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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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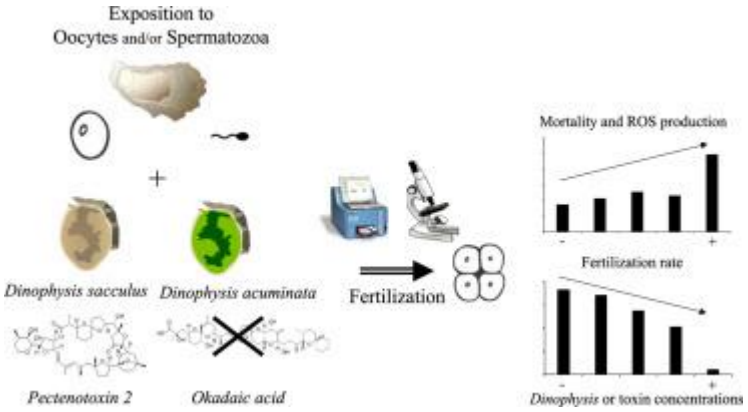
Abstract :

Harmful algal blooms (HABs) of toxic species of the dinoflagellate genus Dinophysis are a threat to human health as they are mainly responsible for diarrheic shellfish poisoning (DSP) in the consumers of contaminated shellfish. Such contamination leads to shellfish farm closures causing major economic and social issues. The direct effects of numerous HAB species have been demonstrated on adult bivalves, whereas the effects on critical early life stages remain relatively unexplored. The present study aimed to determine the in vitro effects of either cultivated strains of D. sacculus and D. acuminata isolated from France or their associated toxins (i.e. okadaic acid (OA) and pectenotoxin 2 (PTX2)) on the quality of the gametes of the Pacific oyster Crassostrea gigas. This was performed by assessing the ROS production and viability of the gametes using flow cytometry, and fertilization success using microscopic counts. Oocytes were more affected than spermatozoa and their mortality and ROS production increased in the presence of D. sacculus and PTX2, respectively. A decrease in fertilization success was observed at concentrations as low as 0.5 cell mL−1 of Dinophysis spp. and 5 nM of PTX2, whereas no effect of OA could be observed. The effect on fertilization success was higher when both gamete types were concomitantly exposed compared to separate exposures, suggesting a synergistic effect. Our results also suggest that the effects could be due to cell-to-cell contact. These results highlight a potential effect of Dinophysis spp. and PTX2 on reproduction and recruitment of the Pacific oyster.

Graphical abstract



Highlights

► *Dinophysis sacculus* and *D. acuminata* increased mortality of *Crassostrea gigas* oocytes. ► Exposure of oocytes and spermatozoa to 0.5 cells mL^{-1} of *D. sacculus* decreased subsequent fertilization success. ► Oyster gametes were negatively affected by exposure to whole culture or resuspended cells of *Dinophysis* spp.5 nM of PTX2 decreased fertilization success of oocytes while OA showed no effect; 500 nM of PTX2 increased ROS production of oocytes. ► Observed effects may be due to either cell to cell contact or PTX2 or other bioactive compounds or a combination.

Keywords : *Dinophysis* spp., Okadaic acid, Pectenotoxins, Oyster gametes, Fertilization success

Abbreviations

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

Introduction

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

Shellfish farming is an important economic sector worldwide. In France, the Pacific oyster, *Crassostrea gigas* (= *Magallana gigas*; Thunberg, 1793) represents the majority of annual

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

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

102 While HABs have mostly been studied in relation to public health, another fundamental
103 issue is the direct effect they have on filter-feeding bivalves (Landsberg, 2002; Matsuyama et al.,
104 2001; Shumway and Cucci, 1987; Sandra E. Shumway, 1990). National monitoring programs
105 along the French Atlantic coast indicate that spawning, development and recruitment of larvae
106 may co-occur with *Dinophysis* spp. (Figure S2; Pouvreau et al., 2019; REPHY, 2019). While

adults and juvenile bivalves can mechanically escape toxic microalgae by cessation of filtration and closing their shells (Hégaret et al., 2007), the planktonic early life stages such as gametes and embryos are directly exposed to HABs and their toxins in the water column and appear more sensitive than adults (Castrec et al., 2019; Glibert et al., 2007; Stoecker et al., 2008; Wang et al., 2006; Yan et al., 2001).

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

The present study investigated the *in vitro* effects of whole culture, resuspended cells and culture filtrate of *Dinophysis sacculus* (Stein, 1883), whole culture of *D. acuminata* (Claparède and Lachmann, 1859) and certified standards of OA and PTX2 on (i) gamete cellular

characteristics (i.e., ROS production, mortality, and morphology), and (ii) fertilization success of oocytes and spermatozoa of the Pacific oyster.

Materials and methods

Microalgal cultures

Monoclonal cultures of *D. sacculus* (Stein, 1883) (strain IFR-DSA-01Lt) and *D. acuminata* (Claparède and Lachmann, 1859) (strain IFR-DAU-02Ar) were isolated in Arcachon, France, in 2015 and 2018, respectively. These mixotrophic species were cultivated in 0.2 µm filter-sterilized natural seawater (FSSW) for *D. sacculus* and L1/20-Si + K/2-Si (Hernández-Urcera et al., 2018) for *D. acuminata* at salinity 35 and fed every two days with ciliate prey *Mesodinium rubrum* (Lohmann, 1908) (strain MBL-DK2009) at a ratio of 1: 1 (predator: prey) according to Park et al. (2006). The ciliate *M. rubrum* was fed three times a week with the cryptophyte *Teleaulax amphioxeia* (Conrad) (Hill, 1992) (strain AND-0710). Both *M. rubrum* and *T. amphioxeia* were cultivated in flasks respectively in L1/20-Si and L1-Si (Guillard and Hargraves, 1993) and diluted every two days for the ciliates and every week for the cryptophyte. All cultures were maintained at 17.8 ± 0.6 °C, at a light intensity of ~ 100 µmol photons m^{-2} s^{-1} provided by cool-white and pink fluorescent tubes (fluora and cool-white fluorescent light, Osram, Munich, Germany) and a 12: 12 (L: D) cycle (Table S1). To increase the biomass of *Dinophysis* spp., cultures were fed at a ratio of 1: 10 (predator: prey) for 4 months before the experiment. One week before the experiment, cultures of *Dinophysis* spp. were filtered on a nylon sieve (mesh 11 µm) and gently rinsed with 75 mL of FSSW to remove any cryptophyte and ciliate. Cultures

were then resuspended in 20 mL of FSSW and starved for one week before the experiment to obtain cells from the mid exponential growth phase on the day of the experiment.

Experimental design

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

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

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

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

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

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

- (iv) The effects of whole cultures of *D. sacculus* on fertilization success was also investigated by exposing gametes only during fertilization. Oocytes, spermatozoa and *D. sacculus* (at final concentrations of 0 (control), 0.5, 5, 50 and 500 cells mL^{-1}) were put in

contact simultaneously in FSSW (n = 11 pools of gametes, each from 3 different organisms).

Briefly, for (i), (ii) and (iii); oocytes or spermatozoa were exposed for 2 h in glass vials at 20 ± 1 °C to the whole culture of *D. sacculus* at a final concentration of 0 (control), 0.5, 5, 50 and 500 cells mL^{-1} in FSSW. These *D. sacculus* cell concentrations were selected to mimic the cell densities in natural blooms occurring in France, from typical (1×10^2 cells L^{-1}) to exceptionally dense blooms (8×10^5 cells L^{-1}, Figure S2, REPHY, 2019).

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

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

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

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

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

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

Toxin analyses of Dinophysis *spp. strains*

Toxin analysis was adapted from Sibat et al. (2018) and García-Portela et al. (2018) and performed on 1 mL (n=3) sub samples of *Dinophysis* spp. cultures collected in exponential growth phase. After centrifugation (3500 *g*, 4 °C, 15 min), both cells and culture filtrates

(supernatants) were extracted. The pellet (intracellular toxins) was extracted with 0.5 mL methanol and sonicated at 25 kHz for 15 min. Extracellular toxins were recovered from the supernatant after liquid-liquid extraction with dichloromethane, which was evaporated under nitrogen and resuspended in 0.5 mL of methanol. Subsequently, samples were filtered (0.2 µm, Nanosep, MF, Pall, Northborough, MA, USA). Analyses were performed using ultra high performance liquid chromatography coupled to low resolution tandem mass spectrometry (UHPLC-LRMS/MS) with a UHPLC system (UFLC XR Nexera, Shimadzu, Tokyo, Japan) coupled to a triple quadrupole/ion-trap mass spectrometer (API 4000 QTrap, ABSciex, Redwood City, CA, USA), equipped with a turboV® ESI source (see details in García-Portela et al. 2018). Certified calibration solutions of PTX2, OA, dinophysistoxin 1 and 2 (DTX1 and DTX2) were obtained from the National Research Council Canada (NRCC, Halifax, NS, Canada). Intracellular (intra) and total (sum of intracellular and extracellular) toxin contents were expressed on a per cell basis (pg $cell^{-1}$) while extracellular (extra) as equivalent (eq) pg $cell^{-1}$. Pectenotoxin 2 eq (PTX2eq) was the sum of pectenotoxin 2, pectenotoxin 2b, pectenotoxin 2 seco-acid and 7-*epi*-pectenotoxin 2 seco-acid, all quantified with PTX2 standard by assuming similar molar responses.

Sampling and maintenance of oysters

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

239

240 *Collection of gametes*

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

248

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

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

255 For cell morphology measurements, values of the forward scatter (FSC) and side scatter (SSC),
256 respectively proxies of cell size and complexity, were used to estimate cell morphology of
257 spermatozoa and oocytes. Spermatozoa viability was assessed using double staining spermatozoa
258 solution with 2 µL of propidium iodide (PI) and 2 µL of SYBR-14 (Live/Dead® Sperm Viability
259 Kit, Molecular Probes, Eugene, USA) at final concentrations of 2 µg mL^{-1} and 1 µM during 10

min in the dark (Le Goïc et al., 2013). For oocyte viability, PI and SYBR-Green-1 (1/10,000 of the commercial solution; Molecular Probes, Eugene, USA) were used (Le Goïc et al., 2014).

ROS production of gametes was measured by staining 200 µL of oocytes or spermatozoa solution with 2 µL (final concentration of 10 µM) of dye 2'7,7'-dichlorofluorescein diacetate DCFH-DA (Sigma, St Quentin Fallavier, France) for 1 h in the dark (Le Goïc et al., 2014; Vignier et al., 2017).

ROS production was expressed as percentage of control (a.u.). For both viability (expressed as mortality) and ROS production measurements, oocytes and spermatozoa concentrations were 5 x 10^4 and 5 x 10^6 cells mL^{-1}, respectively.

Microscopy analysis – fertilization success assessment

To assess fertilization success, oocytes and spermatozoa were inoculated in FSSW (20 ± 1 °C) at 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 mL FSSW (Boulais et al., 2017). When fertilized oocytes in the control (i.e. non-exposed oocytes and spermatozoa) reached 80 % or, after 2 h of incubation, samples were fixed with 1 % formaldehyde (final concentration). Fertilization success (%) was assessed under an inverted light microscope (Axio observer.Z1, Zeiss, Oberkochen, Germany) by counting fertilized and unfertilized oocytes (polar body extrusion from 2 to 8-cell stages vs. no polar body). For the fertilization success measurements in all experiments except conditions (iv), the concentration of *Dinophysis* spp. or toxins were minimum 30-fold lower due to dilution in FSSW and their potential contribution to the observed effect were considered not significant.

Statistical analyses

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

Results

Toxin contents of Dinophysis *spp. cultures*

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

For Exp. 1 and Exp. 2, *D. sacculus* produced similar amounts of total OA (4.5 ± 1.4 and 6.0 ± 0.46 pg cell^{-1}) and total PTX2eq, i.e. sum of concentrations (95 ± 36 and 76 ± 19 pg cell^{-1}; Table 1). The majority of OA was in the extracellular compartment in contrast to PTX2 which was mainly intracellular. For Exp. 3 with *D. acuminata*, the total OA content per cell was 16-fold higher (87 ± 11 pg cell^{-1}) than *D. sacculus* and >90 % was intracellular ($P < 0.001$; Table 1).

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

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

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

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

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

325

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

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

332

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

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

337 A decrease in fertilization success was observed with (i) oocytes exposed for 2 h to 20 and 50 nM
338 of PTX2 compared to control (16 and 0 % vs. 77 %, respectively; $P < 0.001$; Figure 4 A).
339 Similarly, (ii) spermatozoa exposed to 20 and 50 nM of PTX2 decreased fertilization success
340 compared to control (35 and 25 % vs. 81 %; $P < 0.001$; Figure 4 B). However, oocytes exposed
341 to 50 nM, exhibited a more pronounced effect than exposed spermatozoa on fertilization ($P <$
342 0.05; Figure 4 A-B). Exposure of (iii) both oocytes and spermatozoa to 5, 10, 20 and 50 nM of
343 PTX2 reduced the fertilization success by 25, 37, 78 and 97 % compared to control ($P < 0.01$;
344 Figure 4 C). The fertilization in presence of toxins (iv) was significantly diminished only at a
345 concentration of 50 nM of PTX2 (32 %) compared to control (76 %; $P < 0.001$; Figure 4 D).

Neither OA (at any of the concentrations tested) nor MeOH in the control affected the fertilization success.

Discussion

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

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

The toxin exposure concentrations (i.e. from 5 to 50 nM) corresponded to the maximum amounts of OA and PTX2, respectively, produced by 500 cells of *D. sacculus* in our experixcment. Noteworthy, PTX2 caused an increase in ROS production of oocytes exposed to 50 nM, which could reflect a stimulation of oocyte metabolism or cellular stress. Indeed, the production of ROS is a key mechanism involved in stress responses (Kadomura et al., 2006), but an excess of ROS could lead to cellular toxic effects such as destruction of membrane integrity by lipid peroxidation, DNA damage and associated alteration of cell functioning and ultimately cell death

(Cavallo et al., 2003; Landsberg, 2002; Lesser, 2006). This may explain the observed reduced fertilization success of oocytes exposed to PTX2. Similarly, Le Goïc et al. (2014) observed an increased ROS production of oocytes in the presence of the toxic dinoflagellate *Alexandrium minutum*, and suggested this increase production may have reduced oocyte quality.

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

Using mammalian and finfish cell lines (e.g. human, rat, rabbit, salmon), PTXs have been shown to interfere with actin assembly/disassembly, thereby affecting cell cytoskeletal functions and leading to cell death, at concentrations ranging from nM to µM (Ares et al., 2005; Dominguez et al., 2010; Spector et al., 1999). It has been shown that PTX2 causes actin depolymerization (Dominguez et al., 2010), sequestration of monomeric actin (at a concentration of 20 nM; Spector et al., 1999), disrupted F-actin (Hori et al., 1999), and inhibited actin polymerization by a capping process at the barbed-end of F- and G-actin (Allingham et al., 2007). The reduced fertilization success observed in this study could be associated with impairment of the oocyte and spermatozoan cytoskeleton by PTX2, as well as fertilization itself since actin polymerization is a crucial mechanism in oysters, involved in spermatozoan motility and the penetration of the oocyte (Ledu and McCombie, 2003). In the literature, the indirect evidence of the involvement of *Dinophysis* spp. in mortalities observed in the natural environment (reviewed in Landsberg 2002,

391 Basti et al., 2015b) were almost exclusively related to *D. caudata*, a producer of high cellular
392 contents of PTX2 (Basti et al., 2015b, 2015c; Luisa Fernández et al., 2006; Marasigan et al.,
393 2001). The action of PTX2 on actin in oyster gametes would be worthy of investigation in further
394 studies.

395

396 Okadaic acid, in contrast to PTX2, affected neither the gametes nor fertilization success, at
397 concentrations up to50 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

on the cytoskeleton (neither is OA). Interestingly, PTX2 has a high structural similarity with goniodomin A, another algal macrocyclic lactone, which has also been reported to affect the cytoskeleton via F-actin (Espiña et al., 2016). In addition, it should be noted that among the three species of *Alexandrium* that produce goniodomins (Harris et al., 2020), *A. monilatum* has been clearly associated with fish kills since the middle of the last century (Howell, 1953) and more recently also with shellfish mortalities (Harding et al., 2009; May et al., 2010).

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

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

Contact with *Dinophysis* spp. cells, or by the mean of feeding peduncle (Ojamäe et al., 2016), may have resulted in (a) mechanical damage to the membranes of oyster gametes, as suggested by the increase in FSC morphological parameter of oocytes. This proxy of cell size could indicate a swelling of the cells, when exposed to 500 cells mL^{-1} of *D. sacculus* associated to an increase in mortality.

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

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

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

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

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

Some preliminary results also indicate that when gametes were exposed to *D. sacculus*, abnormal development of D-shaped larvae could be observed (personal communications), leading to the question of the effects on early life stages of *C. gigas* and ultimately, recruitment. Similarly, when *C. gigas* larvae were exposed to *A. minutum*, anomalies in swimming behavior, feeding and growth were observed which led to a decrease in survival and settlement of older larvae stages (Castrec et al., 2020).

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

Conclusion

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

The adverse effects observed on oyster fertilization success and gamete cellular characteristics were similar for both *Dinophysis* species. Whether this activity is a general trait of the genus *Dinophysis* and results from similar mechanisms requires further investigation. Future studies should also include other *Dinophysis* spp., as this is a very diverse genus with species showing different toxin profiles, including some that do not produce toxins. It is important to explore the

intraspecific and interspecific diversity of this activity and its broader impacts on shellfish reproduction.

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

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Tables and figures

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

		OA				PTX2eq			
	Exp.	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})
D. sacculus	1	2.2 ± 0.52^{a}	2.3 ± 1.1^{a}	4.5 ± 1.4^{a}	2.8 ± 0.9^{a}	73 ± 26	22 ± 9.0	95 ± 34	55 ± 20
	2	2.1 ± 0.17^{a}	3.9 ± 0.29^{a}	6.0 ± 0.46^{a}	3.7 ± 0.3^{a}	64 ± 16	12 ± 2.9	76 ± 19	44 ± 11
D. acuminata	3	80 ± 9.0^{b}	7.5 ± 1.9^{b}	87 ± 11^{b}	54 ± 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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		Oocytes				Spermatozoa			
		FSC	SSC	ROS (%)	Mortality (%)	FSC	SSC	ROS (%)	Mortality (%)
Exp. 1 *D. sacculus* (cells mL^{-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 *D. acuminata* (cells mL^{-1})	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	*n.a*	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 Toxins (nM)	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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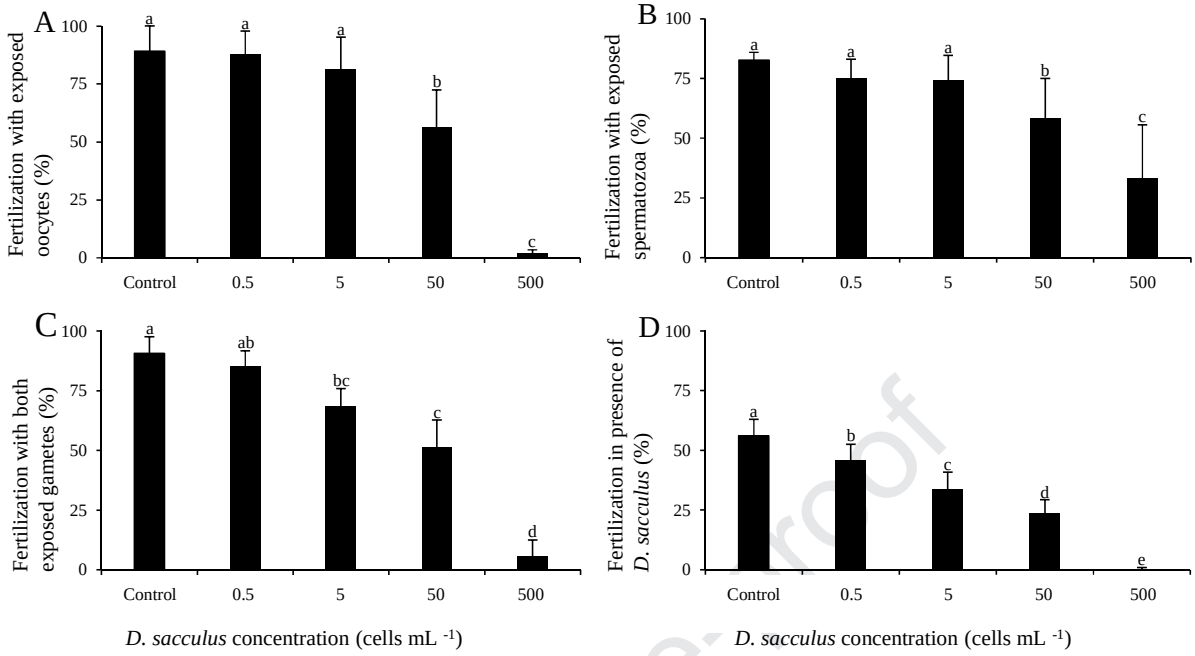

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838 Figure 1: Effect of a gradient of concentration of *D. sacculus* on gamete fertilization (Exp. 1):
839 Mean percentage of fertilized eggs with **A.** oocytes exposed, **B.** spermatozoa exposed, **C.** both
840 gametes exposedand **D.** fertilization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

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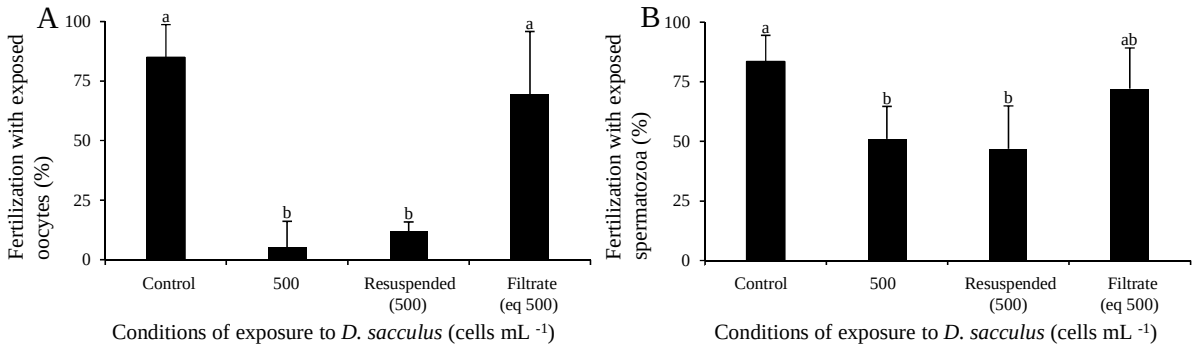

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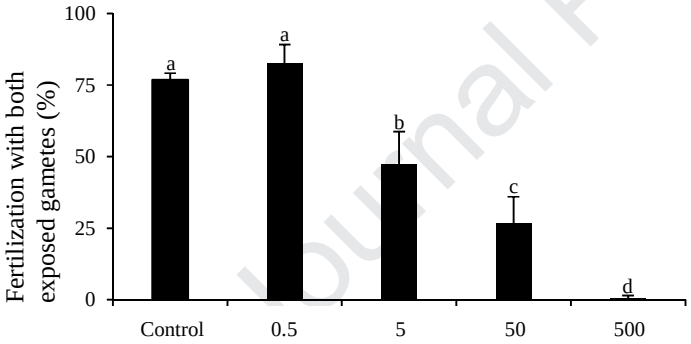
849 Figure 2: Effect of whole culture (500 cells mL^{-1}), resuspended cells (500 cells mL^{-1}) and culture
850 filtrate (eq 500 cells mL^{-1}) of *D. sacculus* on gamete fertilization (Exp. 2). Mean percentage of
851 fertilized eggs (%) with **A.** oocytes exposed and **B.** spermatozoa exposed. Values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



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

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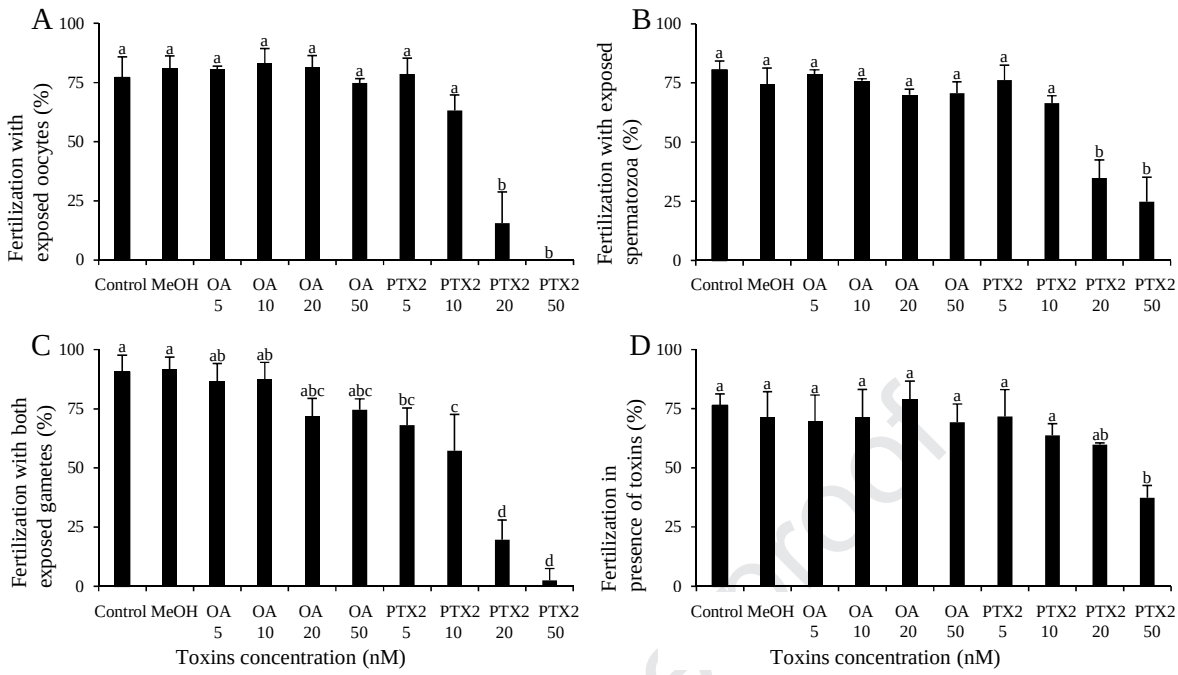

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861 Figure 4: Effect a gradient of concentration of okadaic acid (OA) or Pectenotoxin 2 (PTX2) on
862 gamete fertilization (Exp. 4): Mean percentage of fertilized eggs (%) with **A.** oocytes exposed, **B**
863 spermatozoa exposed, **C.** both gametes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

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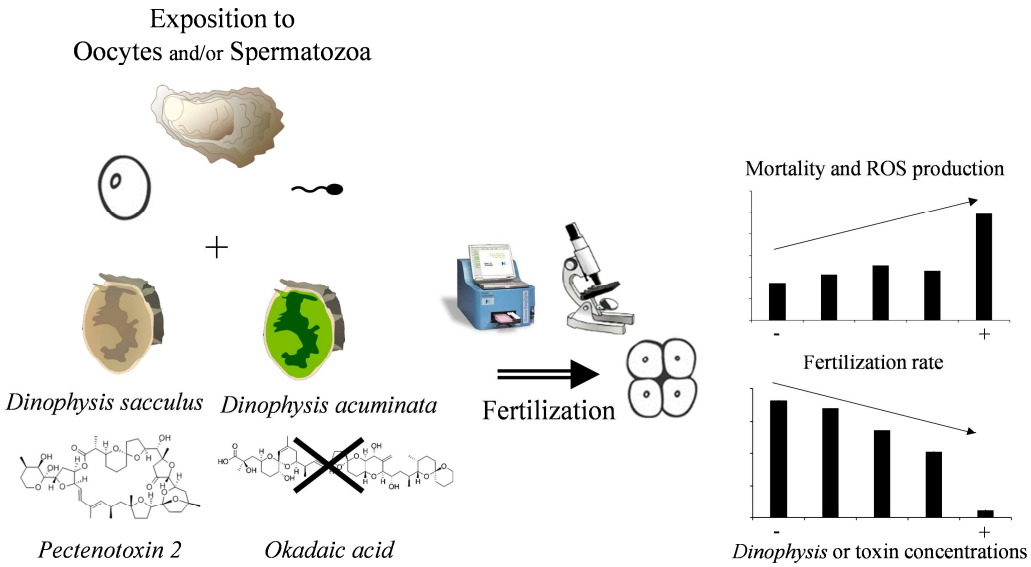

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873 Figure X: Graphical abstract

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Conflict of interest

Authors declare no conflicts of interest.