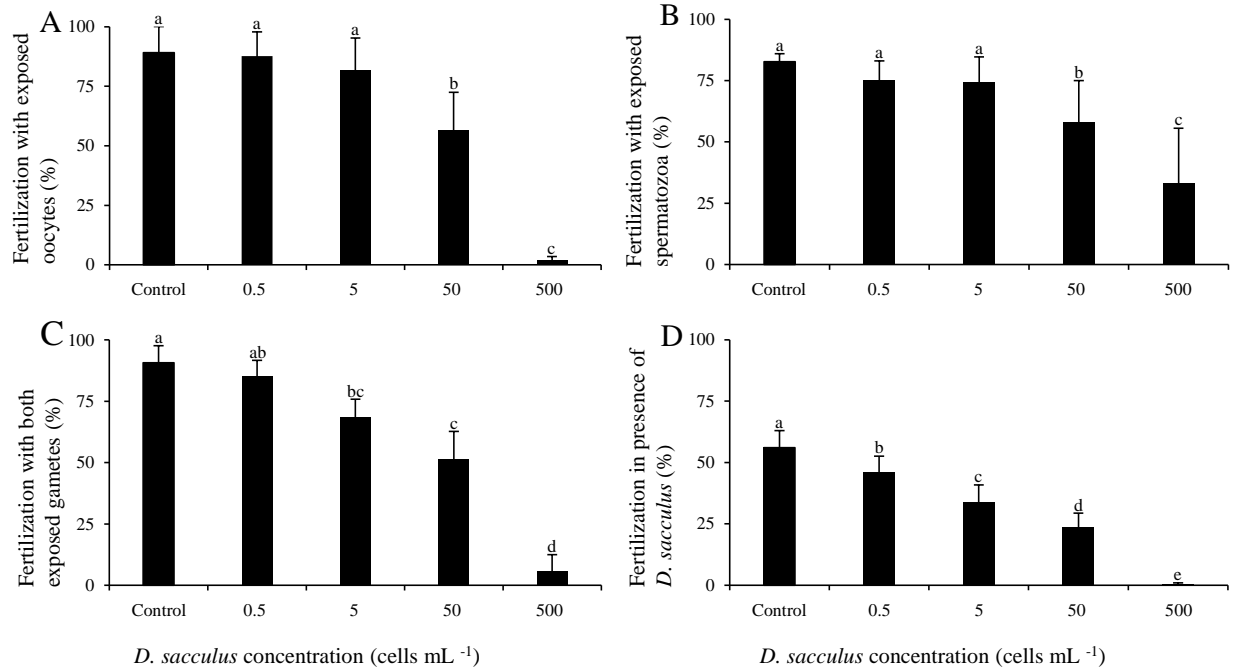
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		Oocytes				Spermatozoa			
		FSC	SSC	ROS (%)	Mortality (%)	FSC	SSC	ROS (%)	Mortality (%)
Exp. 1	Control	359 ± 27^a	1752 ± 65	100	1.7 ± 1.5^a	79.7 ± 2.1	57 ± 1.7	100	2.8 ± 1.3
	0.5	360 ± 28 ^a	1737 ± 70	91 ± 23	2.1 ± 1.6 ^{ab}	79.9 ± 1.8	55 ± 3.1	123 ± 21	4.8 ± 1.9
	5	360 ± 28 ^a	1738 ± 66	96 ± 37	2.6 ± 1.9 ^{ab}	79.8 ± 1.6	55 ± 2.9	134 ± 21	3.5 ± 1.2
	50	371 ± 26 ^a	1746 ± 66	92 ± 8.8	2.3 ± 1.5 ^{ab}	80.1 ± 1.7	56 ± 2.0	131 ± 25	2.8 ± 1.1
	500	473 ± 30^b	1733 ± 66	106 ± 43	4.9 ± 1.6^b	79.3 ± 1.5	56 ± 1.6	118 ± 25	3.5 ± 2.0
Exp. 3	Control	300 ± 11^a	1780 ± 38	100	3.8 ± 0.8 ^a	97.6 ± 1.6	74 ± 1.7	100	16 ± 4.4
	0.5	303 ± 7.9 ^a	1781 ± 42	110 ± 42	3.7 ± 0.6 ^a	97.8 ± 2.5	75 ± 2.9	92 ± 27	14 ± 2.2
	5	304 ± 17 ^{ab}	1783 ± 36	113 ± 90	3.7 ± 0.4 ^a	98.7 ± 1.6	75 ± 1.7	91 ± 14	17 ± 5.8
	50	323 ± 7.6 ^{ab}	1761 ± 40	136 ± 45	<i>n.a</i>	96.9 ± 2.0	76 ± 1.1	108 ± 32	19 ± 4.5
	500	348 ± 38^b	1756 ± 69	113 ± 58	10.4 ± 2.5^b	96.9 ± 0.9	75 ± 1.0	69 ± 12	18 ± 6.5
Exp. 4	Control	326 ± 11	1676 ± 76	100^a	1.4 ± 1.0	98.1 ± 1.5	77 ± 2.4	100	4.9 ± 2.4
	MeOH	329 ± 10	1677 ± 76	120 ± 32 ^{ab}	2.3 ± 0.9	97.9 ± 1.7	77 ± 1.2	163 ± 124	6.2 ± 2.3
	OA 5	329 ± 8.8	1688 ± 71	100 ± 46 ^a	1.6 ± 0.9	97.5 ± 1.5	78 ± 1.4	206 ± 56	4.3 ± 2.3
	OA 10	330 ± 7.8	1681 ± 69	75 ± 25 ^a	1.5 ± 0.4	97.7 ± 1.8	78 ± 2.1	193 ± 51	4.5 ± 2.8
	OA 20	328 ± 9.4	1680 ± 69	91 ± 30 ^a	1.4 ± 0.5	97.9 ± 2.1	78 ± 1.3	168 ± 82	5.2 ± 3.8
	OA 50	328 ± 8.4	1672 ± 58	74 ± 35 ^a	2.2 ± 0.5	97.7 ± 1.7	77 ± 1.7	155 ± 61	5.7 ± 5.6
	PTX2 5	329 ± 9.6	1679 ± 67	94 ± 36 ^a	2.4 ± 1.1	97.6 ± 1.5	78 ± 1.1	199 ± 68	4.0 ± 2.7
	PTX2 10	332 ± 10	1674 ± 71	90 ± 28 ^a	2.3 ± 1.0	97.6 ± 1.5	77 ± 1.8	179 ± 52	4.4 ± 2.1
	PTX2 20	333 ± 9.7	1678 ± 68	105 ± 52 ^a	2.8 ± 0.9	97.6 ± 1.8	77 ± 2.1	173 ± 95	5.1 ± 3.3
PTX2 50	353 ± 14	1678 ± 65	203 ± 108^b	3.1 ± 0.8	97.2 ± 2.8	76 ± 2.1	193 ± 77	4.8 ± 3.5	



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839 Figure 1: Effect of a gradient of concentration of *D. sacculus* on gamete fertilization (Exp. 1): Mean percentage of fertilized eggs with
 840 **A.** oocytes exposed, **B.** spermatozoa exposed, **C.** both gametes exposed and **D.** fertilization in presence of *D. sacculus* (without gametes
 841 exposure) from 0.5 to 500 cell mL⁻¹ and to a sea water control. Values are expressed as mean ± SD (n = 8 – 11). Treatments with
 842 different superscript letters were significantly different.

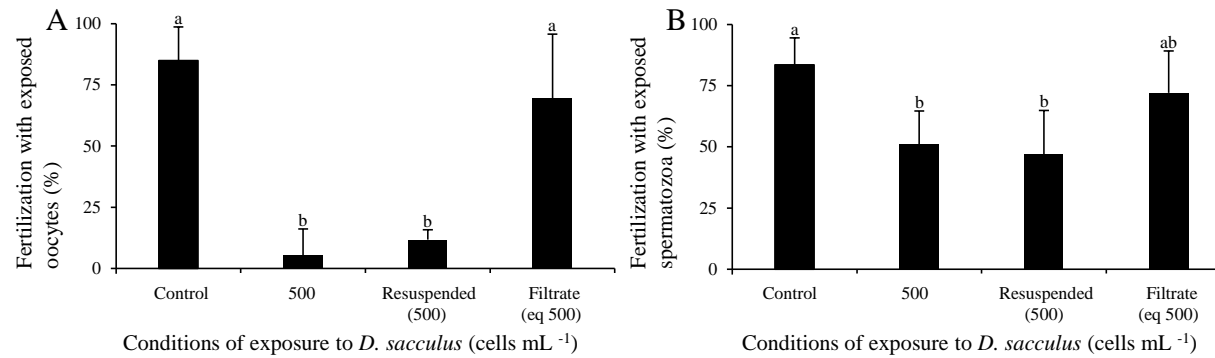
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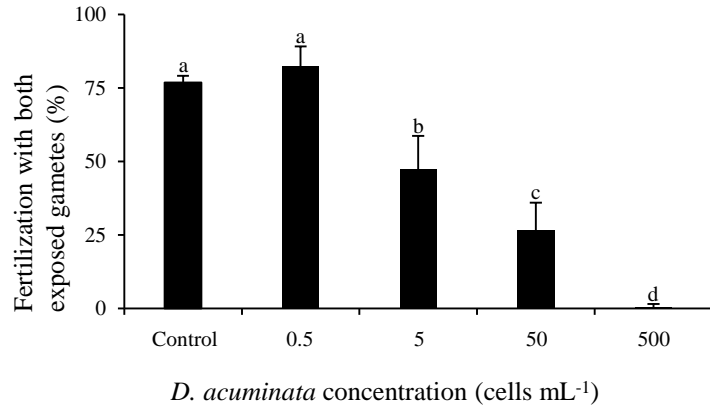
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849 Figure 2: Effect of whole culture (500 cells mL⁻¹), resuspended cells (500 cells mL⁻¹) and culture filtrate (eq 500 cells mL⁻¹) of *D.*
850 *sacculus* on gamete fertilization (Exp. 2). Mean percentage of fertilized eggs (%) with **A.** oocytes exposed and **B.** spermatozoa
851 exposed. Values are expressed as mean \pm SD (n = 5). Treatments with different superscript letters were significantly different.

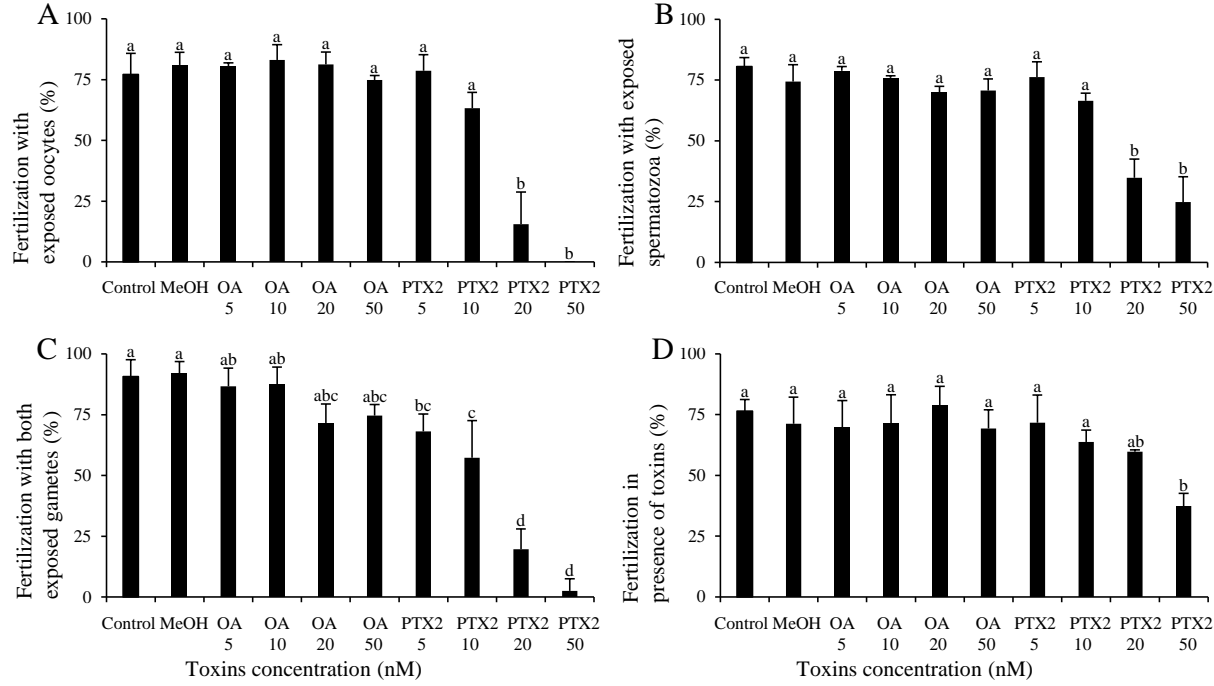


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853 Figure 3: Mean percentage of fertilized eggs (% , Exp. 3) with exposure of both oocytes and spermatozoa to a gradient of concentration
854 of *D. acuminata* from 0.5 to 500 cell mL⁻¹ and to a sea water control. Values are expressed as mean \pm SD (n = 4). Treatments with
855 different superscript letter were significantly different.

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859 Figure 4: Effect a gradient of concentration of okadaic acid (OA) or Pectenotoxin 2 (PTX2) on gamete fertilization (Exp. 4): Mean
 860 percentage of fertilized eggs (%) with **A.** oocytes exposed, **B** spermatozoa exposed, **C.** both gametes exposed and **D.** fertilization in
 861 presence of OA or PTX2 (without gametes exposure) from 5 to 50 nM, including sea water and methanol controls (MeOH). Values are
 862 expressed as mean \pm SD (n = 3 – 4). Treatments with different superscript letters were significantly different.

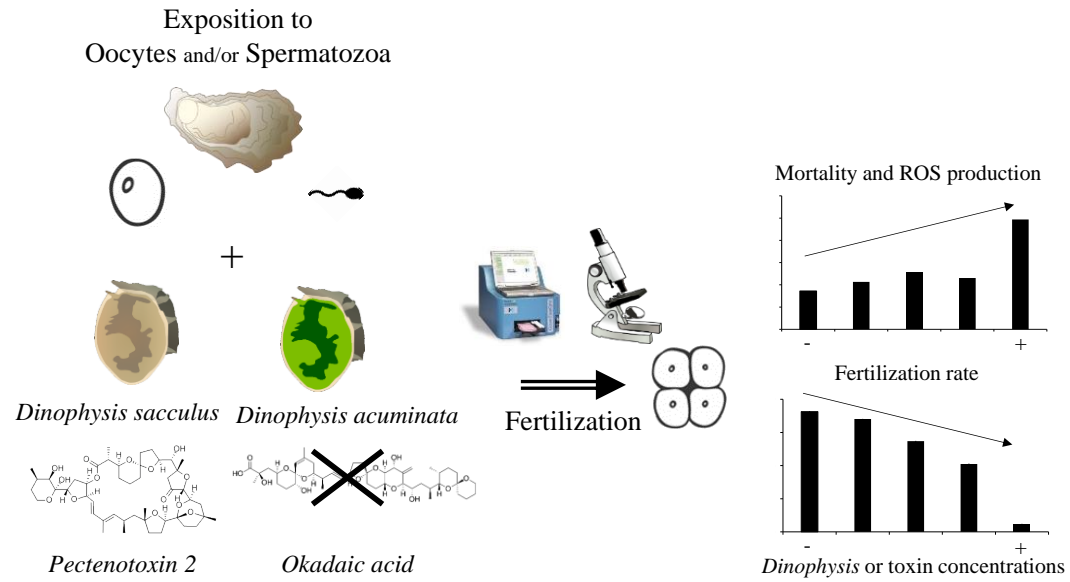
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869 Figure X: Graphical abstract

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871 Table S1: Culture conditions of strains used in this study

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Species	Origin	Strain ID	Medium	Temperature (°C)	Irradiance ^a (μmol photons m ⁻² s ⁻¹)
<i>Teleaulax amphioxeia</i>	Huelva (Spain)	AND-A0710	L1-Si	17.8 ± 0.6	~ 100
<i>Mesodinium rubrum</i>	Helsingør Harbor (Denmark)	MBL-DK2009	L1/20-Si	17.8 ± 0.6	~ 100
<i>Dinophysis sacculus</i>	Arcachon (France)	IFR-DSA-01Lt	Sterilized sea water	17.8 ± 0.6	~ 100
<i>Dinophysis acuminata</i>	Arcachon (France)	IFR-DAU-02Ar	L1/20-Si + K/2-Si	17.8 ± 0.6	~ 100



























873 ^a Cultures were subjected to light in the PAR domain during a circadian cycle 12 h: 12 h (light: dark)

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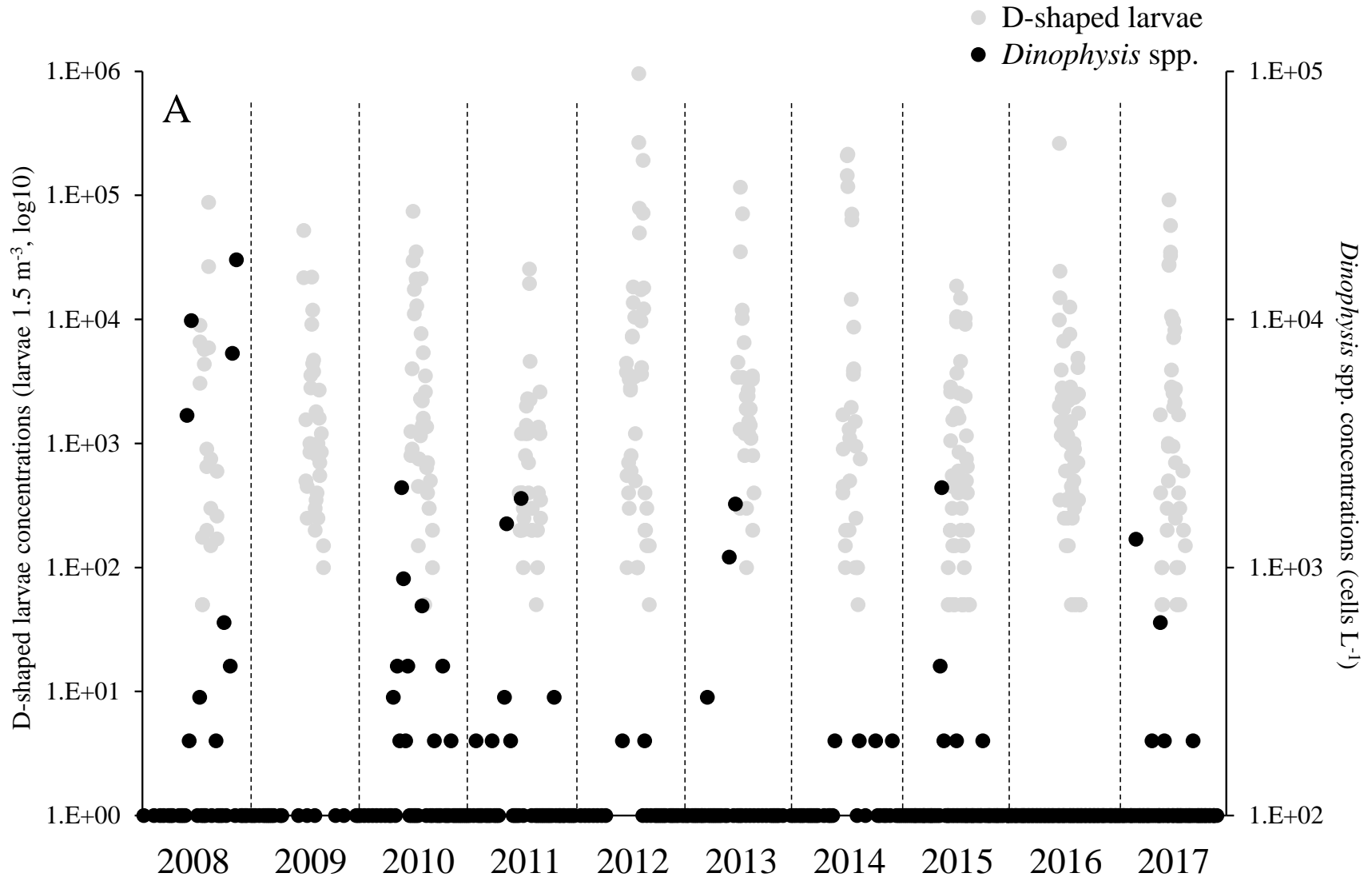
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Experiments	Gamete expositions	FCM Cellular characteristics	Fertilization success	
Exp. 1	(i)  + 	X	X	+ 
	(ii)  + 	X	X	+ 
	(iii)  + 	X	X	Cross together
	(iv)			 +  + 
Exp. 2	(i)  +  resuspended or filtrate	X		+ 
	(ii)  +  resuspended or filtrate	X		+ 
Exp. 3	 + 	X	X	Cross together
Exp. 4	(i)  + OA or PTX2	X	X	+ 
	(ii)  + OA or PTX2	X	X	+ 
	(iii)  + OA or PTX2	X	X	Cross together
	(iv)			OA or PTX2 +  + 

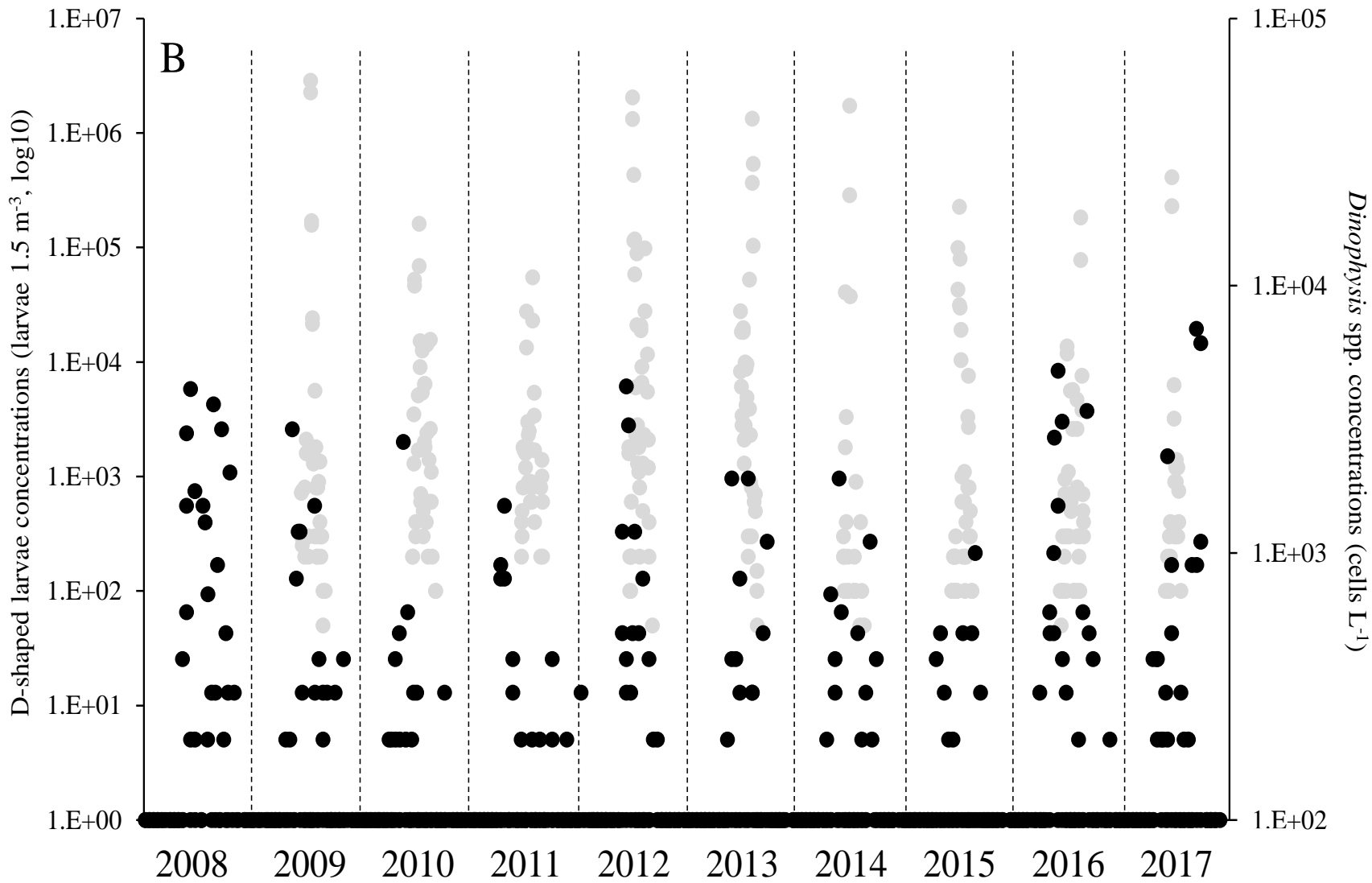
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879 Figure S1: Experimental design. Oysters gametes were exposed, or not, to a gradient of concentration from 0.5 to 500 cells mL⁻¹ of
880 (Exp. 1) *Dinophysis sacculus* and (Exp. 3) *D. acuminata*, to (Exp. 2) *D. sacculus* (500 cells mL⁻¹), resuspended cells (500 cells mL⁻¹)
881 or to culture filtrate (eq 500 cells mL⁻¹) and to (Exp. 4) a gradient of concentration from 5 to 50 nM of okadaic acid (OA) and
882 pectenotoxin 2 (PTX2). Cellular characteristics (morphology, mortality and ROS production) were then analyzed, or not, by flow
883 cytometry (FCM). Finally, depending on each case, exposed gametes were fertilized with non-exposed gametes, with exposed gametes
884 or fertilization were made in presence of *D. sacculus* or toxins.



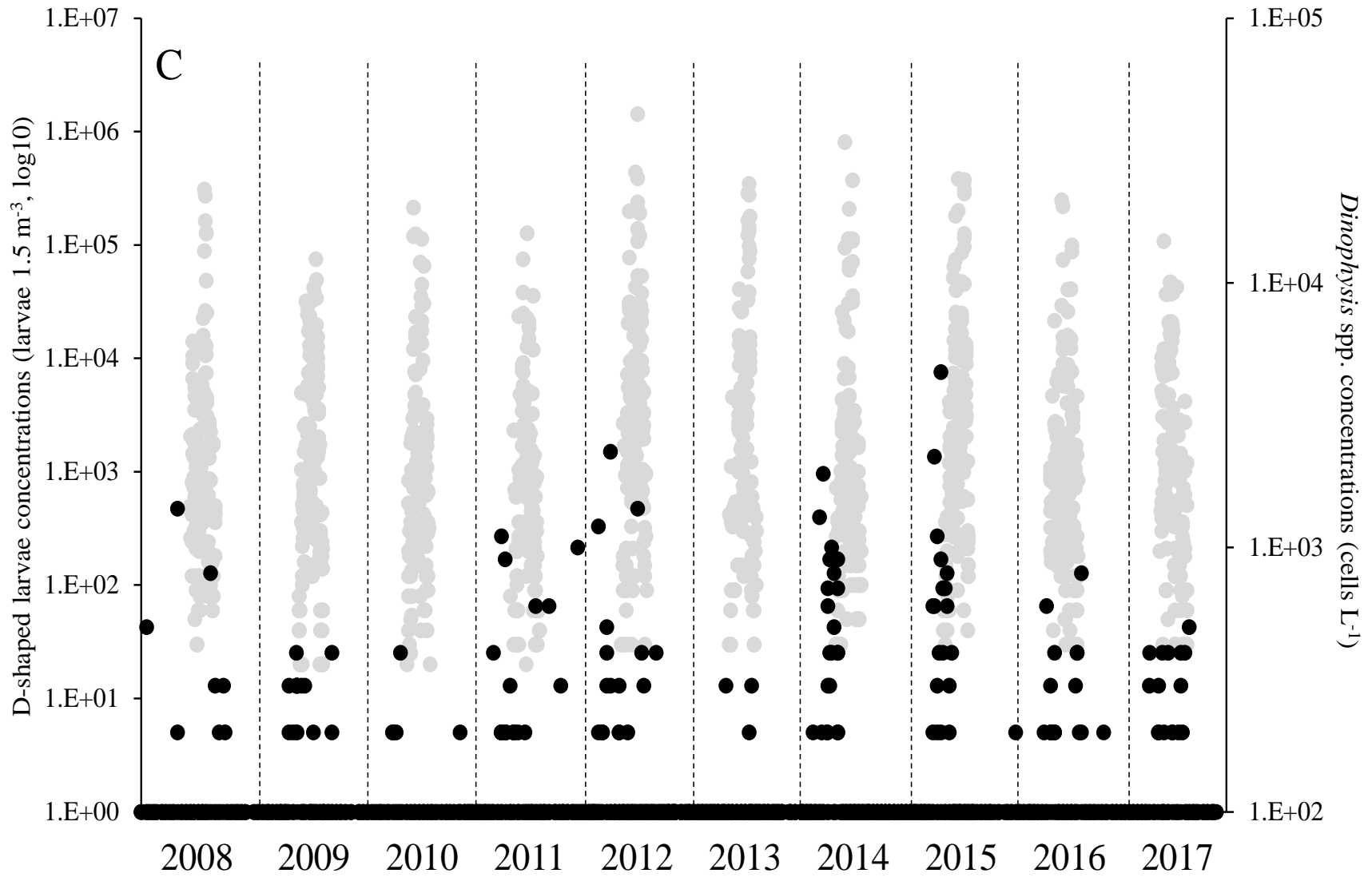
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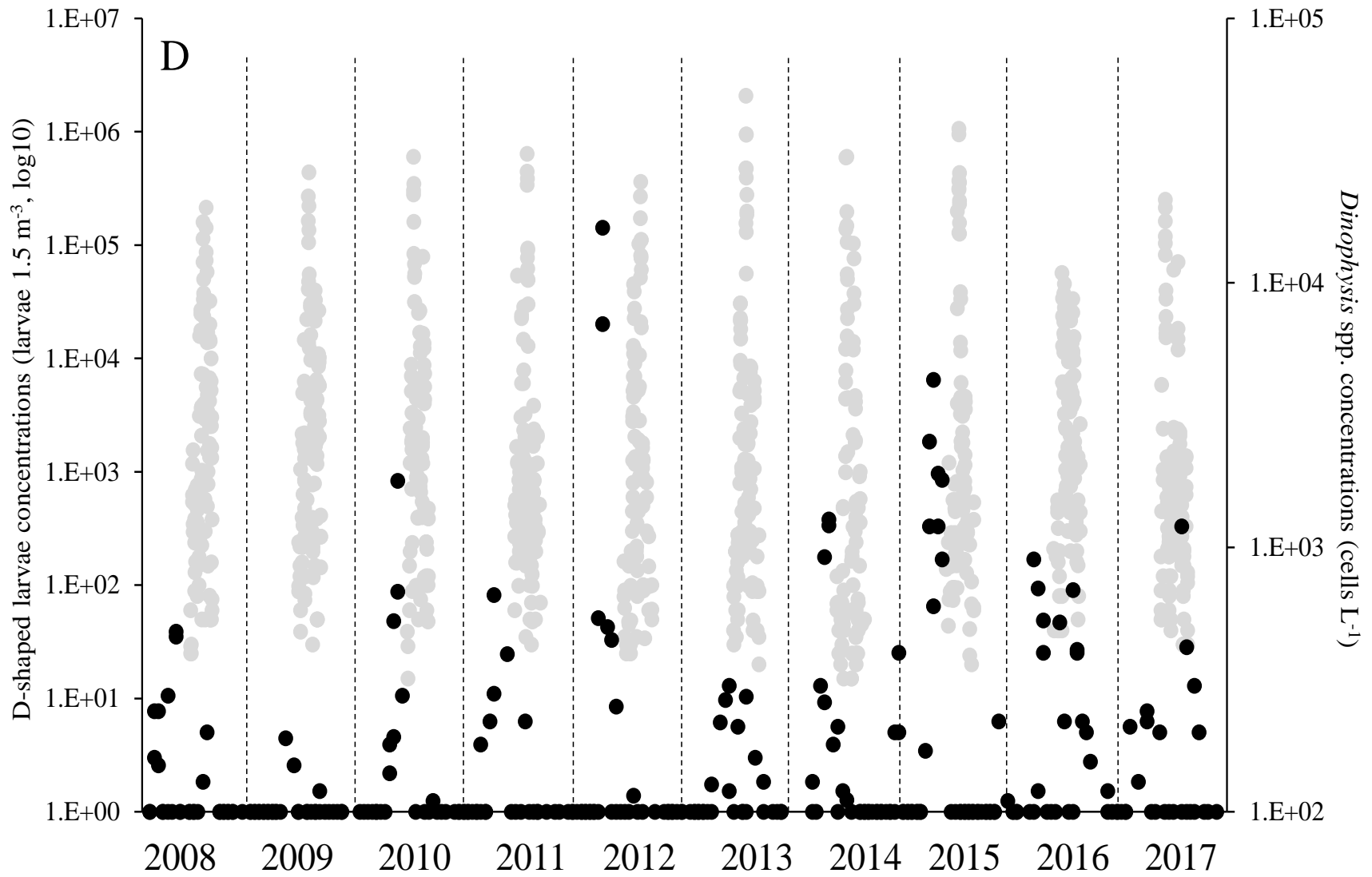
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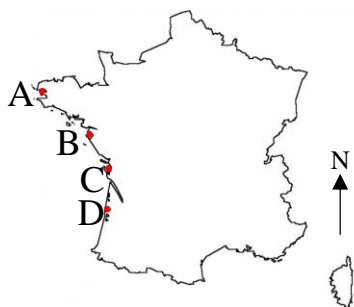
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893 Figure S2: Concentration of *Dinophysis* spp. (black dots; cells L^{-1} ; log10) and D-shaped larvae

894 (grey dots; larvae $1.5\ m^{-3}$; log10) of *Crassostrea gigas* from **A.** Bay of Brest, **B.** Bay of

895 Bourgneuf, **C.** Marennes-Oléron, and **D.** Bay of Arcachon, respectively, from the North of the

896 Bay of Biscay to the South. Data are occurrences from 2008 to 2017 extracted from the REPHY

897 and VELYGER databases. Notes that $100\ cells\ L^{-1}$ of *Dinophysis* spp. is the limit of detection

898 (placed on the x-axis) and occurrences $< LOD$ are not included in the data set. VELYGER

899 observatories are not available for the year 2008 at **B.** Bay of Bourgneuf.

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