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

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Abstract

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

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

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

1. Introduction

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

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

MPs are ubiquitous in marine water with reported concentrations ranging from 1.31 to 102,000 particles per m^3 (Van Cauwenberghe *et al.,* 2015; Auta *et al.,* 2017). PP and PE are the most common polymers in all environmental compartments (Isobe et al., 2014; Enders et al., 2015; Frere et al., 2017). These plastics persist in the environment due to their resistance to biodegradation (Yoshida *et al.,* 2016) but also to chemical, photochemical and mechanical 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 (25-50µm) 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 µm
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 Gallager, 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 (Détrée *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 µm) 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 µm)
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

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

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

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

2. Material and methods

2.1 Animal collection

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

2.2 Chemicals and seawater

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

Cu ($CuSO_4$) and formaldehyde were purchased from Sigma-Aldrich Chemical (St. Quentin Fallavier, France). Three sizes of PE MPs (HDPE) were used (4-6 µm MPP-635 XF, 11-13

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

2.3 Exposure solutions analysis

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

Chemical analyses of Cu were carried out in sandy sediments (surface and semi-depth 5 cm) and in coastal seawater close to shellfish areas. This gave us an indication of the environmental concentrations found. The water was acidified to 5% and then analyzed and diluted to one-third. For the sediment, 200 mg was weighed and then acidified with 6 mL of nitric acid and 2 mL of hydrochloric acid. The sediment was then mineralized by microwave (rise in temperature at 120 °C and then plateau at 120 °C for 30 minutes, then cooling to 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 $\mu g.L^{-1}$.
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 thought 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)

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

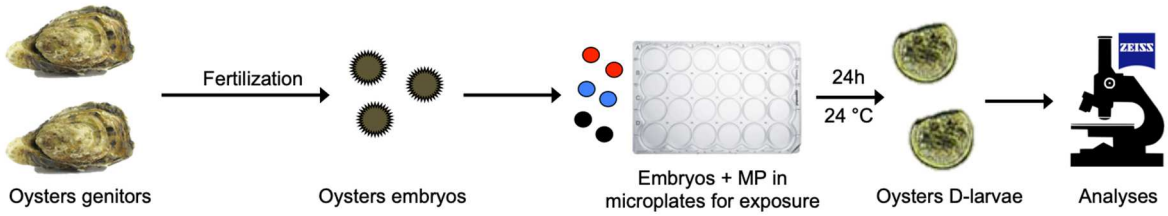


Figure 1. Experimental design. After a 24h-exposure to Cu or MPs, oyster D-larvae malformations and swimming behaviour were analysed. A 2 min-video was recorded for each batch of larvae and then abnormal D-larvae were quantified after formaldehyde fixation.

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

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

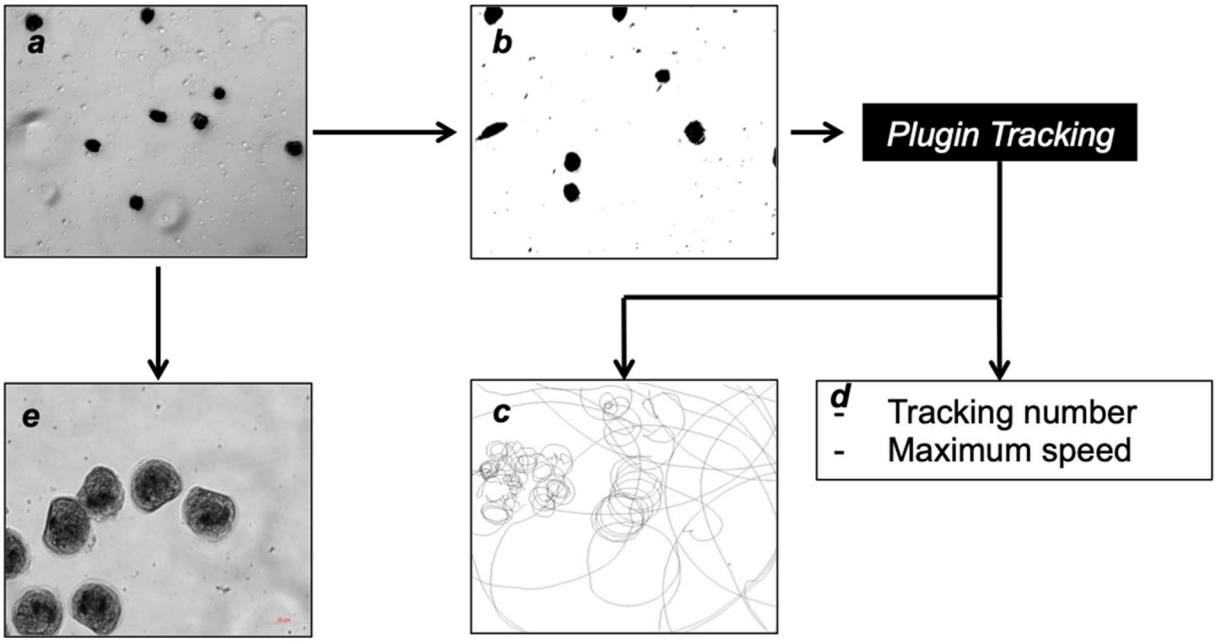
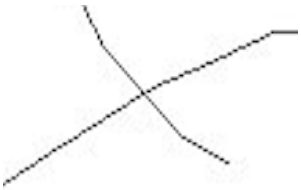
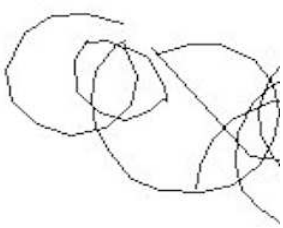


Figure 2. Procedure for swimming behaviour analysis of oyster D-larvae. (**a)** Original image after conversion. (**b)** Binary image. (**c)** Traces of larval path. (**d)** Parameters studied. (**e)** Quantification of abnormal D-larvae and developmental arrest after fixation with formaldehyde (1%).

Table 1. Paths analysis of oyster D-larvae with three categories of trajectories: rectilinear, circular and 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
		

2.6 Developmental abnormalities

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

2.7 Statistics

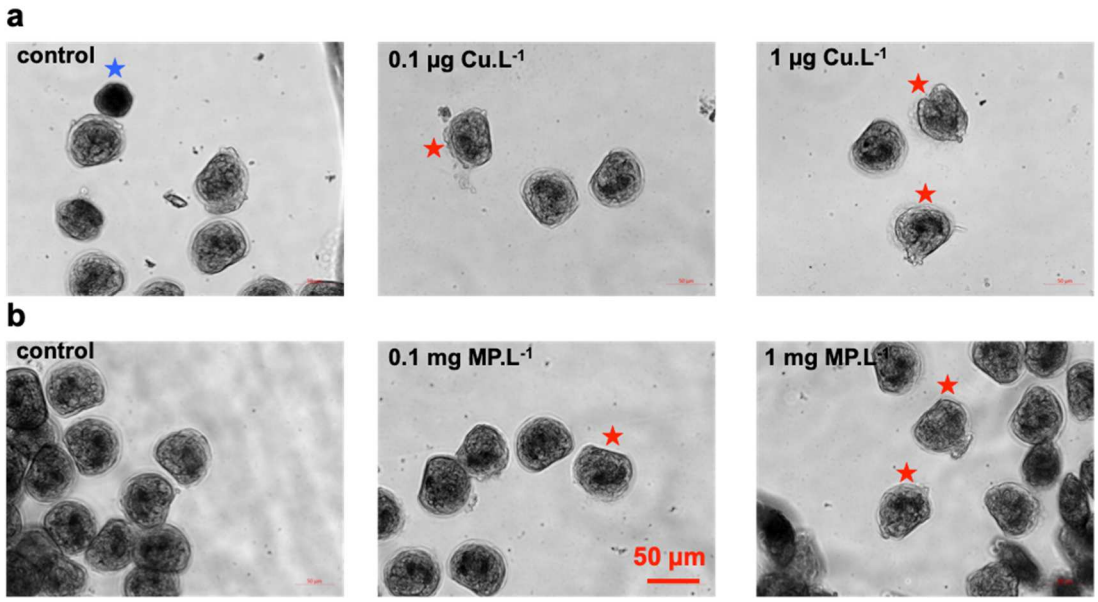
All data are expressed as means ± standard error of the mean (SEM). If data did not follow a normal distribution, it was transformed using the formula: $P'=\arcsin\sqrt{r}$; P corresponds to raw data (frequency of abnormalities) specified in P values from 0 to 1 (Legendre and Legendre, 1998). Homogeneity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

3. RESULTS

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

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

239 developmental arrest was significant from 0.1 µg $Cu.L^{-1}$ (12.9±2.5%) and increased to
240 37.8±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±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±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).
249



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 µg.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

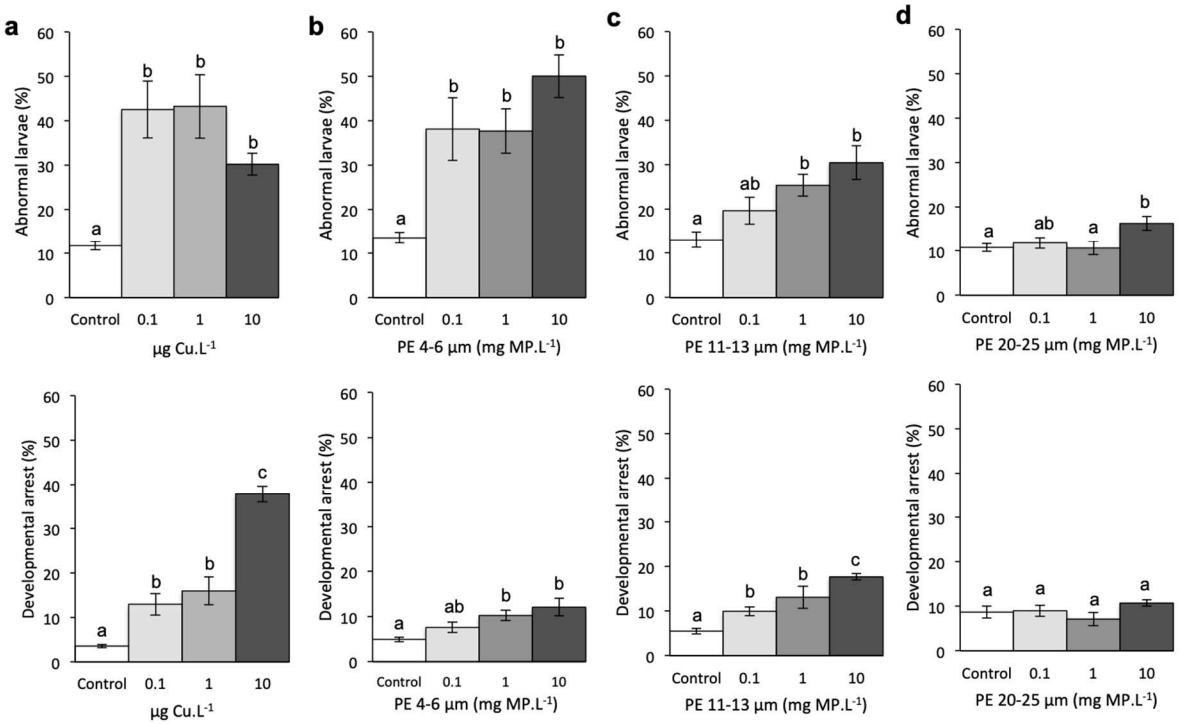


Figure 4. Abnormal larvae (%) vs. concentrations (Mean ± SEM) of oyster D-larvae of *C. gigas* (above) and developmental arrest (%) vs. concentrations (bottom) following 24h oyster embryonic exposure to different concentrations of Cu **(a)** and PE MPs of 4-6 µm **(b)**, 11-13 µm **(c)** and 20-25 µm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

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

In control condition (filtered seawater), the average speed of oyster D-larvae was 109±3 $\mu m.s^{-1}$ and the maximum speed was 191±7 $\mu m.s^{-1}$ (supplementary data, Table S2) while 66.4±13.7% of the D-larvae swam following a rectilinear trajectory and 27.3±16.0% shown circular paths (Figure 6). Motionless larvae were rare within the control group with 6.4±11.1% (Figure 5). After 1 µg $Cu.L^{-1}$ exposure, the maximum speed significantly decreased compared to control and the percentage of circular and motionless trajectories increased 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 MPs.L^{-1} (with respectively 151±5 and 119±6 µm.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 µm.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).

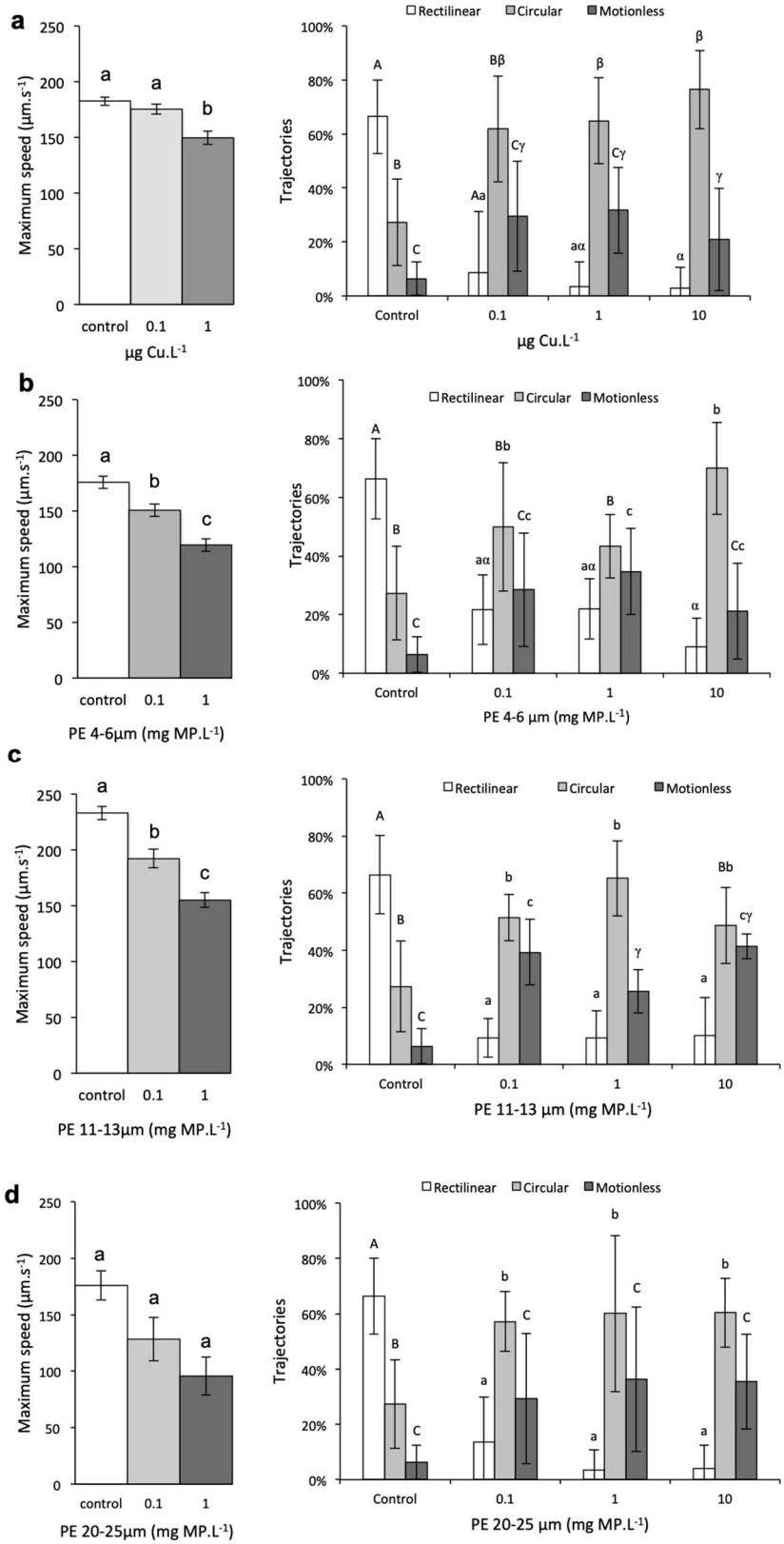


Figure 5. Maximum speed and trajectories of D-larvae of *C. gigas* following 24h embryonic exposure to different concentrations of Cu **(a)** and PE MPs of 4-6 µm **(b)**, 11-13 µm **(c)** and 20-25 µm **(d)**. Control (without pollutants): 0.1, 1 and 10 correspond to concentrations of Cu (µg $Cu.L^{-1}$)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

4. Discussion

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

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

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

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

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

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

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

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

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

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

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

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

attaching to their substrate when in the presence of MPs (Green *et al.,* 2019). More recently, Yin *et al.,* 2019, have shown that black rockfish, *Sebastes schlegelii*, exposed to PS MPs of 15 µm to 0.19 mg.L^{-1}, had drastic reduction in their swimming speeds as well as reduction of range of movement, which may affect hunting behavior and exploration competence.

The impact of PE MPs on swimming behaviour could be explained by interactions of these particles with larval cilia (Figure 6). Beiras *et al.* (2018) reported that PE particles (4-6 µm) can stuck on villi of the chorion of *Oryzias melastigma* embryos. Agglomerates of MPs tend to settle and stick along the mantle of organisms, as with nanoplastics on brine shrimp *A. franciscana* on their antennules and abdomen (Bergami *et al.,* 2016). Nanosized latex particles (39.4 nm) have shown to be adsorbed on the fertilized egg surface of Japanese medaka, *Oryzias latipes* (Kashiwada, 2006). Like Medaka egg chorions, oyster D-larvae have cilia to move in the water column. This would explain the fact that D-larvae exposed to MPs exhibited altered swimming behaviour.

Altogether, these results support the hypothesis that the smallest MPs at high concentrations can trigger deleterious effects on early life stage of Pacific oyster over short-term exposure. Future studies are needed to verify whether LDPE MPs have similar effects on D-larvae and investigate the impacts of ingestion of nanometer-sized PE particles on D-larvae. It would also be particularly relevant to evaluate the long-term effects of chronic exposure to environmental concentrations of MPs.

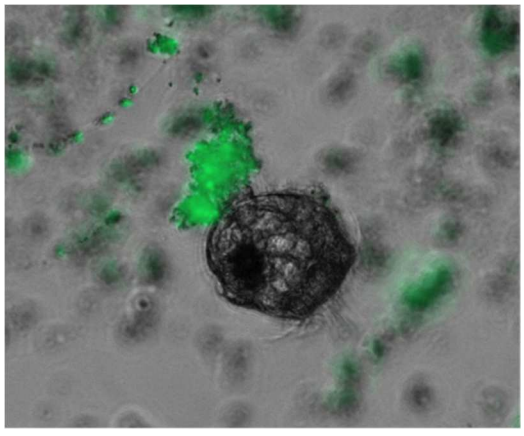


Figure 6. D-larva with MP stuck in her locomotor eyelashes. Screenshot from a video recorded with the ZEISS Axio Observer Z1 microscope (x20). In order to observe MP behaviour, we used fluorescent microbeads (1-5 µm, Cospheric).

5. Conclusions

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

Supplementary data

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

Acknowledgements

A. Bringer was recipient of a PhD grant (Comité Régional de la Conchyliculture de la Charente Maritime CRC17) and for financial support by Région Nouvelle Aquitaine and Comité Départemental de la Charente Maritime (CD17) to develop this research. This work was supported by grants from the AQUAECOs (**A**mélioration de la **QUA**lité **E**nvironnementale sur les zones **CO**nchylicole**s** des Pertuis Charentais) project funded by partnership CRC17, AFB (Agence Française de la Biodiversité, *Parc naturel marin de l'estuaire de la Gironde et de la mer des Pertuis*). Funding was also supported in part by the La Rochelle University, the University of Bordeaux, the Centre National de la Recherche Scientifique (France). The authors greatly thank France Naissain for providing the oysters

genitors specimens & James Emery for its kind contribution to English review of the manuscript. We thanks' all collaborators for their works and their help during this study.

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SUPPLEMENTARY DATA

Table S1. Frequency of abnormal D-larvae (%) and developmental arrest (%) in D-larvae of *C. gigas* exposed to Cu or PE MPs. Control (without pollutants): 0.1, 1 and 10 correspond to concentrations of copper (µg Cu.L-1) or PE MP (mg MP.L-1) using for exposures. Means values ± SEM. Different letters indicated significant differences between different concentrations ($p<0.05$).

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

Table S2. Maximum and average speeds (µm.s^{-1}) recorded for D-larvae of *C. gigas* exposed for 24h to different concentrations of Cu or PE MPs. Control (without pollutants): 0.1, 1 and 10 correspond to concentrations of copper (µg Cu.L^{-1}) or PE MP (mg MP.L^{-1}) used for exposures. Means values ± SEM. Different letters indicated significant differences between different concentrations ($p<0.05$).

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}

Graphical abstract