

## Accepted Manuscript

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PII: S0304-3894(18)30661-7  
DOI: <https://doi.org/10.1016/j.jhazmat.2018.07.101>  
Reference: HAZMAT 19610

To appear in: *Journal of Hazardous Materials*

Received date: 16-3-2018  
Revised date: 13-7-2018  
Accepted date: 26-7-2018

Please cite this article as: Beiras R, Bellas J, Cachot J, Cormier B, Cousin X, Engwall M, Gambardella C, Garaventa F, Keiter S, Le Bihanic F, López-Ibáñez S, Piazza V, Rial D, Tato T, Vidal-Liñán L, Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton, *Journal of Hazardous Materials* (2018), <https://doi.org/10.1016/j.jhazmat.2018.07.101>

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## Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton

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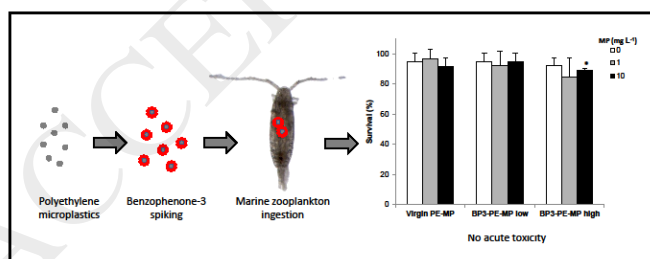
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Declarations of interest: none

### GRAPHICAL ABSTRACT



## HIGHLIGHTS

- PE-MP are ingested by organisms representative of zooplanktonic marine taxa
- Virgin or BP-3 spiked PE-MP do not cause toxic effects on marine zooplankton
- BP-3 spiked PE-MP cause premature hatching of fish eggs compared with virgin PE-MP
- PE-MP do not pose remarkable environmental risk on marine plankton communities

## Abstract

Toxicity of polyethylene microplastics (PE-MP) of size ranges similar to their natural food to zooplanktonic organisms representative of the main taxa present in marine plankton, including rotifers, copepods, bivalves, echinoderms and fish, was evaluated. Early life stages (ELS) were prioritized as testing models in order to maximize sensitivity. Treatments included particles spiked with benzophenone-3 (BP-3), a hydrophobic organic chemical used in cosmetics with direct input in coastal areas. Despite documented ingestion of both virgin and BP-3 spiked microplastics no acute toxicity was found at loads orders of magnitude above environmentally relevant concentrations on any of the invertebrate models. In fish tests some effects, including premature or reduced hatching, were observed after 12 d exposure at  $10 \text{ mg L}^{-1}$  of BP-3 spiked PE-MP. The results obtained do not support environmentally relevant risk of microplastics on marine zooplankton. Similar approaches testing more hydrophobic chemicals with higher acute toxicity are needed before these conclusions could be extended to other organic pollutants common in marine ecosystems. Therefore, the replacement of these polymers in consumer products must be carefully considered.

**Keywords:** polyethylene, marine litter, marine zooplankton, Benzophenone-3, Embryo-larval bioassays

## Introduction

According to recent estimations, between 4.8 and 12.7 million Tons of plastic litter enter the global oceans every year [1]. Conventional plastic polymers are not biodegradable and they are supposed to persist in the environment for hundreds of years. However, plastic debris both

floating in open ocean [2], or sunk in the bottom [3] do not show increasing temporal trends. The combination of thermo-oxidative breakage of the polymeric chains, photodegradation, and leaching of plasticizing additives make plastic objects increasingly brittle and susceptible to mechanical abrasion, especially in high energy environments such as the exposed coastline. Therefore, fragmentation of plastic objects into particles within the microns range, termed microplastics (MP), has been suggested to explain this paradox. In fact abundance of MP on surface waters has increased over the last few decades [4], although no clear temporal trends have also been reported [5]. The ingestion of MP by marine zooplanktonic organisms is well documented [6, 7], and it has been suggested to be a significant input of plastics into the marine trophic webs [8]. Toxicity of MP to planktonic organisms though is a debatable issue, and both reports on acute toxicity [9-11] and lack of effects [12-14] have been published, with endpoints related to motility being specially sensitive [15].

Polyethylene (PE) is the plastic polymer with the highest global production [16], and the most frequently identified in marine litter [16, 17]. PE shows a very low glass transition temperature and plasticizers are not needed in PE objects. However it is susceptible to photo-oxidation, and UV stabilizers are added in outdoor applications. Benzophenone-3 (BP-3, CAS: 131-57-7), IUPAC name (2-hydroxy-4-methoxyphenyl)-phenylmethanone, is a common UV filter used in cosmetics such as sunscreen body creams in amounts between 5-10% of the final product, and as light stabilizer in plastics. The use in sunscreens represents a direct input in coastal waters, where maximum levels reported reached from 0.4 [18] to 3.3  $\mu\text{g L}^{-1}$  [19]. The hydrophobicity of this chemical ( $\log K_{ow}=3.79$ ) predicts its sorption to MP.

Early life stages (ELS) of copepods, bivalves, sea-urchins and fish are extremely sensitive to chemical pollutants, with acute toxicity thresholds frequently in the order of 10-100  $\mu\text{g L}^{-1}$  [20-22]. For this reason, ELS are frequently used for the assessment of pollution in the marine environment, and internationally accepted standard methods are available [23]. According to

literature, MP loads in seawater are within the  $\mu\text{g L}^{-1}$  range, and the highest MP loads reported in the sea are around 0.08 to 0.3  $\text{mg L}^{-1}$  [24, 25]. Following the precautionary principle, we have tested MP effects at densities up to 10-100  $\text{mg L}^{-1}$ , far above current environmental concentrations.

The aim of this study was to test whether the ingestion of, or direct contact with, virgin or BP-3-spiked PE MP caused acute toxicity to a battery of species representative of the main systematic groups present in marine zooplankton, including rotifers, bivalve, crustacean, echinoderm and chordate ELS.

## 1. Materials and methods

### 1.1. Microplastics

Not fluorescent low-density PE microplastics (hereafter PE-MP), with 0.96 to 0.99  $\text{g/cm}^3$  density and particle sizes ranging from 1 to 500  $\mu\text{m}$  were purchased from Micro Powders Inc. (NY-USA), Cospheric (CA, USA), and Rotogal (Boiro, Galicia, Spain). These included CPMS-0.96 (size range 1-4  $\mu\text{m}$ ), MPP-635XF (4-6  $\mu\text{m}$ ), MPP-635G (11-13  $\mu\text{m}$ ), Aquatex-325 (11-15  $\mu\text{m}$ ), Aquamatte (6-8.5  $\mu\text{m}$ ), MPP-1241 (20-25  $\mu\text{m}$ ), and Rotogal micronized PE (<500  $\mu\text{m}$ ), the latter sieved by 40 or 63  $\mu\text{m}$  prior to toxicity testing. In addition, fluorescent green (FMG-1.3, nominal size 1-5  $\mu\text{m}$ , 1.3  $\text{g/cm}^3$  density, 414 nm excitation/515 nm emission) and fluorescent red (FMR-1.3, nominal size 1-5  $\mu\text{m}$ , 1.3  $\text{g/cm}^3$  density, 575 nm excitation/607 nm emission) PE particles purchased from Cospheric were used for documenting particle ingestion in rotifers, copepod and mussel larvae.

### 1.2. Chemicals

Potassium dichromate (VI) ( $\text{K}_2\text{Cr}_2\text{O}_7$ ), BP-3 (purity  $\geq 98\%$ ), and polyoxyethylene (20) polyethylene glycol sorbitan monolaurate (Tween<sup>®</sup>20,) were purchased from Sigma-Aldrich (Schnelldorf, Germany). Other chemicals were of analytical grade, and included copper

standard solution from Fischer Scientific (Portugal), toluene, 3,5-dichlorophenol (3,5-DCP), and dimethyl sulfoxide (DMSO,) from Sigma-Aldrich (Germany). Internal standards as 2,3,4,5,6-pentadeuteriobenzoic acid, 1-bromo-4-phenylbenzene, 1,2,3,4,5,6,7,8-octadeuterionaphthalene and 1,2,3,4,5-pentachloro-6-(2,3,4,5,6-pentachlorophenyl)benzene were purchased from Sigma Aldrich, and 1,2,3,4,5,6,7,8,9,10-decadeuteriophenanthrene was purchased from CDN Isotopes. Solvent used for the extraction of BP-3 were purchased from Promochem, ethyl acetate and cyclohexane. Sartorius provided syringe filter RC4, for the filtration of microplastics. A stock solution of BP-3 was prepared by dilution in DMSO, and diluted in distilled water and in natural 0.22  $\mu\text{m}$ -filtered sea water (FSW), 37‰ salinity. The final concentration of DMSO never exceeded the NOEC previously found for this chemical for each testing organism.

### 1.3. BP-3 spiking

For the preparation of BP-3 spiked MP (hereafter BP3-PE-MP), we aimed at two concentrations that represented, according to literature data, a concentration most likely showing effects (hereafter termed 'high' concentration), and a more environmental relevant concentration ('low' concentration)[18, 26-29]. To create the different concentrations, 25 g of MP particles were transferred into each of 5 separate 250 mL narrow-mouth Septa bottles (Thermo scientific), for a final MP concentration of 125 g L<sup>-1</sup>. BP-3 concentrations in the stock solutions (0.1% toluene) were 20  $\mu\text{g L}^{-1}$  for the high concentration, and 200 ng L<sup>-1</sup> for the low concentration. The bottles were placed on a rotary shaker at the lowest speed (20 rpm) for 2 days. Filtration of the samples was done using a funnel and glass microfiber filter (1.0  $\mu\text{m}$ , Whatman® glass microfiber filters, GE Healthcare Life Sciences). Samples were transferred on the filter, and plastic debris were rinsed with double-deionized water and dried by vacuum evaporation on a ceramic funnel. After 2 days the concentrations on the plastic were determined using an aliquot of the sample (0.3 g) spiked with internal standards (i.a. ,3,4,5,6-

pentadeuteriobenzoic acid, 1-bromo-4-phenylbenzene, 1,2,3,4,5,6,7,8-octadeuterionaphthalene, 1,2,3,4,5-pentachloro-6-(2,3,4,5,6-pentachlorophenyl)benzene, and 1,2,3,4,5,6,7,8,9,10-decadeuteriophenanthrene) and concentrated with a factor of 10.

The samples were extracted with a solvent mixture: ethyl acetate/cyclohexane (1:1). Additionally, an external calibration standard, a blank sample and a recovery sample were prepared. The extracts were measured with GC/MS/MS, electron impact ionization (GC: Agilent 7890A, MS/MS: Agilent 7000 Multiple-Reaction Monitoring (MRM) mode) using a splitless injection of 2  $\mu$ L (column DB5-MS, 30m x 0.25 mm x 0.25  $\mu$ m) Identification and quantification was performed against the retention time and the external calibration using the compound specific Selected Reaction Monitoring (SRM, one quantifier and one qualifier).

#### 1.4. Toxicity testing

##### 1.4.1. *Brachionus plicatilis*

Acute test toxicity tests were performed with the rotifer *Brachionus plicatilis*, following standard methods [30, 31]. Rotifers were obtained by hatching cysts purchased from MicroBioTests, Inc. (Gent, Belgium). Briefly, cysts were placed into Petri dishes ( $\varnothing$  6 cm) containing 10 ml of FSW at 20‰ salinity and incubated at 25°C at an illumination level of 1000 to 3000 lux. The stock was considered suitable for testing when approximately 50% of cysts have hatched in 24 h. For the bioassay, 24 h-hatched rotifers were transferred by Pasteur pipette into a beaker in a final concentration of 10–15 organisms per ml. Then, they were placed in glass vials containing 1 mL of reference toxicant ( $K_2Cr_2O_7$ ), control seawater, or serial dilutions of BP-3, PE-MP and BP3-PE-MP (see Tables 1 and 2). Rotifers were placed in an orbital shaker set at 1 rpm and incubated at 25°C for 48 h. No surfactant was added. The reference toxicant  $K_2Cr_2O_7$  was used to check the biological quality of the stock at the following concentrations: 0, 0.12, 0.25, 0.5-1 mg L<sup>-1</sup>. Endpoints recorded after 48 h were mortality and immobility. Dead organisms were characterized by a lack of movement, including mastax and

foot movement, for 5 s, and immobile organisms were considered those that do not swim nor shift their barycentre but move their appendages. Each treatment, including the control, was prepared in triplicates.

In addition, 1-4  $\mu\text{m}$  polyethylene green fluorescently labelled particles were employed for evaluating MP accumulation in rotifers after 48 h. Tests were performed as described above. Following exposure, the organisms were removed and washed with fresh FSW three times to remove MP bound to the exoskeleton: in this way, according to Nasser and Lynch [32], only ingested particles would be assessed. Organisms were fixed in 4% paraformaldehyde solution in phosphate-buffered saline (PBS, pH 7.4) and observed under an Olympus BX61 epi-fluorescence microscope. Images were acquired, stored and displayed with a software program, using TIFF image format.

#### 1.4.2. *Tigriopus fulvus*

Acute toxicity tests with the crustacean *T. fulvus*, a harpacticoid copepod widely distributed in the Mediterranean Sea, were performed following the UNICHIM protocol [33], based on the international standard ISO14669 [34]. *T. fulvus* adults were maintained in laboratory culture inside 0.5 L polystyrene tissue culture flasks at  $20 \pm 2$  °C with a 16:8 light/dark photoperiod. Nauplii originating from synchronised cultures (24h) were obtained from ovigerous females [35].

For the bioassay set-up, 1 mL of nauplii (15/20 organisms per mL) were transferred by using a Pasteur pipette into small glass vials containing 1 mL of reference toxicant ( $\text{Cu}^{2+}$ ), control FSW, or serial dilutions of BP3, PE-MP and BP3-PE-MP (see Tables 1 and 2). The reference toxicant  $\text{Cu}^{2+}$  was used to check the biological quality of the stock at the following concentrations: 0-0.12-0.25-0.5-1  $\text{mg L}^{-1}$  by evaluating and comparing the  $\text{EC}_{50}$ . Vials were then placed into 50 mL conical centrifuge tubes (5 vials/tube), fixed on a rotatory wheel (speed 2 rpm) and kept at 20°C. No surfactant was added. After 48 h, nauplii were transferred from glass vials into a



multiwell plate, to allow their observation with a stereomicroscope. The percentage of immobile organisms was evaluated, considering as immobile organisms that were unable to move any appendage in a period of up to 20 s of observation and light stimulation of well solution. Each treatment, including the control, was prepared in triplicates.

As reported in the previous paragraph, MP accumulation was also evaluated in copepods after 48 h by exposing nauplii to 1-4  $\mu\text{m}$  Cospheric polyethylene green fluorescently labelled particles.

#### 1.4.3. *Acartia clausi*

Acute lethal toxicity tests with *A. clausi* were conducted following standard methods [34] adapted to nauplius larvae. Biological quality of the stock was checked using 3,5-DCP as reference toxicant. The 48-h  $\text{LC}_{50}$  cannot depart from the range of 0.5 to 1.5  $\text{mg L}^{-1}$  [34]. Additional acceptability criterion was percentage mortality of controls  $\leq 10\%$ . From 48 to 72 h before the start of the test, *A. clausi* mature adults obtained from a laboratory stock maintained by ECIMAT (University of Vigo) were maintained in 2 L jars and fed with cell suspension of *Rhodomonas lens* in an isothermal room at 20 $^{\circ}$  C with bubbling filtered air. Using a 40  $\mu\text{m}$  mesh,  $\leq 24$  h nauplii were collected and placed in a 250 mL beaker. Under binocular stereoscope 10 nauplii were delivered by glass pipette in each of the 20 mL glass vials with no air space. A total of 5 vials per treatment were used. Treatments included control FSW and serial dilutions of PE-MP and BP3-PE-MP (see Tables 1 and 2). The vials were placed in a rotatory wheel set at 1 rpm at a temperature of 20 $\pm$ 1  $^{\circ}$ C and under an 18/6 h light/dark photoperiod. No surfactant was added. Copepod survival was recorded after 48 h of exposure.

#### 1.4.4. *Mytilus galloprovincialis*

Mature mussels were purchased in local markets and gametes were obtained by thermal induction of spawning, according to the methods described by Beiras and Bellas [36]. Mature

oocytes were transferred to 50 mL measuring cylinders, ca. 100  $\mu$ L of diluted sperm were added, and the mixture was shaken gently to facilitate fertilization. Fertilized eggs were transferred before the first cleavage into 25 mL glass vials filled with no head space, at a density of 40 per mL. The vials were filled to the top to avoid the presence of bubbles and placed in a rotatory wheel set at 9 rpm, except for the preliminary test (see below), at a temperature of  $20\pm 1$  °C in the dark. After 48 h incubation, vials were fixed with two drops of 40% formalin and percentage of normal larvae (n=100 per vial) was recorded under an inverted microscope (Carl Zeiss, Axiovert 40 CFL). Mussel larvae were considered abnormal when they did not reach veliger stage, showed irregular shape, convex hinge, and/or protruding mantle [37]. Control normality was always > 75%.

A preliminary experiment was conducted to determine the most appropriate stirring method to evaluate the toxicity of microplastics to mussel embryos. With that aim, three PE concentrations: 20, 50 and 100 mg L<sup>-1</sup> plus a plastic free control (n = 4), were tested in static conditions, on a rotatory wheel (9 rpm), and on an orbital shaker (200 rpm). Volume of experimental vials was 10 mL in static treatment and orbital shaker, and the remaining conditions were the same as described above. No surfactant was added. This experiment showed that the orbital shaker conditions significantly affected the development of larvae mussel, measured as the number of the total larvae and as the percentage of D-veliger larvae, in all concentrations tested of the microplastics. Subsequent experiments were conducted following above described methods except using rotatory mixing only. Control seawater and serial dilutions of PE-MP and BP3-PE-MP (see Tables 1 and 2) were tested.

#### 1.4.5. *Paracentrotus lividus*

Adult sea-urchins were collected from the outer part of Ría de Vigo (NW Iberian Peninsula), maintained in 250 L glass fiber tanks with circulating sand-filtered seawater at a rate of 9 L min<sup>-1</sup>, and fed two times per week with natural collected algae. Temperature was  $18\pm 2$  °C, salinity

34±2 psu and oxygen >5 mg L<sup>-1</sup>. Biological quality of the stock was checked using a certified (1000±4 mg L<sup>-1</sup>) Cu standard solution. The Cu EC<sub>50</sub> values obtained cannot depart more than 30% from the historical chart data. According to the methods described by Beiras, Durán, Bellas and Sánchez-Marín [38], sea-urchin gametes were obtained by dissection of ripe adults, mature oocytes were transferred to 50 mL measuring cylinders, ca. 1 µL of undiluted sperm were added, and the mixture was shaken gently to facilitate fertilization. Fertilized eggs were transferred before the first cleavage into 25 mL glass vials filled with no head space, at a density of 40 per mL. The vials were placed in a rotatory wheel set at 1 rpm at a temperature of 20±1 °C in the dark. Serial dilutions of PE-MP and BP3-PE-MP plus FSW and (when needed) solvent or dispersant controls were carried out. Four replicates per treatment were tested. In non-spiked MP experiments dispersion of the particles was facilitated by using 0.03 µL of Tween®20 per mg MP. This yields a maximum Tween®20 concentration of 3 µL L<sup>-1</sup>, below the experimental NOEC of 7 µL L<sup>-1</sup> obtained for this chemical in preliminary trials.

After 48 h incubation, vials were fixed with four drops of 40% formalin and length (maximum linear dimension in n=35 per vial) was recorded using an inverted microscope (Leica DMI 4000B) and Leica QWIN image analysis software. Acceptability criteria were percentage of fertilized eggs > 98 % and size increase in controls > 253 µm [39].

#### 1.4.6. *Oryzias melastigma*

*O. melastigma* adults were reared at IFREMER from a stock obtained as embryos in 2007 from Doris Au's laboratory (State Key Laboratory in Marine Pollution, City University of Hong Kong, China). Juveniles and adult fish were reared in recirculating systems in water at 25 psu salinity in an isothermal room at 27 ± 1 °C with a 14/10 h light/dark photoperiod. Eggs were collected by siphoning tanks bottom within hours after light onset and quickly cleaned. Then eggs were rolled on sandpaper (p.2000) as described for Japanese medaka [40], extensively rinsed in

order to remove debris and finally transferred in Petri dish filled with artificial 20  $\mu\text{m}$ -filtered synthetic artificial seawater (ASW) (Instant Ocean; salinity 25‰, pH=8) containing methylene blue. Embryo-larval exposures have then been conducted within EPOC laboratory facility (University of Bordeaux). One day post fertilization (dpf) embryos, at morula stage, were exposed to virgin PE-MP at 1 and 10  $\text{mg L}^{-1}$  until 13 dpf, at the end of eleutheroembryo phase. Control condition consisted in embryos reared in sea water only. Exposure units were 70 mm diameter glass Petri dishes, 25 mL of ASW and 30 embryos. Each treatment condition was replicated at least 3 times. The experiment took place in temperature-controlled chambers (Snijders Scientific, Tilburg, The Netherlands) at  $28 \pm 0.3$  °C with a 14:10 light:dark photoperiod, 5000 lx white light, under 27 rpm. rotation (horizontal shaker) to imitate low ocean hydrodynamics and synchronize hatching [41]. Half of the exposure medium was renewed daily. After hatching, yolk-sac larvae were transferred in glass beakers containing 20 mL of exposure medium. Yolk-sac larvae were not fed during the experiment. No surfactant was added. Dissolved oxygen was daily checked to be >90% saturation using a fiber-optic oxygen mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburg, Germany). Dead embryos and larvae were daily recorded and immediately removed to avoid alteration of the medium. Acute toxicity has been monitored using a stereomicroscope according to endpoints defined in OECD guideline TG236.

This study was conducted in accordance with the French and European policies on animal testing [43].

### **1.5. Statistical methods**

Statistical analyses were conducted using IBM SPSS statistics software. Normal distribution and homoscedasticity of the data was checked using the Shapiro-Wilk's and Levene's tests respectively. When significant differences ( $p < 0.05$ ) among groups were found using ANOVA then each treatment was compared to the control using Dunnett's post hoc test to calculate

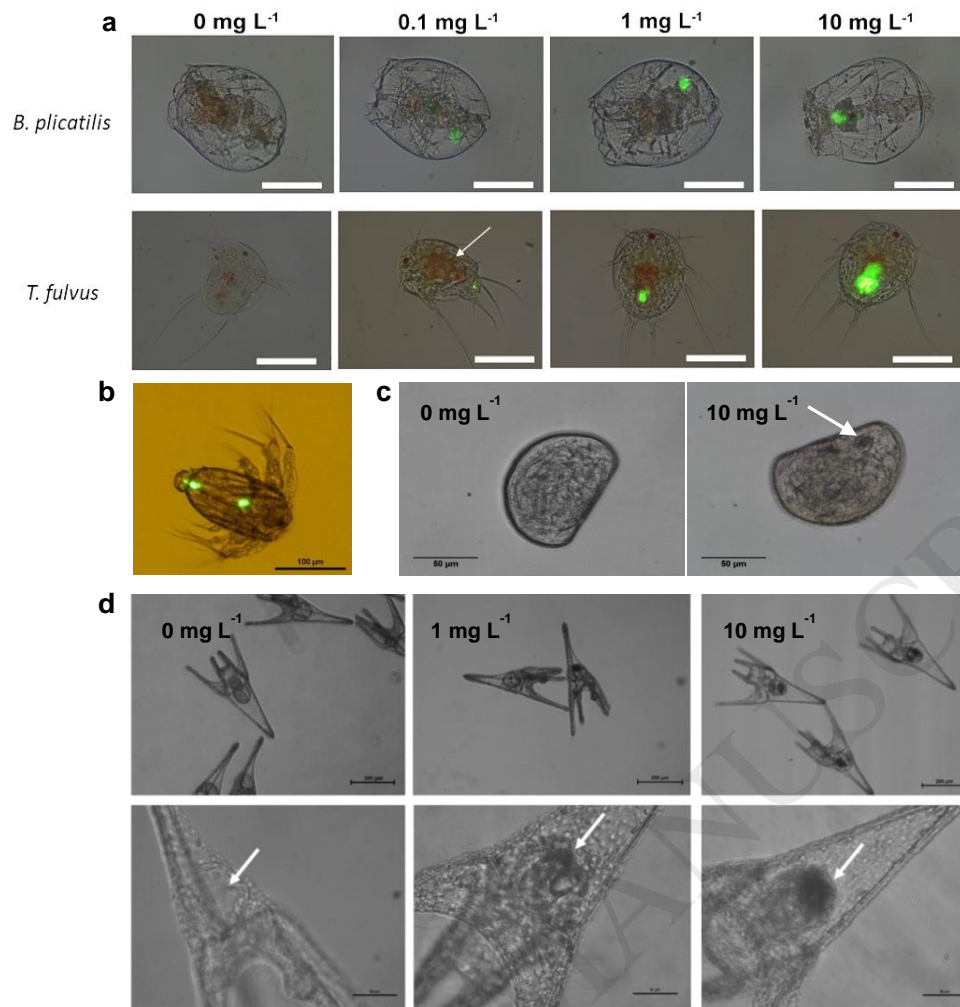
the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC). Non-parametric post hoc tests were used for heteroscedastic data. Lethal ( $LC_{50}$ ) and Effective median concentrations ( $EC_{50}$ ), i.e. concentrations causing a 50% reduction in survival or the sublethal endpoint recorded, and their 95% confidence limits, were calculated using Probit or Trimmed Spearman Karber analysis [44].

## 2. Results

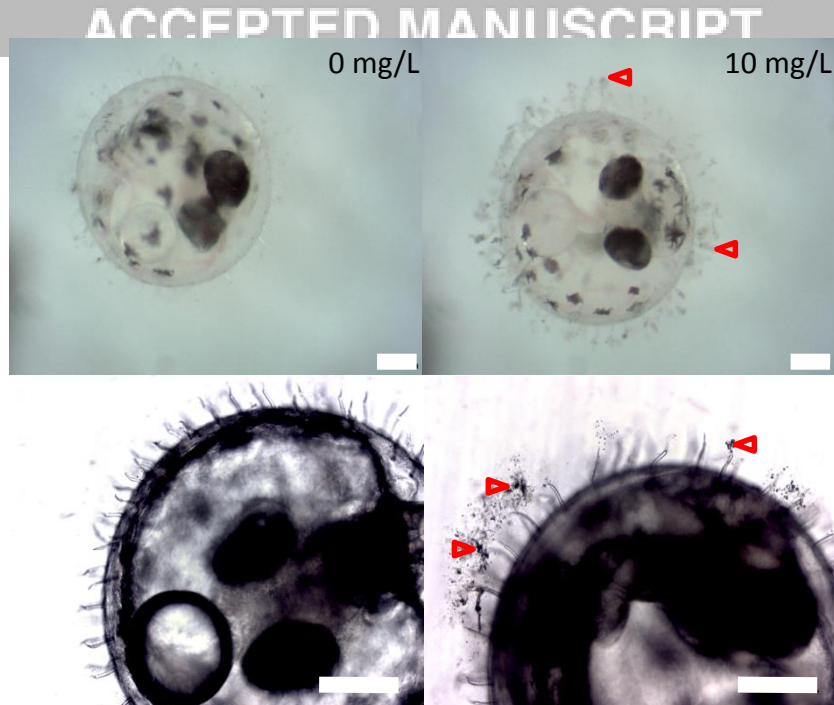
### 2.1. MP ingestion

The rotifers, copepod nauplii, bivalve veliger, and the sea-urchin *plutei* larvae all actively ingested the MP offered at 1 and 10 mg L<sup>-1</sup> (Fig. 1b-d), except for Rotogal PE that even after sieving seemed too large to be ingested by the biological models used. In rotifers and *T. fulvus* nauplii MP were also offered at 0.1 mg L<sup>-1</sup>. In those treatments ingested MP were not observed in *T. fulvus*, and only a small percentage (<30%) of rotifers showed ingestion. Some differences in the distribution of ingested MP in the digestive system across taxonomic groups were noticed. MP preferentially accumulated at the end of the gut in *copepod* larvae (Fig. 1b), throughout the whole digestive tract in bivalve veliger and rotifers (Fig. 1a and c), and in the stomach in *plutei larvae* (Fig.1 d).

In the fish tests used in this study, embryos and larvae feed on their vitellus reserves during the time window tested (from 0 to 13 dpf). Microscope observations support the lack of internalization, but show direct contact of PE-MP onto embryos (Fig. 2). Particles stuck to the villi (short non-attaching filaments) at the surface of the chorion.



**Fig. 1 Ingestion of microplastics.** a, *B. plicatilis* and *T. fulvus* nauplii revealing fluorescent polyethylene microparticles (PE-MP) inside. Note that copepods did not ingest PE-MP at 0.1 mg L<sup>-1</sup> (arrow). Scale bars are 100 μm. b, *A. clausi* nauplius larvae with fluorescent PE-MP accumulated on the gut. Scale bar 100 μm. c, *M. edulis* larvae revealing PE-MP inside. Scale bar 50 μm. d, *P. lividus* larvae, notice the transparent stomachs in 0 mg L<sup>-1</sup> in contrast with the dark filled stomachs in the other treatments. Top images x10 magnification, scale bars 200 μm and down images x40 magnification, scale bars are 50 μm.

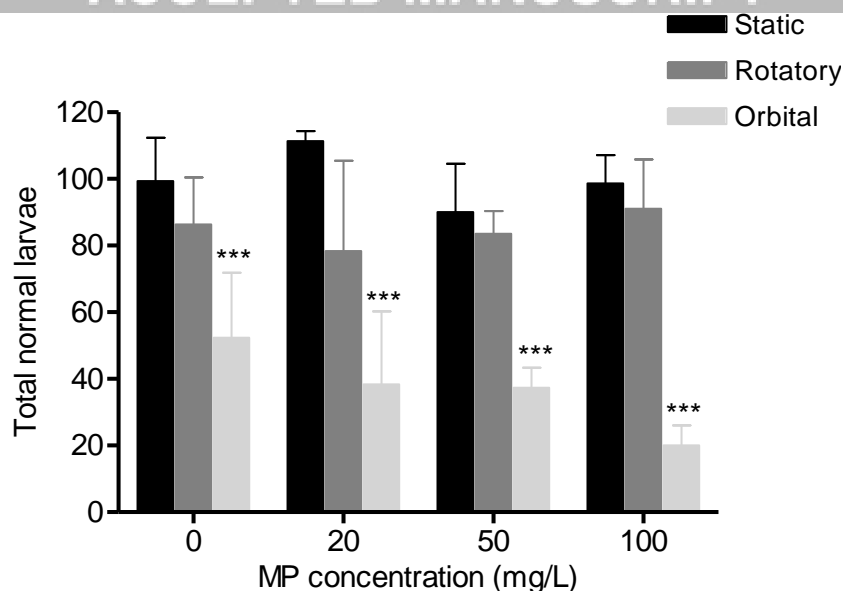


**Fig. 2** *Oryzias melastigma* embryos exposed to 0 and 10 mg PE-MP L<sup>-1</sup> (4-6 μm). Embryos are in direct contact with microplastics stuck on villi at the surface of the chorion. White scale bars 200 μm.

## 2.2. Toxicity tests

### 2.2.1. 'Virgin' PE

When tested in static conditions or in a rotatory wheel, there was no significant effect of any of the tested plastic materials on the mussel embryonic development, either measured as the total number of larvae or as the percentage of D-veliger larvae (Fig. 3). No interaction between MP concentration and mixing conditions was either observed in mussel larvae, according to results of a 2-way ANOVA. However, orbital shaking at the power needed to keep MP in suspension (200rpm) significantly reduced the number of normal D-veliger larvae at the end of the exposure period. Therefore, this kind of agitation causing abnormal development in the control was discarded for further testing.



**Fig 3.** Mussel larval development after exposure of embryos to PE-MP (4-6  $\mu\text{m}$ ) under static conditions, rotatory, and orbital shaking. Results are expressed as total normal larvae observed after 48 h. The same number of embryos was transferred into each experimental vial. Bars represent mean  $\pm$  SD (n=4). Asterisks refer to significant differences with 'static' treatments. \*\*\*  $p < 0.001$ .

The non-spiked PE-MP did not cause any significant effect at any of the concentrations tested below 30  $\text{mg L}^{-1}$  in any of the biological models (Table 1), with the exception of the 1-4  $\mu\text{m}$  particles, which showed a LOEC=0.01  $\text{mg L}^{-1}$  for *B. plicatilis* immobility, a LOEC=1  $\text{mg L}^{-1}$  for *B. plicatilis* mortality ( $\text{LC}_{50} > 10 \text{ mg L}^{-1}$ ), and a LOEC= 1  $\text{mg L}^{-1}$  for *T. fulvus* mortality ( $\text{LC}_{50} = 1.82 \text{ mg L}^{-1}$ ; 95% CI: 1.34-2.48).

**Table 1.** Toxicity testing conditions and results obtained with non-spiked polyethylene microparticles (PE-MP). <sup>(1)</sup> mortality <sup>(2)</sup> immobility <sup>(3)</sup> mortality and immobility.

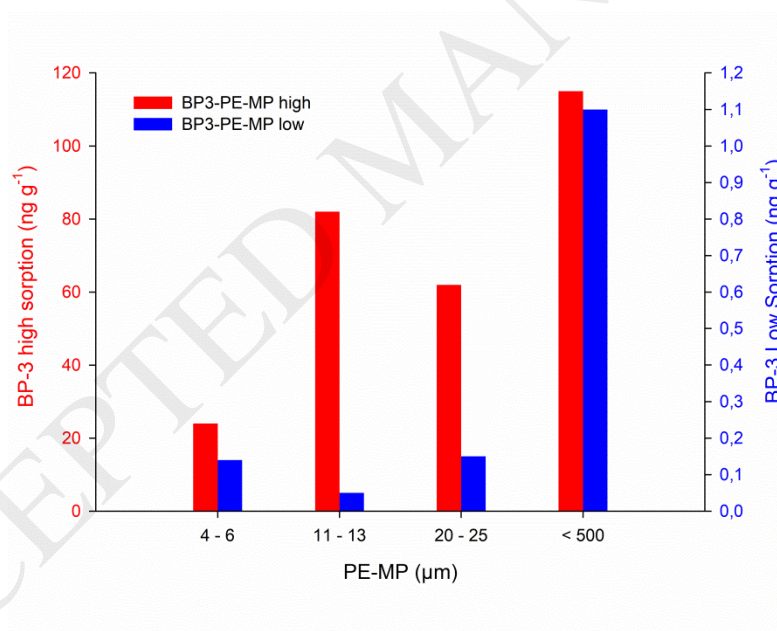
Test spp	MP size	MP loads tested ( $\text{mg L}^{-1}$ )	NOEC/LOEC ( $\text{mg L}^{-1}$ )	Stirring method
<i>B. plicatilis</i>	1-4 $\mu\text{m}$	0, 0.01, 0.1, 1, 10	LOEC=1 <sup>(1)</sup> LOEC=0.01 <sup>(2)</sup>	Horizontal shaker
	4-6 $\mu\text{m}$	0, 0.01, 0.1, 1, 10	LOEC>10 <sup>(3)</sup>	Horizontal shaker
<i>T. fulvus</i>	1-4 $\mu\text{m}$	0, 0.01, 0.1, 1, 10	LOEC=1	Rotatory wheel
	4-6 $\mu\text{m}$	0, 0.01, 0.1, 1, 10	LOEC>10	Rotatory wheel
<i>A. clausi</i>	4-6 $\mu\text{m}$	0, 1, 3, 10, 30	LOEC>30	Rotatory wheel
<i>M. gallop.</i>	1-4 $\mu\text{m}$	0, 20, 50, 100	LOEC >100	Rotatory wheel
	4-6 $\mu\text{m}$	0, 20, 50, 100	LOEC >100	Rotatory wheel
	6-8.5 $\mu\text{m}$	0, 20, 50, 100	LOEC >100	Rotatory wheel
	11-13 $\mu\text{m}$	0, 20, 50, 100	LOEC >100	Rotatory wheel
	<63 $\mu\text{m}$	0, 20, 50, 100	LOEC >100	Rotatory wheel
<i>P. lividus</i>	4-6 $\mu\text{m}$	0, 1, 3, 10, 30, 100	NOEC = 30 LOEC = 100	Rotatory wheel



	11-13 $\mu\text{m}$	0, 1, 3, 10, 30, 100	LOEC>100	Rotatory wheel
	11-15 $\mu\text{m}$	0, 1, 3, 10, 30, 100	NOEC=30	Rotatory wheel
	<40 $\mu\text{m}$	0, 1, 3, 10, 30, 100	LOEC=100	Rotatory wheel
<i>O. melastigma</i>	4-6 $\mu\text{m}$	0, 1, 10	LOEC>100	Rotatory wheel
	4-6 $\mu\text{m}$	0, 1, 10	LOEC >10	Horizontal shaker

### 2.2.2. BP-3 and BP-3-spiked PE

Chemical analyses of BP-3 on the different MP particles (fig. 4) show that a 100-fold increase in dissolved BP-3 concentration reflects in a similar increase in measured BP-3 sorbed to the particles, except for the 11-13  $\mu\text{m}$  particles with the low BP-3 treatment, which yielded sorbed BP-3 concentrations below the limit of detection (LOD=0.05 ng/g). The BP-3 concentration in the non-spiked MP was always <LOD.



**Fig. 4** BP-3 sorption on the different polyethylene microparticles (PE-MP) incubated at low and high BP-3 concentrations.

**Table 2.** Toxicity testing with BP-3-spiked polyethylene microparticles (BP3-PE-MP). The particles used for spiking were 4-6  $\mu\text{m}$  PE-MP except when otherwise stated. Low and high concentrations of spiking correspond to incubations at 0.2 and 20  $\mu\text{g L}^{-1}$  BP-3

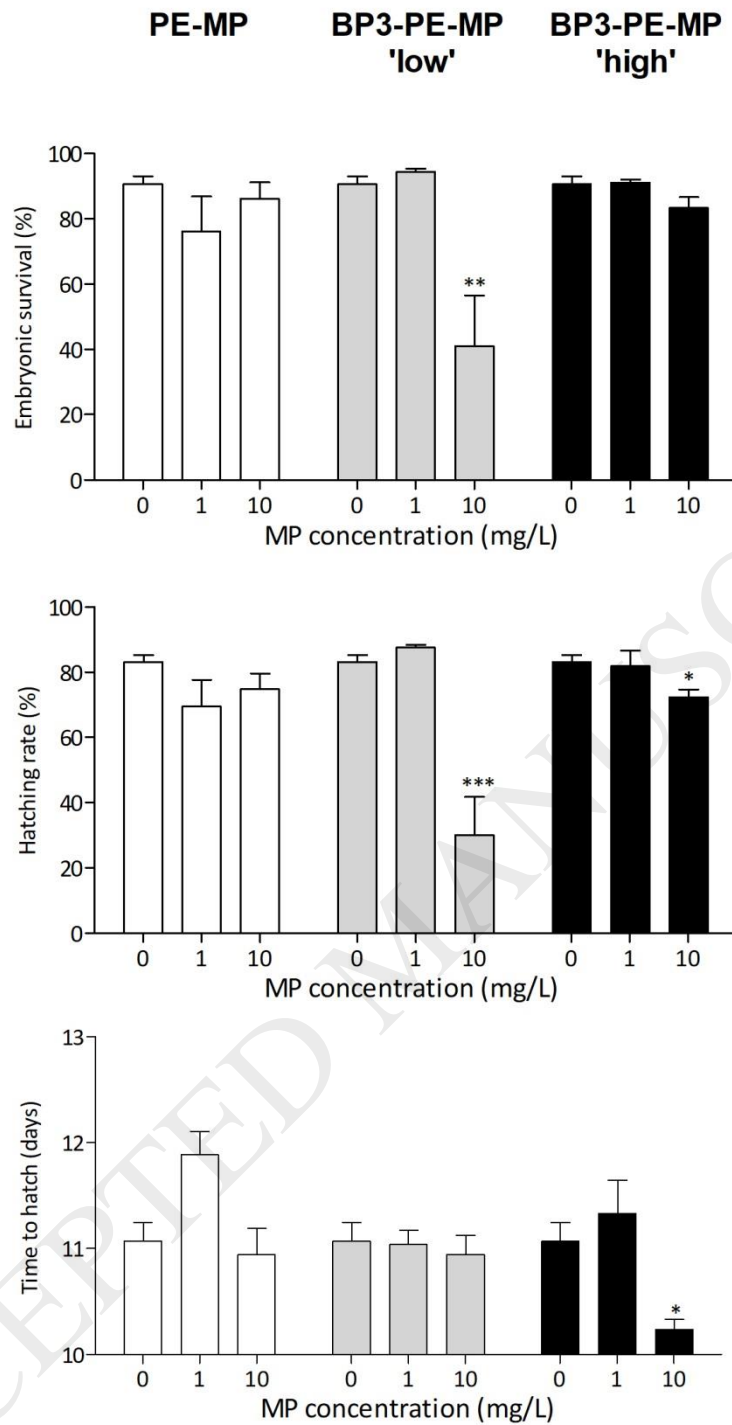
respectively. For *B. plicatilis*, LOEC values refer to both immobility and mortality endpoints.

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Test spp	Testing material	Treatments tested (mg L <sup>-1</sup> )	LOEC (mg L <sup>-1</sup> )	Stirring method
<i>B. plicatilis</i>	Waterborne BP-3	0, 0.5, 1, 2.5, 5, 10	LOEC>10	Horizontal shaker
	BP3-PE-MP low	0, 0.01, 0.1, 1, 10	LOEC>10	Horizontal Shaker
	BP3-PE-MP high	0, 0.01, 0.1, 1, 10	LOEC>10	Horizontal Shaker
<i>T. fulvus</i>	Waterborne BP-3	0, 0.1, 1, 10	LOEC = 1	Rotatory wheel
	BP3-PE-MP low	0, 0.01, 0.1, 1, 10	LOEC>10	Rotatory wheel
	BP3-PE-MP high	0, 0.01, 0.1, 1, 10	LOEC>10	Rotatory wheel
<i>A. clausi</i>	Waterborne BP-3	0, 0.01, 0.05, 0.25, 0.5, 1, 2	LOEC = 1	Rotatory wheel
	BP3-PE-MP low	0, 1, 10	LOEC>10	Rotatory wheel
	BP3-PE-MP high	0, 1, 10	LOEC>10	Rotatory wheel
<i>M. galloprovincialis</i>	BP3-PE-MP low	0, 1, 10	LOEC>10	Rotatory wheel
	BP3-PE-MP high	0, 1, 10	LOEC>10	Rotatory wheel
	BP3-PE-MP (11-13µm) low	0, 1, 10	LOEC>10	Rotatory wheel
	BP3-PE-MP (11-13 µm) high	0, 1, 10	LOEC>10	Rotatory wheel
<i>P. lividus</i>	Waterborne BP-3	0, 0.1, 0.5, 1, 2, 4, 8, 10	LOEC = 1	Rotatory wheel
	BP3-PE-MP low	0, 1, 10	LOEC>10	Rotatory wheel
	BP3-PE-MP high	0, 1, 10	LOEC>10	Rotatory wheel
<i>O. melastigma</i>	Waterborne BP-3	0, 24x10 <sup>-9</sup> , 24x10 <sup>-8</sup> , 24x10 <sup>-6</sup> , 2.4x10 <sup>-3</sup> , 0.024, 0.24, 2.4, 24	LOEC=0.24x10 <sup>-6</sup>	Horizontal shaker
	BP3-PE-MP low	0, 1, 10	LOEC = 10	Horizontal shaker
	BP3-PE-MP high	0, 1, 10	LOEC = 10	Horizontal shaker

Acute toxicity tests with waterborne BP-3 were conducted using *B. plicatilis*, *T. fulvus*, *A. clausi*, *P. lividus* and *O. melastigma*. BP-3 EC<sub>50</sub> values obtained were 1.8 mg L<sup>-1</sup> (95% CI: 1.43-2.32) for *T. fulvus*, 1.3 mg L<sup>-1</sup> for *A. clausi*, 4.11 mg L<sup>-1</sup> (95% CI: 2.14-6.09) for *P. lividus*, and >10 mg L<sup>-1</sup> for *B. plicatilis*, far above those commonly found even in the most polluted waters. In the case of *O. melastigma*, LOEC was 0.24 ng L<sup>-1</sup>, and hatching rate was reduced to about a half at 24 ng L<sup>-1</sup>. However, the response was not monotonic and EC<sub>50</sub> values were not calculable.

There were no significant effects of BP3-PE-MP at any of the spiking concentrations tested on any of the invertebrate planktonic organisms used (Table 2). In contrast, acute toxicity has been observed after 13-d exposure to MP-PE when spiked with BP-3 in fish embryos (Fig. 5). At low BP-3 concentration, 10 mg L<sup>-1</sup> BP3-PE-MP decreased embryonic survival and prevented hatching. At high BP-3 concentration, 10 mg L<sup>-1</sup> did not significantly reduce embryonic survival but prevented hatching and induced premature hatching.



**Fig 5. Toxicity of polyethylene microplastics to *Oryzias melastigma* embryos.** Treatments included non-spiked polyethylene microplastics (PE-MP), and PE-MP spiked with BP-3 at 'low' and 'high' concentrations (BP3-PE-MP). Embryonic survival, hatching rate, and time to hatch was recorded after 12 days of exposure. Bars represent mean  $\pm$ SD, N=3-6. Asterisks refer to significant differences to the control treatment (C). \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .

### 3. Discussion

Oceans cover approximately 70 percent of the Earth's surface. Oceanic food webs are supported by plankton communities, important also for biodiversity as they host a wide variety of species and life forms. Plankton communities are composed not only by organisms such as rotifers and copepods that spend their whole life duration in the plankton (holoplankton), but also others such as bivalves and echinoderms that have planktonic larvae (meroplankton) although adult life stages inhabit the benthos. Furthermore, many pelagic fish lay their eggs in the neuston, the surface layer of the water, and larvae undergo a planktonic life. Therefore, potential effects of pollution on planktonic organisms will also affect the benthic and pelagic habitats.

ELS of copepods, bivalves, sea-urchins and fish are extremely sensitive to chemical pollutants, which explain their use in regulatory tools for the marine environment. Due to their low density, PE and other polymer MP accumulate in the surface layer of the water. Both weathering and biofouling may affect the buoyancy of these particles, which can be found also in the water column. Therefore, planktonic organisms are readily exposed to MP. In addition, planktonic suspension feeders such as rotifers, copepods, bivalve and echinoderm larvae are capable to ingest particles within the same size fractions that the MP used in this study, from 1 to 25  $\mu\text{m}$ . Microplastic toxicity testing with water column organisms demands experimental set-up allowing homogeneous dispersion of the particles in the water column. We have found that smooth rotatory shaking allows MP dispersion and subsequent ingestion by zooplankton with no need of surfactants that may interfere in toxicity and depart from environmentally relevant conditions. For mussel larvae, rotatory shaking provided a homogeneous plastic suspension with both positive and negative buoyancy particles, and did not increase background mortality compared to static controls. In contrast orbital shaking caused unacceptably high background abnormalities in control treatments, probably due to physical

damage caused at the high orbital speed (200rpm) needed to keep MP in suspension by this method of stirring. In the case of medaka eggs, a smooth shaking allowed both normal development of embryos and exposure (contact) of MPs to egg chorions.

Maximum densities on MP in marine surface waters range from 0.0002 and 0.32 mg L<sup>-1</sup>, with median of 0.0047 mg L<sup>-1</sup> (reviewed [45]). We have tested toxicity at densities up to 100 mg L<sup>-1</sup> and did not find any effects at concentrations up to 10 mg L<sup>-1</sup> except for 1-4 µm particles for rotifers (LOEC=0.01-1 mg L<sup>-1</sup>) and *Tigriopus* (LOEC=1 mg L<sup>-1</sup>). Therefore the experimental evidence here provided does not support a significant effect of PE MP on a wide range of marine planktonic organisms at environmentally relevant conditions. This is in line with the several of the latest reports published testing effects of MP on marine zooplanktonic organisms [12-14].

When very high MP concentrations were tested we did find some effects on behavioral endpoints related to motility. Using different zooplankton species, Gambardella et al. [15] had already pointed out that behavioral responses were orders of magnitude more sensitive to MP than mortality. Other sublethal trait such as ingestion rate has also been reported to be affected by MP [11]. These findings support the use of behavioral endpoints as a suitable tool for detecting high MP contamination.

Microplastics have been hypothesized to play a role as vectors of hydrophobic organic compounds (HOC) to marine organisms upon ingestion, since they accumulate HOC concentrations far above those in the water column [46] [47]. Other more recent studies question this role on the basis of simple equilibrium partitioning or more sophisticated thermodynamics models [48-51]. In order to test the effects of MP as vectors of HOC to zooplankton, we used BP-3 as model organic, and intended to obtain MP spiked with two distinct concentrations of sorbed BP-3, termed 'high' and 'low'. To the best of our knowledge there are no data available on environmental concentrations of BP-3 in plastic or on toxicity of

BP-3 sorbed to plastics. Therefore, the spiking protocol was designed using BP-3 concentrations in the water representative of toxic and environmentally relevant concentrations, respectively, according to the literature. The method was successful and, despite the comparatively short exposure time, the two distinctly different BP-3 concentrations were achieved, with final concentrations in the MPs ranging from 24 to 115 ng g<sup>-1</sup> for the high concentration, and from 0.14 to 1.1 ng g<sup>-1</sup> for the low concentration, except for the low concentration with the 11-13 µm particles. However, the variation in BP-3 concentrations on the different MP size classes did not follow the expected pattern of a higher sorption rate in smaller (and thus with a higher surface to volume ratio) particles. This might be due to heterogeneity in size and shape within each class of commercial particles (see Supplementary Fig. 1). Although HOC sorption kinetics is a complex issue not addressed by the present experimental design and highly dependent on both inherent polymer properties and environmental weathering, we have found that the ingestion of MP spiked at BP-3 concentrations 100 times higher than those found in the most polluted coastal waters did not cause toxicity to the biological models tested, with the exception of fish ELS. This difference may be due to a higher sensitivity of these organisms and/or to the longer duration of the exposure. Similar approaches testing HOC with higher acute toxicity and  $K_{ow}$  values are needed before these conclusions could be extended to other organic pollutants common in marine ecosystems.

#### **4. Conclusions**

All feeding stages of zooplanktonic organisms representative of marine taxa tested ingested PE-MP within the 1 to 10 µm size range. Acute exposures of zooplanktonic marine invertebrates, including sensitive early life stages, to either virgin or BP-3 spiked polyethylene MP at densities above those reported in the most polluted marine waters did not cause any

toxic effects. In contrast, fish ELS in contact with BP-3 spiked MP showed premature hatching and significantly decreased hatching rates compared to those in contact with virgin MP.

There is still a healthy debate in the scientific community as to whether MP pose an unacceptable risk to the receiving environment. The current study indicates that PE-MP do not pose a significant risk to marine zooplankton, and we recommend careful re-evaluation of the need to replace these polymers in consumer products, especially when little is known about the potential impacts of the alternatives.

### **Acknowledgements**

The authors acknowledge D. Costas, C. Clérandeau, J.C. Crebassa and all the staff of ECIMAT, IFREMER and EPOC laboratories for their helpful technical support.

Funding: This research was conducted in the frame of the JPI-Oceans EPHEMARE Project, funded by the Spanish Government (MINECO) (PCIN-2015-187-C03-03 and CTM2016-77945-C3); the French National Research Agency (Agence National de La Recherche) (ANR-15-JOCE-0002-05); the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (942-2015-1865). R.B. benefited from the Grant “Program of Consolidation and structuring of competitive research groups in the University system of Galicia” by the Galician Government (ED431C 2017/46).



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