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Nanoplastics impaired oyster free living stages, gametes and embryos

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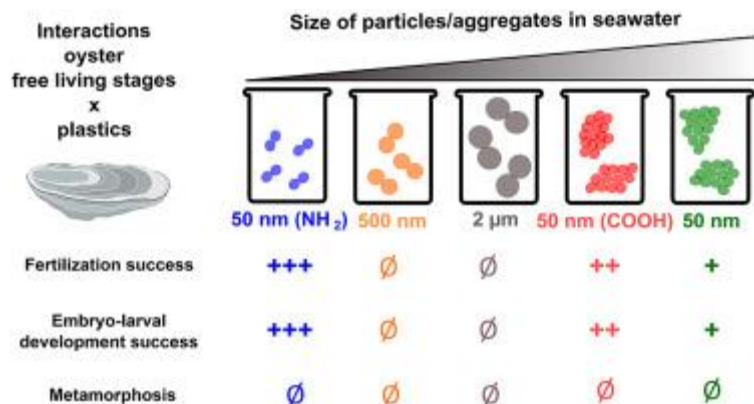
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Abstract :

In the marine environment, most bivalve species base their reproduction on external fertilization. Hence, gametes and young stages face many threats, including exposure to plastic wastes which represent more than 80% of the debris in the oceans. Recently, evidence has been produced on the presence of nanoplastics in oceans, thus motivating new studies of their impacts on marine life. Because no information is available about their environmental concentrations, we performed dose-response exposure experiments with polystyrene particles to assess the extent of micro/nanoplastic toxicity. Effects of polystyrene with different sizes and functionalization (plain 2- μm , 500-nm and 50-nm; COOH-50 nm and NH₂-50 nm) were assessed on three key reproductive steps (fertilization, embryogenesis and metamorphosis) of Pacific oysters (*Crassostrea gigas*). Nanoplastics induced a significant decrease in fertilization success and in embryo-larval development with numerous malformations up to total developmental arrest. The NH₂-50 beads had the strongest toxicity to both gametes (EC₅₀ = 4.9 $\mu\text{g}/\text{mL}$) and embryos (EC₅₀ = 0.15 $\mu\text{g}/\text{mL}$), showing functionalization-dependent toxicity. No effects of plain microplastics were recorded. These results highlight that exposures to nanoplastics may have deleterious effects on planktonic stages of oysters, presumably interacting with biological membranes and causing cyto/genotoxicity with potentially drastic consequences for their reproductive success.

Graphical abstract



Highlights

► Oyster gametes, embryos and larvae were exposed to nano- and microplastics. ► Nanoplastics caused significant decrease in fertilization and embryogenesis success. ► Nanoplastics functionalization influences their behavior and toxicity. ► No effect of plain microplastics was demonstrated on all endpoints.

Keywords : Oyster, Embryos, Gametes, Microplastics, Nanoplastics

35 **Introduction**

36 Mismanagement of plastic wastes is one of the major concerns of the scientific community in the
37 21st century (Galloway et al., 2017). The exponential use of plastics by human society since 1950
38 has led to a significant release of wastes into the environment (Cole et al., 2011; Geyer et al.,
39 2017). Between 13,200 and 34,800 tons of plastic debris were estimated to have been introduced
40 daily into the oceans in 2010, and this amount is expected to increase by an order of magnitude
41 by 2025 (Jambeck et al., 2015). Today, plastic debris are widespread and ubiquitous in marine
42 environments from the sea surface (Eriksen et al., 2014) to the sediment (Van Cauwenberghe et
43 al., 2015), including in remote areas such as polar regions (Cózar et al., 2017), deep-sediments
44 (Bergmann et al., 2017) and desert islands (Lavers and Bond, 2017).

45 Microplastics (MP) are defined as particles with a size less than 5 mm (Galloway et al., 2017),
46 originating from manufactured beads/fibers (primary MP) or weathering of larger waste
47 (secondary MP). They represent the most abundant plastic items in oceans in terms of the number
48 of particles per unit of water (>92% of floating plastics) (Cole et al., 2011; Eriksen et al., 2014).
49 Recently, a new class of debris was described, namely nanoplastics (NP), defined as particles
50 <100 nm (Galloway et al., 2017) or <1000 nm (Gigault et al., 2018). The definition used in the
51 present study (<100 nm) refers to the usual definition of nanoscale; *i.e.* the point where the
52 properties of a material change (higher surface area effect and interaction with biological
53 membranes) (Klaine et al., 2012). Their production has been demonstrated by mechanical
54 fragmentation (Lambert and Wagner, 2016), photo-degradation (Gigault et al., 2016) or
55 biodegradation (Dawson et al., 2018) of larger items. Likewise, similarly to MP, primary NP
56 from cosmetics (Hernandez et al., 2017), 3D-printing wastes (Stephens et al., 2013), lubricants
57 (Dubey et al., 2015) or drugs (Lusher et al., 2017) are suspected to enter the oceans directly. The

58 increase of NP used in such industries – and thus their release in environment – is suspected
59 although very little information is available regarding the actual quantities used and discarded. At
60 sea, the presence of plastic particles lower than 1 μm has been recently argued in the Atlantic
61 Gyre (Ter Halle et al., 2017). However, owing to a lack of methods, no or very little information
62 is currently available about the environmental concentrations of NP and small MP particles (<100
63 μm), respectively (e.g. Huvet et al., 2016). Their environmental concentrations can only be
64 estimated, for example following a power-law increase (around a 2.2 factor) from sea surface
65 samples as recently proposed (Erni-Cassola et al., 2017).

66 Despite the lack of knowledge concerning MP and NP distributions in the oceans, a consensus
67 exists about the threat posed by small plastic particles for aquatic life (GESAMP, 2015). Effects
68 of MP on feeding behavior (Cole et al., 2013; Ogonowski et al., 2016), energy balance (Wright et
69 al., 2013; Watts et al., 2015), reproduction (Sussarellu et al., 2016; Gardon et al., 2018), and
70 immune system (Avio et al., 2015; Paul-Pont et al., 2016) were demonstrated and ecological
71 impacts can be discerned (Rochman et al., 2015; Green et al., 2016; Galloway et al., 2017).
72 Furthermore, at the nanoscale, specific effects are expected as result of the physico-chemical
73 properties of NP (Mattsson et al. 2015a; da Costa et al., 2016). Nanoparticles have a much greater
74 surface/volume ratio than microparticles – the number of surface atoms increases when size
75 decreases – which enhances their reactivity in aquatic environments (Mattsson et al. 2015a,
76 Rocha et al. 2015). Likewise, the risk of translocation and overall transfer into the tissues of
77 organisms increases at the nanoscale. For instance, fluorescent nano-polystyrene beads (NP-PS;
78 50 nm) seemed dispersed in the body of *Paracyclopsina nana* after ingestion, while MP (500 nm
79 and 6 μm) remained in the digestive tract with a shorter retention time (Jeong et al., 2017). Initial
80 assessments of NP toxicity highlighted risks to survival, feeding activity, embryogenesis, the

81 immune system, fecundity (number of offspring and/or pregnancy rate), metabolism (changes in
82 amino acid composition, liver dysfunctions and energy balance) and behavior at a wide range of
83 trophic levels including phytoplankton (Besseling et al., 2014), echinoderms (Della Torre et al.,
84 2014), rotifers (Jeong et al., 2016), crustaceans (Cui et al., 2017; Jeong et al., 2017), bivalves
85 (Wegner et al., 2012 ; Canesi et al., 2016) and fish (Mattsson et al. 2015b, Mattsson et al. 2017).

86 In the adult Pacific oyster *Crassostrea gigas* (Bayne et al., 2017), polystyrene microbeads of 2
87 and 6 μm were shown to interfere considerably with gametogenesis, in terms of quantity and
88 quality of produced gametes, leading to undesirable effects on the performance of offspring
89 despite no direct exposure (Sussarellu et al., 2016). Because *C. gigas* has external fertilization,
90 the free-living stages (*i.e.* gametes, embryos and larvae) must cope with the stress occurring in
91 estuarine and coastal marine habitats where oysters live. To date only one study has investigated
92 the impacts of plastic debris exposure to Pacific oyster larvae using 1 and 10 μm MP with no
93 effect on their growth rate or survival (Cole and Galloway, 2015). These authors also studied the
94 ingestion of polystyrene particles spanning 70 nm to 20 μm in size, but no toxic endpoint was
95 monitored following exposure to this size class. For gametes, carboxylic nanoplastics (100 nm)
96 induced oxidative stress in oyster spermatozoa linked to an increase in ROS production
97 (González-Fernández et al., 2018). In this context, the present study aims to assess the potential
98 adverse effects of plastic items on Pacific oyster free-living stages, targeting specifically the
99 essential steps of fertilization, embryo-larval development and metamorphosis, so as to provide a
100 view over the complete life cycle in addition to the adult exposure of Sussarellu et al. (2016) (Fig.
101 1). Here, oyster gametes, embryos and larvae were exposed to five types of polystyrene particles,
102 varying in size from NP to MP (50 nm; 500 nm; 2 μm) and in functionalization (no functional
103 group, or presence of carboxyl or amine groups) to examine a size effect between MP and NP

104 (plain particles), as well as a surface properties effect between NP exhibiting different
105 functionalization. The behavior of the particles was measured in seawater using Dynamic Light
106 Scattering (DLS) to assess particle aggregation and modifications of the mean surface charge.

107 **Materials and methods**

108 **Micro- and nanoplastic**

109 Five commercially available polystyrene (PS) beads were purchased from Polysciences/Bangs
110 Laboratories and stored at 4°C prior to experiments: 50-nm, 500-nm and 2- μ m beads without
111 functionalization (Plain), and 50-nm beads coated with carboxyl (COOH-50) or amine groups
112 (NH₂-50). Before each handling, particles were vortexed to prevent particle aggregation and
113 insure good suspension homogenization. Commercial suspensions were in ultrapure water (UW)
114 with Tween-20[®] surfactant (<0.1%) to limit aggregation; Tween-20[®] had previously been
115 demonstrated to be innocuous for marine invertebrates at this dose (Ostroumov, 2003). Raman
116 microspectroscopy analysis confirmed the PS nature of the polymer for all beads and no
117 additional features were observed in the PS spectra across all particles. (Fig. S1). All tests (DLS
118 and exposures) were performed with the same batch of particles.

119 **Dynamic Light Scattering (DLS) analysis**

120 DLS (Zetasizer NanoZS; Malvern Instruments; United Kingdom) was used to determine the
121 aggregation state (polydispersity index – PDI; Arbitrary Units (A.U.)), the mean size of
122 particles/aggregates (hydrodynamic diameter; nm) and the mean surface charge (ζ -potential; mV)
123 of MP/NP in two media: UW, as delivered by the supplier, and natural filtered seawater collected
124 from the Bay of Brest (FSW; 1- μ m filtered and UV-treated; pH 8.1 and 34 PSU). When PDI
125 exceeds 0.2, particles were considered to be aggregated. Measurements were performed in

126 triplicate at 20°C (similar to the T°C used for bioassays) and a concentration of 100 µg/mL at T0
127 and T24h, each containing 13 runs (10 sec.measure⁻¹) for PDI and hydrodynamic diameter, and
128 40 runs (10 sec.measure⁻¹) for ζ-potential as conducted by González-Fernández et al. (2018). This
129 concentration was used for DLS analysis owing to the presence of artifacts at lower
130 concentrations.

131 **Suspensions of MP/NP for bioassays**

132 MP and NP stock suspensions were prepared in UW at 1,000 µg/mL, while working suspensions
133 were prepared in FSW. Four concentrations of plastic were tested: 0.1, 1, 10 and 25 µg/mL, plus
134 a control group (0 µg/mL), in order to identify toxicity thresholds. A total of 25 treatments (5
135 particle types × 5 concentrations) were then tested on the three early stages (gametes, embryos
136 and larvae; see below).

137 **Biological material**

138 Oysters from 2 cohorts, produced in 2014 and 2015 according to Petton et al. (2015), were
139 deployed in 2016 in the bay of Brest and in the Marennes-Oléron basin (France). In the summer
140 of 2017, oysters were randomly sampled to collect their gametes for assays on gametes and
141 embryo-larval development. For the metamorphosis assay, pediveliger larvae (21 days old) were
142 purchased from a commercial hatchery (Société Atlantique de Mariculture, France).

143 **Gamete assay**

144 Sperm from two males and oocytes from three females were collected by stripping the gonad.
145 This was repeated in five replicates, involving a total of 10 males and 15 females. Sperm were
146 then sieved at 100 µm, and oocytes at 100 µm then 20 µm to eliminate debris (Steele and
147 Mulcahy, 1999). Oocytes were diluted in 2 L and sperm in 100 mL of FSW maintained at 21°C ±

148 1°C (mean \pm SD). Spermatozoa mobility and round shape of oocytes, used as proxies of gamete
149 quality, were checked by microscopy (Olympus BX51; $\times 10$ -20 magnification with phase contrast
150 for sperm) (Fabbri et al., 2014). Spermatozoa and oocyte concentrations were estimated by flow
151 cytometry (EasyCyte Plus cytometer; Millipore Corporation; USA) (Le Goïc et al., 2014, 2013).
152 Gametes (1,000 oocytes/mL; 100:1 spermatozoa:oocyte ratio) were placed at the same time in 40
153 mL glass vials filled with 30 mL of FSW at $21^{\circ}\text{C} \pm 1^{\circ}\text{C}$, containing the MP or NP suspensions (5
154 particle types \times 5 concentrations; 5 replicates per treatment).

155 After 1.5 h, samples were fixed with a formaldehyde-seawater solution (0.1% final) to estimate
156 the fertilization yield under a microscope (Zeiss Axio Observer Z1; $\times 10$ -40 magnification;
157 observation of 150 oocytes per vial). The fertilization yield was defined as: (number of fertilized
158 oocytes / [number of fertilized and unfertilized oocytes]) \times 100 (Martínez-Gómez et al., 2017).
159 An oocyte was considered to be fertilized when polar bodies and cell divisions were observed.

160 **Embryo-larval assay**

161 The standardized AFNOR procedure (AFNOR XP-T-90-382) was used to perform this assay.
162 Fertilization was achieved in five replicates with gametes collected from five males and five
163 females per replicate (total: 25 males and 25 females) following the procedure described above.
164 Once fertilization was achieved in a 2-L glass beaker filled with 1.5 L of FSW with high
165 fertilization yields ($>90\%$; verified under a Zeiss Axio Observer Z1; $\times 10$ -40 magnification),
166 1,500 embryos were collected per replicate and diluted at a concentration of 60 embryos/mL in
167 40 mL glass vials filled with 25 mL of FSW ($21^{\circ}\text{C} \pm 1^{\circ}\text{C}$) containing the MP or NP suspensions
168 (5 particle types \times 5 concentrations; 5 replicates per treatment). After 36 h in dark conditions,
169 samples were fixed with a formaldehyde-seawater solution (0.1% final) to evaluate the D-larval

170 yield under a microscope (Zeiss Axio Observer Z1; $\times 10$ -63 magnification; observation of 100
171 larvae per vial). The D-larval yield was defined as: (number of normal D-larvae / number of
172 normal and abnormal D-larvae) $\times 100$ (Di Poi et al., 2014). A normal D-larvae indicated
173 embryogenic success, while an abnormal larva presented mantle, shell and/or hinge
174 malformations, or developmental arrest at the embryonic stage (Mottier et al., 2013).

175 **Metamorphosis assay**

176 The bioassay at the metamorphosis stage was performed as described in Di Poi et al. (2014).
177 Briefly, a total of 65 ± 15 pediveliger larvae per treatment were exposed to plastic particles in 12-
178 well microplates (NUNC© with the Nunclon™ Delta surface treatment) filled with 1.5 mL of
179 FSW containing the MP or NP suspensions (5 particle types \times 5 concentrations; 6 replicates per
180 treatment) for 24 h at $21^\circ\text{C} \pm 1^\circ\text{C}$. Metamorphosis of oyster larvae was stimulated by adding 10^{-4}
181 M epinephrine (Sigma-Aldrich; CAS number: 51-43-4) (Coon et al., 1990) immediately after the
182 start of the exposure (Di Poi et al., 2014). After the 24 h incubation, samples were fixed with a
183 formaldehyde-seawater solution (0.1% final) to determine the metamorphosis yield under a
184 microscope (Leica DM-IRB; $\times 10$ magnification; all larvae were observed). The metamorphosis
185 yield was defined as: (number of metamorphosed larvae / total number of larvae) $\times 100$. A
186 metamorphosed larva is characterized by a significant growth of shell and gills, and loss of the
187 velum and foot (Di Poi et al., 2014).

188 **Statistical analyses**

189 Statistical analyses and graphical representations were produced using the R software.
190 Percentages were analyzed after angular transformation. Normality and homogeneity of variance
191 were verified by the Shapiro-Wilk and Levene methods, respectively. The Student's *t*-test was

192 used to compare particle behavior (size and ζ -potential) between UW and FSW. For effects of
193 particle concentrations on fertilization, embryo-larval development and metamorphosis success,
194 parametric (ANOVA) or non-parametric (Kruskal-Wallis) analyses of variance were followed by
195 post-hoc methods (Tukey or Conover) for pairwise comparisons when differences were detected.
196 Whenever a dose-response pattern was observed, the package “DRC” was used to determine the
197 half maximal effective concentration (EC_{50}), defined as the concentration of a substance leading
198 to a significant effect in 50% of the population. All data are represented by means \pm standard
199 deviation (SD).

200 **Results**

201 **Particle characterization**

202 The 2- μ m and 500-nm beads formed small aggregates in UW ($PDI > 0.2$), whereas all NP
203 remained in their original form ($PDI < 0.2$; Table 1). For all particles, the aggregation state or size
204 of aggregates increased significantly when added to seawater ($p < 0.01$). Only the NH_2 -50 formed
205 aggregates at the nanometer scale (mean \pm SD; 96.5 ± 2.0 nm) in FSW. The Plain-50 ($5951.0 \pm$
206 264.3 nm) and $COOH$ -50 (3735.0 ± 443.8 nm) formed larger aggregates than the 2- μ m ($3113.7 \pm$
207 32.3 nm) and 500-nm (1620.7 ± 188.8 nm) beads in FSW. All particles presented a negative
208 surface charge in UW and FSW, with the exception of NH_2 -50 that exhibited a positive surface
209 charge in all media. The seawater systematically buffered the charge of all MP/NP with mean
210 surface charge values decreasing towards zero in seawater compared to UW ($p < 0.01$; Table 1).
211 No significant changes ($p > 0.05$) of charge and aggregation were observed between T0 and T24h
212 in FSW for all particles except the Plain-50 which formed bigger aggregates exceeding 10 μ m in
213 FSW ($p < 0.05$; Table S1).

214 Gamete assay

215 The control treatment (0 plastic) presented a high fertilization yield (mean \pm SD; $92.3 \pm 1.0\%$),
216 demonstrating the good quality/maturity of the gametes and the quality of the FSW. The 2- μm
217 (Fig. 2A) and 500-nm (Fig. 2B) particles had no effect on the fertilization yield relative to the
218 control group ($p > 0.05$). All NP significantly impaired the fertilization yield in a dose-response
219 manner between 1 and 25 $\mu\text{g/mL}$. Exposure to Plain-50 (Fig. 2C) led to significant reductions in
220 fertilization ($p < 0.05$) of 2.7, 55.7 and 72.7% for 1, 10 and 25 $\mu\text{g/mL}$, respectively, associated
221 with an EC_{50} value of $12.3 \pm 7.5 \mu\text{g/mL}$. The COOH-50 particles (Fig. 2D) induced significant
222 decreases ($p < 0.05$) of 3.8, 65.7 and 93.0% with an EC_{50} value of $7.8 \pm 1.1 \mu\text{g/mL}$. The NH_2 -50
223 exhibited the strongest toxicity inducing significant decreases ($p < 0.05$) in the fertilization yield of
224 6.3, 75.4 and 91.2% for increasing doses of NP associated with an EC_{50} value of $4.9 \pm 0.9 \mu\text{g/mL}$
225 (Fig. 2E; Fig. S2).

226 Embryo-larval assay

227 Exposure to 2- μm (Fig. 3A) and 500-nm (Fig. 3B) did not cause any significant effect on
228 embryo-larval development compared with the control treatment (mean \pm SD; $93.3 \pm 1.5\%$) at 36
229 hours post-fertilization (hfp). The D-larval yield was significantly reduced ($p < 0.01$) by exposure
230 to 10 and 25 $\mu\text{g/mL}$ of Plain-50 (Fig. 3C) leading to a mean reduction of 9.2 and 16.9%,
231 respectively. This was insufficient to estimate a robust EC_{50} value for the Plain-50 (Fig. S3).
232 Exposure to COOH-50 led to a mean reduction of 32.2 and 100% after exposure to 10 and 25
233 $\mu\text{g/mL}$, respectively (Fig. 3D) with an EC_{50} value of $11.60 \pm 10.5 \mu\text{g/mL}$. The highest toxicity
234 was observed for the NH_2 -50 with a significant decrease of 6.4% ($p < 0.05$) in the D-larval yield at
235 the lowest concentration (0.1 $\mu\text{g/mL}$), followed by a total inhibition (100% reduction) of the

236 embryo-larval development success for higher doses giving an EC₅₀ value of $0.15 \pm 0.4 \mu\text{g/mL}$
237 (Fig. 3E; Fig. S3).

238 Compared to the control group where D-larvae appeared healthy (Fig. 4A), Plain-50 (10 and 25
239 $\mu\text{g/mL}$) and COOH-50 (10 $\mu\text{g/mL}$) caused numerous mantle or/and shell malformations (Fig.
240 4A-B). Only dead embryos/larvae were observed at the highest concentration of COOH-50 (Fig.
241 4D) whereas mainly cell debris were observed upon exposure to the three highest doses of NH₂-
242 50 (Fig. 4E-F). In both cases, this represents evidence of developmental arrest.

243 **Metamorphosis assay**

244 A high metamorphosis yield was observed in all treatments, ranging from 81.5 ± 9.0 to 90.8 ± 2.4
245 (mean \pm SD = $86.6 \pm 3.6\%$), and no significant effect of MP/NP exposure ($p > 0.05$) on
246 metamorphosis success of *C. gigas* was demonstrated, regardless of particle type or
247 concentration. Furthermore, no abnormalities were observed under a microscope for any of the
248 treatments tested.

249 **Discussion**

250 Strong effects of NP were observed on the success of fertilization and embryogenesis of *C. gigas*
251 depending on particle dose and functionalization. Based on the commercial size, a higher toxicity
252 of NP compared to MP was demonstrated here, in agreement with previous observations across a
253 range of species, including copepods (Jeong et al., 2017, 2016; Lee et al., 2013), crustaceans (Ma
254 et al., 2016) and fish (Mattsson et al., 2017). This comparison was only done for plain particles,
255 and the functionalization-dependent toxicity remains to be tested for MP, especially using amine
256 groups displaying the strongest toxicity at the nanoscale. These insights support the purpose that
257 risks of NP may be higher than microscale counterparts (Wright and Kelly, 2017). Indeed, there

258 is a consensus concerning the risk of nanomaterials as a result of their high reactivity and their
259 capacity to cross biological membranes (Nel et al., 2006). It is noteworthy that the short term
260 exposure to plain 500-nm and 2- μ m beads did not show any effect on the two essential planktonic
261 stages of oyster reproduction and development (gametes and embryos), whereas deleterious
262 effects after 2-months of exposure to 2 and 6- μ m plain PS beads were previously demonstrated
263 on gametogenesis of adult oysters leading to subsequent negative impacts on unexposed gametes
264 and offspring (Sussarellu et al., 2016).

265 The dose-response exposure experiments performed here, which are the recommended approach
266 when environmental concentrations are unknown (e.g. Paul-Pont et al., 2018), allowed the
267 estimation of the half maximal effective concentration (EC_{50}) indicating the concentration of a
268 compound when 50% of its maximal effect is observed. The lowest EC_{50} was observed for the
269 NH_2 -50, which was 1.6 to 77 times more toxic for gametes and embryos, respectively, than the
270 $COOH$ -50. The highest EC_{50} in NP exposures was observed for the Plain-50 particles presumably
271 due to a decrease of their bioavailability owing to the presence of aggregates higher than 10 μ m
272 observed in seawater at T24h. Oyster embryos exhibited similar sensitivity as mussel embryos
273 (48h exposures; EC_{50} NH_2 -50: 0.14 μ g/mL)(Balbi et al., 2017), but their sensitivity was higher
274 than that of sea urchin embryos (48h exposures; EC_{50} NH_2 -50: 2.61 μ g/mL)(Della Torre et al.,
275 2014), suggesting inter-species variability. Additionally, biological stage within the same species
276 appears to be an important factor in determining effects, considering the absence of NP toxicity
277 on metamorphosis success. As demonstrated here, oyster larvae seem to withstand MP/NP
278 exposures, in agreement with a previous study showing no effect on growth rate or survival of
279 oyster larvae upon exposure to 1 and 10 μ m PS particles for 8 days (Cole and Galloway, 2015).
280 The absence of toxicity of MP/NP on pediveliger oyster larvae is probably linked to a decrease in

281 the larvae surface/volume ratio, and/or the appearance of a shell protecting larvae from
282 polystyrene particles (Hickman, 1999; Liebig and Vanderploeg, 1995; Schiaparelli et al., 2004).

283 The potential underlying mechanisms of NP toxicity include impairment of biological
284 membranes, sub-cellular toxicity or physical blockages, notably for spermatozoa. These
285 explanatory hypotheses, discussed below, are not mutually exclusive and could all play a role in
286 the observed adverse effects of NH₂-50, Plain-50 and COOH-50 on oyster planktonic stages.

287 The observed toxicity of nano-PS on gametes and embryos may be related to damage caused by
288 membrane breakages (Nel et al., 2009). Indeed, adhesion of nanoplastics on oyster gametes, both
289 oocytes and spermatozoa (González-Fernández et al., 2018), and sea urchin and mussel embryos
290 (Della Torre et al., 2014; Balbi et al., 2017) was recently demonstrated. We can rely on these
291 published data from different models and particles to suggest that NP have stuck on oyster's
292 gametes and embryos. Consequences might be significant for biological membranes: molecular
293 simulations have demonstrated the capacity of nano-PS to perturb lipid membranes (Rossi et al.,
294 2014). Even if metallic and plastic nanoparticles cannot be directly compared, nickel and iron
295 nanoparticles reduced the membrane integrity of *Ciona instinalis* (Gallo et al., 2016) and *Mytilus*
296 *edulis* spermatozoa, leading to a decrease in fertilization success (Kadar et al., 2011). Interactions
297 between nanoparticles and biological membranes are driven by particle aggregation and size.
298 Here, the most toxic nanoplastics (NH₂-50) remained at the nanometer size in seawater and were
299 thus expected to interact more with biological membranes through their higher reactivity and
300 capacity to cross biological membranes (Nel, 2006; Verma and Stellacci, 2010). Similarly,
301 exposure to NH₂-50 led to higher toxicity in sea urchin embryos and shrimp larvae (Bergami et
302 al., 2016; Della Torre et al., 2014), compared to COOH-40 nm forming approximately 1 µm
303 aggregates in seawater. The major differences in aggregation observed for the different

304 nanobeads in ultrapure water and filtered seawater are a result of the characteristics of the
305 nanobeads themselves and the surrounding medium (Nel et al., 2009; Rocha et al., 2015). The
306 high aggregation of COOH-50 and Plain-50 observed here in filtered seawater can be explained
307 by a strong interaction between the negative surface charge of these NP and the abundant cationic
308 ions such as Ca^{2+} in seawater. It is noteworthy that Plain-50 forming micrometric sized
309 aggregates led to significant toxicity on gametes and embryos while plain MP exhibiting lower
310 micrometric size ($2\mu\text{m}$) seemed innocuous. This suggests that nanoparticles remain highly
311 reactive with biological membrane even in the form of large aggregates.

312 The differential interactions between cells and nanoparticles may also be linked to their surface
313 properties, notably the net surface charge (Nel et al., 2009). The so-called buffering effect
314 observed on the net surface charge of MP/NP incubated in filtered seawater compared to
315 ultrapure water is also related to the presence of anions and cations in seawater that would have
316 interacted with their surface layer. The ions brought the ζ -Potential to a neutral surface charge,
317 and as a result, decreased NP stability (El Badawy et al., 2010; Lin et al., 2010). The lowest
318 surface charge observed for the Plain-50 may lead to reduced interaction with gamete and
319 embryo membranes, explaining their lower toxicity compared to other NP. Cationic nanoparticles
320 interact with negative membrane residuals more easily than anionic ones, and this interaction
321 triggers internalization to maintain the overall negative membrane charge, which may eventually
322 induce membrane disruptions (Cho et al., 2009; Fröhlich, 2012). Furthermore, a chemical effect
323 of the functionalization cannot be excluded and will be dependent on the commercial products
324 and their manufacturers.

325 Impairment of membrane integrity during cell divisions can lead to developmental arrest during
326 embryogenesis (Rossi et al., 2014), in agreement with the numerous malformations we observed

327 upon NP exposure. These results call for detailed microscopic analyses of exposed cell
328 membranes, coupled with lipidomic approaches to assess lipid membrane composition upon NP
329 exposure, in order to better understand the effects of NP on membrane integrity.

330 Given the high aggregation (3–10 μ m) observed in our data for the Plain-50 and COOH-50,
331 congestion of gametes is a possibility; our previous study demonstrated the adhesion of
332 carboxylic nanopolystyrene (100 nm) on oyster spermatozoa (González-Fernández et al., 2018).
333 This congestion may hamper spermatozoa internalization into oocytes, leading to negative effects
334 on the fertilization yield as observed upon exposure to Plain-50 and COOH-50.

335 Among the numerous physicochemical properties of the particles, including size, surface charge,
336 aspect ratio, porosity that impact *in vivo* behavior of MP and NP, surface corona is of real
337 importance (e.g. Galloway et al., 2017). Indeed, corona formation on nano-PS can fluctuate
338 depending on the surface properties of the particle, *i.e.* functionalization and charge (Lundqvist et
339 al., 2008). It can affect the particle chemical identity with significant consequences on ingestion
340 and interaction with cells and organs (Hristov et al., 2015; Canesi et al., 2016; Nasser & Lynch,
341 2016). Further studies are required to characterize the NP-cell interactions (entry, adhesion and
342 membrane impairments) in oyster gametes and embryos and to understand the toxic pathways
343 involved.

344 At the same time, the NP toxicity observed here could be related to sub-cellular toxicity upon
345 internalization and/or membrane disruption of gametes/embryos. For instance, NH₂-50 was better
346 internalized in human cell lines representing various organs, and led to more cytotoxic effects
347 than COOH-50 and Plain-50 (Anguissola et al., 2014; Bannunah et al., 2014). Similar
348 mechanisms, involving NP entry leading to sub-cellular toxicity, could also be hypothesized here
349 requiring fine microscopical observations using fluorescent NP. In the present study, the

350 exclusive occurrence of developmental arrest during exposure at the highest concentrations of
351 NH₂-50 and COOH-50 could indicate the involvement of apoptosis pathways, as described in the
352 sea urchin *Paracentrotus lividus* (Della Torre et al., 2014; Pinsino et al., 2017). The intermediate
353 situation, where malformed larvae (mantle, shell and hinge malformations) were observed upon
354 exposure to Plain-50, intermediate concentrations of COOH-50 and the lowest concentration of
355 NH₂-50, could be a result of dysregulation of genes involved in shell mineralization, as
356 previously demonstrated by transcriptional analysis in mussel embryos exposed to 0.15 µg/mL of
357 NH₂-50. Another toxic effect, previously characterized during exposure to chemical agents and
358 nanoplastics, involves a decrease in DNA integrity or a disruption of the cell oxidative balance of
359 oyster gametes and embryos (Akcha et al., 2012; Behrens et al., 2016; Vignier et al., 2017;
360 González-Fernández et al., 2018). Nanoparticles can interfere with electron transfer of the
361 intracellular medium, inducing a production of ROS (superoxide anion / hydroxyl radical,
362 hydrogen peroxide) and generating disruption of redox functions (Fu et al., 2014). This
363 overproduction of ROS results in several types of damage, such as lipid peroxidation or DNA
364 breakages leading to embryotoxicity (Xie et al., 2017). In agreement with these observations we
365 previously suggested that spermatozoa may lose their ability to fertilize oocytes as a consequence
366 of an oxidative stress induced by exposure of oyster spermatozoa to carboxylic nanopolystyrene
367 (100 nm) (González-Fernández et al., 2018).

368 The lowest concentration (0.1 µg/mL) used here was five times higher than the mass
369 concentration of MP used in the study of Sussarellu et al. (2016) based on equivalent mass
370 concentration of >333µm plastics debris hotspots. At this concentration, only exposure of oyster
371 embryos to NH₂-50 had a significant effect, which suggests that the probability of oyster
372 planktonic stages suffering fertilization and embryo-larval development disruptions due to NP

373 exposure is low in nature at the present time. However, taking into account the calculations of
374 Besseling et al. (2014), the toxic effects observed here began at lower concentrations than the
375 highest mass concentration of plastic debris (16.9 $\mu\text{g}/\text{mL}$) estimated at the water-sediment
376 interface. This location is known for its high plastic contamination and where wild adult oysters
377 live and spawn (Martin et al., 2017). Furthermore, the power-law increase in MP concentration
378 with decreasing particle size in sea surface samples suggests that small MP are increasingly
379 abundant, and that MP concentrations will be underestimated if the smallest fraction is not
380 properly quantified (Bergmann et al., 2017; Erni-Cassola et al., 2017).

381 With regards to the increase of (nano)plastics used in industry (GESAMP 2015), the recent
382 estimation of their mismanagement and release into oceans worldwide, as well as the continuous
383 breakdown of plastic waste at the nanometer scale, better management of end-of-life plastics is
384 should be strongly recommend to enable a transition to a circular economy (Brink et al. 2017)
385 and limit or prevent accidental releases. For instance, nano-TiO₂ levels are expected to reach up
386 to 1 $\mu\text{g}/\text{mL}$ in nature (Holden et al., 2014), although its estimated accidental release in the marine
387 environment is much lower (between 2 and 6 million tons over the next 10 years (Haynes et al.,
388 2017)) than that estimated for plastic wastes. The latter were estimated between 4.8 and 12.7
389 million tons in 2010 alone, with an expected increase of an order of magnitude by 2025 (Jambeck
390 et al., 2015).

391 **Conclusion**

392 Our study is the first demonstration of adverse effects of nanoplastics on oyster early-life stages,
393 with the fertilization/embryogenesis steps being particularly sensitive. The combination of fine
394 microscopy and Omics (lipidomics, transcriptomics) tools is now needed to fully understand the
395 underlying toxicity mechanisms that likely include both membrane disruption and sub-cellular

396 toxicity. Significant ecological implications can be expected as effects on gametes, fertilization
397 and embryo-larval development determine recruitment, population stability and ecosystem
398 structure. Indeed, oysters sustain the formation of reefs providing micro-habitats for a large
399 community of invertebrates and nursery areas for pelagic organisms (Bayne, 2017). We suggest
400 that direct effects on early-life stages should be integrated into the “adverse outcome pathway”
401 (AOP) scheme describing microplastic toxicity pathways in aquatic organisms (Galloway and
402 Lewis, 2016). Indeed, this additional pathway may influence the offspring viability and the
403 overall reproductive output. In this context, our work highlights the interest of using oysters as a
404 model to describe the risk of plastic debris in coastal and estuarine areas where a high spatial
405 variability of contamination is expected.

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413 **References**

- 414 Akcha, F., Spagnol, C., Rouxel, J., 2012. Genotoxicity of diuron and glyphosate in oyster
415 spermatozoa and embryos. *Aquat. Toxicol.* 106–107, 104–113.
416 <https://doi.org/10.1016/j.aquatox.2011.10.018>
- 417 Anguissola, S., Garry, D., Salvati, A., O’Brien, P.J., Dawson, K.A., 2014. High content analysis
418 provides mechanistic insights on the pathways of toxicity induced by amine-modified
419 polystyrene nanoparticles. *PLoS One* 9. <https://doi.org/10.1371/journal.pone.0108025>

- 420 Avio, C.G., Gorbi, S., Milan, M., Benedetti, M., Fattorini, D., D'Errico, G., Pauletto, M.,
421 Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from
422 microplastics to marine mussels. *Environ. Pollut.* 198, 211–222.
423 <https://doi.org/10.1016/j.envpol.2014.12.021>
- 424 Balbi, T., Camisassi, G., Montagna, M., Fabbri, R., Franzellitti, S., Carbone, C., Dawson, K.,
425 Canesi, L., 2017. Impact of cationic polystyrene nanoparticles (PS-NH₂) on early embryo
426 development of *Mytilus galloprovincialis*: Effects on shell formation. *Chemosphere* 186, 1–
427 9. <https://doi.org/10.1016/j.chemosphere.2017.07.120>
- 428 Bannunah, A.M., Vllasaliu, D., Lord, J., Stolnik, S., 2014. Mechanisms of Nanoparticle
429 Internalization and Transport Across an Intestinal Epithelial Cell Model: Effect of Size and
430 Surface Charge. *Mol. Pharm.* 11, 4363–4373. <https://doi.org/10.1021/mp500439c>
- 431 Bayne, B., 2017. Biology of oyster 1st Edition. *Eds Academic Press*, 862 pp.
- 432 Bayne, B.L., Ahrens, M., Allen, S.K., D'auriac, M.A., Backeljau, T., Beninger, P., Bohn, R.,
433 Boudry, P., Davis, J., Green, T., Guo, X., Hedgecock, D., Ibarra, A., Kingsley-Smith, P.,
434 Krause, M., Langdon, C., Lapègue, S., Li, C., Manahan, D., Mann, R., Perez-Paralle, L.,
435 Powell, E.N., Rawson, P.D., Speiser, D., Sanchez, J.-L., Shumway, S., Wang, H., 2017. The
436 Proposed Dropping of the Genus *Crassostrea* for All Pacific Cupped Oysters and Its
437 Replacement by a New Genus *Magallana*: A Dissenting View. *J. Shellfish Res.* 36, 545–
438 547. <https://doi.org/10.2983/035.036.0301>
- 439 Behrens, D., Rouxel, J., Burgeot, T., Akcha, F., 2016. Comparative embryotoxicity and
440 genotoxicity of the herbicide diuron and its metabolites in early life stages of *Crassostrea*
441 *gigas*: Implication of reactive oxygen species production. *Aquat. Toxicol.* 175, 249–259.
442 <https://doi.org/10.1016/j.aquatox.2016.04.003>
- 443 Bergami, E., Bocci, E., Luisa, M., Monopoli, M., Salvati, A., Dawson, K.A., Corsi, I., 2016.
444 Nano-sized polystyrene affects feeding, behavior and physiology of brine shrimp *Artemia*
445 *franciscana* larvae. *Ecotoxicol. Environ. Saf.* 123, 18–25.
446 <https://doi.org/10.1016/j.ecoenv.2015.09.021>
- 447 Bergmann, M., Wirzberger, V., Krumpfen, T., Lorenz, C., Primpke, S., Tekman, M.B., Gerdt, G.,
448 2017. High quantities of microplastic in Arctic deep-sea sediments from the
449 HAUSGARTEN observatory. *Environ. Sci. Technol.* [acs.est.7b03331](https://doi.org/10.1021/acs.est.7b03331).
450 <https://doi.org/10.1021/acs.est.7b03331>
- 451 Besseling, E., Wang, B., Lüring, M., Koelmans, A.A., 2014. Nanoplastic affects growth of *S.*
452 *obliquus* and reproduction of *D. magna*. *Environ. Sci. Technol.* 48, 12336–12343.
453 <https://doi.org/10.1021/es503001d>

- 454 Canesi, L., Ciacci, C., Fabbri, R., Balbi, T., Salis, A., Damonte, G., Cortese, K., Caratto, V.,
455 Monopoli, M.P., Dawson, K., Bergami, E., Corsi, I., 2016. Interactions of cationic
456 polystyrene nanoparticles with marine bivalve hemocytes in a physiological environment:
457 Role of soluble hemolymph proteins. *Environ. Res.* 150, 73–81.
458 <https://doi.org/10.1016/j.envres.2016.05.045>
- 459 Cho, E.C., Xie, J., Wurm, P.A., Xia, Y., 2009. Understanding the role of surface charges in
460 cellular adsorption versus internalization by selectively removing gold nanoparticles on the
461 cell surface with a I²/KI etchant. *Nano Lett.* 9, 1080–1084.
462 <https://doi.org/10.1021/nl803487r>
- 463 Cole, M., Galloway, T.S., 2015. Ingestion of Nanoplastics and Microplastics by Pacific Oyster
464 Larvae. *Environ. Sci. Technol.* 49, 14625–14632. <https://doi.org/10.1021/acs.est.5b04099>
- 465 Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T.S.,
466 2013. Microplastic ingestion by zooplankton. *Environ. Sci. Technol.* 47, 6646–6655.
467 <https://doi.org/10.1021/es400663f>
- 468 Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the
469 marine environment: A review. *Mar. Pollut. Bull.* 62, 2588–2597.
470 <https://doi.org/10.1016/j.marpolbul.2011.09.025>
- 471 Coon, S.L., Walch, M., Fitt, W.K., Weiner, R.M., Bonar, D.B., 1990. Ammonia Induces
472 Settlement Behavior in Oyster Larvae. *Biol. Bull.* 179, 297–303.
473 <https://doi.org/10.2307/1542321>
- 474 Cózar, A., Martí, E., Duarte, C.M., García-de-Lomas, J., van Sebille, E., Ballatore, T.J., Eguíluz,
475 V.M., González-Gordillo, J.I., Pedrotti, M.L., Echevarría, F., Troublè, R., Irigoien, X., 2017.
476 The Arctic Ocean as a dead end for floating plastics in the North Atlantic branch of the
477 Thermohaline Circulation. *Sci. Adv.* 3, e1600582. <https://doi.org/10.1126/sciadv.1600582>
- 478 Cui, R., Kim, S.W., An, Y.-J., 2017. Polystyrene nanoplastics inhibit reproduction and induce
479 abnormal embryonic development in the freshwater crustacean *Daphnia galeata*. *Sci. Rep.*
480 7, 12095. <https://doi.org/10.1038/s41598-017-12299-2>
- 481 da Costa, J.P., Santos, P.S.M., Duarte, A.C., Rocha-Santos, T., 2016. (Nano)plastics in the
482 environment - Sources, fates and effects. *Sci. Total Environ.* 566–567, 15–26.
483 <https://doi.org/10.1016/j.scitotenv.2016.05.041>
- 484 Dawson, A.L., Kawaguchi, S., King, C.K., Townsend, K.A., King, R., Huston, W.M., Bengtson
485 Nash, S.M., 2018. Turning microplastics into nanoplastics through digestive fragmentation
486 by Antarctic krill. *Nat. Commun.* 9, 1001. <https://doi.org/10.1038/s41467-018-03465-9>

- 487 Della Torre, C., Bergami, E., Salvati, A., Faleri, C., Cirino, P., Dawson, K.A., Corsi, I., 2014.
488 Accumulation and Embryotoxicity of Polystyrene Nanoparticles at Early Stage of
489 Development of Sea Urchin Embryos *Paracentrotus lividus*. Environ. Sci. Technol. 48,
490 12302–12311. <https://doi.org/10.1021/es502569w>
- 491 Di Poi, C., Evariste, L., Serpentine, A., Halm-Lemeille, M.P., Lebel, J.M., Costil, K., 2014.
492 Toxicity of five antidepressant drugs on embryo–larval development and metamorphosis
493 success in the Pacific oyster, *Crassostrea gigas*. Environ. Sci. Pollut. Res. 21, 13302–13314.
494 <https://doi.org/10.1007/s11356-013-2211-y>
- 495 Dubey, M.K., Bijwe, J., Ramakumar, S.S. V, 2015. Nano-PTFE: New entrant as a very promising
496 EP additive. Tribol. Int. 87, 121–131. <https://doi.org/10.1016/j.triboint.2015.01.026>
- 497 El Badawy, A.M., Luxton, T.P., Silva, R.G., Scheckel, K.G., Suidan, M.T., Tolaymat, T.M.,
498 2010. Impact of Environmental Conditions (pH, Ionic Strength, and Electrolyte Type) on the
499 Surface Charge and Aggregation of Silver Nanoparticles Suspensions. Environ. Sci.
500 Technol. 44, 1260–1266. <https://doi.org/10.1021/es902240k>
- 501 Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F.,
502 Ryan, P.G., Reisser, J., 2014. Plastic Pollution in the World's Oceans: More than 5 Trillion
503 Plastic Pieces Weighing over 250,000 Tons Afloat at Sea. PLoS One 9, 1–15.
504 <https://doi.org/10.1371/journal.pone.0111913>
- 505 Erni-Cassola, G., Gibson, M.I., Thompson, R.C., Christie-Oleza, J.A., 2017. Lost, but Found
506 with Nile Red: A Novel Method for Detecting and Quantifying Small Microplastics (1 mm
507 to 20 μm) in Environmental Samples. Environ. Sci. Technol. 51, 13641–13648.
508 <https://doi.org/10.1021/acs.est.7b04512>
- 509 Fabbri, R., Montagna, M., Balbi, T., Raffo, E., Palumbo, F., Canesi, L., 2014. Adaptation of the
510 bivalve embryotoxicity assay for the high throughput screening of emerging contaminants in
511 *Mytilus galloprovincialis*. Mar. Environ. Res. 99, 1–8.
512 <https://doi.org/10.1016/j.marenvres.2014.05.007>
- 513 Fröhlich, E., 2012. The role of surface charge in cellular uptake and cytotoxicity of medical
514 nanoparticles. Int. J. Nanomedicine 7, 5577. <https://doi.org/10.2147/IJN.S36111>
- 515 Fu, P.P., Xia, Q., Hwang, H.-M., Ray, P.C., Yu, H., 2014. Mechanisms of nanotoxicity:
516 generation of reactive oxygen species. J. food drug Anal. 22, 64–75.
517 <https://doi.org/10.1016/j.jfda.2014.01.005>
- 518 Gallo, A., Boni, R., Buttino, I., Tosti, E., 2016. Spermotoxicity of nickel nanoparticles in the
519 marine invertebrate *Ciona intestinalis* (ascidians). Nanotoxicology 10, 1096–1104.
520 <https://doi.org/10.1080/17435390.2016.1177743>

- 521 Galloway, T.S., Cole, M., Lewis, C., Atkinson, A., Allen, J.I., 2017. Interactions of microplastic
522 debris throughout the marine ecosystem. *Nat. Ecol. Evol.* 1, 116.
523 <https://doi.org/10.1038/s41559-017-0116>
- 524 Galloway, T.S., Lewis, C.N., 2016. Marine microplastics spell big problems for future
525 generations. *Proc. Natl. Acad. Sci. USA* 113, 2331–2333.
526 <https://doi.org/10.1073/pnas.1600715113>
- 527 Gardon, T., Reisser, C., Soyeux, C., Quillien, V., & Le Moullac, G., 2018. Microplastics Affect
528 Energy Balance and Gametogenesis in the Pearl Oyster *Pinctada margaritifera*.
529 *Environmental Science & technology*, 52(9), 5277–5286.
- 530 GESAMP (2015). “Sources, fate and effects of microplastics in the marine environment: a global
531 assessment” (Kershaw, P. J., ed.). (IMO/FAO/UNESCO-
532 IOC/UNIDO/WMO/IAEA/UN/UNEP/UNDP Joint Group of Experts on the Scientific
533 Aspects of Marine Environmental Protection). Rep. Stud. GESAMP No. 90, 96.
- 534 Geyer, R., Jambeck, J.R., Law, K.L., 2017. Production, use, and fate of all plastics ever made.
535 *Sci. Adv.* 3, e1700782. <https://doi.org/10.1126/sciadv.1700782>
- 536 Gigault, J., Ter Halle, A., Baudrimont, M., Pascal, P., Gauffre, F., Phi, T.-L., El Hadri, H., Grassl,
537 B., Reynaud, S., 2018. Current opinion: What is a nanoplastic? *Environ. Pollut.* 235, 1030–
538 1034. <https://doi.org/10.1016/j.envpol.2018.01.024>
- 539 Gigault, J., Pedrono, B., Maxit, B., Ter Halle, A., 2016. Marine plastic litter: the unanalyzed
540 nano-fraction. *Environ. Sci. Nano* 3, 346–350. <https://doi.org/10.1039/C6EN00008H>
- 541 González-Fernández, C., Tallec, K., Le Goïc, N., Lambert, C., Soudant, P., Huvet, A., Suquet,
542 M., Berchel, M., Paul-Pont, I., 2018. Cellular responses of Pacific oyster (*Crassostrea*
543 *gigas*) gametes exposed in vitro to polystyrene nanoparticles. *Chemosphere* 208, 764–772.
544 <https://doi.org/10.1016/j.chemosphere.2018.06.039>
- 545 Green, D.S., Boots, B., O’Connor, N.E., Thompson, R., 2016. Microplastics affect the ecological
546 functioning of an important biogenic habitat. *Environ. Sci. Technol.* 51, 68–77.
547 <https://doi.org/10.1021/acs.est.6b04496>
- 548 Haynes, V.N., Ward, J.E., Russell, B.J., Agrios, A.G., 2017. Photocatalytic effects of titanium
549 dioxide nanoparticles on aquatic organisms—Current knowledge and suggestions for future
550 research. *Aquat. Toxicol.* 185, 138–148. <https://doi.org/10.1016/j.aquatox.2017.02.012>
- 551 Hernandez, L.M., Yousefi, N., Tufenkji, N., 2017. Are There Nanoplastics in Your Personal Care
552 Products? *Environ. Sci. Technol. Lett.* 4, 280–285.
553 <https://doi.org/10.1021/acs.estlett.7b00187>

- 554 Hickman, C.S., 1999. Adaptive Function of Gastropod Larval Shell Features. *Invertebr. Biol.*
555 118, 346–356. <https://doi.org/10.2307/3227006>
- 556 Holden, P.A., Klaessig, F., Turco, R.F., Priester, J.H., Rico, C.M., Avila-Arias, H., Mortimer, M.,
557 Pacpaco, K., Gardea-Torresdey, J.L., 2014. Evaluation of Exposure Concentrations Used in
558 Assessing Manufactured Nanomaterial Environmental Hazards: Are They Relevant?
559 *Environ. Sci. Technol.* 48, 10541–10551. <https://doi.org/10.1021/es502440s>
- 560 Hristov, D.R., Rocks, L., Kelly, P.M., Thomas, S.S., Pitek, A.S., Verderio, P., Mahon, E.,
561 Dawson, K.A., (2015). Tuning of nanoparticle biological functionality through controlled
562 surface chemistry and characterisation at the bioconjugated nanoparticle surface. *Sci. Rep.* 5,
563 17040. <https://doi.org/10.1038/srep17040>.
- 564 Huvet, A., Paul-Pont, I., Fabioux, C., Lambert, C., Suquet, M., Thomas, Y., Robbens, J.,
565 Soudant, P., Sussarellu, R., 2016. Reply to Lenz et al.: Quantifying the smallest
566 microplastics is the challenge for a comprehensive view of their environmental impacts.
567 *Proc. Natl. Acad. Sci. USA* 113, E4123–E4124. <https://doi.org/10.1073/pnas.1607221113>
- 568 Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R.,
569 Law, K.L., 2015. Plastic waste inputs from land into the ocean. *Science* 347, 768–771.
570 <https://doi.org/10.1126/science.1260352>
- 571 Jeong, C.B., Kang, H.M., Lee, M.C., Kim, D.H., Han, J., Hwang, D.S., Souissi, S., Lee, S.J.,
572 Shin, K.H., Park, H.G., Lee, J.S., 2017. Adverse effects of microplastics and oxidative
573 stress-induced MAPK/Nrf2 pathway-mediated defense mechanisms in the marine copepod
574 *Paracyclopina nana*. *Sci. Rep.* 7, 1–11. <https://doi.org/10.1038/srep41323>
- 575 Jeong, C.B., Won, E.J., Kang, H.M., Lee, M.C., Hwang, D.S., Hwang, U.K., Zhou, B., Souissi,
576 S., Lee, S.J., Lee, J.S., 2016. Microplastic Size-Dependent Toxicity, Oxidative Stress
577 Induction, and p-JNK and p-p38 Activation in the Monogonont Rotifer (*Brachionus*
578 *koreanus*). *Environ. Sci. Technol.* 50, 8849–8857. <https://doi.org/10.1021/acs.est.6b01441>
- 579 Kadar, E., Tarran, G.A., Jha, A.N., Al-Subiai, S.N., 2011. Stabilization of engineered zero-valent
580 nanoiron with Na-acrylic copolymer enhances spermotoxicity. *Environ. Sci. Technol.* 45,
581 3245–3251. <https://doi.org/10.1021/es1029848>
- 582 Klaine, S.J., Koelmans, A.A., Horne, N., Carley, S., Handy, R.D., Kapustka, L., Nowack, B., von
583 der Kammer, F., 2012. Paradigms to assess the environmental impact of manufactured
584 nanomaterials. *Environ. Toxicol. Chem.* 31, 3–14. <https://doi.org/10.1002/etc.733>
- 585 Lambert, S., Wagner, M., 2016. Characterisation of nanoplastics during the degradation of
586 polystyrene. *Chemosphere* 145, 265–268.
587 <https://doi.org/10.1016/j.chemosphere.2015.11.078>

- 588 Lavers, J.L., Bond, A.L., 2017. Exceptional and rapid accumulation of anthropogenic debris on
589 one of the world's most remote and pristine islands. *Proc. Natl. Acad. Sci. USA* 114, 6052–
590 6055. <https://doi.org/10.1073/pnas.1619818114>
- 591 Le Goïc, N., Hégaret, H., Boulais, M., Béguel, J.-P., Lambert, C., Fabioux, C., Soudant, P., 2014.
592 Flow cytometric assessment of morphology, viability, and production of reactive oxygen
593 species of *Crassostrea gigas* oocytes. Application to Toxic dinoflagellate (*Alexandrium*
594 *minutum*) exposure. *Cytom. Part A* 85, 1049–1056. <https://doi.org/10.1002/cyto.a.22577>
- 595 Le Goïc, N., Hégaret, H., Fabioux, C., Miner, P., Suquet, M., Lambert, C., Soudant, P., 2013.
596 Impact of the toxic dinoflagellate *Alexandrium catenella* on Pacific oyster reproductive
597 output: application of flow cytometry assays on spermatozoa. *Aquat. Living Resour.* 26,
598 221–228. <https://doi.org/10.1051/alr/2013047>
- 599 Lee, K., Shim, W.J., Kwon, O.Y., Kang, J., 2013. Size-Dependent Effects of Micro Polystyrene
600 Particles in the Marine Copepod *Tigriopus japonicus*. *Environ. Sci. Technol.* 47, 11278–
601 11283. <https://doi.org/dx.doi.org/10.1021/es401932b>
- 602 Liebig, J.R., Vanderploeg, H.A., 1995. Vulnerability of *Dreissena polymorpha* Larvae to
603 Predation by Great Lakes Calanoid Copepods: the Importance of the Bivalve Shell. *J. Great*
604 *Lakes Res.* 21, 353–358. [https://doi.org/10.1016/S0380-1330\(95\)71046-2](https://doi.org/10.1016/S0380-1330(95)71046-2)
- 605 Lin, D., Tian, X., Wu, F., Xing, B., 2010. Fate and Transport of Engineered Nanomaterials in the
606 Environment. *J. Environ. Qual.* 39, 1896. <https://doi.org/10.2134/jeq2009.0423>
- 607 Lundqvist, M., Stigler, J., Elia, G., Lynch, I., Cedervall, T., Dawson, K.A, 2008. Nanoparticle
608 size and surface properties determine the protein corona with possible implications for
609 biological impacts. *Proc. Natl. Acad. Sci. USA* 105, 14265–14270.
610 <https://doi.org/10.1073/pnas.0805135105>
- 611 Lusher, A. L., Hollman, P. C. H., Mendoza-Hill, J. J., 2017. Microplastics in Fisheries and
612 Aquaculture: Status of Knowledge on their Occurrence and Implications for Aquatic
613 Organisms and Food Safety. *FAO Fisheries and Aquaculture Technical Paper*. No. 615, 147
614 pp.
- 615 Ma, Y., Huang, A., Cao, S., Sun, F., Wang, L., Guo, H., Ji, R., 2016. Effects of nanoplastics and
616 microplastics on toxicity, bioaccumulation, and environmental fate of phenanthrene in fresh
617 water. *Environ. Pollut.* 219, 166–173. <https://doi.org/10.1016/j.envpol.2016.10.061>
- 618 Martin, J., Lusher, A., Thompson, R.C., Morley, A., 2017. The Deposition and Accumulation of
619 Microplastics in Marine Sediments and Bottom Water from the Irish Continental Shelf. *Sci.*
620 *Rep.* 1–9. <https://doi.org/10.1038/s41598-017-11079-2>

- 621 Martínez-Gómez, C., León, V.M., Calles, S., Gomáriz-Olcina, M., Vethaak, A.D., 2017. The
622 adverse effects of virgin microplastics on the fertilization and larval development of sea
623 urchins. *Mar. Environ. Res.* 130, 69–76. <https://doi.org/10.1016/j.marenvres.2017.06.016>
- 624 Mattsson, K., Hansson, L.-A., Cedervall, T., 2015a. Nano-plastics in the aquatic environment.
625 *Environ. Sci. Process. Impacts* 17, 1712–1721. <https://doi.org/10.1039/C5EM00227C>
- 626 Mattsson, K., Ekvall, M.T., Hansson, L.A., Linse, S., Malmendal, A., Cedervall, T., 2015b.
627 Altered behavior, physiology, and metabolism in fish exposed to polystyrene nanoparticles.
628 *Environ. Sci. Technol.* 49, 553–561. <https://doi.org/10.1021/es5053655>
- 629 Mattsson, K., Johnson, E. V., Malmendal, A., Linse, S., Hansson, L.-A., Cedervall, T., 2017.
630 Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered
631 through the food chain. *Sci. Rep.* 7, 11452. <https://doi.org/10.1038/s41598-017-10813-0>
- 632 Mottier, A., Kientz-Bouchart, V., Serpentine, A., Lebel, J.M., Jha, A.N., Costil, K., 2013. Effects
633 of glyphosate-based herbicides on embryo-larval development and metamorphosis in the
634 Pacific oyster, *Crassostrea gigas*. *Aquat. Toxicol.* 128–129, 67–78.
635 <https://doi.org/10.1016/j.aquatox.2012.12.002>
- 636 Nasser, F., Lynch, I., 2016. Secreted protein eco-corona mediates uptake and impacts of
637 polystyrene nanoparticles on *Daphnia magna*. *J. Proteomics* 137, 45–51.
638 <https://doi.org/10.1016/j.jprot.2015.09.005>
- 639 Nel, A., Xia, T., Mädler, L., Li, N., 2006. Toxic Potential of Materials at the Nanolevel. *Science*.
640 311, 622–627. <https://doi.org/10.1126/science.1114397>
- 641 Nel, A.E., Mädler, L., Velegol, D., Xia, T., Hoek, E.M. V., Somasundaran, P., Klaessig, F.,
642 Castranova, V., Thompson, M., 2009. Understanding biophysicochemical interactions at the
643 nano-bio interface. *Nat. Mater.* 8, 543–557. <https://doi.org/10.1038/nmat2442>
- 644 Ogonowski, M., Schür, C., Jarsén, Å., Gorokhova, E., 2016. The Effects of Natural and
645 Anthropogenic Microparticles on Individual Fitness in *Daphnia magna*. *PLoS One* 11,
646 e0155063. <https://doi.org/10.1371/journal.pone.0155063>
- 647 Ostroumov, S.A., 2003. Studying effects of some surfactants and detergents on filter-feeding
648 bivalves. *Hydrobiologia* 500, 341–344. <https://doi.org/10.1023/A:1024604904065>
- 649 Paul-Pont, I., Tallec, K., González Fernández, C., Lambert, C., Vincent, D., Mazurais, D.,
650 Zambonino-Infante, J-L., Brotons, G., Lagarde, F., Fabioux, F., Soudant, P., Huvet, A.,
651 2018. Constraints and Priorities for Conducting Experimental Exposures of Marine
652 Organisms to Microplastics. *Front. Mar. Sci.* 5, 252.
653 <https://doi.org/10.3389/fmars.2018.00252>

- 654 Paul-Pont, I., Lacroix, C., González Fernández, C., Hégaret, H., Lambert, C., Le Goïc, N., Frère,
655 L., Cassone, A.-L., Sussarellu, R., Fabioux, C., Guyomarch, J., Albentosa, M., Huvet, A.,
656 Soudant, P., 2016. Exposure of marine mussels *Mytilus* spp. to polystyrene microplastics:
657 Toxicity and influence on fluoranthene bioaccumulation. *Environ. Pollut.* 216, 724-737.
658 <https://doi.org/10.1016/j.envpol.2016.06.039>
- 659 Petton, B., Boudry, P., Alunno-Bruscia, M., Pernet, F., 2015. Factors influencing disease-induced
660 mortality of Pacific oysters *Crassostrea gigas*. *Aquac. Environ. Interact.* 6, 205–222.
661 <https://doi.org/10.3354/aei00125>
- 662 Pinsino, A., Bergami, E., Della Torre, C., Vannuccini, M.L., Addis, P., Secci, M., Dawson, K.A.,
663 Matranga, V., Corsi, I., 2017. Amino-modified polystyrene nanoparticles affect signalling
664 pathways of the sea urchin (*Paracentrotus lividus*) embryos. *Nanotoxicology* 11, 201–209.
665 <https://doi.org/10.1080/17435390.2017.1279360>
- 666 Rocha, T.L., Gomes, T., Sousa, V.S., Mestre, N.C., Bebianno, M.J., 2015. Ecotoxicological
667 impact of engineered nanomaterials in bivalve molluscs: An overview. *Mar. Environ. Res.*
668 111, 74–88. <https://doi.org/10.1016/j.marenvres.2015.06.013>
- 669 Rochman, C.M., Browne, M.A., Underwood, A.J., van Franeker, J.A., Thompson, R.C., Amaral-
670 Zettler, L.A., 2015. The ecological impacts of marine debris: unraveling the demonstrated
671 evidence from what is perceived. *Ecology* 96, 301–312. <https://doi.org/10.1890/14-2070.1>
- 672 Rossi, G., Barnoud, J., Monticelli, L., 2014. Polystyrene nanoparticles perturb lipid membranes.
673 *J. Phys. Chem. Lett.* 5, 241–246. <https://doi.org/10.1021/jz402234c>
- 674 Schiaparelli, S., Cattaneo-Vietti, R., Mierzejewski, P., 2004. A “protective shell” around the
675 larval cocoon of *Cephalodiscus densus* Andersson, 1907 (Graptolithoidea, Hemichordata).
676 *Polar Biol.* 27, 813–817. <https://doi.org/10.1007/s00300-004-0661-x>
- 677 Steele, S., Mulcahy, M.F.O., 1999. Gametogenesis of the oyster *Crassostrea gigas* in southern
678 Ireland. *J. Mar. Biol. Assoc. UK.* 79, 673–686.
- 679 Stephens, B., Azimi, P., El Orch, Z., Ramos, T., 2013. Ultrafine particle emissions from desktop
680 3D printers. *Atmos. Environ.* 79, 334–339. <https://doi.org/10.1016/j.atmosenv.2013.06.050>
- 681 Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M.E.J., Le Goïc, N.,
682 Quillien, V., Mingant, C., Epelboin, Y., Corporeau, C., Guyomarch, J., Robbens, J., Paul-
683 Pont, I., Soudant, P., Huvet, A., 2016. Oyster reproduction is affected by exposure to
684 polystyrene microplastics. *Proc. Natl. Acad. Sci. USA* 201519019.
685 <https://doi.org/10.1073/pnas.1519019113>
- 686 ten Brink, P., Schweitzer J.-P., Watkins, E., De Smet, M., Leslie, H., Galgani, F., 2017. T20 Task

- 687 Force Circular Economy Circular Economy Measures to Keep Plastics and Their Value in
688 the Economy. G20 Insights, 1–12.
- 689 Ter Halle, A., Jeanneau, L., Martignac, M., Jardé, E., Pedrono, B., Brach, L., Gigault, J., 2017.
690 Nanoplastic in the North Atlantic Subtropical Gyre. *Environ. Sci. Technol.* 51, 13689–
691 13697. <https://doi.org/10.1021/acs.est.7b03667>
- 692 Van Cauwenberghe, L., Devriese, L., Galgani, F., Robbins, J., Janssen, C.R., 2015. Microplastics
693 in sediments: A review of techniques, occurrence and effects. *Mar. Environ. Res.* 111, 5–17.
694 <https://doi.org/10.1016/j.marenvres.2015.06.007>
- 695 Verma, A., Stellacci, F., 2010. Effect of surface properties on nanoparticle-cell interactions.
696 *Small* 6, 12–21. <https://doi.org/10.1002/sml.200901158>
- 697 Vignier, J., Volety, A.K., Rolton, A., Le Goïc, N., Chu, F.L.E., Robert, R., Soudant, P., 2017.
698 Sensitivity of eastern oyster (*Crassostrea virginica*) spermatozoa and oocytes to dispersed
699 oil: Cellular responses and impacts on fertilization and embryogenesis. *Environ. Pollut.* 225,
700 270–282. <https://doi.org/10.1016/j.envpol.2016.11.052>
- 701 Vogeler, S., Bean, T. P., Lyons, B. P., Galloway, T. S. (2016). Dynamics of nuclear receptor gene
702 expression during Pacific oyster development. *BMC Dev. Biol.* 16, 33.
703 <https://doi.org/10.1186/s12861-016-0129-6>
- 704 Watts, A.J.R., Urbina, M.A., Corr, S., Lewis, C., Galloway, T.S., 2015. Ingestion of Plastic
705 Microfibers by the Crab *Carcinus maenas* and Its Effect on Food Consumption and Energy
706 Balance. *Environ. Sci. Technol.* 49, 14597–14604. <https://doi.org/10.1021/acs.est.5b04026>
- 707 Wegner, A., Besseling, E., Foekema, E.M., Kamermans, P., Koelmans, A.A., 2012. Effects of
708 nanopolystyrene on the feeding behavior of the blue mussel (*Mytilus edulis* L.). *Environ.*
709 *Toxicol. Chem.* 31, 2490–2497. <https://doi.org/10.1002/etc.1984>
- 710 Wright, S.L., Kelly, F.J., 2017. Plastic and Human Health: A Micro Issue? *Environ. Sci. Technol.*
711 51, 6634–6647. <https://doi.org/10.1021/acs.est.7b00423>
- 712 Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013. Microplastic ingestion decreases
713 energy reserves in marine worms. *Curr. Biol.* 23, R1031–R1033.
714 <https://doi.org/10.1016/j.cub.2013.10.068>
- 715 Xie, J., Yang, D., Sun, X., Cao, R., Chen, L., Wang, Q., Li, F., Wu, H., Ji, C., Cong, M., Zhao, J.,
716 2017. Individual and Combined Toxicities of Benzo[a]pyrene and 2,2",4,4"-
717 Tetrabromodiphenyl Ether on Early Life Stages of the Pacific Oyster, *Crassostrea gigas*.
718 *Bull. Environ. Contam. Toxicol.* 99, 582–588. <https://doi.org/10.1007/s00128-017-2164-9>
- 719

720 **Table legend**

721 **Table 1.** Mean size (in nm), aggregation state (PDI in arbitrary units, A.U.) and charge (ζ -
722 Potential in mV) of polystyrene particles in ultrapure water (UW), and UV-treated 1- μ m filtered
723 seawater (FSW). Analyses were performed by Dynamic Light Scattering (DLS) at 20°C in
724 triplicate and data are represented as means \pm SD. Comparisons were made between media using
725 the Student's t-test; * : $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

726 **Figure legends**

727 **Fig. 1** Life cycle of oyster showing the results of exposures on different stages to MP/NP. This
728 scheme was modified from Vogeler et al. (2016).

729 **Fig. 2** Fertilization yield (%) after 1.5 h exposure of oyster gametes (1,000 oocytes.mL⁻¹; 100:1
730 spermatozoa:oocyte ratio) to (A) 2- μ m, (B) 500-nm, (C) Plain-50 nm, (D) COOH-50 nm, (E)
731 NH₂-50 nm polystyrene beads at five concentrations: 0, 0.1, 1, 10 and 25 μ g/mL. The assay was
732 replicated five times and data are represented as means \pm SD. Multiple comparisons were made
733 between treatments using Tukey's HSD (500-nm, NH₂-50) or Conover (Plain-50, COOH-50)
734 methods at the 5% alpha level; homogeneous groups are indicated by the same letter.

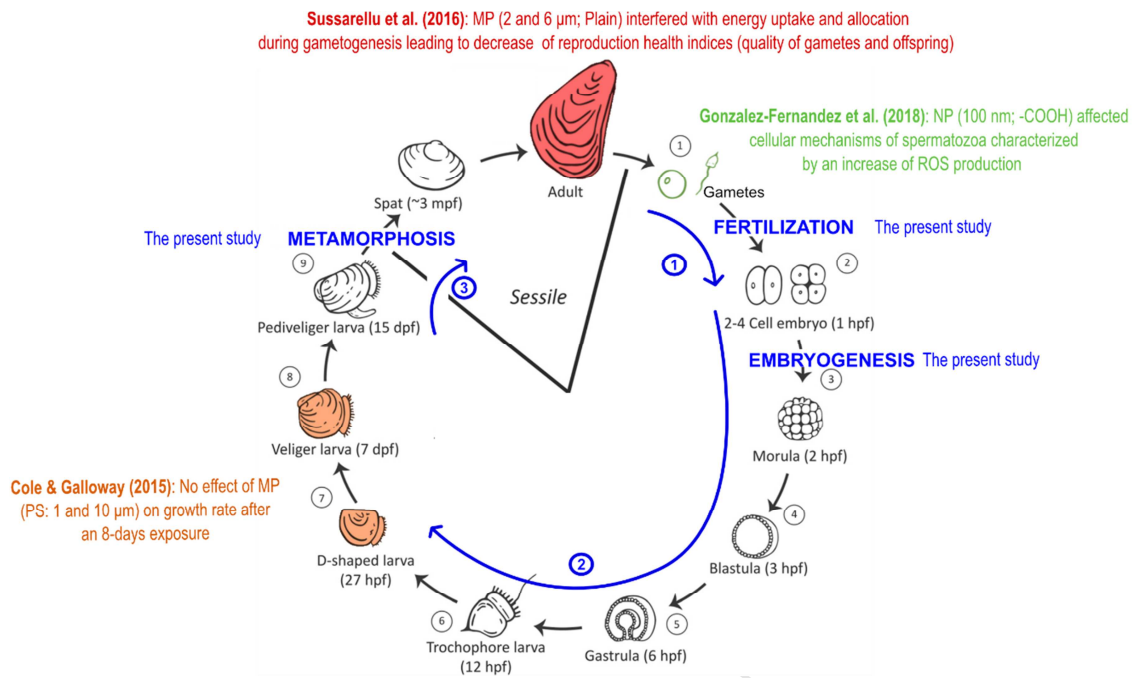
735 **Fig. 3** D-larval yield (%) after 36 h exposure of fertilized eggs to (A) 2- μ m, (B) 500-nm, (C)
736 Plain-50 nm, (D) COOH-50 nm, (E) NH₂-50 nm polystyrene beads at five concentrations: 0, 0.1,
737 1, 10 and 25 μ g/mL. The assay was replicated five times and data are represented as mean \pm SD.
738 Multiple comparisons were made between treatments using Tukey's HSD (Plain-50, COOH-50)
739 or Conover (NH₂-50) methods at the 5% alpha level; homogeneous groups are indicated by the
740 same letter.

741 **Fig. 4** Microscopy panel of embryo-larval development success after 36 h exposure to
742 polystyrene nanobeads compared with normal D-larvae observed in the control treatment (A),
743 larvae with shell and/or mantle malformations after exposure to Plain-50 (25 μ g/mL) (B), and
744 COOH-50 (10 μ g/mL) (C). Only developmental arrest, dead larvae and cell debris were observed
745 for all embryos following exposure to 25 μ g/mL of COOH-50 (D) and from 1 to 25 μ g/mL of
746 NH₂-50 (E and F). Size in μ m is represented by the scale bar.

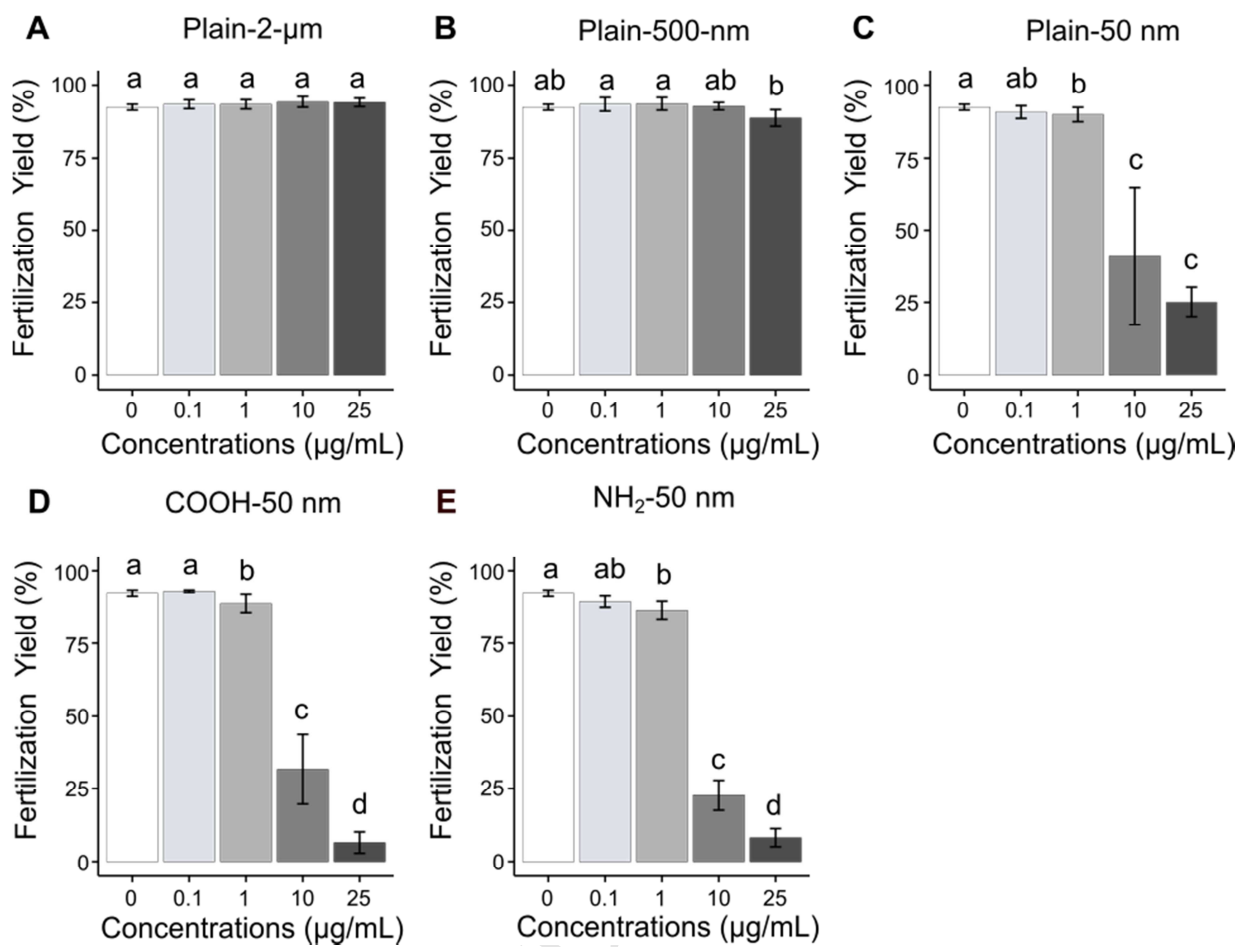
747 **Table 1**

Media	Particles	Commercial Size (nm)	Particle/Aggregate Size (nm)	PDI (A.U.)	ζ -Potential (mV)
UW	2- μ m	2,000	2681.0 \pm 50.5	0.35 \pm 0.01	-44.8 \pm 0.9
	500-nm	500	774.3 \pm 29.3	0.46 \pm 0.05	-67.8 \pm 7.0
	COOH-50	50	55.9 \pm 0.4	0.06 \pm 0.01	-62.1 \pm 0.4
	Plain-50	50	49 \pm 0.4	0.03 \pm 0.02	-70.1 \pm 1.4
	NH ₂ -50	50	53.3 \pm 2.3	0.12 \pm 0.02	44.0 \pm 1.5
FSW	2- μ m	2,000	3113.7 \pm 32.3***	0.42 \pm 0.02	-30.5 \pm 1.5***
	500-nm	500	1620.7 \pm 188.8*	0.66 \pm 0.08	-28.3 \pm 0.6**
	COOH-50	50	3735.0 \pm 443.8**	0.48 \pm 0.01	-13.8 \pm 0.8***
	Plain-50	50	5951.0 \pm 264.3***	0.60 \pm 0.05	-31.3 \pm 4.4**
	NH ₂ -50	50	96.5 \pm 2.0***	0.52 \pm 0.01	15.6 \pm 2.7***

748

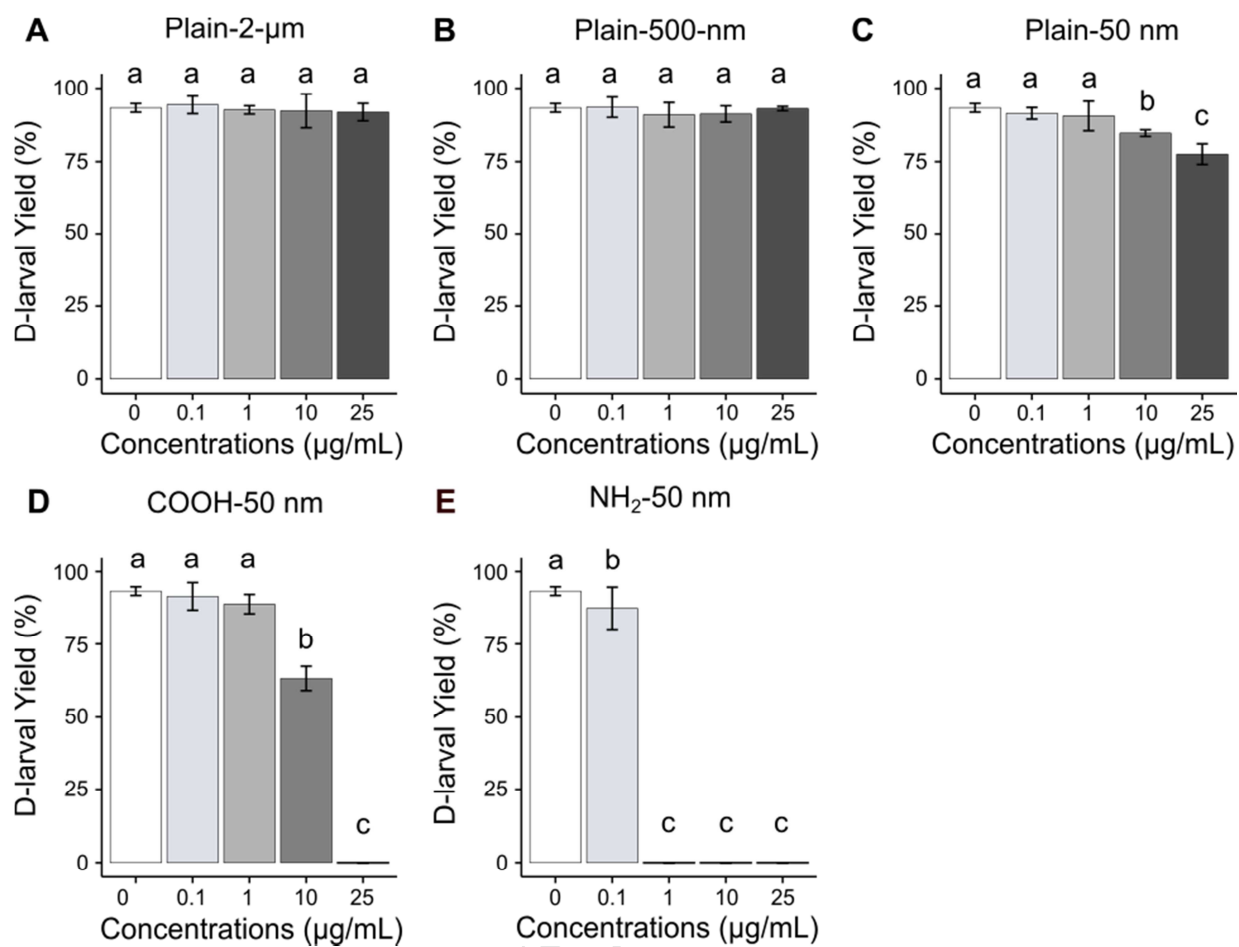


749
750 **Fig. 1**



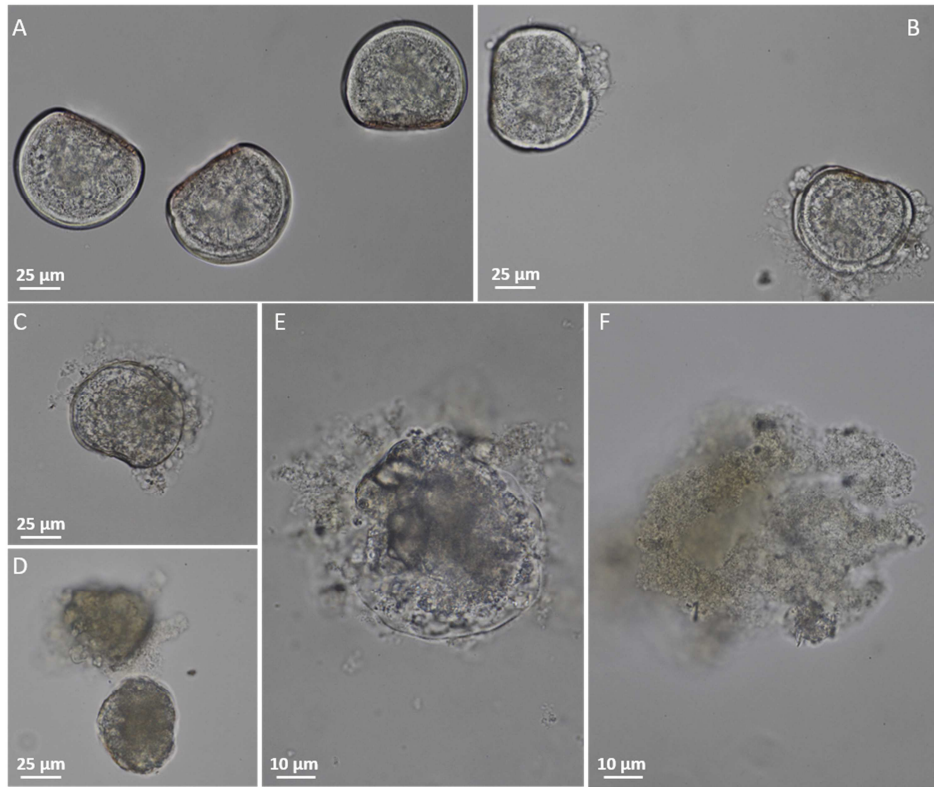
751

752 **Fig. 2**



753

754 **Fig. 3**



755

756 **Fig. 4**