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1 **High density polyethylene (HDPE) microplastics impair development and swimming**
2 **activity of Pacific oyster D-larvae, *Crassostrea gigas*, depending on particle size**

3

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6

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12

13 **Abstract**

14 Understanding the effects of plastic debris on marine ecosystems is essential in encouraging
15 decision-makers to take action. The present study investigates the effect of a 24h
16 experimental exposure to high density polyethylene (HDPE) microplastics (MPs) of different
17 sizes (4-6, 11-13 and 20-25 μm) and at three concentrations (0.1, 1 and 10 mg MP.L^{-1}) on
18 the development and locomotor activity of early stages of Pacific oyster, *Crassostrea gigas*.
19 The bivalve embryo-larval assay (AFNOR, XP T90-382) was used in this study but with
20 additional toxicity criteria: the developmental arrests, abnormal D-larvae, maximum speed
21 and swimming trajectory. Copper (Cu), was used as a positive control. Our results show that
22 smaller MPs (4-6 and 11-13 μm) induced higher rates of malformations and developmental
23 arrests than the larger ones (20-25 μm). In addition, a dose-dependent decrease of
24 maximum swimming speed was observed for larvae exposed to MPs of 4-6 and 11-13 μm .
25 On the other hand, there was no significant difference in swimming speed with the largest
26 MPs size tested (20-25 μm). For all three sizes of MP, there was a decrease in straight-line
27 swimming trajectories, and an increase in circular trajectories. This abnormal swimming
28 behaviour could affect larvae survival as well as colonization of new habitats.

29

30 **Keywords:** Polyethylene microplastics; Pacific oyster; early life stage; development;
31 swimming behaviour

32

33 **Capsule:** Polyethylene microplastics of different sizes induce differential deleterious effects
34 on development and swimming activity of oyster D-larvae

35

36 **1. Introduction**

37 Plastic debris pollution is on the increase, and has been identified as major emerging global
38 problem affecting marine organisms and humans alike (Sutherland *et al.*, 2010; Caruso,
39 2015; Wang *et al.*, 2016).

40 In 2017, over 348 million tons of plastic were manufactured (PlasticsEurope, 2018). Eighty
41 percent of plastic production in Europe is made up of 6 main polymers (PlasticsEurope,
42 2016): polypropylene (PP), HDPE and LDPE (high and low-density polyethylene), polyvinyl
43 chloride (PVC), PUR (polyurethane), polyethylene terephthalate (PET) and polystyrene (PS).
44 Jambeck *et al.* (2015) estimated that between 2 and 5% of these plastics are discharged into
45 oceans. In recent years, microplastics (MPs) have been increasingly in the public eye. MPs
46 are defined as any forms of plastic particle between 1 μm and 5 mm size (Cole *et al.*, 2011;
47 Wagner *et al.*, 2014), while nanoplastics are defined as any plastic less than 100 nanometres
48 in length (Lambert and Wagner, 2016; Gigault *et al.*, 2016). While micro and nanoplastics are
49 often the result of fragmentation and degradation of larger volumes of plastic, they can also
50 be released directly into the environment.

51 MPs are ubiquitous in marine water with reported concentrations ranging from 1.31 to
52 102,000 particles per m^3 (Van Cauwenberghe *et al.*, 2015; Auta *et al.*, 2017). PP and PE are
53 the most common polymers in all environmental compartments (Isobe *et al.*, 2014; Enders *et*
54 *al.*, 2015; Frere *et al.*, 2017). These plastics persist in the environment due to their resistance
55 to biodegradation (Yoshida *et al.*, 2016) but also to chemical, photochemical and mechanical
56 degradation (Cooper *et al.*, 2010).

57 During the 2009 International Coastal Cleanup, a number of different types of marine debris
58 were collected from coastlines and waterways around the world: cigarettes, bags, food
59 wrappers, caps, beverage bottles, cups, plates, spoons, etc. (Kershaw *et al.*, 2011). Recent
60 publications have reported a high percentage (80%) of small MPs (25-50 μ m) in surface water
61 or sediment (particles for which few data are available), when compared with larger particles
62 (Enders *et al.*, 2015; Bergmann *et al.*, 2017). The estimated concentration of MPs in surface
63 seawater along European coastlines is 2.505 mg.m⁻³ for sizes between 10 and 1000 μ m
64 (Paul-Pont *et al.*, 2018).

65 Deleterious effects of MPs on feeding processes, behaviour, metabolism anomalies of
66 holoplanktonic, meroplanktonic and benthic aquatic organisms have been reported for PS
67 (Gerritsen and Porter, 1982; Holland *et al.*, 1986; Ward and Targett, 1989; Solow and
68 Gallagher, 1990; Hart, 1991; Mayer, 1994; Ward, 1996; Baer *et al.*, 2008). Only a few studies
69 have focused on PE effects, particularly on blue mussel (*Mytilus sp.*) with effects on tissues
70 and cells (D  tr  e *et al.*, 2017; Von Moos *et al.*, 2012). Green, 2016 has shown that PE can
71 influence the assembly of European oyster populations (*Ostrea edulis*). Au *et al.*, 2015
72 observed that freshwater amphipods, *Hyalella azteca*, exposed to PE (fluorescent blue PE,
73 10 to 27 μ m) showed growth and reproduction alterations after 10 to 42 days of exposure.
74 Beiras *et al.*, 2018 showed that exposure of zooplankton to PE (low-density PE, 1 to 500 μ m)
75 did not cause any significant toxic effects after 12 days of exposure. Cole *et al.*, 2015,
76 observed the ingestion of MPs by the Pacific oyster larvae. In addition, a significant and
77 linear increase in MPs uptake with increasing concentrations was observed at different larval
78 stages of *Mytilus galloprovincialis* (Capolupo *et al.*, 2018).

79 The present study focuses on the potential effects of PE for their high occurrence in the
80 oceans. Indeed, the vast majority of equipment used by aquatic farmers is made of polymers,
81 particularly PE. The French shellfish industry produced 216,917 tons of marketable shellfish
82 during the 2015-2016 year (CNC, 2016), giving it one of the largest outputs in the world. Of
83 that figure, the Poitou-Charente region was the most productive, accounting for 23% of the

84 national total (CNC, 2016). It is therefore essential for better understanding the interaction of
85 Pertuis Charentais' environment with oysters.

86 In addition to plastic pollution, coastal areas undergo several others anthropic pressures
87 such as Copper (Cu) based fungicides widely used in viticulture to combat downy mildew in
88 the Nouvelle-Aquitaine region (Singh, R.S., 2000), along with antifouling paint (Gatidou *et al.*,
89 2007). In this study, copper will serve as a positive control.

90 The objective of this paper is to study the embryo-larval development and swimming
91 behaviour of D-larvae of oysters (*C. gigas*) in response to direct acute exposure to HDPE
92 micro-particles of three different sizes without ingestion. Indeed, oyster embryos and larvae
93 of less than 24 hours old feed exclusively on their vitelline reserves and they cannot ingest
94 particles.

95

96 **2. Material and methods**

97 **2.1 Animal collection**

98 Mature oysters (diploid male and female), *C. gigas* (Bayne *et al.*, 2019; Bayne *et al.*, 2017)
99 came from a commercial hatchery specialized in production of mature oysters (France
100 Naissain, France). Oysters were kept dry at 4°C for two days to get better spawning outside
101 the natural breeding season and then. Oysters were acclimatized in 18 °C filtered seawater
102 one hour before the beginning of experiments.

103

104 **2.2 Chemicals and seawater**

105 Seawater was collected from the Ile de Ré (SW France). After sampling, seawater was
106 filtered using membrane filter of 20 µm, 5 µm, 1 µm and 0.2 µm to eliminate debris and
107 microorganisms. Filtered seawater was stored at 4°C with continuous bubbling and was used
108 within 7 days. A few hours before the experiment, filtered seawater was filtered again at 0.2
109 µm. Water and sand samples were taken from the Atlantic coast.

110 Cu (CuSO₄) and formaldehyde were purchased from Sigma-Aldrich Chemical (St. Quentin
111 Fallavier, France). Three sizes of PE MPs (HDPE) were used (4-6 µm MPP-635 XF, 11-13

112 μm MPP-635 G and 20-25 μm MPP-1241, density 0.96, Micropowders Inc. USA). Stock
113 solutions of $100 \mu\text{g.L}^{-1}$ for Cu and 100 mg MP.L^{-1} for MPs were prepared in pure Milli-Q-
114 water (Milipore) for Cu and in filtered seawater for MPs. Working solutions were obtained by
115 diluting the stock solutions in filtered seawater and conserve at 4°C and in the dark for MPs.
116 Before each exposure, working solutions were centrifuged at low speed for 2 min. Three
117 concentrations of exposure were selected according to previous work for Cu: 0.1, 1 and 10
118 $\mu\text{g.L}^{-1}$ and for MPs: 0.1, 1 and 10 mg.L^{-1} (Beiras *et al.*, 2018). The selected concentrations in
119 MPs are higher than what can be found in the environment. For Cu, the range of
120 experimental concentrations was chosen based on preliminary studies (Mai *et al.*, 2012) in
121 order to get a complete dose-response curve.

122

123 **2.3 Exposure solutions analysis**

124 For quantitative analysis of PE MPs at different concentrations (0.1, 1 and 10 mg MP.L^{-1}),
125 each solution was tested in a flow cytometer (Attune Acoustic Focusing Cytometer). Two-
126 millilitres samples were prepared from the MP solution used for embryo-larval exposures
127 ($n=4/\text{condition}$). The samples were vortexed (StarLab Vortex IR, 12,000 rpm for 20 sec)
128 before being transferred into the cytometer to homogenize the solution. $300 \mu\text{L}$ were taken
129 for flow cytometry analysis. Calibration was performed to obtain an analysis rate of 500
130 $\mu\text{L.min}^{-1}$ and a saturation of 10 000 particles maximum detected. Control analytical samples
131 ($n = 12$) were made of filtered seawater (without the presence of MPs) to obtain a blank and
132 remove background if necessary (particles naturally present in the water).

133 Chemical analyses of Cu were carried out in sandy sediments (surface and semi-depth 5 cm)
134 and in coastal seawater close to shellfish areas. This gave us an indication of the
135 environmental concentrations found. The water was acidified to 5% and then analyzed and
136 diluted to one-third. For the sediment, 200 mg was weighed and then acidified with 6 mL of
137 nitric acid and 2 mL of hydrochloric acid. The sediment was then mineralized by microwave
138 (rise in temperature at 120°C and then plateau at 120°C for 30 minutes, then cooling to
139 room temperature). The resulting mineralization was topped up to 50 mL with ultra-pure

140 water. The prepared samples were then assayed by ICPMS (Inductively Coupled Plasma
141 Mass Spectrometry, Thermofisher, X, Model II). The limit of quantification was equivalent to
142 0.02 $\mu\text{g}\cdot\text{L}^{-1}$.

143

144 **2.4 Experimental design**

145 Mature oysters were cleaned to avoid any external contaminations and remove the
146 microorganisms attached to them. Male and female oysters were induced to spawn by
147 thermal stimulation (alternating immersion in filtered seawater of 18 °C and 28 °C for 30 min)
148 or by stripping the gonad where thermal stimulation was ineffective (Mai *et al.*, 2013 and
149 Gamain *et al.*, 2017). Spawning males and females were individually isolated in beakers (500
150 mL of filtered seawater) at their respective spawning temperatures (LM Parker *et al.*, 2009).
151 Individuals were left undisturbed for 10 min and then removed from beakers. Eggs and
152 sperm from two individuals were selected to give a single pairing. Sperms and eggs were
153 sieved separately through 50 μm and 100 μm meshes (Sefar Nitex), respectively to eliminate
154 debris and faeces. Sperm mobility was verified and the number of eggs was counted under
155 stereomicroscope (Nikon) at a magnification of 100.

156 Eggs were fertilized with sperm in ratio of 1:10 (egg:sperm). Fertilization success was
157 verified under a microscope, and embryos were then counted and transferred to a 24-well
158 microplate (Greiner Bio-One, Cellstar) for embryotoxicity assays following previously
159 published protocols (His *et al.*, 1997; Quiniou *et al.*, 2005; NF ISO 17244, 2015). MP
160 solutions were resuspended before injecting them into the microplates. Fertilized eggs
161 (around 350 eggs/well) were exposed in wells containing 2 mL of toxicant solution and were
162 incubated in the climatic chamber during 24h at 24 °C in the dark to obtain an optimized
163 development (Robinson *et al.*, 1992 and AFNOR 2009).

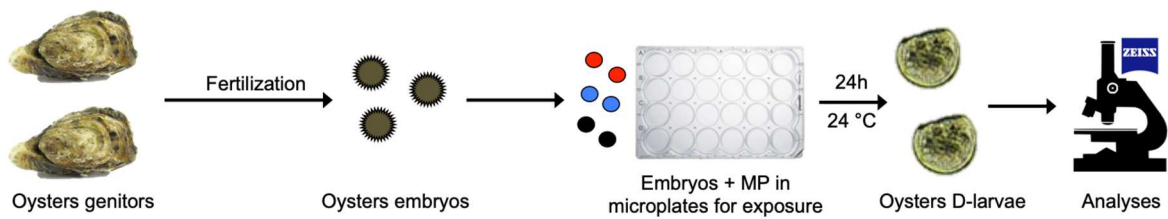
164

165 **2.5 Swimming activity**

166 After 24h (e.g. 1 dpf, day post fertilization) of incubation, the swimming behaviour was
167 recorded under a microscope (ZEISS Axio Observer Z1, UMR LIENSs, La Rochelle, France)

168 with a magnification of 100 in natural light condition and in air conditioned-room at 24 °C.
169 Two-minute videos were recorded (2 or 3 videos per condition, and per replicate). In this
170 experiment, a replicate corresponds to a pair of oysters (couple) having obtained fertilization
171 success. Three different couples were used and four analytical replicates were performed for
172 each condition. The experimental design presented above is shown schematically in Figure
173 1.

174



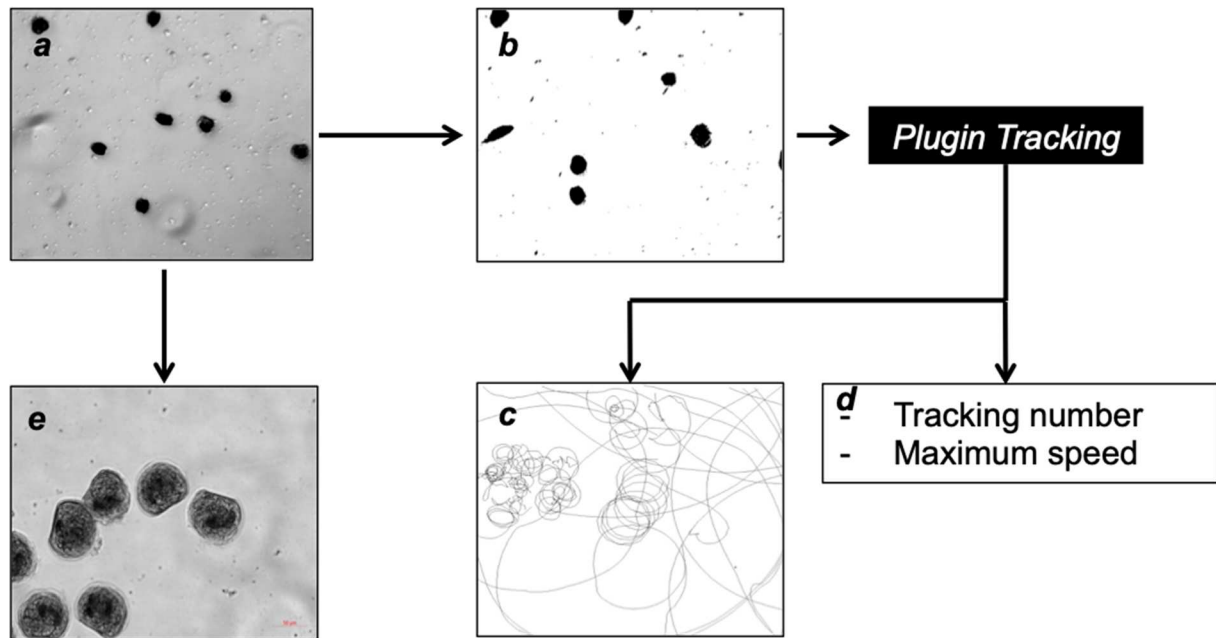
175

176 **Figure 1.** Experimental design. After a 24h-exposure to Cu or MPs, oyster D-larvae malformations
177 and swimming behaviour were analysed. A 2 min-video was recorded for each batch of larvae and
178 then abnormal D-larvae were quantified after formaldehyde fixation.

179

180 A freeware application (VirtualDub, Windows) for video conversion was used to subsample
181 films to 4fps and convert them to AVI format (Gamain *et al.*, 2019). ImageJ (1,52a software)
182 was used to analyse different videos. Videos (AVI format) were converted to grayscale image
183 (Figure 2a), then converted into a binary stack of images (Figure 2b). Swimming parameters
184 (Figure 2d) of each individual D-larvae numbered as maximum speed (pixel/s) and swimming
185 trajectories were calculated using the wrMTrck plugin (Figure 2d). As a result, each tracked
186 larva was assigned a number, with each number used to identify the tracked larvae in the
187 results file (Figure 1). We identified three different types of larval path: (1) rectilinear; (2)
188 circular and (3) motionless following protocol published by Gamain *et al.*, 2019 with
189 improvements (Table 1). The use of a graphic tablet (Wacom Bamboo Pen&Touch) and
190 image processing software (Photos, Windows 10) allowed swimming trajectories to be
191 quantified and characterised. In this study, we used a single condition (control) without

192 contaminant for trajectory analysis. Once the videos were processed with the plugin, we take
 193 back video analysis to ensure that the same larvae were not detected more than once.
 194



195
 196 **Figure 2.** Procedure for swimming behaviour analysis of oyster D-larvae. (a) Original image after
 197 conversion. (b) Binary image. (c) Traces of larval path. (d) Parameters studied. (e) Quantification of
 198 abnormal D-larvae and developmental arrest after fixation with formaldehyde (1%).

199
 200 **Table 1.** Paths analysis of oyster D-larvae with three categories of trajectories: rectilinear, circular and
 201 motionless.

Rectilinear	Circular	Motionless
Straight or lightly curved lines + paths without any pattern	Large circles + paths with a repeating pattern or loops	Small circles + large points

212 **2.6 Developmental abnormalities**

213 After video recording, 25 μ L of 1% buffered formalin were added to each well, and the
214 percentage of abnormal oyster larvae (Figure 2e) and developmental arrest were recorded
215 (His *et al.*, 1999; Quiniou *et al.*, 2005). Abnormal larvae and developmental arrest were
216 directly observed an inverted microscope (Nikon eclipse). An important prerequisite for this
217 test is the presence, in control condition of less than 20% of abnormal larvae (Quiniou *et al.*,
218 2005). Three different couples were used and four replicates were performed for each
219 condition.

220

221 **2.7 Statistics**

222 All data are expressed as means \pm standard error of the mean (SEM). If data did not follow a
223 normal distribution, it was transformed using the formula: $P' = \arcsin \sqrt{r}$; P corresponds to raw
224 data (frequency of abnormalities) specified in P values from 0 to 1 (Legendre and Legendre,
225 1998). Homogeneity of variance (Levene's test) was verified and statistical analysis was
226 performed by Kruskal-Wallis test. Differences between tested concentrations means were
227 then performed using Kruskal Nemenyi Post-hoc test (equivalent to the Tukey test for non-
228 parametric data). Significance difference was accepted when p-value < 0.05. Statistical
229 analysis was conducted using R, and graphs prepared using Microsoft Excel.

230

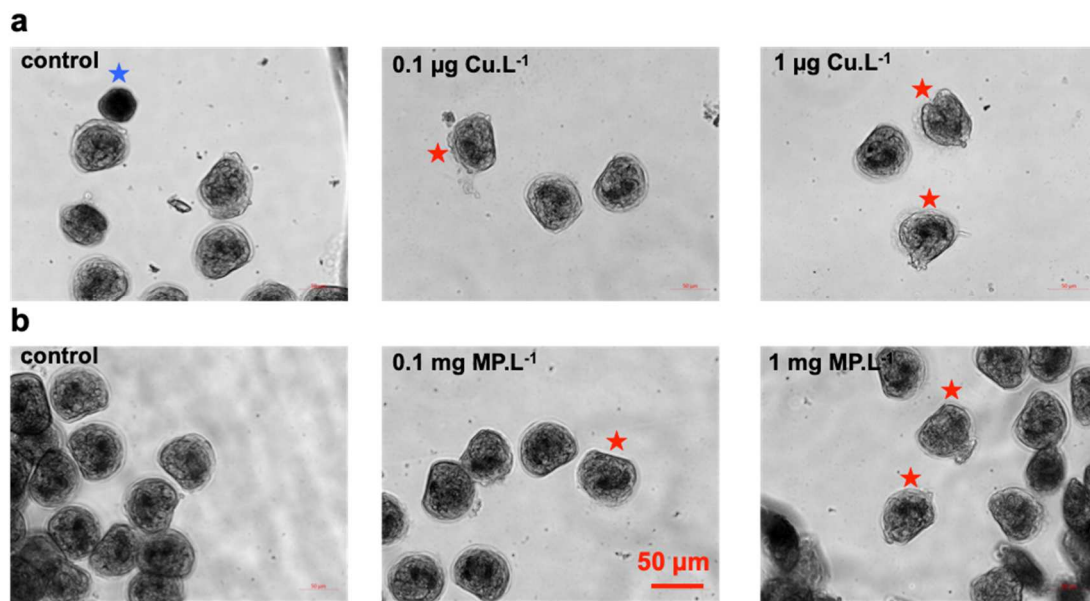
231 **3. RESULTS**

232 **3.1 Effects of different sizes of polyethylene microplastics on embryo-larval** 233 **development (abnormal D-larvae of *C. gigas*)**

234 Different abnormalities (abnormal shell, convex shell, scalloped shell, incomplete shell and
235 mantle abnormality) observed in oyster D-larvae (Mottier *et al.*, 2013) were recorded after
236 24h exposure to copper (Figure 3.a) and PE MPs (Figure 3.b and c). Abnormal larvae and
237 developmental arrest average frequencies were respectively $12.8 \pm 1.3\%$ and $4.6 \pm 0.5\%$ for
238 control conditions (Figure 4 and supplementary data, Table S1). The percentage of

239 developmental arrest was significant from 0.1 $\mu\text{g Cu.L}^{-1}$ ($12.9\pm 2.5\%$) and increased to
240 $37.8\pm 9.7\%$ (Figure 4.a and supplementary data Table S1). For the exposure to different sizes
241 of PE MPs, the number of malformed oyster D-larvae increased significantly from the lowest
242 PE MPs concentration (0.1 mg MP.L^{-1}) for the 4-6 μm (Figure 4.b) and 11-13 μm particle
243 sizes (Figure 4.c). The percentage of developmental arrest showed a dose-dependent
244 increase for the 11-13 μm PE MPs (Figure 4.c) with a maximum effect at the highest tested
245 concentration ($17.7\pm 0.7\%$ at 10 mg MP.L^{-1}). For the larger MPs (20-25 μm), significantly
246 different malformation rates were observed from the highest concentration at 10 mg MP.L^{-1}
247 ($16.3\pm 1.5\%$, Figure 4.d). There are no significant differences in developmental arrest with
248 embryo-larval exposure to MP 20-25 μm (Figure 4.d).

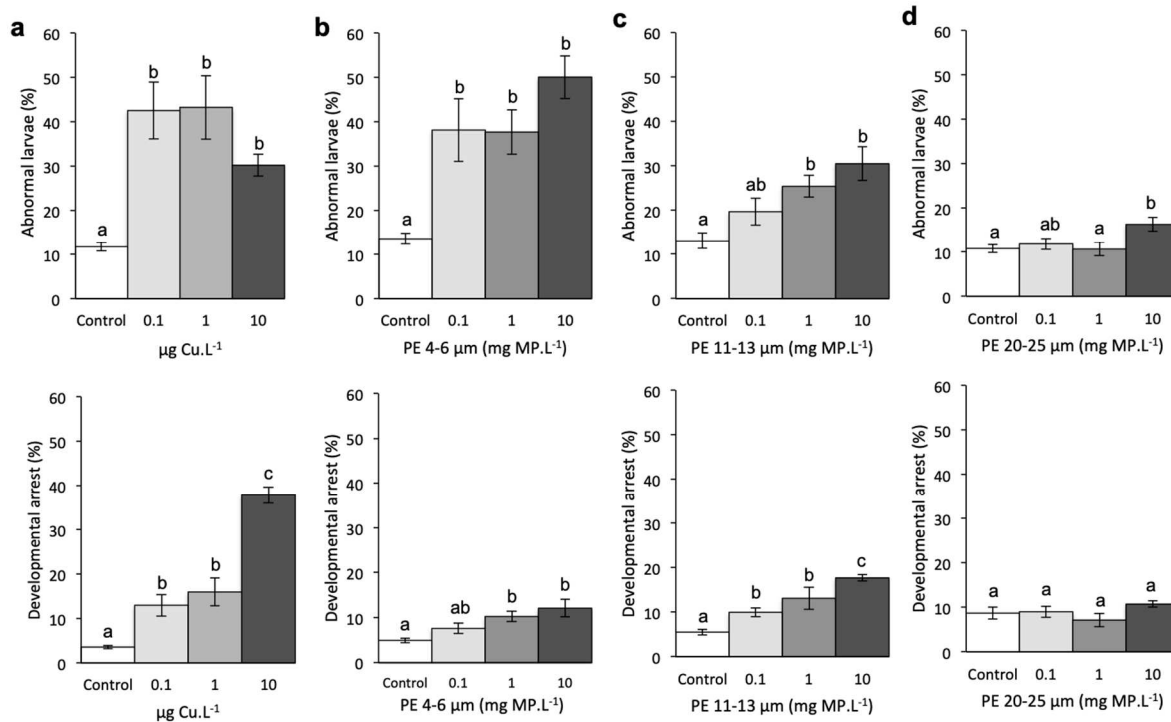
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250

251 **Figure 3.** Photographs of normal, abnormal D-larvae (red star) of *C. gigas* and developmental arrest
252 (blue star) after 24h of exposure to 0.1 and 1 $\mu\text{g.L}^{-1}$ of Cu (**a**) or to 0.1 and 1 mg.L^{-1} of PE MPs 4-6 μm
253 (**b**). ZEISS Axio Observer Z1 photographs (x20).

254



255

256

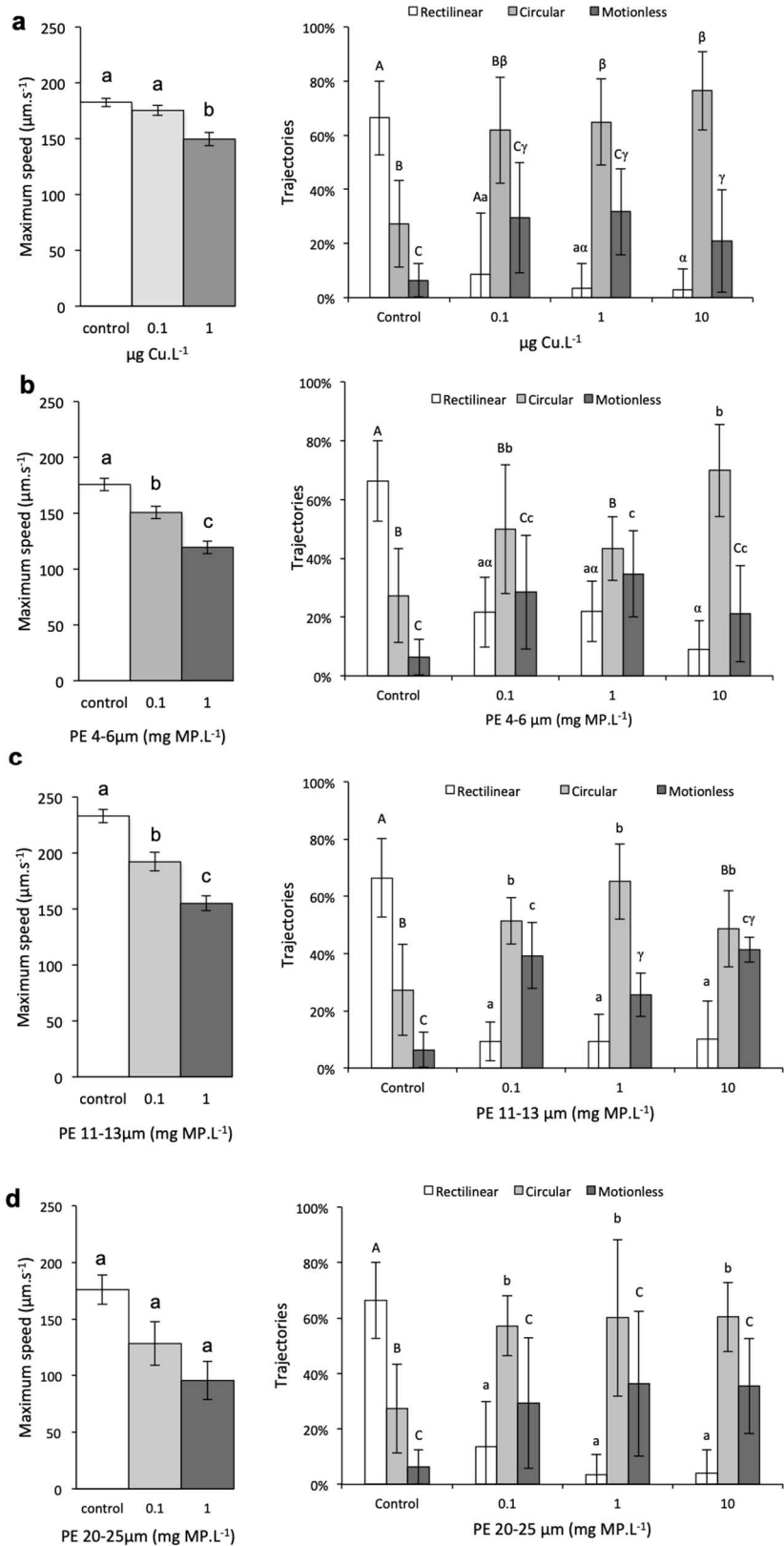
257 **Figure 4.** Abnormal larvae (%) vs. concentrations (Mean \pm SEM) of oyster D-larvae of *C. gigas*
 258 (above) and developmental arrest (%) vs. concentrations (bottom) following 24h oyster embryonic
 259 exposure to different concentrations of Cu (a) and PE MPs of 4-6 μm (b), 11-13 μm (c) and 20-25 μm
 260 size (d). Control (without pollutants): 0.1, 1 and 10 correspond to concentrations of Cu ($\mu\text{g Cu.L}^{-1}$) and
 261 PE MPs using for exposures (mg MP.L^{-1}). Values are mean \pm SEM for three replicates. Different
 262 letters indicated significant differences between different concentrations ($p < 0.05$).

263

264 3.2 Effects of different sizes of polyethylene microplastics on D-larvae swimming 265 activities (maximum speed recorded and swimming trajectories) of *C. gigas*

266 In control condition (filtered seawater), the average speed of oyster D-larvae was 109 ± 3
 267 $\mu\text{m.s}^{-1}$ and the maximum speed was 191 ± 7 $\mu\text{m.s}^{-1}$ (supplementary data, Table S2) while
 268 $66.4 \pm 13.7\%$ of the D-larvae swam following a rectilinear trajectory and $27.3 \pm 16.0\%$ shown
 269 circular paths (Figure 6). Motionless larvae were rare within the control group with $6.4 \pm 11.1\%$
 270 (Figure 5). After $1 \mu\text{g Cu.L}^{-1}$ exposure, the maximum speed significantly decreased
 271 compared to control and the percentage of circular and motionless trajectories increased
 272 significantly, whereas rectilinear trajectory declined (Figure 5.a). After exposure to MP 4-6

273 μm , a decrease in maximum recorded swimming speeds was observed for the
274 concentrations at 0.1 and 1 mg MP.L⁻¹ (with respectively 151±5 and 119±6 $\mu\text{m}\cdot\text{s}^{-1}$, Figure
275 5.b). We also observed a strong increase in circular trajectories for MP-exposed larvae, with
276 49.9±22.0% at 0.1 mg MP.L⁻¹ and 69.8±15.6% at 10 mg MP.L⁻¹ condition (Figure 5.b). For
277 11-13 μm MPs, the same trend can be seen with a significant decrease in maximum
278 swimming speed. Effectively, at 0.1 and 1 mg MP.L⁻¹ we find respectively 192±8 and 155±6
279 $\mu\text{m}\cdot\text{s}^{-1}$ (Figure 5.c). At concentrations 0.1 and 1 mg MP.L⁻¹, there is a significant increase in
280 circular trajectories (with respectively 51.4±8.1 and 65.1±13.1%, Figure 5.c). While the
281 straight paths decrease significantly for all 11-13 μm MP concentrations. Motionless
282 trajectories, on the other hand, increase with concentrations in MP. However, there were no
283 significant differences in maximum speed recorded of D-larvae exposed to MP 20-25 μm
284 (Figure 5.d). The trajectories analysed show an increase in "circular" and a reduction in
285 straight-line trajectories (Figure 5.d).



286

287 **Figure 5.** Maximum speed and trajectories of D-larvae of *C. gigas* following 24h embryonic exposure
 288 to different concentrations of Cu (**a**) and PE MPs of 4-6 µm (**b**), 11-13 µm (**c**) and 20-25 µm (**d**).
 289 Control (without pollutants): 0.1, 1 and 10 correspond to concentrations of Cu (µg Cu.L⁻¹) or PE MPs

290 (mg MP.L⁻¹) used for exposures. Values are mean ± SEM for three replicates. Different letters
291 indicated significant differences between different concentrations (p<0.05).

292

293 **3.3 Exposure solution analysis**

294 Concentrations of the different sizes of PE MPs were measured using flow cytometry in the
295 filtered seawater working solutions, (Table 2). For 4-6 µm MPs we found dilution factors of
296 8.7 and 9.3. These calculated dilution factors are consistent with theoretical concentrations.
297 For 11-13 µm MPs, the first dilution factor was 3 for 0.1 mg MP.L⁻¹, which is quite low
298 compared to the theoretical dilution factor of 10. For 20-25 µm MPs, the dilution factors of 6
299 and 6.8 are in adequacy with the theoretical concentrations of exposures.

300

301 **Table 2.** Theoretical and measured MP concentrations (mean±SEM) in the working solutions at the
302 beginning of the embryotoxicity test.

303

Conditions	Theoretical (mg.L ⁻¹)	Measured (MP.µL ⁻¹)	Measured (MP.mL ⁻¹)	Dilution factor
MP 4-6 µm	0.1	0.3±0.0	300	-
	1	2.6±0.1	2600	8.7
	10	24.1±0.7	24100	9.3
MP 11-13 µm	0.1	0.3±0.0	300	-
	1	0.9±0.0	900	3
	10	16.1±0.1	16100	17.9
MP 20-25 µm	0.1	0.2±0.0	200	-
	1	1.2±0.0	1200	6
	10	8.2±0.2	8200	6.8

304

305

306

307 **4. Discussion**

308 The present study is the first to focus on swimming behavior of oyster larvae (*C. gigas*)
309 exposed to PE MPs. It is an experimental integrative approach, designed to complement the
310 previously published results on the effects of PS MPs on oyster embryos and larvae (Tallec
311 *et al.*, 2018; Cole *et al.*, 2015; Sussarellu *et al.*, 2016).

312 Through this study, the main objective is to evaluate the impact of MP exposure (HDPE
313 spherical particles) on the development and swimming activity of oyster embryos and larvae
314 (early stages of development). It is important to understand that our swimming analyses
315 consider two-dimensional rather than three-dimensional movement (Gamain *et al.*, 2019). A
316 potential way of improving this in the future will be to capture behaviour in three dimensions,
317 thus covering the whole water column.

318 A flow cytometry verification was set up to determine actual concentrations of MP particles in
319 the exposure media. Long *et al.*, 2017, had also determined MP concentrations through flow
320 cytometry. Apart from the concentration at 1 mg MP.L⁻¹ for PE 11-13 µm, the other
321 concentrations seem consistent to the expected theoretical concentrations. The
322 concentrations used in this study are high compared to what is currently found in the marine
323 environment (Van Cauwenberghe *et al.*, 2015; Auta *et al.*, 2017).

324 In filtered seawater, oyster D-larvae adopted mainly a straight-line trajectory as already
325 reported by Gamain *et al.* (2019) for the same species. Circular and motionless trajectories
326 are considered as an abnormal swimming behaviour of D-larvae (His *et al.*, 1999; Gamain *et*
327 *al.*, 2019). The average swimming speed recorded for this study was 100 µm.s⁻¹ lower than
328 this recorded by Gamain *et al.*, (2019). These differences of behaviour could be explained by
329 the fact that fertilization was carried out during the spawning period (June-August) whereas
330 in our study, the fertilizations were realized during the October-November period. The
331 temperature when recording videos plays also an important role and was maintained during
332 our experimentation to 24 °C.

333 For all controls, abnormal D-larvae was below 20% and the mean rate was 12.8±1.3%.
334 Exposure to Cu induced a significant increase of abnormal D-larvae from the first

335 concentration tested e.g. $0.1 \mu\text{g Cu.L}^{-1}$ which is above the current water concentration of Cu
336 ($0.06 \mu\text{g Cu.L}^{-1}$) detected in the coastal waters of Pertuis Charentais in 2019. Regarding
337 development arrests, a significant increase was observed from $0.1 \mu\text{g Cu.L}^{-1}$. These results
338 are higher than in the paper of Gamain *et al.*, 2016 for the same copper concentrations but is
339 in adequacy with Gamain *et al.*, 2019.

340 Greater toxicity of smaller plastic particles were reported by Tallec *et al.*, 2018 for PS
341 nanoplastics (500 and 50 nm) compared to MP ($2 \mu\text{m}$) on fertilization success and embryo-
342 larval development of oyster *C. gigas*. With the same tested concentrations of PE particles,
343 we observed a significant increase of developmental anomalies. From the results of our
344 study, we can conclude that the smaller the PE MPs are, the more deformities and
345 developmental arrests are visible on the oyster larvae. Different impacts on D-larvae have
346 been reported following the exposure of spawners. A decrease in size and growth of oyster
347 larvae were reported following exposure to PS MPs (Sussarellu *et al.*, 2016). Spherical
348 particles are the most commonly used in laboratory studies, but fibers and fragments are the
349 most common forms detected in wild organisms (de Sá *et al.*, 2018). Mesaric *et al.* (2015)
350 investigated acute toxicity and performed swimming tests on *Artemia salina* larvae with MP
351 concentrations between 0.01 and 1 mg.L^{-1} . They reported that nanomaterials can bind on
352 external surfaces of *A. salina* larvae and affect their swimming activity. In addition to testing
353 the effect of PE MPs on the early stages of *C. gigas*, copper exposures were performed in
354 order to have the presence of additional, better referenced control (MacInnes *et al.*, 1979;
355 Wang *et al.*, 2011; Mai *et al.*, 2012; Gamain *et al.*, 2017; Sussarellu *et al.*, 2018).

356 With respect to swimming behaviour (recording of maximum speed), larvae for control
357 conditions (1 dpf) had a maximum swimming speed between 160 and $182 \mu\text{m.s}^{-1}$. Larvae
358 exposed to the Pertuis Charentais environmental concentration had no altered swimming
359 speed. A decrease in swimming speed was observed from $1 \mu\text{g Cu.L}^{-1}$. Contrary to this
360 result, Gamain *et al.*, 2019 did not observe any effects on swimming behaviour at any of
361 these Cu concentrations.

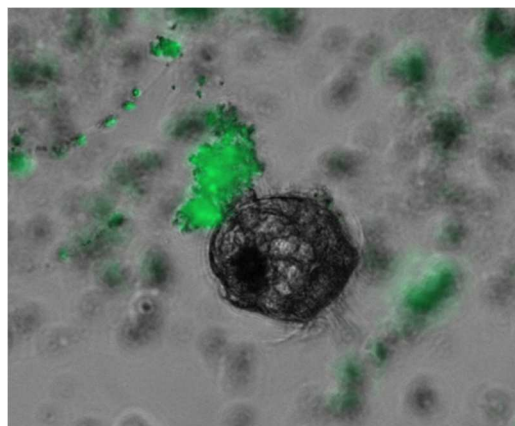
362 In our MP exposure conditions (24 °C, salinity of 33 usi, in the dark), the average speed and
363 maximum swimming speed recorded for oyster larvae are respectively 109 $\mu\text{m}\cdot\text{s}^{-1}$ and 192
364 $\mu\text{m}\cdot\text{s}^{-1}$ for control condition (without MP). According to Suquet *et al.*, 2012, the average
365 speed recorded for oyster larvae without contamination was 105 $\mu\text{m}\cdot\text{s}^{-1}$. The results would
366 seem rather similar, despite the fact that they used a development temperature of 19 °C,
367 whereas ours was 24 °C. In comparison with Gamain *et al.*, 2019, which found swimming
368 values much higher at 144 and 297 $\mu\text{m}\cdot\text{s}^{-1}$ for average speed and maximum swimming
369 speed, respectively. The differences in values could be explained by the quality of the
370 parents but also the period of the year and the day when the videos are recorded. We also
371 kept the optimal temperature of development during the video recording which may be the
372 cause of the differences in swimming speed between these two studies.

373 For the small (4-6 μm) and medium size (11-13 μm) of PE particles, a dose-dependent
374 decrease in swimming speed was observed, while for the larger ones (20-25 μm), no effect
375 was reported. Lee *et al.* (2013) tested three different sizes of PS MPs (0.05, 0.5 and 6 μm in
376 diameter) on the marine copepod, *Tigriopus japonicus* in a two-generation chronic toxicity
377 test. Their results highlighted that nanoplastics (0.05 μm and 0.5 μm) but not microplastics (6
378 μm) affect the survival of nauplii and copepodites in the F0 and F1 generations.

379 The analysis of the trajectories taken by the larvae during their displacements shows, for all
380 the tested MPs, a decrease in straight-line trajectories and an increase in circular
381 trajectories. This alteration of swimming behavior could lead to behavioral differences in
382 larvae's ability to predate but especially in their ability to react to a predator. It seems that the
383 smaller the plastics, the more the effects are noticeable on swimming behavior but also on
384 developmental anomalies. This is comparable to the results of Tallec *et al.*, 2018, where
385 nanoplastics had more effects on larval development than coarser MPs.

386 There may also be effects on the survival, fitness, dispersion of larvae and colonization of
387 new habitats (Gamain *et al.*, 2019). The observed effects of early embryo-larval exposure to
388 MPs in oysters could have affected the following developmental stages (pediveliger, spat and
389 adults). Indeed, a recent publication shows that blue mussels, *Mytilus edulis*, have difficulty

390 attaching to their substrate when in the presence of MPs (Green *et al.*, 2019). More recently,
391 Yin *et al.*, 2019, have shown that black rockfish, *Sebastes schlegelii*, exposed to PS MPs of
392 15 μm to 0.19 $\text{mg}\cdot\text{L}^{-1}$, had drastic reduction in their swimming speeds as well as reduction of
393 range of movement, which may affect hunting behavior and exploration competence.
394 The impact of PE MPs on swimming behaviour could be explained by interactions of these
395 particles with larval cilia (Figure 6). Beiras *et al.* (2018) reported that PE particles (4-6 μm)
396 can stuck on villi of the chorion of *Oryzias melastigma* embryos. Agglomerates of MPs tend
397 to settle and stick along the mantle of organisms, as with nanoplastics on brine shrimp *A.*
398 *franciscana* on their antennules and abdomen (Bergami *et al.*, 2016). Nanosized latex
399 particles (39.4 nm) have shown to be adsorbed on the fertilized egg surface of Japanese
400 medaka, *Oryzias latipes* (Kashiwada, 2006). Like Medaka egg chorions, oyster D-larvae
401 have cilia to move in the water column. This would explain the fact that D-larvae exposed to
402 MPs exhibited altered swimming behaviour.
403 Altogether, these results support the hypothesis that the smallest MPs at high concentrations
404 can trigger deleterious effects on early life stage of Pacific oyster over short-term exposure.
405 Future studies are needed to verify whether LDPE MPs have similar effects on D-larvae and
406 investigate the impacts of ingestion of nanometer-sized PE particles on D-larvae. It would
407 also be particularly relevant to evaluate the long-term effects of chronic exposure to
408 environmental concentrations of MPs.
409



410
411

412 **Figure 6.** D-larva with MP stuck in her locomotor eyelashes. Screenshot from a video recorded with
413 the ZEISS Axio Observer Z1 microscope (x20). In order to observe MP behaviour, we used
414 fluorescent microbeads (1-5 μm , Cospheric).

415

416 **5. Conclusions**

417 In conclusion, our experimental results highlight that small (4-6 μm) and medium sizes (11-13
418 μm) HDPE particles at high concentrations are toxic for *C. gigas* early life stage. However, in
419 comparison to the effects of Cu, the toxicity of HDPE MPs is much lower. Our data suggest
420 that small HDPE particles (4-6 μm) are the most toxic to the embryo-larval development of
421 oyster. PE of 20-25 μm had very little effect on development anomalies and developmental
422 arrest. With regard to swimming behaviour, D-larvae of *C. gigas* are more sensitive to PE
423 particles < 15 μm . Further research on early life stages of bivalves and other invertebrates, is
424 crucial in strengthening the knowledge base required to establish recommendations and
425 potential long-term effects of using plastics in coastal areas and estuaries.

426

427 **Supplementary data**

428 Supplementary data associated with this article can be found in the online version.

429

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442

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657 **SUPPLEMENTARY DATA**

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659 **Table S1.** Frequency of abnormal D-larvae (%) and developmental arrest (%) in D-larvae of
 660 *C. gigas* exposed to Cu or PE MPs. Control (without pollutants): 0.1, 1 and 10 correspond to
 661 concentrations of copper ($\mu\text{g Cu.L}^{-1}$) or PE MP (mg MP.L^{-1}) using for exposures. Means
 662 values \pm SEM. Different letters indicated significant differences between different
 663 concentrations ($p < 0.05$).

664

Conditions	Abnormal larvae	Developmental arrest
Control	12.8 \pm 1.3 ^a	4.6 \pm 0.5 ^a
Cu 0,1	42.5 \pm 6.4 ^b	12.9 \pm 2.5 ^b
Cu 1	43.2 \pm 7.2 ^b	15.9 \pm 3.2 ^b
Cu 10	30.2 \pm 3.3 ^b	37.8 \pm 9.7 ^c
MP (4-6 μm) 0,1	38.1 \pm 7.1 ^b	7.6 \pm 1.1 ^{ab}
MP (4-6 μm) 1	37.7 \pm 5.0 ^b	10.2 \pm 1.1 ^b
MP (4-6 μm) 10	50.1 \pm 4.8 ^b	12.1 \pm 1.9 ^b
MP (11-13 μm) 0,1	19.7 \pm 3.0 ^{ab}	9.9 \pm 1.0 ^b
MP (11-13 μm) 1	25.4 \pm 2.4 ^b	13.1 \pm 2.5 ^b
MP (11-13 μm) 10	30.5 \pm 3.8 ^b	17.7 \pm 0.7 ^c
MP (20-25 μm) 0,1	11.8 \pm 1.0 ^{ab}	9.0 \pm 1.3 ^a
MP (20-25 μm) 1	10.6 \pm 1.5 ^a	7.1 \pm 1.5 ^a
MP (20-25 μm) 10	16.3 \pm 1.5 ^b	10.7 \pm 0.7 ^a

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671 **Table S2.** Maximum and average speeds ($\mu\text{m}\cdot\text{s}^{-1}$) recorded for D-larvae of *C. gigas* exposed
 672 for 24h to different concentrations of Cu or PE MPs. Control (without pollutants): 0.1, 1 and
 673 10 correspond to concentrations of copper ($\mu\text{g Cu}\cdot\text{L}^{-1}$) or PE MP ($\text{mg MP}\cdot\text{L}^{-1}$) used for
 674 exposures. Means values \pm SEM. Different letters indicated significant differences between
 675 different concentrations ($p < 0.05$).
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Conditions	Maximum speed	Average speed
Control	192 ± 7^a	109 ± 3^a
Cu 0,1	175 ± 4^a	120 ± 3^b
Cu 1	150 ± 6^b	97 ± 4^c
MP (4-6 μm) 0,1	151 ± 5^b	88 ± 4^b
MP (4-6 μm) 1	119 ± 6^c	66 ± 4^c
MP (11-13 μm) 0,1	192 ± 8^b	125 ± 5^b
MP (11-13 μm) 1	155 ± 6^c	100 ± 4^c
MP (20-25 μm) 0,1	128 ± 19^a	61 ± 8^b
MP (20-25 μm) 1	96 ± 17^a	49 ± 9^c

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Graphical abstract

