1 **Experimental ingestion of fluorescent microplastics by Pacific oysters, *Crassostrea gigas*, and**

2 **their effects on the behaviour and development at early stages**

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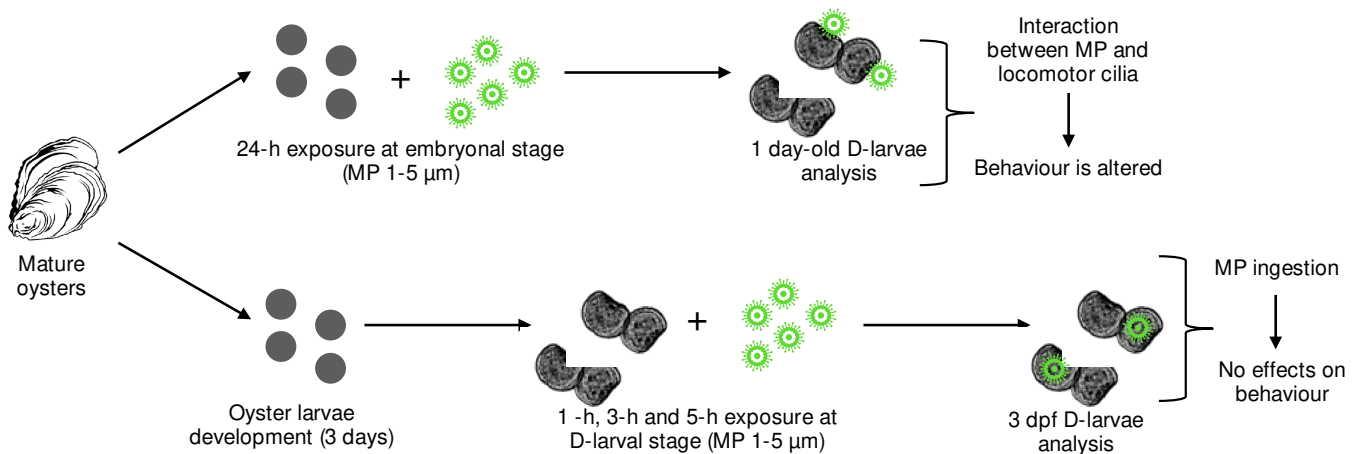
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14 **ABSTRACT**

15 Plastics are persistent synthetic polymers that accumulate as waste in the marine

16 environment. Microplastics (MPs, <5 mm) can be found either as microbeads in body care and

17 some industrial products or as plastic debris through degradation. Plastic microbeads (1-5 μm,

18 fluorescent, Cospheric) were used to characterise the MP ingestion and determine their

19 potential harmful effects on both the swimming behaviour and development of oyster D-larvae

20 (*Crassostrea gigas*). For 24 hours, embryos were first exposed to MPs at a temperature of 24 °C.

21 In addition, 3 day-old D-larvae were exposed to the same temperature for 1, 3 and 5 hours.

22 Three concentrations of MPs were used: 0.1, 1 and 10 mg MP. L^{-1}. After a 24-hour period of

embryonic exposure, we noted that MP agglomerates were stuck to the D-larvae coat and locomotor eyelashes. We also observed a significant increase in severe malformations and developmental arrests for larvae exposed to MPs ranging from 1 mg MP. L^{-1}. In terms of swimming behaviour, the maximum speed recorded was lower for larvae exposed at 0.1 and 1 mg MP. L^{-1}. After an acute exposure to MPs, particles were found in the digestive tract of 3 dpf (days post fertilization) D-larvae. After one-hour exposure, the concentrations tested (0.1, 1 and 10 mg MP. L^{-1}) resulted in respectively 38%, 86% and 98%. The larvae swimming behaviour was recorded and analysed. Unlike the results observed at the embryo-larval stage, 3-dpf larvae showed significant impacts with no dose-response effect.

ABSTRACT GRAPHIC

Mature oysters

24-h exposure at embryonal stage (MP 1-5 µm)

1 day-old D-larvae analysis

Interaction between MP and locomotor cilia

Behaviour is altered

Oyster larvae development (3 days)

1 -h, 3-h and 5-h exposure at D-larval stage (MP 1-5 µm)

3 dpf D-larvae analysis

MP ingestion

No effects on behaviour

38

1. INTRODUCTION

Microplastic particles (0.1 µm - 5 mm) are the most numerically abundant form of solid waste on Earth (Ericksen *et al.,* 2014). In 2017, the world's plastic production nearly reached 350 million tons, including more than 64 million tons in Europe (PlasticsEurope, 2018). Each year, approximately 4.8 to 12.7 million tons end in the oceans (Jambeck *et al.,* 2015). Although there are significant regional differences in plastic use and disposal (Kershaw *et al.,* 2011), at a worldwide level, the most used polymers are: polyethylene (PE), polypropylene (PP), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polystyrene (PS) and polyamide (PA) (Andrady and Neal 2009, PlasticsEurope 2010).

Microplastics are plastic particles that are smaller than 5 mm in diameter (Arthur *et al.,* 2009). The presence of MPs has been reported in almost all studied habitats around the globe (Ivar do Sul and Costa, 2014; Andrady *et al.,* 2011; Law and Thompson, 2014). In 2013, the average concentration of MPs in the Northeast Atlantic was of 2.46 MP.m^3. 89% of these MPs were smaller than 5 mm (Lusher *et al.,* 2014). Microplastics can either be suspended in the water column (Lattin *et al.,* 2004), buoy at surface water (Moore *et al.,* 2001; Morét-Ferguson *et al.,* 2010) or accumulate in sediments (Brown *et al.,* 2011) in coastal waters (Ng and Obbard, 2006), rivers (Sadri and Thompson, 2014), estuaries (Browne *et al.,* 2010) and ocean gyres (Maximenko et al., 2012). In aquatic ecosystems, the presence of plastics of different sizes and forms could negatively impact all levels of the marine food web, including the zooplankton one. For instance, marine zooplankton (copepods, decapods, bivalve larvae, etc.) can ingest MPs directly or by trophic transfer (Lee *et al.,* 2013; Setala *et al.,* 2014; Cole *et al.,* 2013; Cole *et al.,*

60 2015). MPs can also be transferred to higher trophic levels via mesozooplankton (Setala *et al.,*

61 2014 and Beiras *et al.,* 2018). In addition, several studies documented the MP effects on the

62 zooplankton's feeding behaviour, growth, development, reproduction and lifespan (Cole *et al.,*

63 2015; Botterell *et al.,* 2019).

64 Our study supports the idea that bivalves, including the Pacific oyster, *Crassostrea gigas*, are

65 promising sentinel species to study the impacts of small MPs (<200 µm) (Bonanno *et al.,* 2018).

66 The embryo-larval stage of oysters is frequently used as a bioassay in ecotoxicology, as this life

67 stage is easy to obtain and is very sensitive to environmental pollutants (His *et al.,* 1999; Mai *et*

68 *al.,* 2012; Mottier *et al.,* 2013 and Tallec *et al.,* 2018). Oyster larvae can ingest micro and

69 nanoplastics in the early stages (3-24 dpf) of their development (Cole *et al.,* 2015). In the work

70 carried out by Sussarellu *et al.*, 2016, adult oysters *C. gigas* that were exposed to 2-6 µm

71 polystyrene microplastics were used as oyster genitors. The authors then studied the MP

72 impacts on larval malformations and growth and concluded to noticeable effects on larvae.

73 Through their work, Bringer *et al.* 2020, demonstrated that polyethylene MPs had detrimental

74 impact on the *C. gigas* swimming behaviour and larval development.

75 In this study, the effects of 1-5 µm MPs on two early stages of oyster development were

76 assessed: the embryo-larval stage at 24 hours and D-larvae stage at 72 hours. Two batches of

77 larvae were used to test four concentrations (0, 0.1, 1 and 10 mg MP. L^{-1}). In the first phase,

78 embryo larvae were exposed to MPs. Then, the MP effects on the D-larvae (24 hour-old)

79 malformations, developmental arrests and swimming behaviour were analysed. In the second

80 phase, D-larvae were raised for 3 days – e.g. a stage with exogenous feeding (His *et al.,* 1988) –

81 and were then exposed to MPs at different exposure time intervals (1, 3 and 5 hours).

82   There are several advantages in using fluorescent plastic microspheres. First, commercial MPs

83   benefit from homogeneous sizes and shapes. Second, many plastic particles can be purchased

84   with embedded fluorophores or pigments allowing easy visualisation in biodeposits and tissues

85   (Ward *et al.,* 2019). For both experiments, the D-larvae swimming behaviour (including the

86   maximum speed and trajectories) was analysed. Our results provided relevant information on

87   both the development and swimming behaviour of two different embryo-larval stages after

88   acute exposure to MPs (1-5 µm).

89

90   **2. MATERIAL AND METHODS**

91   **2.1 Animal collection**

92   Mature oysters (*Crassostrea gigas*, Bayne *et al.,* 2017; Bayne *et al.,* 2019) came from a

93   commercial hatchery specialised in the year-round production of mature oysters (France

94   Naissain, France). During the September-October 2018 period, the oysters were kept at

95   approximately 4 °C for two days (which enabled us to have better quality laying eggs for the

96   first tests to be carried out) and acclimatised for 2 hours in filtered sea water (FSW) before

97   being fertilised in laboratory conditions.

98

99   **2.2 Seawater**

100 Seawater was collected from the French island of Île de Ré (South West of France). After

101 sampling, seawater was filtered using a membrane filter of 20 µm, 5 µm, 1 µm and 0.2 µm and

102 a pump generating UVs to eliminate debris and microorganisms. FSW was stored at 4 °C with

103 continuous bubbling and was used within 7 days. A few hours before the experiment, FSW was

104 filtered again at 0.2 µm.

105

106 **2.3 Gonad retrieval and fertilisation**

107 Oyster genitors were spawn by thermal stimulation (alternating immersion in 18 °C and 28 °C

108 seawater for 30 minutes) or by stripping the gonad (Mai *et al.,* 2013 and Gamain *et al.,* 2017).

109 At the time of the gamete expulsion, spawning males and females were individually isolated in

110 beakers with 0.2 µm FSW. After analysing the gamete morphology under a microscope at 10X

111 magnification (Nikon Eclipse), both eggs and sperm were selected for a single pairing. To

112 eliminate faeces and debris, these were separately sieved through 50 µm and 100 µm meshes

113 (Sefar Nitex). Sperm mobility was assessed and the number of eggs counted under a

114 microscope at 20X magnification (Nikon Eclipse). Eggs were fertilised with sperm using a ratio of

115 1:10 (egg:sperm) and homogenised every five minutes with an agitator to prevent polyspermy.

116 Fertilisation success was observed under a microscope, and embryos were then counted and

117 transferred. The first batch was exposed to MPs for 24 hours in order to observe their effects

118 on the embryo-larval development. Embryos were transferred to 24-well microplates (Greiner

119 Bio-One, Cellstar free of detectable DNase, RNase, human DNA and pyrogens. Content non-

120 cytotoxic). The second batch was held in development for 72 hours before being exposed to

121 MPs for 1, 3 and 5 hours. Oyster larvae began to feed on exogenous phytoplankton organisms

122 from 48-72 hours, before that they fed on their vitelline reserves (His *et al.,* 1999). Embryos

123 were transferred to 12-well microplates (Greiner Bio-One, Cellstar free of detectable DNase,

124 RNase, human DNA and pyrogens. Content non-cytotoxic) for further development and

125   exposure. For both batches, approximately 350-400 fertilised eggs were transferred in each

126   well containing 2 mL of FSW (Figure 1). These microplates were incubated in the dark at 24 °C

127   to reach standard development (Robinson *et al.,* 1992 and AFNOR 2009). The first batch was

128   incubated for 24 hours and the second for 72 hours (including additional stages of 1, 3 and 5

129   hours for ingestion).

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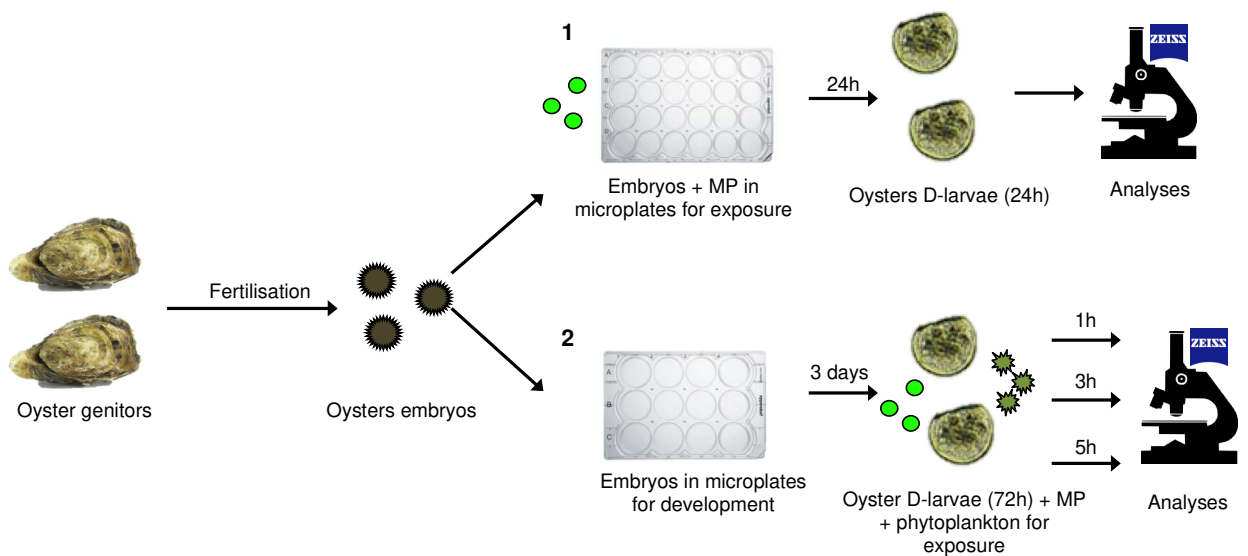
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132   **Figure 1.** Experimental design of both batches schematised, **(1)** embryonic exposure and analysis after a 24-hour

133   development; **(2)** larval exposure after 3 dpf and analysis after a 1, 3 and 5-hour exposure.

134

135   **2.4 Microplastic preparation and exposure conditions**

136   The MPs used were fluorescent (510 nm excitation) and ranged between 1 and 5 µm (FMG -

137   Green Fluorescent Microspheres 1.3g/cc 1-5um, Proprietary Polymer, Cospheric, California,

138   USA). For the experiment, MP particles were scattered in the water column.

139   MP solutions were prepared in FSW 0.2 µm and stored in the dark at 2 °C to limit microbial

140   growth. For the first batch, 2 mL of MP solution was put in the microplates with the embryos

141 for a 24-hour exposure. For the second batch, the 3-dpf D-larvae were exposed to MP

142 microbeads for 1, 3 and 5 hours. 2 mL of MP solution and 50 μL of algae mixture (Roland *et al.,*

143 1988; 50.000 cells. mL^{-1}) were injected in 12-well microplates containing 2 mL of larvae (3 dpf)

144 suspended in FSW. A mix of *Isochrysis spp.* (*T.iso* strain) and *Skeletonema costatum* (5:1 ratio;

145 France Naissain, France; Cole *et al.,* 2015) was used for feeding and growth experiments. Algal

146 size, biovolume and population density were assessed using a Nikon Eclipse microscope (x20).

147 For both batches, three different concentrations were used: 0.1 mg MP. L^{-1}; 1 mg MP. L^{-1} and 10

148 mg MP. L^{-1} excluding control treatment.

149

150 **2.5 Chemical analyses**

151 To carry out chemical analyses of 1-5 μm fluorescent MPs at 0.1; 1 and 10 mg MP. L^{-1}, each

152 solution was assayed using a flow cytometer (Attune Acoustic Focusing Cytometer). 2 mL

153 samples of MP solution used in embryo-larval exposure were vortexed (StarLab Vortex IR,

154 12,000 rpm for 20 sec) before going into a cytometer to homogenise the solution

155 (n=4/condition). A volume of 300 μl was sampled using the cytometer, which operated at a flow

156 rate of 500 μl.min^{-1}. Control analytical samples (n=12) contained 0.2 μm FSW (MPs excluded)

157 to remove naturally-present particles. The maximum threshold to detect particles was set at

158 10,000 events. Mili-Q water samples (n=12) were also assessed to check the relevance of the

159 detected events (<200 events). Then, a calibration was performed for MPs of different sizes

160 (HDPE MPs 4-6 μm MPP-635 XF, 11-13 μm MPP-635 G and 20-25 μm MPP-1241, density 0.96,

161 Micropowders Inc. USA) to select the relevant ones to the study (1-5 μm and fluorescent).

162

2.6 Swimming behaviour (maximum speed recorded and trajectories)

Post-exposure (meaning after 24 hours for the first batch and respectively 1, 3 and 5 hours for the second batch), 2-minute videos of D-larvae were recorded using a 24 °C thermoregulated box (ZEISS Axio Observer Z1, x5) to determine their swimming behaviour (maximum speed and trajectories, Bringer *et al.,* 2020). Then, oyster D-larvae were fixed (1% buffered formaldehyde) for abnormal larvae analysis. Formaldehyde was purchased from Sigma-Aldrich Chemical (St. Quentin Fallavier, France). A video conversion freeware (VirtualDub, Windows) set the frame rate at 4 FPS and converted it to AVI format. ImageJ (1,52a software) was used to analyse the videos. Videos (.avi) were opened and converted first into grayscale images, and then into binary images. For each video, a plugin was installed to deduce the swimming parameters of the recorded D-larvae. As a result, each larva was provided with a tracking number to easily identify them in the result file. It also included the maximum speed recorded (pixel. s^{-1}). In addition, swimming trajectories were assessed using an image file. Consequently, three different types of larval path were identified: (1) rectilinear, (2) circular and (3) motionless (Bringer *et al.,* 2020). The use of a graphic tablet (Wacom Bamboo Pen &Touch) and image processing software (Photos, Windows 10) enabled swimming trajectories to be quantified and characterised. The same larva can be detected several times, entering and exiting the visual field (Gamain *et al.,* 2019 and Bringer *et al.,* 2020).

The video created to assess the swimming behaviour is available upon request (additional data). The results displayed are expressed in mean ± SEM for the three replicates (three different couples of oyster genitors). There are seven videos by couple, resulting in n=21 videos/treatment.

185

2.7 Developmental abnormalities in D-larvae

After having recorded the D-larvae swimming behaviour, 25 µL of 1% buffered formalin was added to each well. A biometric analysis of larvae exposed for 24 hours (n = 15) was carried out on the ImageJ software, using photos obtained by microscopy (ZEISS Axio Observer, Z1, x20). The larvae were measured, by drawing a dummy line lengthwise (Talmage *et al.,* 2009 and Helm *et al.,* 2006). Then, the percentage of abnormal oyster larvae and developmental arrest was assessed (His *et al.,* 1999 and Quiniou *et al.,* 2005). Both phenomena were observed under an inverted microscope (Nikon eclipse, x10). This test required to have less than 20% of abnormal larvae in control conditions (absence of contamination). Three different couples were used and four replicates each were performed. Screenshots of the recorded videos were taken (VLC Media Player, Windows) to observe the contiguous MPs sticking on D-larvae (after a 24-hour period). The results displayed are expressed in mean ± SEM for the three replicates (three different couples of oyster genitors). Twelve wells for each condition were analysed using approximately 200 D-larvae in each well. This resulted in n=2,400 D-larvae analysed/treatment.

2.8 MP ingestion by 3-dpf D-larvae

To determine the MP presence or absence in 3-dpf D-larvae and the percentage of larvae ingesting microbeads, a digital reconstruction of the wells was created by superimposing pictures (GFC fluorescence, ZEISS Axio Observer Z1, x5 objective). By zooming well by well, a count of larvae was carried out (Figure S1). Three replicates (three different couples of oyster genitors) and four wells of each were analysed, resulting in n=12 wells analyses/treatment.

207 The number of microspheres ingested by each larva was assessed through fluorescence

208 intensity measurements. To do so, the ratio between the mean fluorescence intensity of each

209 isolated bead (n=30) and the fluorescence intensity in the D-larvae digestive tract

210 (n=6/treatment) was calculated. Not only the fluorescence intensity of the MP beads in the

211 larval gut indicated if the particles were ingested or not (Figure S2) but it enabled to precisely

212 determine where these were located in the digestive tract. For this experiment, the 3-dpf

213 larvae were individually and independently tested for MP ingestion, meaning that larvae for

214 each exposure time interval were different. Three replicates (three different couples of oyster

215 genitors) were analysed, resulting in n=6 for each couple and n=18/treatment.

216

217 **2.9 Statistics**

218 The statistical analysis was conducted using R and graphs on Microsoft Excel, except the PCA-

219 Biplot which was created on R Studio using the "FactoMineR" package. Homogeneity of

220 variance (Levene's test) and normality of distribution (Shapiro-Wilk) were assessed. In order to

221 compare the various treatments, an analysis of variance (ANOVA) using Tukey's post-hoc test

222 was performed. For data not entering in the scope of a parametric test, a statistical analysis was

223 carried out through the Kruskal-Wallis Test. Differences in tested concentration means were

224 then assessed using the Kruskal Nemenyi Post-hoc test with the PMCMR package (the Tukey

225 test equivalent for non-parametric data). When $p<0.05$, significance was tolerated. Data are

226 written as mean ± SEM.

227

228 **3. RESULTS**

3.1 M

Theoretical (mg MP.L^{-1})	Measured (MP.mL^{-1})	Dilution factor
0.1	7 211±602	X
1	68 733±337	9.5
10	54 3528±42 646	7.9

230

Table 1

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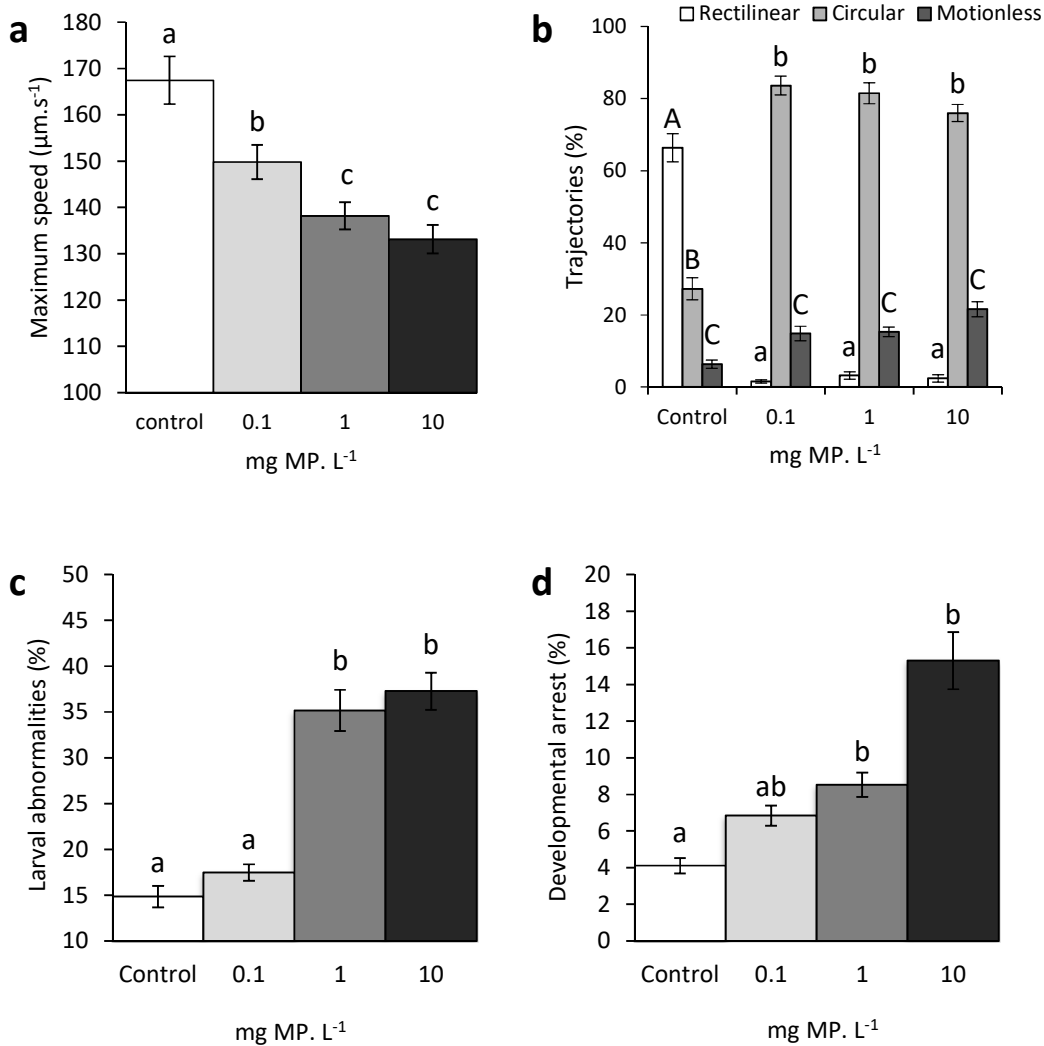
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248   length at 10 mg MP. L^{-1} was of 55.9±6.3 μm. These results differed from other conditions,

249   particularly from control larvae which had a length of 73.6±6.7 μm (Table S3).

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251

252

253   **Figure 2. (a)** Maximum speed recorded, **(b)** swimming trajectories, **(c)** percentage of abnormal D-larvae and **(d)**

254   developmental arrest of oyster D-larvae exposed for 24 hours to various concentrations (0; 0.1; 1; 10 mg MP. L^{-1})

255   of fluorescent MPs (1-5 μm). Values are expressed as mean ± SEM for the three replicates. A variation in letters

256   indicates significant differences between concentrations ($p<0.05$, Kruskal-Wallis for swimming behaviour and

257   ANOVA for abnormal and developmental arrest quantification).

258

259 Regarding the malformations and developmental arrests of oyster D-larvae (Figure 2.c & d), a

260 dose-response effect to exposure concentrations was noted. An abnormal larval rate was

261 observed at the two highest concentrations of 1 and 10 mg MP. L^{-1}, with respectively 35.2±2.2

262 % and 37.3±2.0% (Table S3). In contrast, malformed larvae in control conditions had a rate of

263 14.8±1.2%. In terms of developmental arrests, the highest rate was assessed at 10 mg MP. L^{-1},

264 with 15.3±1.6% (Table S3). The higher the MP concentration was, the higher the rate of

265 developmental arrests. On these screenshots (Figure S4), MPs formed agglomerates. In

266 addition, they stuck on the mantle and the locomotor eyelashes of the 24-hour D-larvae. On

267 this picture (Figure S4, on the right), some MP agglomerates were larger than the larvae.

268

269 **3.3 MP ingestion assessment and quantification in 3-dpf larvae at different time intervals**
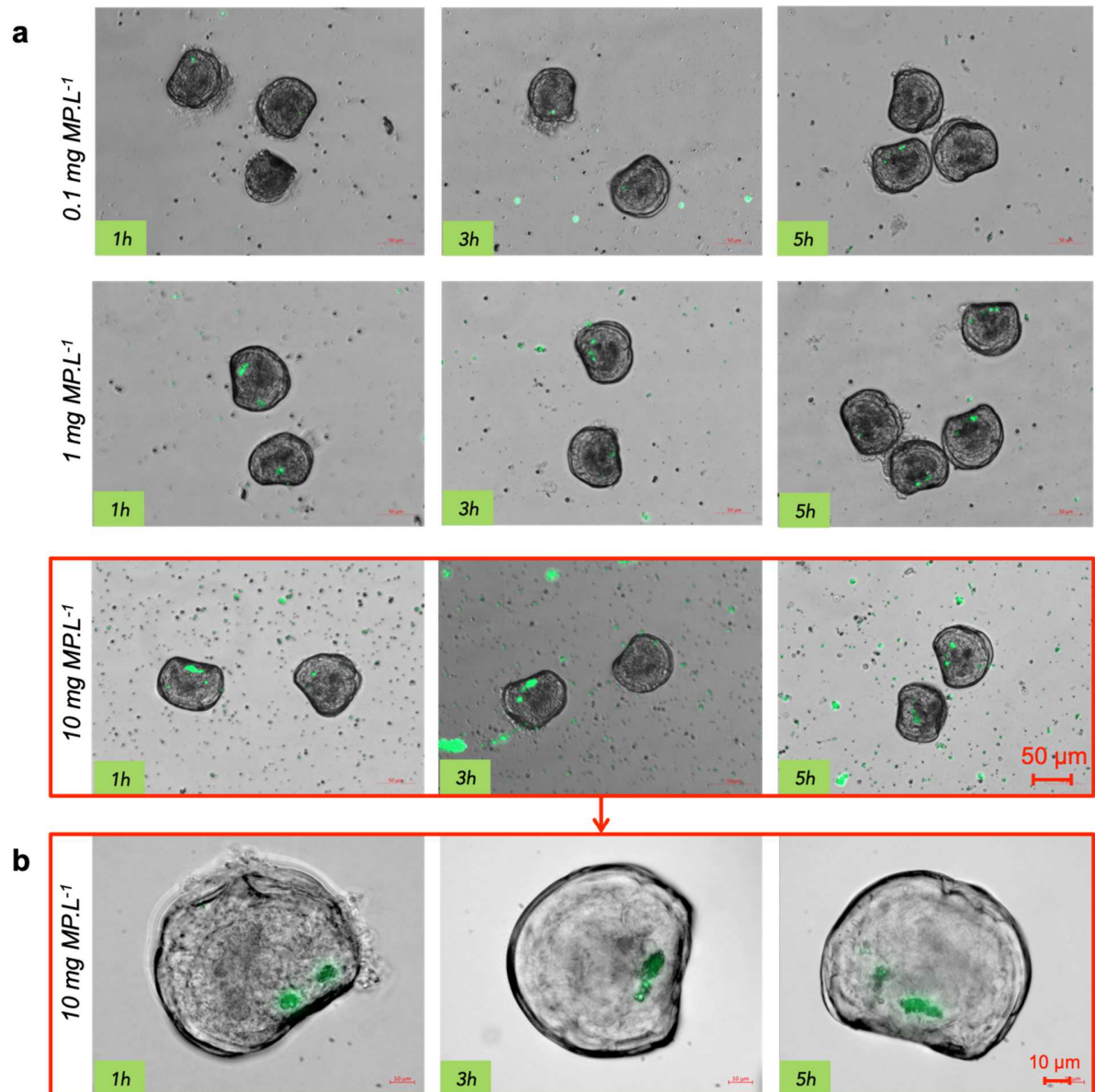
Figure 3. Microscopic pictures ZEISS Axio Observer Z1, magnification x20 **(a)** and microscopic zoomed pictures on 10 mg MP. L^{-1}, ZEISS Axio Observer Z1, magnification x60 **(b)** of 3-dpf oyster D-larvae exposed at different time intervals (1, 3 and 5 hours) and facing different concentrations of fluorescent MPs.

The ingestion of fluorescent MPs by 3-dpf oyster D-larvae was assessed. Larvae ingested beads after 1, 3 and 5 hours of fluorescent MP exposure (Figure 3.a). We noted that the tested MPs tended to accumulate in the larvae digestive tract (Figure S2). However, there was no

278 difference between the exposure time intervals for a given concentration (Figure 3.b). When

279 reconstructing the wells of microplates, we managed to quantify the number of D-larvae that

280 ingested MPs and the ones who did not (Figure 3). A clear dose-response effect was observed.

281 However, no impact was highlighted on the exposure time intervals (Figure 4). The larvae that

282 were exposed to 10 mg MP. L^{-1} were more likely to have ingested MPs (98%) than the ones

283 exposed to 1 mg MP. L^{-1} (84%) or 0.1 mg MP. L^{-1} (47%).
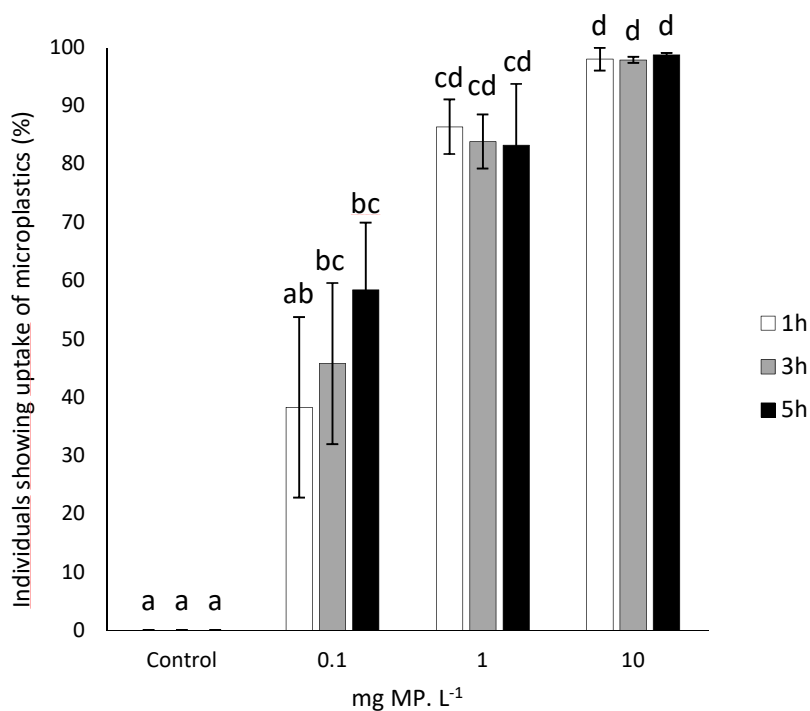

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285 **Figure 4.** MP ingestion by oyster D-larvae according to exposure time intervals (1, 3 and 5 hours) and MP

286 concentrations (0; 0.1; 1; 10 mg MP. L^{-1}). Bars are expressed as mean values ± SEM for the three replicates. A

287 variation in letters indicates significant differences ($p < 0.05$, ANOVA).

288

289 These results could be directly linked to the number of beads ingested by the oyster D-larvae

290 and present in their digestive tract. Assessing the fluorescent content and intensity in larvae

291 enabled to determine the number of beads ingested by each individual (Figure 5.a). Almost all

292   oyster larvae exposed to 10 mg MP. L^{-1} ingested MPs (98%, red circle, Figure 5.a). The different

293   exposure time intervals were grouped in the graph as there was no significant difference for

294   this parameter. Larvae exposed to 0.1 mg MP.L^{-1} ingested an average of 14.7±0.8 MP.larvae^{-1}

295   (grey circle, Figure 5.a), while the ones exposed to 1 mg MP.L^{-1} (blue circle, Figure 5) and 10 mg

296   MP.L^{-1} respectively ingested 35.7±4.1 and 141.8±5.2 MP.larvae^{-1}. There was no significant

297   difference between the three MP concentrations for the amount of microbeads ingested by the

298   D-larvae.

a

b

299
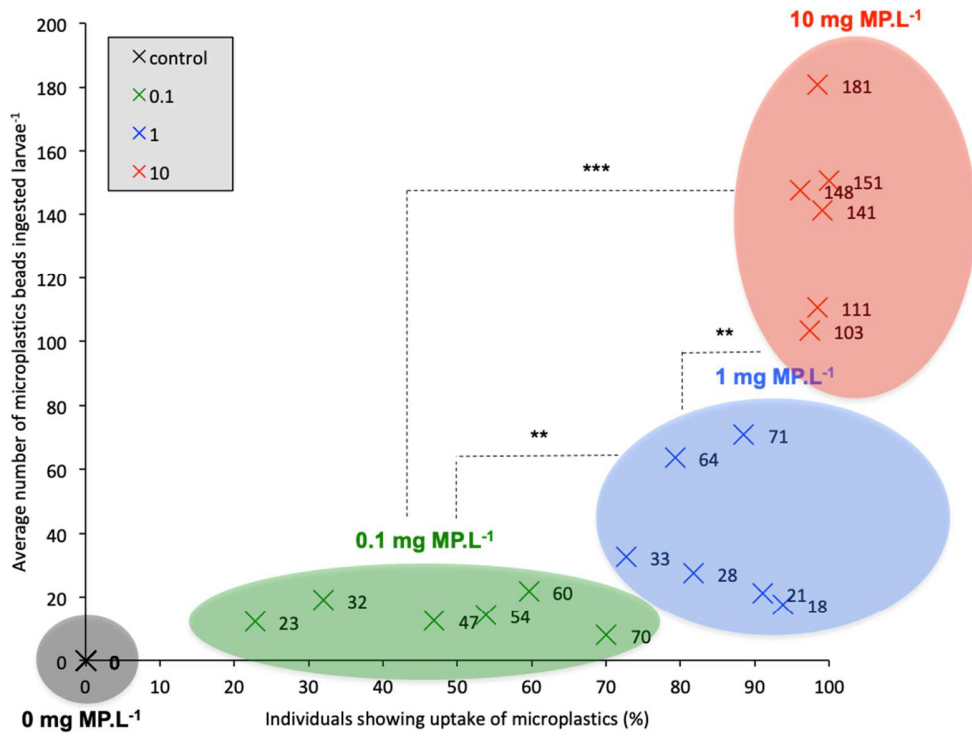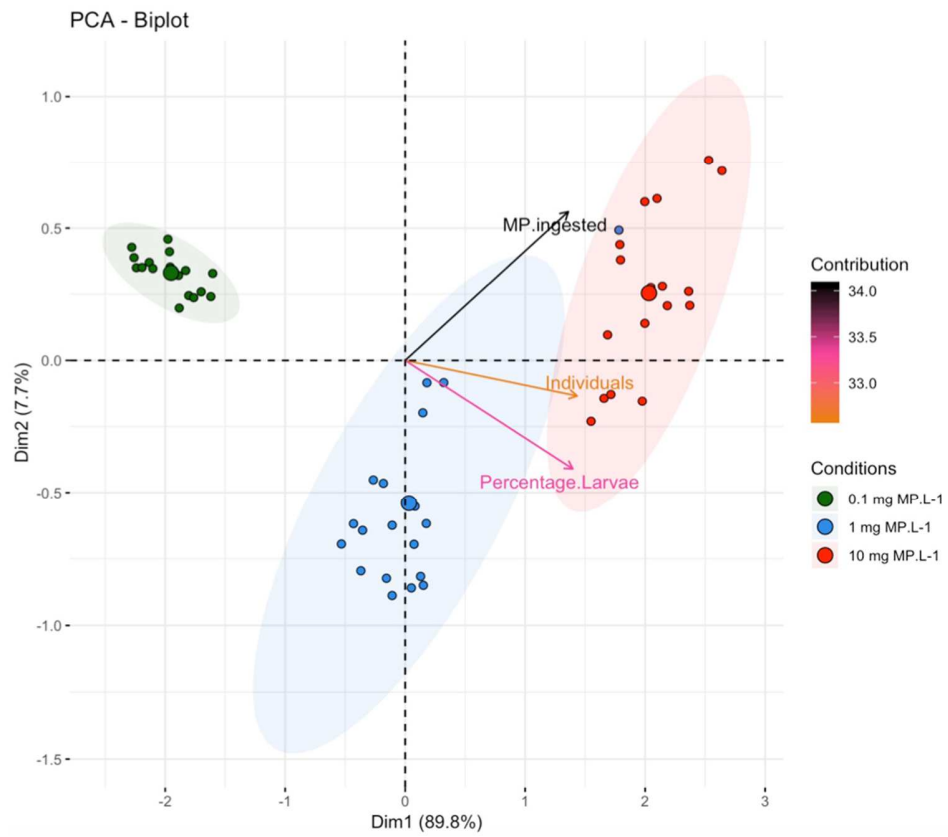
Figure 5. Relationship between the number of oyster D-larvae that ingested MPs (%) and the mean number of

301 microbeads ingested per larva **(a)** after 1, 3 and 5 hours of exposure (n=18). Asterisks indicate significant

302 differences (Kruskal Wallis Test). Levels of significance: $p<0.1$="."; $p<0.05$="*"; $p<0.01$="**"; $p<0.001$="***" and **(b)**

303 correlation plot (PCA-Biplot) between different variables: individuals, MPs ingested by larvae and percentage of

304 larvae (%) presenting MPs in their digestive tract.

305

306 To correlate the 3 variables, a PCA-Biplot was created (Figure 5.b). Individuals who ingested

307 high concentrations of MPs (fluorescence found in their digestive tract) were correlated to the

308 percentage of larvae that ingested MPs. Groups of individuals (depending on their exposure

309 conditions) were well distributed on the PCA. Moreover, "contribution" of variables highlighted

310 that the number of MPs ingested by larvae was the variable that contributed the most to
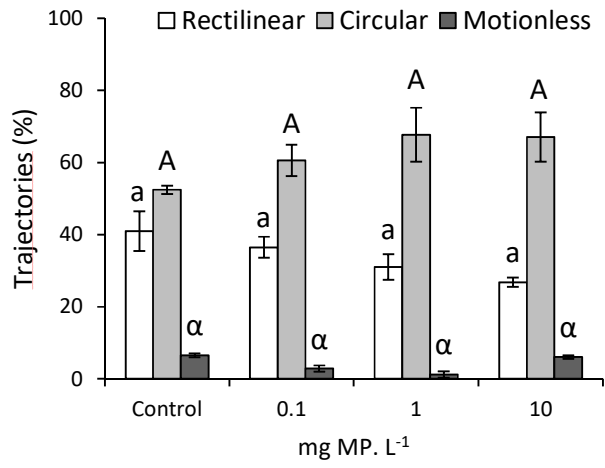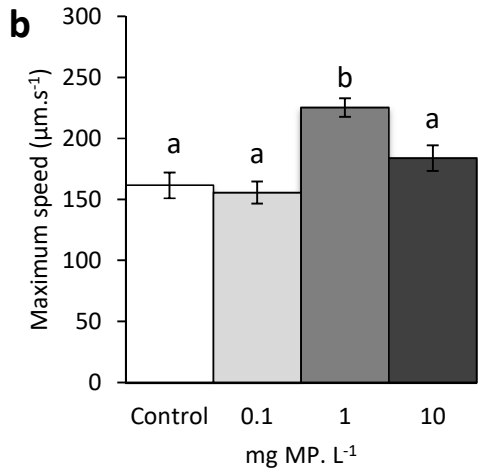
311 creating correlations.

312

313 **3.4 Effects of MP exposure on 3-dpf larvae**

314 For this experiment, D-larvae were bred for 3 dpf (72 hours). Then, they were exposed to MPs

315 (1-5 µm) for 1, 3 and 5 hours. The effects of MP exposure to the larvae swimming behaviour
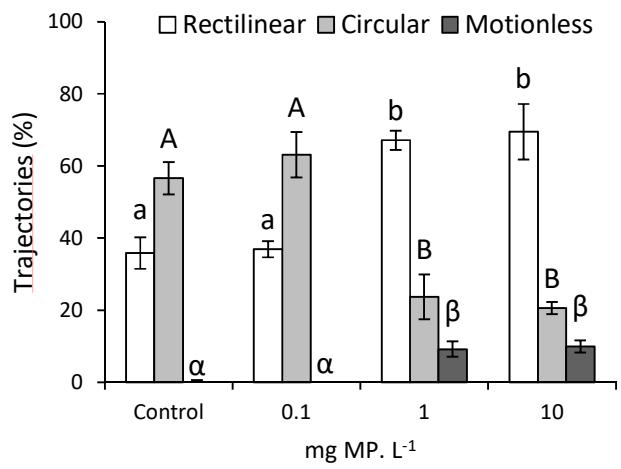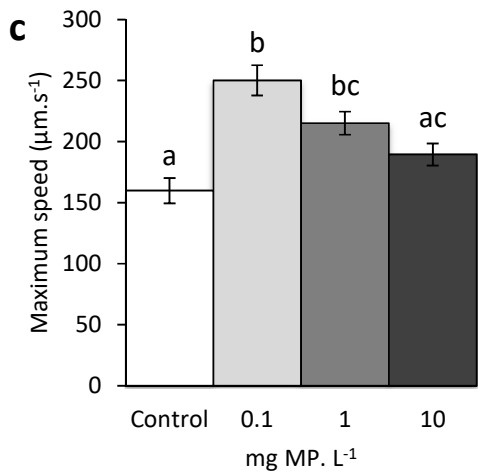
316 was then analysed (Figure 6).

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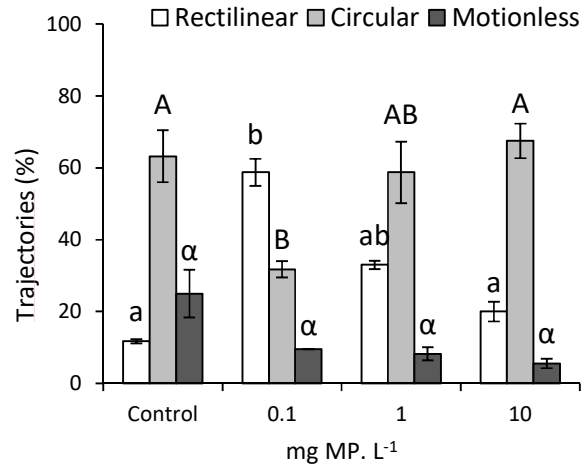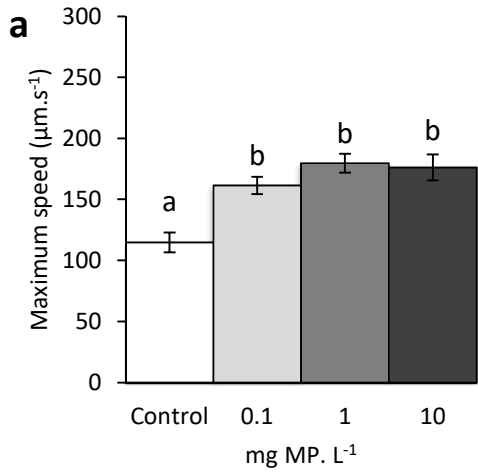
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321 **Figure 6.** Maximum speed recorded and swimming trajectories of 3-dpf oyster D-larvae after a 1 **(a),** 3 **(b)** and 5-

322 hour **(c)** exposure to fluorescent MPs at different concentrations (0; 0.1; 1; 10 mg MP. L^{-1}). Values are expressed as

323 mean values ± SEM for the three replicates. A variation in letters indicates significant differences between

324 different concentrations ($p<0.05$, Kruskal-Wallis test).

325

326 After a 1-hour exposure to different MP concentrations, the maximum speed recorded

327 increased significantly for exposed D-larvae (Figure 6.a) in comparison to control larvae.

328 However, there are no differences observed in terms of MP concentrations. After 3 and 5 hours

329 of exposure (Figure 6.b and 6.c), the results were quite heterogeneous; no trend could be

330 defined. Regarding their swimming trajectories, control larvae had predominant circular

331 trajectories (Figure 6.a). After 3 hours, exposed larvae tended more towards circular

332 trajectories when increasing MP concentration, although this is not significant. After a 5-hour

333 exposure, we noted a significant difference ($p>0.05$) in swimming. Indeed, there was an

334 increase in motionless trajectories ($p> 0.05$). At this stage of development (72-hour D-larvae),

335 swimming trajectories seemed not to be as strongly impacted by MPs.

336

337 **4. DISCUSSION**

338 For the whole study, we examined two different developmental stages of oysters exposed to 1-

339 5 μm fluorescent MPs: (1) embryos after being exposed for 24 hours and (2) 3-dpf D-larvae

340 after being exposed for 1, 3 and 5 hours.

341 First of all, after a 24-hour exposure, D-larvae exposed at the embryonic stage had MPs stuck

342 on their locomotor cilia and their coat. They did not show any sign of MP ingestion. When

343 embryos were directly in contact with fluorescent MPs, the (24-hour) larvae body length was

344   impacted. Exposed oyster D-larvae were smaller than unexposed ones. The consequences on

345   larval size were previously documented by Sussarellu *et al*. (2016). When oyster broodstocks

346   were exposed to 2 and 6 µm polystyrene beads, the larvae were smaller in size. Similarly, after

347   being exposed to MPs (20 µm polystyrene beads), copepod eggs (*Calanus helgolandicus*) were

348   smaller (Cole *et al.,* 2015). However, these biometric results and growth abnormalities might be

349   due to developmental delays and malformations directly impacting the D-larvae size. As

350   mentioned, exposed oyster larvae showed abnormalities and developmental arrests. Some

351   studies highlighted that contaminants such as heavy metals and pesticides caused

352   developmental defects (Mai *et al.,* 2012; Gamain *et al.,* 2017; Gamain *et al.,* 2016). We

353   observed similar phenomena when species were exposed to 4-6 µm polyethylene MPs (Bringer

354   *et al.,* 2020). In addition, a recent study has underlined that blue mussel larvae, *Mytilus edulis*,

355   had malformations and developmental defects after being exposed to 100 nm and 2 µm

356   polystyrene microbeads (Rist *et al.,* 2019). In this study, we noted a sharp increase in

357   malformed larval rate from 1 mg MP. L^{-1} and a dose-dependent increase in developmental

358   arrests up to 15% at 10 mg MP. L^{-1}. These results echoed a previous study using 4-6 µm PE MPs

359   (Bringer *et al.,* 2020). Our findings were consistent with the results of Lei *et al.* (2018), who

360   demonstrated that MP toxicity was size-dependant rather than polymer-dependant. The study

361   highlighted that the swimming behaviour of D-larvae, in particular their maximum speed

362   recorded and trajectories, was impacted by MPs. Exposed larvae swam slower and had

363   predominant circular swimming trajectories. Gamain *et al.* (2019) also reached similar

364   conclusions when studying oyster larvae exposed to copper and pesticides. In addition, a

365   reduced swimming speed was observed in oyster D-Larvae exposed to different sizes of PE MPs

366   (Bringer *et al.,* 2020). According to our microscopic observations, MPs stuck on the larvae cilia.

367   Several studies documented that MPs tended to bind on aquatic species and disturb their

368   swimming behaviour. For instance, brine shrimps *Artemia franciscana* had MPs stuck to their

369   antennas and consequently an abnormal swimming behaviour (Bergami *et al.,* 2016). MPs also

370   stuck on the tentacles of *Hydra attenuata,* which could impair feeding by restricting its ability to

371   move and capture prey (Murphy *et al.,* 2018). The observations on larvae exposed (*H.*

372   *attenuata*) for 24 hours underlined that MPs *quanta* (Alimi *et al.,* 2018) were likely to impact

373   their swimming behaviour, mainly their speed and trajectories.

374   In a second phase, 3-dpf larvae were analysed and exposed to MPs at different time intervals

375   (1, 3 and 5 hours). In previous studies, authors noticed that oyster larvae could ingest and

376   accumulate MPs in their digestive tract (Jones and Jones, 1988; Cole *et al.,* 2015). Capture

377   efficiency of small particles (ca. 1–6 µm) is species-specific and depends on the gill architecture

378   and structure of the latero frontal cilia or cirri (Silverman *et al.,* 1995; Ward and Shumway 2004;

379   Rosa *et al.,* 2017, 2018). We noted that the higher the MP concentration was, the more

380   particles the larvae ingested. However, no significant difference was observed in terms of

381   exposure times. This implies that D-larvae ingested MPs faster than they egested (Cole *et al.,*

382   2016). The correlations in the PCA-Biplot highlighted that the larvae ingested and stored large

383   quantities of MPs in their digestive tract when the experimental unit was loaded with particles.

384   A previous study demonstrated that oyster (*C. gigas*) and slipper limpet (*C. fornicata*) larvae

385   could deplete the nutrient stocks in their environment in no time (Blanchard *et al.,* 2008).

386   MP ingestion and accumulation in the digestive tract of 3dpf D-larvae indicated an increase in

387   their maximum swimming speed under certain contamination conditions. MP ingestion altered

388　the maximum swimming speed and thus, to a lesser extent, the trajectories. However, this

389　phenomenon was not observed for all contaminated conditions. One cannot clearly conclude in

390　a trend in duration nor in a dose-response effect.

391　In this study, we noticed that MPs tended to agglomerate and stick to the locomotor eyelashes

392　of the 24 hour-old larvae, which were not able to filter exogenous food and did not show MP

393　ingestion. However, MPs were ingested by the 72-hour D-larvae (3 dpf). This impacted their

394　swimming behaviour as larvae with MPs clung on their eyelashes showed behavioural

395　abnormalities while larvae that had ingested MPs did not demonstrate behavioural differences.

396　This study underlined that the swimming behaviour and the development of the oyster were

397　affected when exposed at embryonic stage to small PE MPs. For older larva exposed to MPs at 3

398　dpf, their swimming activity tended to increase when exposure time lasted up to 1 hour. The

399　larvae did not show significant difference in their swimming trajectories after being exposed to

400　MPs.

401　Another study demonstrated that egestion rate kinetic of 1-5 μm fluorescent MPs by oyster

402　larvae was fairly rapid (< 3 h) and depended on the particle size (Ward *et al.,* 2019). Indeed, one

403　could investigate the larvae MP egestion and exclude the possibility of re-ingestion (Ris *et al.,*

404　2019) by developing a relevant protocol.

405

406　**5. CONCLUSIONS**

407　In this study, MPs stuck on the locomotor cilia of the D-larvae exposed for a 24-hour period at

408　the embryo-larval stage, modifying their maximum swimming speed and to, a lesser extent,

409　their trajectories. We concluded that being exposed to small fluorescent MPs at the embryo-

410 larval stage was detrimental for D-larvae (*C. gigas*), causing developmental abnormalities,

411 changes in their swimming behaviour (both speed and trajectories) as well as developmental

412 arrests after a 24-hour period. The effects observed were dose-dependent.

413 In addition, 3-dpf exposed D-larvae ingested MPs, which were then observed in their digestive

414 tracts. The higher the MP concentration was, the more MP particles the D-larvae tended to

415 ingest. Regarding their swimming behaviour, we noted an increase in speed, especially for

416 larvae exposed for a 1-hour period. The swimming trajectories did not seem to be impacted by

417 MPs. Further research on *Crassostrea gigas* and other bivalve species, such as *Mytillus edulis*, is

418 essential to build up on our knowledge. This would help establish recommendations on the

419 ecological risks of MPs in coastal and estuarine ecosystems.

420

421 **SUPPORTING INFORMATION**

422 Video: the oyster D-larvae swimming behaviour was recorded on a ZEISS microscope, both after

423 a 24-hour exposure at the embryo-larval stage, after 3 dpf of development and a 3-hour

424 exposure at 0.1 mg MP. L^{-1}.

425

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441 **REFERENCES**

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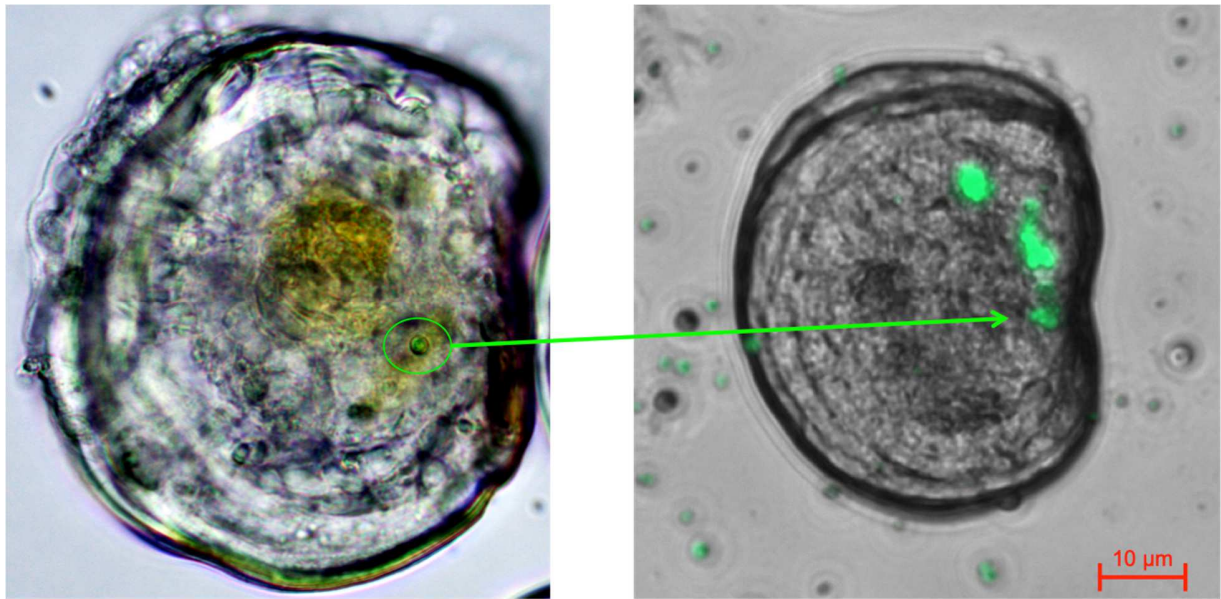611   microplastic pollution. *Environmental science & technology*.

612

613   **ADDITIONAL DATA**
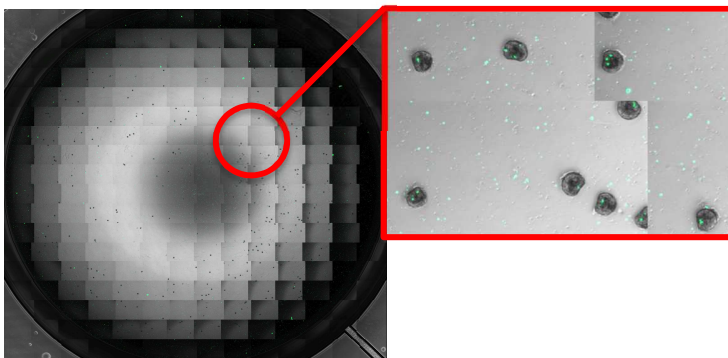
615   **Figure S1.** Counting method (superimposing pictures to reconstruct wells) to determine MP presence/absence in

616   D-larvae using a zooming function.

617

618

Figure. S2. Microscopic pictures (ZEISS Axio Observer Z1) of oyster-D larvae exposed to fluorescent MPs (1-5 µm).
On the left picture: fluorescent microbead in the larva digestive tract (described by Jones and Jones, 1988). On the
right picture: larva with agglomerates MP microbeads with exposure of 1 mg MP. L^{-1}.

Conditions	Body length	Abnormal	Developmental arrest	Maximum speed
Control	73.6±6.7 [a]	14.8±1.2 [a]	4.1±0.4 [a]	167.4±5.1 [a]
0.1 mg MP.L^{-1}	60.3±0.7 [b]	17.5±0.9 [a]	6.8±0.6 [ab]	148.5±3.7 [b]
1 mg MP.L^{-1}	58.3±3.7 [bc]	35.2±2.2 [b]	8.5±0.7 [b]	135.6±3.0 [c]
10 mg MP.L^{-1}	55.9±6.3 [c]	37.3±2.0 [b]	15.3±1.6 [b]	137.9±3.1 [c]

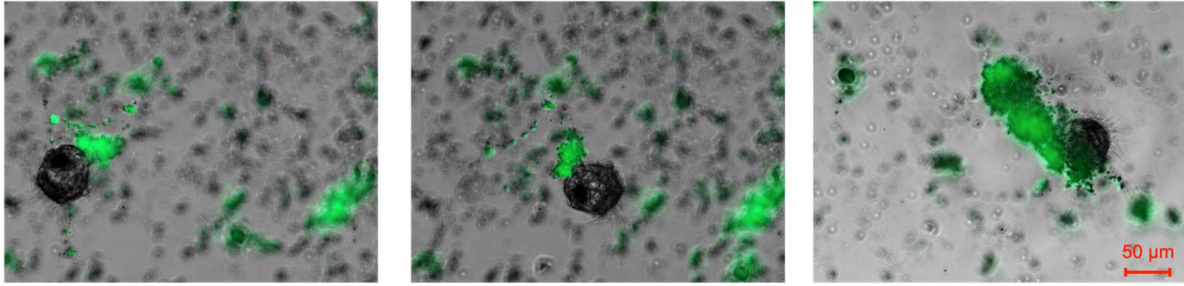**Figure S4.** Screenshots taken from the recorded videos (ZEISS Axio Observer Z1) of oyster D-larvae exposed to fluorescent MPs for 24 hours (1 mg MP. L^{-1}) at the embryonic stage. Presence of fluorescent MP as agglomerates close to the eyelashes and the larval mantle.