



Toxicity assessment of pollutants sorbed on environmental microplastics collected on beaches: Part II-adverse effects on Japanese medaka early life stages

Pauline Pannetier, Bénédicte Morin, Christelle Clérandeau, Jennifer Laurent, Coline Chapelle, Jérôme Cachot

► To cite this version:

Pauline Pannetier, Bénédicte Morin, Christelle Clérandeau, Jennifer Laurent, Coline Chapelle, et al.. Toxicity assessment of pollutants sorbed on environmental microplastics collected on beaches: Part II-adverse effects on Japanese medaka early life stages. *Environmental Pollution*, 2019, 248, pp.1098 - 1107. 10.1016/j.envpol.2018.10.129 . hal-03486956

HAL Id: hal-03486956

<https://hal.science/hal-03486956>

Submitted on 20 Dec 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

1 Toxicity assessment of pollutants sorbed on environmental microplastics collected on beaches: Part
2 II-adverse effects on Japanese medaka early life stages.

3 Pauline Pannetier, Bénédicte Morin*, Christelle Clérandeau, Jennifer Laurent, Coline Chapelle and
4 Jérôme Cachot

5 Univ. Bordeaux, EPOC, UMR 5805, F-33400 Talence, France

6 *Corresponding author: benedicte.morin@u-bordeaux.fr; Tel: +33 (0)5 40 00 22 56 / Fax: +33 (0)5 40
7 00 87 19

8

9 **Abstract**

10 While microplastics are present in great abundance across all seas and oceans, little is known about
11 their effects on marine life. In the aquatic environment, they can accumulate a variety of chemicals
12 and can be ingested by many marine organisms including fish, with chronic physical and chemical
13 effects. The purpose of this paper is to evaluate the toxic effects of pollutants sorbed at the surface
14 of environmental microplastics (MPs), collected on various beaches from three islands of the Pacific
15 Ocean. Developmental toxicity of virgin MPs or artificially coated with B[a]P and environmental MPs
16 from Easter Island, Guam and Hawaii was evaluated on embryos and prolarvae of Japanese medaka.
17 Mortality, hatching success, biometry, malformations, EROD activity and DNA damage were analysed
18 after exposure to DMSO extracts. No toxicity was observed for extracts of virgin MPs whatever the
19 endpoint considered. Extracts of virgin MPs coated with 250 ng.g⁻¹ of B(a)P induced lethal effects
20 with high embryo mortality (+81%) and low hatching rate (-28%) and sublethal effects including
21 biometry and swimming behavior changes, increase of EROD activity (+94%) and DNA damage
22 (+60%). Environmental MPs collected on the three selected islands exhibited different polymer,
23 pollutant and toxicity patterns. The highest toxicity was detected for MPs extract from Hawaiï with
24 head/body length and swimming speed decreases and induction of EROD activity and DNA strand
25 breaks. This study reports the possible sublethal toxicity of organic pollutants sorbed on MPs to fish
26 early life stages.

27
28 **Capsule**

29 This study highlighted the developmental toxicity of environmental and BaP-spiked microplastic
30 extracts on early life stages of Japanese medaka.

31 **Key words:** Environmental microplastic, pollutants, fish early life stages, embryotoxicity, genotoxicity

1 Introduction

First identified as an issue of concern in the 1950s, plastic contamination in marine environments is now the focus of media, public, and scientific attention. Researchers from a number of different fields, such as polymer science, environmental engineering, ecology, ecotoxicology, marine biology and oceanography have taken great interest in the issue (Law, 2017). In 2010, it was estimated that 4.8 to 12.7 million tons of plastics were released into the world's oceans, mostly as a result of shortcomings in waste management procedures (Jambeck et al., 2015).

Plastics are now one of the main types of debris found in the marine environment (Law, 2017). Their persistence in the aquatic environment is estimated to run to hundreds or even thousands of years depending on the type of plastic (Collignon et al., 2012). Plastic debris tend to fragment into smaller pieces through different chemical, biological or physical processes (Barnes et al., 2009). Plastic debris comes in various shapes (fibers, fragments and granules), colors, and sizes. Microplastics (MPs) are particles between 1µm and 5mm in size (Arthur et al., 2009). These small plastic debris can either be issue of the fragmentation of larger plastics (secondary microplastics) or can be produced under this form like microbeads in personal care products and microfibers from textile (primary microplastics).

A recent large review of literature about contamination of marine life by plastic debris, reported 344 species impacted by plastic entanglement, including marine turtles, seals, whales, seabird and 89 species of fish. Besides, 233 marine species were reported to contain plastic in their digestive tract, including 92 fish species, with different consequences according to plastic type and organism size (Law, 2017).

While the plastic contamination in marine ecosystems has been widely documented, there has been little evidence of the deleterious effects of microplastics on marine life. Two major effects can be triggered by exposure to microplastics: physical effects, leading to a blockage of the digestive tract or a feeling of fullness (Cole et al., 2011; Rochman et al., 2013b) along with ulceration or perforation of the digestive tract wall or the gill epithelium (Peda et al., 2016), and toxicological effects induced by

plastic components such as plastic monomers, plastic additives (PAs) e.g. phthalates, bisphenol A, flame retardant, UV stabilizer, dye, etc. (Koelmans, 2015; Koelmans et al., 2014; Rochman, 2015), or pollutants accumulated during plastic ageing. These chemicals are mostly hydrophobic organic chemicals, which sorb onto particle surfaces. They include (among others) polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and organochlorine pesticides (Derraik, 2002; Karapanagioti et al., 2011; Koelmans et al., 2013, 2016). Many of these substances are known to be persistent, bioaccumulative, and toxic, with at least 78% of the priority pollutants identified by the US EPA known to be associated with plastic marine debris (Rochman et al. 2013a). Few studies have reported the toxic effects of artificially spiked microplastics with pollutants, including endocrine perturbation, hepatic stress, oxidative stress, reduced enzyme activity, cellular necrosis, and alteration of immune system (Browne et al., 2013; Law, 2017; Mazurais et al., 2015; Oliveira et al., 2013; Rochman et al., 2014; Teuten et al., 2009; Pittura et al., 2018). The resulting biological consequences of MP exposure could compromise the survival, the growth and reproduction of organisms and cause malformations, particularly in the early life stages of fish that are particularly sensitive to pollutants (Mazurais et al., 2015).

Fish early life stages have been widely used in ecotoxicology, mainly due to their high sensitivity to a number of chemical substances (Embry et al., 2010; Lammer et al., 2009). Tests using fish embryo and eleutheroembryos (prolarvae) are considered *in vitro* assays, and therefore outside the scope of EU regulations (European Union, 2010). Japanese medaka (*Oryzias latipes*) embryos and prolarvae, due to their sensitivity and ease of use are increasingly used for toxicity testing of chemicals, such as metals (Barjhoux et al., 2012; Eaton et al., 1978; Jezierska et al., 2009), nanoparticles (Bai et al., 2010; George et al., 2012), or organic compounds (Cachot et al., 2007; González-Doncel et al., 2008; Helmstetter and Alden, 1995; Le Bihanic et al., 2014a; Vicquelin et al., 2011) and plastics (Rochman et al., 2014). Several studies reported the negative impacts of microplastics on mollusk as brown mussel larvae (Gandara e Silva et al., 2016) or sea urchin (Nobre et al., 2015) and zooplankton

(Beiras et al., 2018). However, to date, no study has been carried out to analyze the toxicity of environmental samples of MPs.

A worldwide sampling campaign (Race for Water Odyssey 2015), was carried out in 2015 by the Race for Water Foundation to harvest microplastics from beaches on islands located near the main oceanic gyres. Only MP samples from three islands of the Pacific Ocean (North Pacific and South Pacific) were considered in this study. The purpose of this paper was to evaluate and characterize the lethal and sublethal effects of pollutants sorbed at the surface of these microplastics on embryos and prolarvae of fish using a wide range of biomarkers. Same biomarkers were analyzed on commercial MPs (virgin or B(a)P coated) obtained from laboratory plastic items.

2 Materials and methods

2.1 Plastic samples

Virgin microplastic mixture was made up of 40% of Low-Density PolyEthylene (LDPE), 25% of High Density PolyEthylene (HDPE), 25% of PolyPropylene (PP) and 10% of PolyStyrene (PS) from newly purchased laboratory plastic items according to environmental sample of MPs from Swiss lakes (Faure et al., 2015). Virgin microplastics were used as plastic control (C-) or positive plastic control (C+) after coating with benzo(a)pyrene solution (B[a]P, CAS Number: 50-32-8) according to protocol described in Pannetier et al, this issue. Final theoretical concentration of B[a]P on MP particles was 250 $\mu\text{g.g}^{-1}$. Environmental microplastics were collected (Pannetier et al., this issue) from beaches on 9 islands located in the vicinity of the oceanic gyres. This study focused on environmental MP samples collected on sandy beaches from three Pacific islands: Kawa bay, Big Island on Hawaii (Ha), Anakena on Easter Island (Ea) and Pago bay on Guam (Gu). Ea sample was composed of 94% PE and 6% PP, Ha sample of 27% PE, 77% PP and 1% PS and Gu sample of 59% PE, 37% PP and 4% of MP particles could not be identified. Beach plastic samples were analyzed for polycyclic aromatic hydrocarbons (PAHs),

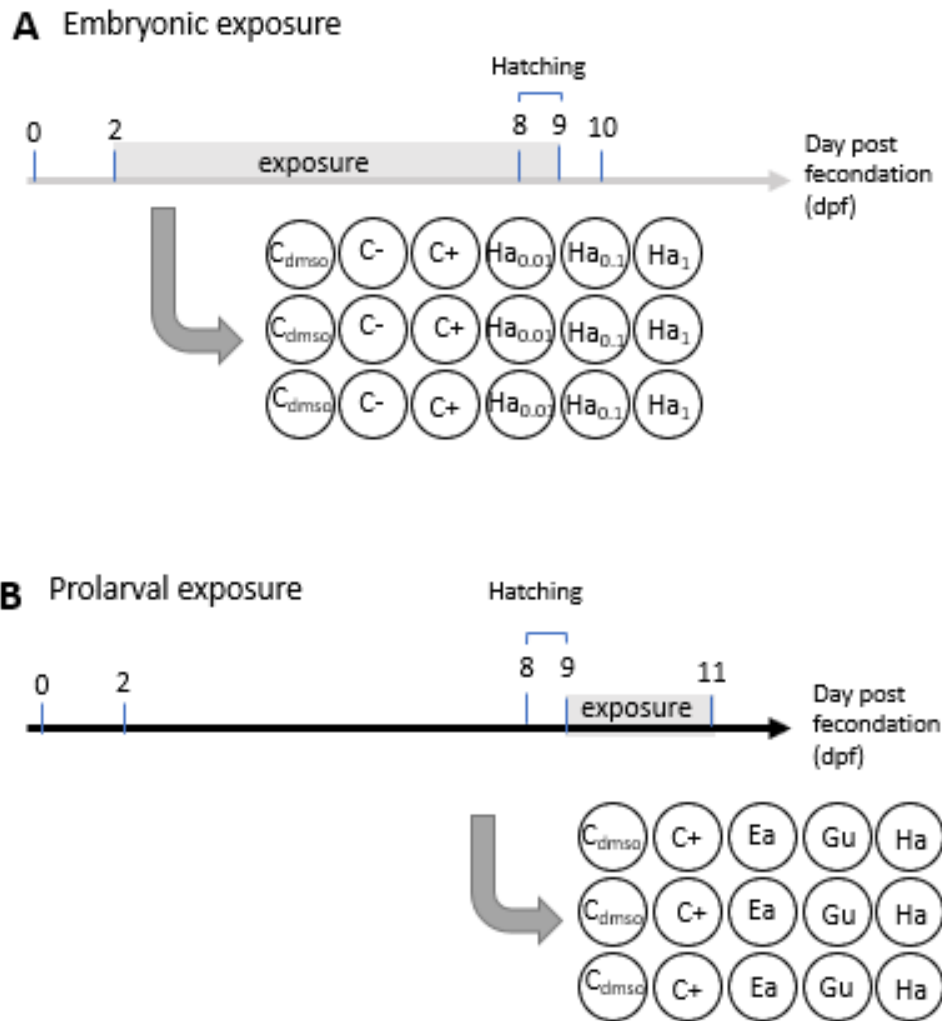
polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT). The concentration of total PCBs ($<5\text{ ng.g}^{-1}$) and DDT ($<4\text{ ng.g}^{-1}$) was under quantification limits for all samples. Concentration of totals PAHs was 18 ng.g^{-1} for Ha, 6 ng.g^{-1} for Gu and 2 ng.g^{-1} for Ea. More information can be found in Part I in this issue (Pannetier et al, this issue).

Environmental and control MPs were grinded and sieved to obtain microparticles measuring less than $600\mu\text{m}$. Organic extracts of MPs were obtained with dimethyl sulfoxide (DMSO) extraction (100mg of MP in 1mL of DMSO, 16h of shaking at 175rpm in dark).

These MPs were used for six treatments in triplicate on embryos : (1) Control solvent (C_{DMSO}), embryos were exposed to 1% of DMSO in Egg Rearing Solution (ERS); (2) negative plastic control (C^-), 1% of virgin microplastic extract in ERS; (3) positive plastic control (C^+), 1% of extract of artificial microplastic coated with $100\text{ }\mu\text{M}$ of B[a]P; (4) 0.01% of Ha microplastic extract in ERS ($\text{Ha}0.01$); (5) 0.1% of Ha microplastic extract in ERS ($\text{Ha}0.1$); (6) 1% of Ha microplastic extract in ERS ($\text{Ha}1$). They were also used for five treatment conditions on prolarvae: (1) Control solvent (C_{DMSO}), just-hatched larvae exposed to 0.1% of DMSO in mixing water; (2) positive plastic control (C^+), 0.1% of virgin microplastic coated with $100\text{ }\mu\text{M}$ of B[a]P extract in mixing water; (3) Easter island (Ea), 0.1% of Ea microplastic extract in mixing water; (4) Guam (Gu), 0.1% of Gu microplastic extract in mixing water; (5) Hawaii (Ha), 0.1% of Ha microplastic extract in mixing water.

2.2 Medaka embryos and prolarvae

Japanese medaka embryos were provided by the UMS Amagen (CNRS, Gif-sur-Yvette, France) at early gastrula stage 14-15 according to Iwamatsu (2004). After reception, embryos were sorted and unfertilized, dead or delayed embryos were carefully removed. Two different experiments were carried out, one lasting 8 days on embryos and another lasting 48h on prolarvae (Fig. 1).



129

130 Figure 1: Experimental design for Japanese medaka embryonic and prolarval exposures to
 131 microplastic extracts

132 The first experiment (Fig. 1A) was an 8-day embryonic-exposure, from 2-day post fertilization (dpf) to
 133 10 dpf, to different dilutions of extracts of MP from Hawaii. After 24h of acclimatization, 2 dpf
 134 embryos were distributed by 25 into a glass petri dish filled with 4 mL of embryo rearing solution,
 135 ERS (1 g NaCl, 0.03 g KCl, 4.04 g CaCl₂ and 0.163 g MgSO₄ in 1L Milli-Q autoclaved water)
 136 amended/not amended with MP-extract (Pannetier et al., this issue). Petri dishes were incubated in
 137 an air-conditioned chamber (Snidjers Scientific, Tilburg, The Netherlands) at 26°C with light/dark
 138 period of 12h/12h at 5000-lx white light. After hatching (8-9 dpf), prolarvae were transferred into 50
 139 mL glass beakers with 40 mL of mixing water (1/3 tap water + 2/3 distilled water). Medium was

renewed every 48h and dissolved oxygen concentration was measured daily with a fiber-optic oxygen mini-sensor Fibox 3 (PreSens Precision Sensor, Regensburg, Germany) and associated software (OxyView v6.02). The pH of ERS solution was also controlled every 48h (WTW, Microprocessor pH Meter, pH 537).

The second experiment was a 48h exposure of prolarvae to MP extracts from the three Pacific islands. At reception, embryos were grown in a Ø 9cm plastic petri dish at 26°C with light/dark period of 12h/12h at 5000-lx white light with ERS renewal each 48h and daily dissolved oxygen control. Petri dishes were gently shaken 24h/24h to synchronize hatching. On peak hatching day, prolarvae were collected and distributed by 50 into 50 mL glass beakers with 40 mL of contaminated or non-contaminated mixing water (1/3 tap water + 2/3 distilled water). Mixing water was renewed every 24h for 48h contamination and dissolved oxygen concentration was measured daily. As no toxicity of negative plastic control (C-) was noted in the first experiment, the 1% of clean microplastic extract in ERS was not tested in this second experiment.

2.3 Biomarkers

2.3.1 Mortality/ Hatching success

Mortality was checked daily and dead prolarvae or embryos were immediately removed to avoid medium degradation. The percentage of mortality was determined according to number of dead embryos/larvae at the end of the exposure compared to number of total embryos/larvae. Mortality was recorded for each stage. Hatching rate was calculated as the number of hatched individuals compared to the total number of embryos. Larval mortality referred to the number of dead prolarvae compared to hatched individuals in cases of embryonal contamination. Dead embryos or prolarvae during acclimatization time (ca. 4%) were not considered.

2.3.2 Biometry/malformations

For embryonic contamination, at hatching, 10 prolarvae per replicate were anesthetized with cold water (4°C). Prolarvae were individually examined to record morphological abnormalities and lesions (spinal, craniofacial deformations and ocular cardiac and yolk sac anomalies, edemas) then photographed at 25X magnification using a stereomicroscope MZ7.5 Leica (Nanterre, France), equipped with a CCD camera DFP420C Leica and associated software Leica Microsystems V3.8. Percentage of abnormal prolarvae was calculated by the number of prolarvae with morphological abnormalities or lesions compared to total number of prolarvae. Photographs were also analyzed to determine total body length (from terminal point of the mouth to the end of caudal fin), head length (from terminal point of the mouth to the rear of operculum) of the hatching prolarvae. The ratio between head length and total body length was also calculated.

2.3.3 EROD activity

The *in vivo* EROD activity of the P4501A protein was quantified in medaka prolarvae by the production of the fluorescent product resorufin. This test was carried out using an *in vivo* quantitative spectrofluorimetric method adapted from Le Bihanic et al., 2013. For each replicate, 5 prolarvae per well were transferred into a 48-wells microplate. Mixing water was removed and replaced with 600 µL of a 1 µM ethoxyresorufin solution (ER). The microplate was then incubated in the dark at 26 °C. After 1h, the solution was removed and replaced with 600 µL of fresh medium containing 1µM of ER. Immediately after 4h of incubation, 100 µL was removed from each well and transferred to a white 96-well microplate. This operation was duplicated. The fluorescence in the 96-microplate is read at 540 nm / 580 nm (λ excitation / λ emission). A resorufin standard range (0 nM, 1.25 nM, 2.5 nM, 5 nM, 10 nM) is used to determine the average resorufin production per well. The *in vivo* EROD activity is calculated in pM of resorufin.larva¹.minute⁻¹.

2.3.4 Comet assay

The Comet assay was performed according to the original protocol of Singh et al. 1988 and adapted to whole medaka larvae with enzymatic dissociation step (Morin et al., 2011). The comet assay is carried out with 5 larvae by replicate. After being anesthetized in benzocaine (200 mg.L⁻¹), prolarvae were crushed and cells dissociated through 45 minutes of shaking in MEM-dispase solution (0.125%) at 37°C. After dissociation, cells were rinsed clean, centrifuged, and fixed between two layers of LMPA (Low melting point agarose, Sigma-Aldrich) on microscope slides pre-coated with of NMPA (Normal melting point agarose) at 200 000 cell.mL⁻¹. Agarose polymerization (2x15 min, 4 °C, dark condition) was followed by lysis at 4 °C in the dark for 1h in lysis solution (2.5 mM NaCl, 100 mM EDTA, 10 mM Tris-HCl, 1% Tritonx100, pH 10). After lysis, slides were placed at 4 °C in an electrophoresis tank containing an alkaline solution (0.3 M NaOH, 1 mM EDTA, pH>13) for 20 min in the dark before electrophoresis at 25 V and 300 mA for 20 min. Electrophoresis was stopped by washing three times in a neutralizing solution (Tris-HCl 0.4 M; pH 7.5) and slides were then transferred into absolute ethanol for 20 min and dried overnight. The prepared slides were stained with 20 µL of ethidium bromide (BET; 20 µg.mL⁻¹) and analyzed at x200 using an epifluorescence microscope (Olympus BX51, Rungis, France) coupled with CCD camera (Zeiss, Germany) and the Comet assay IV software (Instrument Perspective Ltd, Bury St Edmunds, UK). For each sample, 100 randomly selected nucleoids are analyzed on two replicated gels. DNA damage are expressed as percentage tail DNA, which is the percentage of DNA which has migrated from the head (Collins, 2004).

2.3.5 Photomotor assay

The photomotor assay was used to evaluate the locomotion or swimming capacities of prolarvae after light stimulation. Responses were measured on 12 prolarvae per replicate condition at the end of the contamination. This test is based on a published method (Emran et al., 2007) and adapted by Le Bihanic et al., (2014b). Prolarvae were randomly selected and placed into a 48-well microplate

with 500 μ L of mixing water. After 2h (minimum) of larval acclimatization, in the dark and at 26 °C, the microplate was placed in a Daniovision chamber (Noldus, Wageningen, Netherlands) for a second period of acclimatization of 30 min at 26 °C. Prolarvae locomotion was recorded with an IR digital video camera (Ikegami Electronics, Neuss, Germany) and an Ethovision 12.0 image analysis system (Noldus, Wageningen, the Netherlands). Analysis included one period of 20 min in dark followed by 10 min in light and 20 min in dark. Velocity and mobility were calculated for each larva. Velocity data refers to the mean velocity in mm/s. Mobility refers to body movement, independent of spatial displacement, with a threshold of body movement during 20% of time for immobility and 60% for high mobility. For mobility, data were expressed as mean \pm SD for each 10-min period. Velocity was expressed as mean \pm SD for each 10-min period or for each minute of analysis. Prolarvae unable to swim because of severe morphological abnormalities and prolarvae with detection problems during analysis (<10% of subject not found) were discarded.

2.4 Statistics

For all experiments, each exposure condition was replicated 3 times and considered as an independent sample. Solvent treatment was considered as a negative control treatment.

Data is indicated as mean \pm SD. R software was used for statistical analysis. Normality of data distribution was tested on data residues using the Shapiro-Wilk test ($p < 0.05$). Variance homogeneity was evaluated by the Levene's test on data residues ($p < 0.05$). In cases of homogenous variance and normal distribution, data was analyzed by Anova. Where this was not the case, data was transformed by log or Arc sinus transformation. If the hypothesis was still not confirmed, a Kruskal-Wallis non-parametric test ($p < 0.05$) was performed. These tests were combined with a Tukey post-hoc test ($p < 0.05$).

3 Results

3.1 Embryotoxicity of microplastic extracts

3.1.1 Mortality and hatching success

Microplastic extracts did not induce increased embryonic or larval mortality in any set of conditions, except for embryos exposed to B(a)P-spiked MP extracts (Fig. 2A). For this condition, embryonic mortality increased drastically (81.1%) compared with other conditions (between 3.1% and 10.7%). Embryos exposed to B(a)P-spiked MP extracts showed a significant decrease (28%) in hatching rate compared to other conditions (91.5%-100%) (Fig. 2B). Exposure to Ha0.1 extract induced a slight but significant decrease in hatching success (91.5%) compare to C_{DMSO} , but no to other treatments. According to the high mortality rate observed for embryos exposed to B(a)P-spiked MPs extract (>80%), the few living individuals were not analyzed for the other biomarkers.

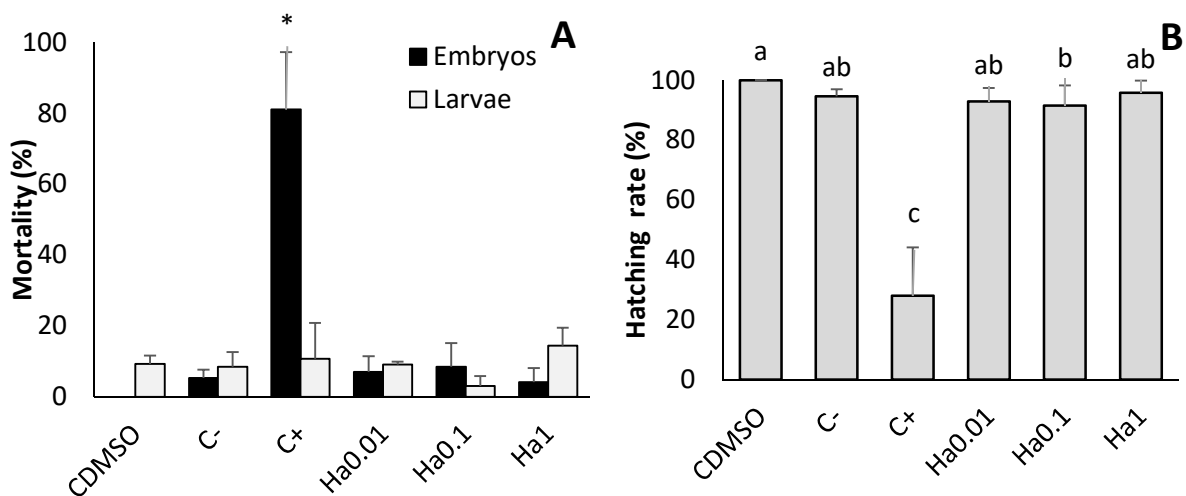


Figure 2: Embryonic and larval mortality rates (A) and hatching rate (B) (%), mean \pm SD, $n=25$, $N=3$) following exposure for 8 days to different concentrations of organic extracts of microplastic from Hawai: CDMSO: 1% of DMSO; C-: 1% of virgin MPs extract, C+, 1% of B(a)P coated MPs extract, Ha0.01, Ha0.1 and Ha1: 0.01%, 0.1% and 1% of Ha MPs extract. * and letters indicate significant differences between treatments (Kruskal-Wallis, $p < 0.05$).

3.1.2 Biometry and malformations

After 8 days of embryonic exposure to MP extracts, significant differences in total body length were highlighted between conditions (Fig. 3). For head length (Fig. 3A) there was no significant difference between conditions. In contrast, the total body length of polarvae exposed to Ha0.1s extract was significantly higher than other conditions. Ratio of head/body length (Fig. 3B) was decreased as extract doses were increased. This decrease was significant for Ha0.1 and Ha1 in comparison to negative control (C_{DMSO}). No significant differences between treatments were observed in terms of malformations. For all conditions, malformations rate was below 10% with similar rate for each concentration: between $2.2 \pm 1.2\%$ for Ha1 and $7.5 \pm 3.5\%$ for control.

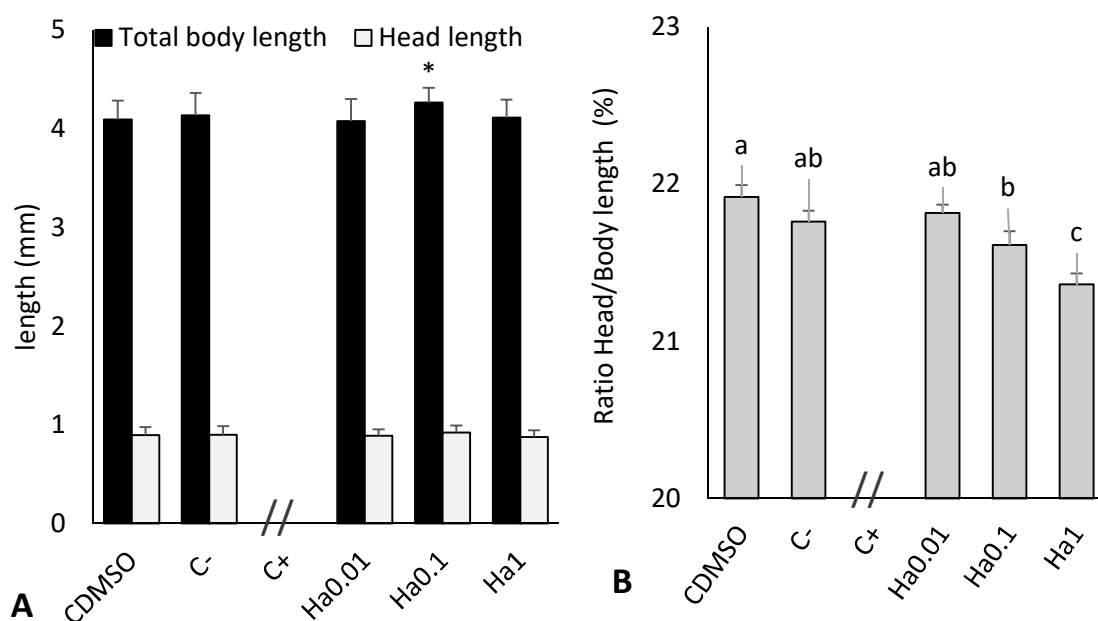


Figure 3: Total body length and head length (A) and ratio head/body length (B) of polarvae at hatching (mean \pm SD, $n=10$, $N=3$) after 8 days of exposure to different concentrations of MP extracts. C_{DMSO} : 1% of DMSO; C-: 1% of virgin extract, C+, 1% of B(a)P coated MPs extract, Ha0.01, Ha0.1 and Ha1: 0.01%, 0.1% and 1% of Ha MPs extract. Letters and * indicate significant differences between treatments (Kruskal-Wallis, $p<0.05$).

3.1.3 EROD and COMET

Exposure to 0.1% et 1% MPs extracts from Hawaii induced significant EROD activity on prolarvae with values increasing from 0.06 ± 0.01 pM of ethoxyresorufine/larva/min for DMSO control to 0.31 ± 0.05 and 0.26 ± 0.02 pM of ethoxyresorufine/larva/min for 0.1% et 1% MPs extracts respectively.

DNA damage was measured using the comet assay. The average tail DNA ranged from $4.25 \pm 2.03\%$ for control and $2.84 \pm 0.98\%$ for Ha0.1 and no significant variation in DNA breaks was highlighted whatever the condition considered.

3.1.4 Behavior

For behavioral analysis, only swimming speed (Fig. 4A) and mobility (Fig. 4B) data are shown here. Monitoring of prolarvae speed following dark/light stimulation (Fig. 4A) highlighted impacts following exposure to environmental MP extracts. Indeed, Ha0.01 exposed prolarvae had lower speed than control prolarvae (solvent) during the second dark period. For Ha0.1, this difference was also significant during the first dark period. Effects of 1% MP extract on behavior were only significant during light periods of analysis. On the other hand, no speed change was caused by embryo exposure to virgin MP extracts. Study of prolarvae exhibiting highly frequent mobility (Fig. 4B) also highlighted impacts of exposure to environmental MP extracts. After exposure to 0.01% and 0.1% MPs extracts, a significant decrease in frequent mobility was observed in all light conditions except Ha0.1. On the other hand, no speed change was observed after exposure of medaka embryos to virgin MP extracts (C-).

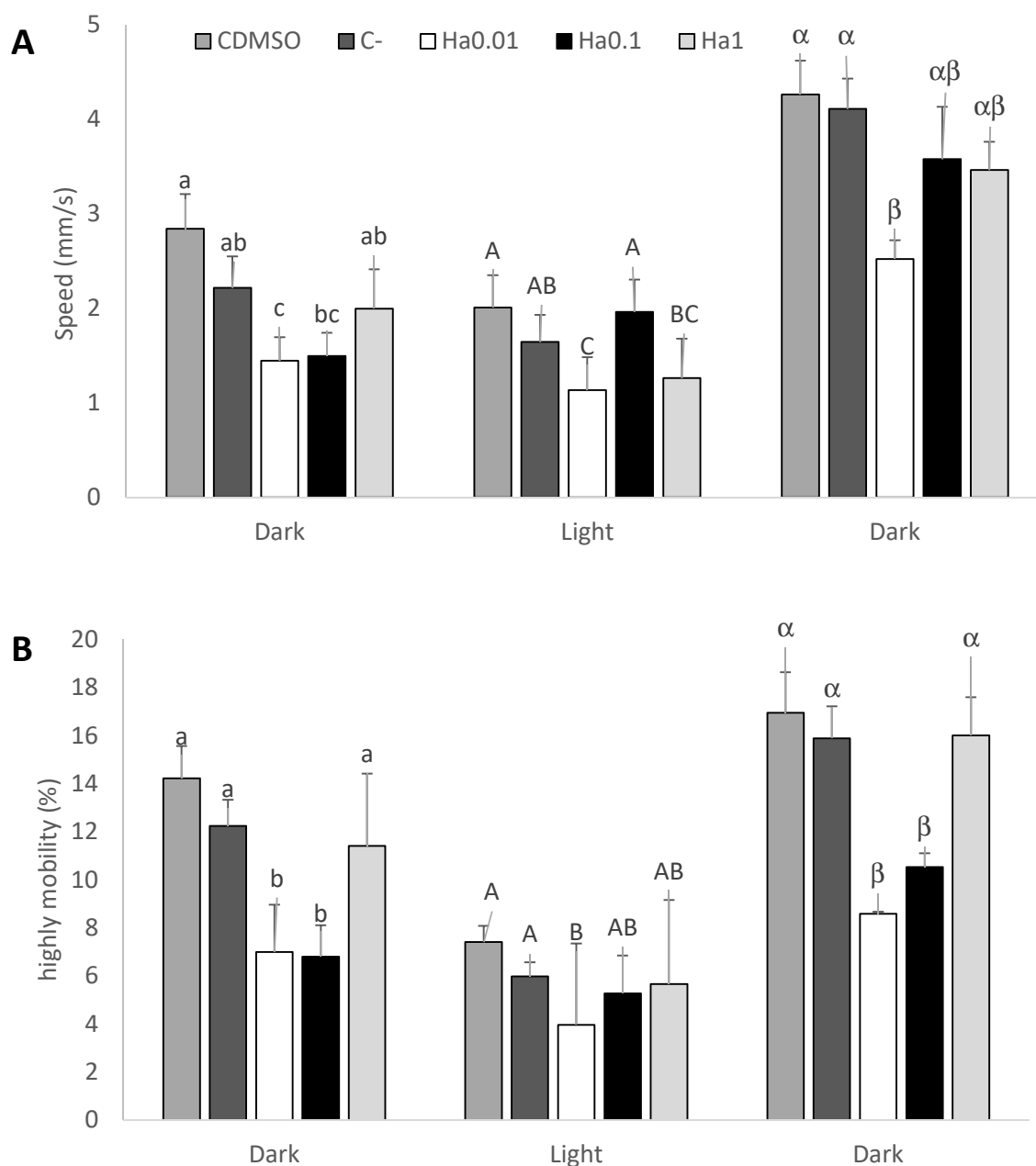


Figure 4: Swimming speed (A) and high mobility (B) variations of Japanese medaka prolarvae during dark/light stimulation (mean \pm SE, n=12, N=3) after exposure to MPs organic extracts. CDMSO: 1% of DMSO; C-: 1% of virgin extract, Ha0.01, Ha0.1 and Ha1: 0.01%, 0.1% and 1% of Ha MPs extract. Letters indicate significant differences between treatments (Kruskal-Wallis, $p < 0.05$).

3.2 Toxicity of microplastic extracts on prolarvae

3.2.1 Mortality

No significant variation in larval mortality was observed in any set of exposure conditions: C_{DMSO} $3.33 \pm 3.33\%$, C+ $2.00 \pm 1.96\%$, Ea $3.00 \pm 3.03\%$, Gu $4.20 \pm 4.17\%$ and Ha $7.90 \pm 3.95\%$.

3.2.2 Biometry and malformations

After 48h of exposure to different MP extracts, no significant difference was observed for prolarvae head and total body length, regardless of conditions (Fig. 5A). However, the ratio of head/body length (Fig. 5B) was significantly decreased for C+ and increased for Ha exposure conditions. No significant differences between conditions were observed in terms of malformations. For all conditions, the malformation rate was low, ranging between 0% and $6.33 \pm 1.37\%$.

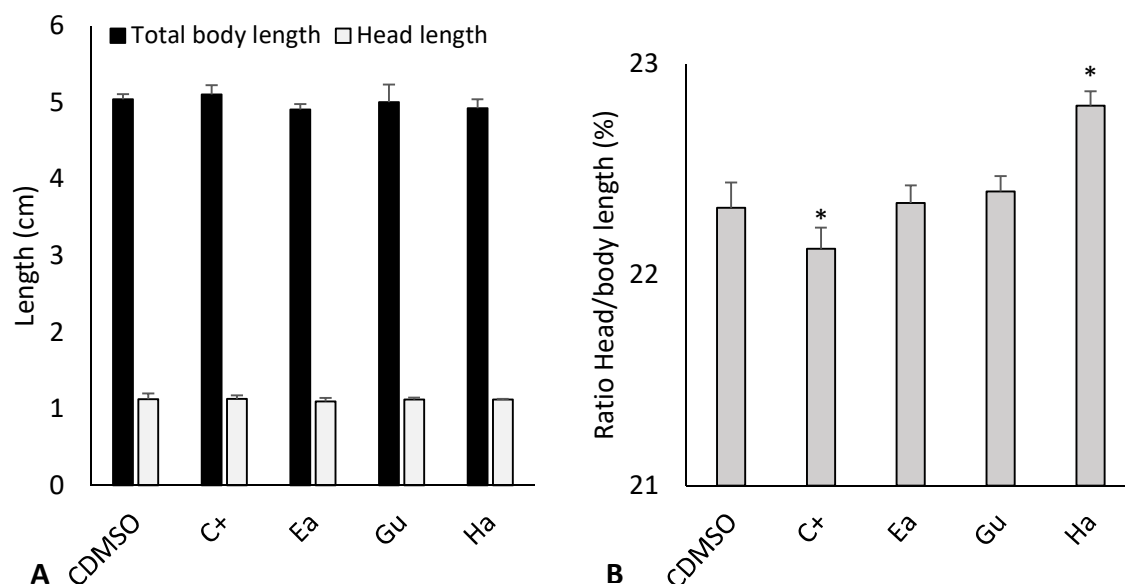
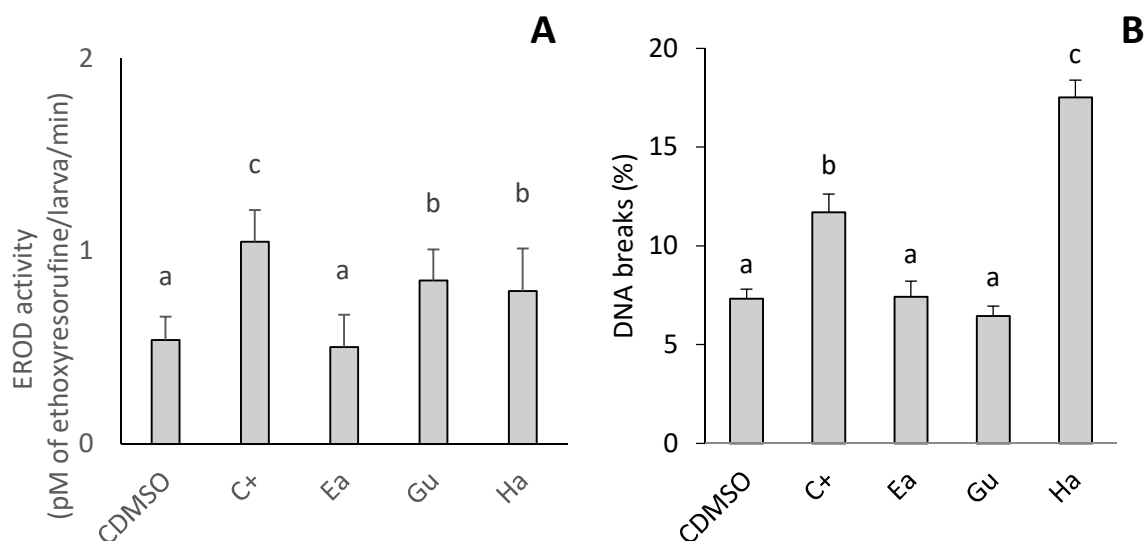


Figure 5: Total body and head length (A) and Ratio head/body length (B) of Japanese medaka prolarvae after 48h of exposure to different MPs extractss (mean \pm SD, n=10, N=3). C_{DMSO} : 0.1% of DMSO; C+, 0.1% of B(a)P coated MPs extract, Ea, Gu and Ha: 0.1% of Ea, Gu and Ha MPs extract. * indicate significant differences (Kruskall-Wallis, $p < 0.05$).

3.2.3 EROD and COMET assay

Larvae showed a significant increase in EROD activity after exposure to C+, Gu and Ha conditions (Fig. 6A). This induction was significantly higher for C+. Besides, a significant increase in DNA strand breaks was observed in prolarvae cells exposed to C+ and Ha extract (Fig. 6B). DNA damage was particularly high in prolarvae exposed to Ha extracts.

319



320

321 *Figure 6: EROD activity (A) and DNA strand breaks (B) (mean±SD, N=3) in Japanese medaka prolarvae after 48h of exposure*
 322 *to different MPs extracts. C_{DMSO}: 0.1% of DMSO; C+, 0.1% of B(a)P coated MPs extract, Ea, Gu and Ha: 0.1% of Ea, Gu and Ha*
 323 *MPs extract. Letters indicate significant differences (Kruskal-Wallis, p<0.05).*

324

325 3.2.4 Behavior

326 As for the first experiment, only swimming speed (Fig. 7A) and high mobility (Fig.7B) data are shown
 327 here. Larvae swimming speed after dark/light stimulation (Fig.7A) was not impacted by
 328 contamination by environmental MP extracts. On the other hand, during the last dark period,
 329 prolarvae exposed to B(a)P coated MPs (C+) had a significant lower speed than prolarvae exposed to
 330 DMSO (C_{DMSO}) and environmental MP extracts (Ea, Gu and Ha). High mobility frequency of prolarvae
 331 (Fig.7B) was not impacted by MP extracts during the first two periods of analysis. In contrast, high
 332 mobility frequency of prolarvae exposed to MPs extracts was lower than control prolarvae during the
 333 last dark period. This difference was only significant for Gu during the last dark period.

334

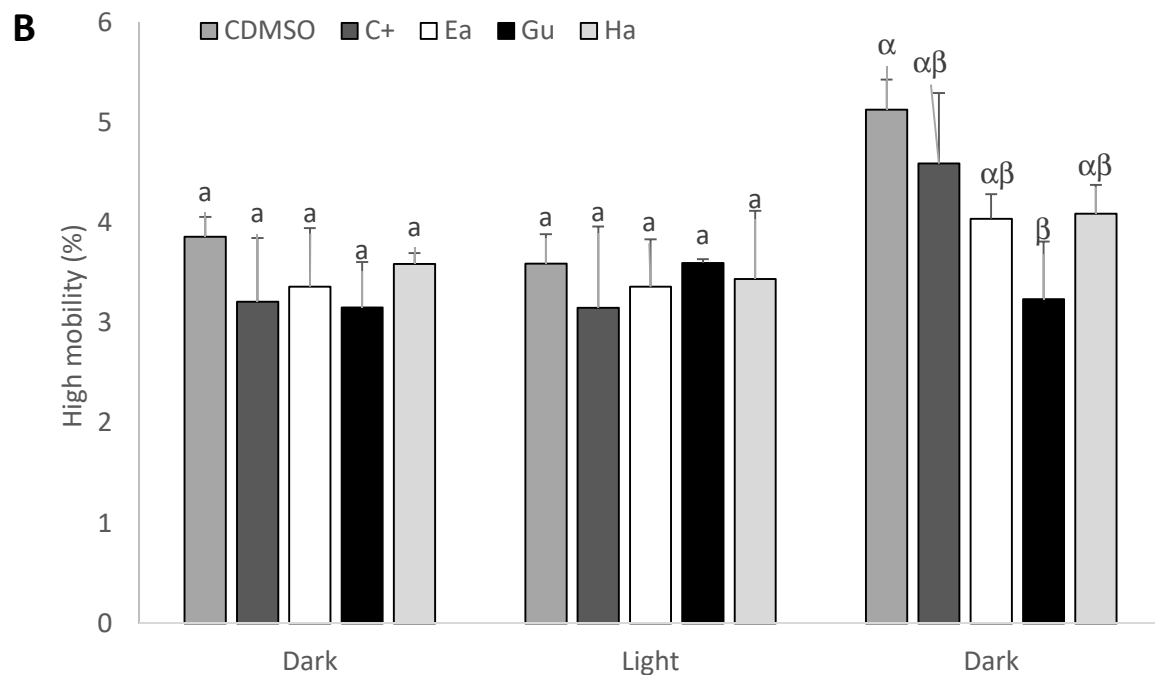
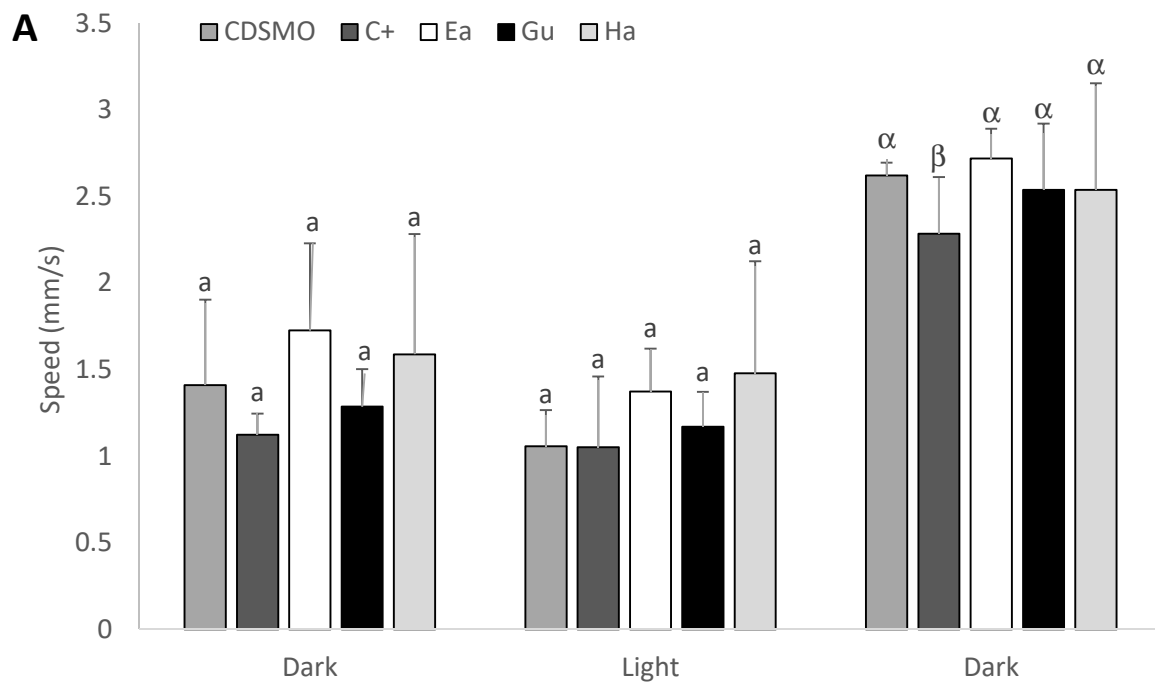


Figure 7: Swimming speed (A) and high mobility (B) variations during dark and light stimulation (mean±SE, n=12, N=3) in Japanese medaka prolarvae after 48h of exposure to different extracts of MPs. C_{DMSO} : 0.1% of DMSO; C+: 0.1% of B(a)P coated MPs extract, Ea, Gu and Ha: 0.1% of Ea, Gu and Ha MPs extract. Letters indicate significant differences between conditions by period (Kruskal-Wallis, $p<0.05$).

4 Discussion

This study included two complementary experiments. In the first one, medaka embryos were exposed to different concentrations from 0.01 to 1% of MPs extract from Hawaii (Ha). In the second one, medaka prolarvae were exposed to one unique concentration of MPs extract (0.1%) from different samples collected in Hawaii (Ha), Easter Island (Ea) and Guam (Gu).

Exposure of medaka embryos to DMSO extract of MPs artificially coated with 1% of B(a)P (C+1%), led to high embryonic mortality and low hatching rate. The acute toxicity of C+ is likely to be triggered by high concentrations of BaP (100 μ M) used for MP spiking and the high effectiveness of DMSO in solubilizing B(a)P. The other positive control extract (C+0.1%) induced no mortality but had some marked sublethal effects on different biological functions. No significant effects on body and head length or development were recorded, which was probably due to the short exposure time. However, impact on head/body ratio was observed. This could have long term impacts on growth (De Meyer et al., 2017). EROD activity and DNA damage were also significant. B(a)P as well as high weight PAHs were known to induce EROD activity and DNA damage (Carlson et al., 2002; Wessel et al., 2010; Woo et al., 2006). Impact on swimming behavior was also observed after light/dark stimulation of prolarvae. Some PAHs as pyrene and BaA or PAHs mixture including BaP, have been shown to alter fish swimming performances (Oliveira et al., 2012; Le Bihanic et al., 2014a, 2014b; Vignet et al., 2014).

Virgin MPs extract (C-) did not produce any significant effects - at least for the endpoints examined in this work. This could be the result of, either poor extraction of plastic additives (PAs), or extraction of certain additives but without any developmental toxicity. Indeed, plastics contain a lot of additives used to improve plastic properties, and these PAs were often described to have negative impacts on organism physiology (Koelmans, 2015; Lusher, 2015; Rochman, 2015). The effects of PAs have been recently reported on early life stage of marine species, including mollusks, crustaceans, echinoderms and chordates (Durán and Beiras, 2017). In addition, zebra fish (*Danio rerio*) prolarvae and embryos exposed to PBDE47, a well-known flame retardant used in plastic composition, displayed delayed

368 hatching, reduced growth, morphological abnormalities, impaired cardiovascular function, and
369 suboptimal flow of cerebrospinal fluid (Lema et al., 2007).

370 Exposure to DMSO extracts of environmental MP samples did not affect mortality rates. Effects on
371 biometry were particularly prevalent in terms of body length and head/body length ratio. Several
372 studies have shown developmental anomalies in fish embryos after exposure to organic pollutants
373 such as PAHs, PCBs and other substances (Gewurtz et al., 2011; González-Doncel et al., 2008; Le
374 Bihanic et al., 2014a; Schmidt et al., 2005). Modifications of head/body ratio could have long term
375 effects on growth (De Meyer et al., 2017).

376 Increased EROD activity highlights exposure of fish to AhR inducers in MP extracts. Many chemicals
377 are known to activate AhR, leading to EROD activity in fish, including some PAHs, PCBs, dioxin-like
378 compounds, OCP, PFOS and PBPE (Koenig et al., 2012; Lyons et al., 2011; Wessel et al., 2010). AhR
379 inducers such as PAHs were detected in most MP-extracts, particularly those from Hawaii (Pannetier
380 et al., this issue). While some PAHs are well-known genotoxicants, no DNA damage was detected in
381 prolarvae. Exposure was probably too short and/or toxicant concentrations too low to cause
382 genotoxic effects (Le Bihanic et al., 2016). When zebrafish embryos were exposed, with varying
383 incubation time, to a mixture of four PAHs in a ratio of 12:1:3:1 (naphtalene/phenanthrene/
384 pyrene/benzo(a)pyrene) at low, medium or high (4524:524:1783:741 $\mu\text{g.L}^{-1}$) concentration levels,
385 significantly elevated levels of DNA damage were observed with 72h exposure and for the highest
386 level of PAH mixture (Sogbanmu et al., 2016). The chorion could also limit the penetration of
387 compounds into the embryo. Indeed, only some PAHs can cross the chorion of medaka, which limits
388 exposure (González-Doncel et al., 2008). In this case, the chorion plays a protective role. Indeed, the
389 chorion has frequently been believed to function as an embryo-specific barrier for toxic substance
390 penetration due to its limited permeability (Henn and Braunbeck, 2011; Strähle et al., 2012).

391 Modifications of swimming behavior manifested themselves as decreases in speed and mobility. Fish
392 prolarvae response to light stimulation after exposition to pollutants depends on the type of

chemical and concentration tested (Irons et al., 2013). Decreases in speed and mobility were reported in literature after exposure of fish embryos or larvae to a large variety of organic pollutants such as PAHs, pesticides and psychotropic drugs (Oliveira et al., 2012; Le Bihanic et al., 2014a; Faria et al., 2015; Chiffre et al., 2016). A study have also reported an increase in fish swimming activity after exposure to environmental concentrations of PAHs (Le Bihanic et al., 2014c). In the present study, prolarvae were exposed to a cocktail of contaminants, and effects on behavior were low and not dose-dependent. These could suggest that the different chemicals in the MP extracts had an antagonistic effect on behavior or that the chemical concentrations were too low. In the natural environment, locomotion impairment can have a significant impact on fish survival (higher predation) or growth rate (feeding efficiency) and can lead to reduced populations (Little and Finge, 1990). Conversely, an increase in swimming activity can lead to high energy consumption, which can in turn impair other physiological pathways. Since in this study no attempt was made to measure energy reserves, the ecological relevance of the behavioral endpoints measured for further development of the larvae is not known. Persistent organic pollutants are known to have impacts on fish and other species' behavior (Scott and Sloman, 2004; Weis et al., 2001). For example, DDT at sublethal concentrations is known to have negative impacts on fish's central nervous system, thus also on their locomotion (Davy et al., 1972). MP extracts from Easter Island did not show any toxic effects on medaka prolarvae, although the chemical contamination profile of Easter Island's plastics was similar to Guam beach at least for the chemicals analyzed. Different types of plastic have shown different contaminant sorption properties or capacities to release harmful additives (Rochman et al., 2013a). Plastic life history, including discharge area and time of transportation, could influence degradation, chemical contamination and ageing of MPs (Rochman, 2015). Ageing of polymer could also have an influence on chemical sorption and desorption (Rochman, 2013a) and could somewhat explain why Ea extract was less toxic than Gu extract.

Results obtained here with embryos or larvae of medaka confirm the toxicity of MP extracts observed previously on fish cell lines (Pannetier et al., this issue). The absence of toxic effect with

the virgin MP extract suggests that observed effects after environmental MP extract exposure are most likely due to the contaminants. A toxic gradient from Hawaii sample to Guam and Easter Island was observed. Even if persistent organic pollutants adsorbed to the plastic surface could explained at least in part the toxicity of the extracts, additives or monomers can also influence this toxicity.

Today, microplastics were found everywhere in hydrosphere (Faure et al, 2015; Dris et al, 2015, Eriksen et al, 2014). Once MPs are ingested by organism, pollutants found on microplastics' surface could be desorbed under digestive enzyme action. The soft DMSO extraction performed in this study was used to mimic the potential transfer of microplastic-associated persistent organic pollutants on the intestinal tract via ingestion. Results obtained here suggest an impact of MP sorbed pollutants and consequently a potential risk for marine organisms.

5 Conclusion

This study highlighted the developmental impacts of environmental and BaP-spiked microplastic extracts on early life stages of Japanese medaka. Soft DMSO extraction of MPs released pollutants and additives that induced sublethal effects affecting various biological functions (growth, swimming behavior) and metabolic pathways (xenobiotic metabolization, DNA integrity). The most prominent effects were detected for microplastics from Hawaii, more than those from Guam and Easter Island. Additional studies using direct exposure of fish to MP particles are still required to evaluate physical and chemical impacts of MPs in more environmentally realistic conditions.

Acknowledgements

Pauline Pannetier received a Ph.D fellowship from the French Ministère de l'Enseignement Supérieur et de la Recherche. The authors wish to thank the Race for Water Foundation for supplying

microplastic samples and EPFL of Lausanne for polymer analysis. The authors would like to thank James Emery for providing English proofreading services.

6 Bibliography

Arthur, C., Baker, J., Bamford, H., 2009. Proceedings of the International Research Workshop on the Occurrence , Effects , and Fate of Microplastic Marine Debris. Group 530.

Bai, W., Zhang, Z., Tian, W., He, X., Ma, Y., Zhao, Y., Chai, Z., 2010. Toxicity of zinc oxide nanoparticles to zebrafish embryo: a physicochemical study of toxicity mechanism. J. Nanoparticle Res. 12, 1645–1654.

Barjhoux, I., Baudrimont, M., Morin, B., Landi, L., Gonzalez, P., Cachot, J., 2012. Effects of copper and cadmium spiked-sediments on embryonic development of Japanese medaka (*Oryzias latipes*). Ecotoxicol. Environ. Saf. 79, 272–282. doi:10.1016/j.ecoenv.2012.01.011

Barnes, D.K.A., Galgani, F., Thompson, R.C., Barlaz, M., Barnes, D.K.A., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and Fragmentation of Plastic Debris in Global Environments. Philos. Trans. R. Soc. Biol. Sci. 364, 1985–1998.

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., 2018. Ingestion and contact with polyethylene microplastics does not cause acute toxicity on marine zooplankton. J. Hazard. Mater. 360, 452–460.

Browne, M.A.A., Niven, S.J.J., Galloway, T.S.S., Rowland, S.J.J., Thompson, R.C.C., 2013. Microplastic moves pollutants and additives to worms, reducing functions linked to health and biodiversity. Curr. Biol. 23, 2388–2392. doi:10.1016/j.cub.2013.10.012

Cachot, J., Law, M., Pottier, D., Peluhet, L., Norris, M., Budzinski, H., Winn, R., 2007. Characterization of toxic effects of sediment-associated organic pollutants using the λ transgenic medaka.

466 Environ. Sci. Technol. 41, 7830–7836. doi:10.1021/es071082v

467 Carlson, E.A., Li, Y., Zelikoff, J.T., 2002. The Japanese medaka (*Oryzias latipes*) model: Applicability for
 468 investigating the immunosuppressive effects of the aquatic pollutant benzo[a]pyrene (BaP).
 469 Mar. Environ. Res. 54, 565–568. doi:10.1016/S0141-1136(02)00175-7

470 Chiffre, A., Clérandeau, C., Dwoinikoff, C., Le Bihanic, F., Budzinski, H., Geret, F., Cachot, J., 2016.
 471 Psychotropic drugs in mixture alter swimming behaviour of Japanese medaka (*Oryzias latipes*)
 472 larvae above environmental concentrations. Environ. Sci. Pollut. Res. 23, 4964–4977.
 473 doi:10.1007/s11356-014-3477-4

474 Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the
 475 marine environment: A review. Mar. Pollut. Bull. 62, 2588–2597.
 476 doi:10.1016/j.marpolbul.2011.09.025

477 Collignon, A., Hecq, J.H., Glagani, F., Voisin, P., Collard, F., Goffart, A., 2012. Neustonic microplastic
 478 and zooplankton in the North Western Mediterranean Sea. Mar. Pollut. Bull. 64, 861–864.
 479 doi:10.1016/j.marpolbul.2012.01.011

480 Collins, a R., 2004. The comet assay for DNA damage and repair: principles, applications, and
 481 limitations. Mol. Biotechnol. 26, 249–261. doi:10.1385/MB:26:3:249

482 Davy, F.B., Kleerekoper, H., Gensler, P., 1972. Effects of Exposure to Sublethal DDT on the Locomotor
 483 Behavior of the Goldfish (*Carassius auratus*). J. Fish. Res. Board Canada 29. doi:10.1139/f72-
 484 202

485 De Meyer, J., Maes, G.E., Dirks, R.P., Adriaens, D., 2017. Differential gene expression in narrow- and
 486 broad-headed European glass eels (*Anguilla anguilla*) points to a transcriptomic link of head
 487 shape dimorphism with growth rate and chemotaxis. Mol. Ecol. 38, 42–49.
 488 doi:10.1111/mec.14155

489 Derraik, J.G., 2002. The pollution of the marine environment by plastic debris: a review. Mar. Pollut.

490 Bull. 44, 842–852. doi:10.1016/S0025-326X(02)00220-5

491 Dris, R., Gasperi, J., Rocher, V., Saad, M., Renault, N., Tassin, B., 2015. Microplastic contamination in
 492 an urban area: a case study in Greater Paris. *Environ. Chem.* 12, 592. doi:10.1071/EN14167

493 Durán, I., Beiras, R., 2017. Acute water quality criteria for polycyclic aromatic hydrocarbons,
 494 pesticides, plastic additives, and 4-Nonylphenol in seawater. *Environ. Pollut.* 224, 384–391.
 495 doi:10.1016/j.envpol.2017.02.018

496 Eaton, J.G., McKim, J.M., Holcombe, W., 1978. Metal toxicity to embryos and larvae of seven
 497 freshwater fish species—i. cadmium. *Bull. Environ. Contam. Toxicol.* 19, 95–103.

498 Embry, M.R., Belanger, S.E., Braunbeck, T. a., Galay-Burgos, M., Halder, M., Hinton, D.E., Léonard, M.
 499 a., Lillicrap, A., Norberg-King, T., Whale, G., 2010. The fish embryo toxicity test as an animal
 500 alternative method in hazard and risk assessment and scientific research. *Aquat. Toxicol.* 97,
 501 79–87. doi:10.1016/j.aquatox.2009.12.008

502 Emran, F., Rihel, J., Adolph, A.R., Wong, K.Y., Kraves, S., Dowling, J.E., 2007. OFF ganglion cells cannot
 503 drive the optokinetic reflex in zebrafish. *Proc. Natl. Acad. Sci.* 104, 19126–19131.
 504 doi:10.1073/pnas.0709337104

505 Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borerro, J.C., Galgani, F., Ryan, P.G.,
 506 Reisser, J., 2014. Plastic Pollution in the World’s Oceans: More than 5 Trillion Plastic Pieces
 507 Weighing over 250,000 Tons float at Sea. *PLoS One* 9, 1–15. doi:10.1371/journal.pone.0111913

508 European Union, 2010. Directive 2010/63/EU of the European Parliament and of the Council of 22
 509 September 2010 on the protection of animals used for scientific purposes. *Off. J. Eur. Union* 33–
 510 79. doi:10.1016/L0063

511 Faria, M., Garcia-Reyero, N., Padrós, F., Babin, P.J., Sebastián, D., Cachot, J., Prats, E., Arick, M., Rial,
 512 E., Knoll-Gellida, A., Mathieu, G., Le Bihanic, F., Escalon, B.L., Zorzano, A., Soares, A.M.V.M.,
 513 Raldúa, D., 2015. Zebrafish Models for Human Acute Organophosphorus Poisoning. *Sci. Rep.* 5,

514 15591. doi:10.1038/srep15591

515 Faure, F., Demars, C., Wieser, O., Kunz, M., De Alencastro, L.F., 2015. Plastic pollution in Swiss surface
516 waters: Nature and concentrations, interaction with pollutants. *Environ. Chem.* 12, 582–591.
517 doi:10.1071/EN14218

518 Gandara e Silva, P.P., Nobre, C.R., Resaffe, P., Pereira, C.D.S., Gusmão, F., 2016. Leachate from
519 microplastics impairs larval development in brown mussels. *Water Res.* 106, 364–370.
520 doi:10.1016/j.watres.2016.10.016

521 George, S., Lin, S., Ji, Z., Thomas, C.R., Li, L., Mecklenburg, M., Meng, H., Wang, X., Zhang, H., Xia, T.,
522 Hohman, J.N., Lin, S., Zink, J.I., Weiss, P.S., Nel, A.E., 2012. Surface Defects on Plate-Shaped
523 Silver Nanoparticles Contribute to Its Hazard Potential in a Fish Gill Cell Line and Zebrafish
524 Embryos. *ACS Nano* 6, 3745–3759. doi:10.1021/nn204671v

525 Gewurtz, S.B., Bhavsar, S.P., Fletcher, R., 2011. Influence of fish size and sex on mercury/PCB
526 concentration: Importance for fish consumption advisories. *Environ. Int.* 37, 425–434.
527 doi:10.1016/j.envint.2010.11.005

528 González-Doncel, M., González, L., Fernández-Torija, C., Navas, J.M., Tarazona, J.V., 2008. Toxic
529 effects of an oil spill on fish early life stages may not be exclusively associated to PAHs: Studies
530 with Prestige oil and medaka (*Oryzias latipes*). *Aquat. Toxicol.* 87, 280–288.
531 doi:10.1016/j.aquatox.2008.02.013

532 Helmstetter, M.F., Alden, R.W., 1995. following topical and immersion exposures to
533 pentachlorophenol. *Aquat. Toxicol.* 32, 15–29.

534 Henn, K., Braunbeck, T., 2011. Dechoriation as a tool to improve the fish embryo toxicity test (FET)
535 with the zebrafish (*Danio rerio*). *Comp. Biochem. Physiol. - C Toxicol. Pharmacol.* 153, 91–98.
536 doi:10.1016/j.cbpc.2010.09.003

537 Irons, T.D., Kelly, P.E., Hunter, D.L., MacPhail, R.C., Padilla, S., 2013. Acute administration of

538 dopaminergic drugs has differential effects on locomotion in larval zebrafish. *Pharmacol.*
539 *Biochem. Behav.* 103, 792–813. doi:10.1016/j.pbb.2012.12.010

540 Iwamatsu, T., 2004. Stages of normal development in the medaka *Oryzias latipes*. *Mech. Dev.* 121,
541 605–618. doi:10.1016/j.mod.2004.03.012

542 Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R., Law, K.L.,
543 2015. Plastic waste inputs from land into the ocean. *Science*. 347, 768–771.
544 doi:10.1126/science.1260352

545 Jezierska, B., Ługowska, K., Witeska, M., 2009. The effects of heavy metals on embryonic
546 development of fish (a review). *Fish Physiol. Biochem.* 35, 625–640.

547 Karapanagioti, H.K., Endo, S., Ogata, Y., Takada, H., 2011. Diffuse pollution by persistent organic
548 pollutants as measured in plastic pellets sampled from various beaches in Greece. *Mar. Pollut.*
549 *Bull.* 62, 312–317. doi:10.1016/j.marpolbul.2010.10.009

550 Koelmans, A.A., Besseling, E., Wegner, A., Foekema, M., 2013. Plastic as a carrier of POPs to aquatic
551 organisms : A model analysis . *Environ. Sci. Technol.* 47, 7812–7820. doi:10.1021/es401169n

552 Koelmans, A.A., Besseling, E., Foekema, E.M., 2014. Leaching of plastic additives to marine
553 organisms. *Environ. Pollut.* 187, 49–54. doi:10.1016/j.envpol.2013.12.013

554 Koelmans, A., 2015. Modeling the role of microplastics in Bioaccumulation of organic chemicals to
555 marine aquatic organisms. A Critical Review, in: Bergmann, M., Gutow, L., Klages, M. (Eds.),
556 *Marine Anthropogenic Litter*. Berlin, pp. 309–324.

557 Koelmans, A.A., Bakir, A., Burton, G.A., Janssen, C.R., 2016. Microplastic as a Vector for Chemicals in
558 the Aquatic Environment: Critical Review and Model-Supported Reinterpretation of Empirical
559 Studies. *Environ. Sci. Technol.* 50, 3315–3326. doi:10.1021/acs.est.5b06069

560 Koenig, S., Fernández, P., Solé, M., 2012. Differences in cytochrome P450 enzyme activities between

561 fish and crustacea: Relationship with the bioaccumulation patterns of polychlorobiphenyls
 562 (PCBs). *Aquat. Toxicol.* 108, 11–17. doi:10.1016/j.aquatox.2011.10.016

563 Lammer, E., Carr, G.J., Wendler, K., Rawlings, J.M., Belanger, S.E., Braunbeck, T., 2009. Is the fish
 564 embryo toxicity test (FET) with the zebrafish (*Danio rerio*) a potential alternative for the fish
 565 acute toxicity test? *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* 149, 196–209.
 566 doi:10.1016/j.cbpc.2008.11.006

567 Law, K.L., 2017. Plastics in the Marine Environment. *Annu. Rev. Mar. Sci.* 9, 205–229.
 568 doi:10.1146/annurev-marine-010816-060409

569 Le Bihanic, F., Couillard, C.M., Rigaud, C., Légaré, B., 2013. A simple and reliable in vivo EROD activity
 570 measurement in single *Fundulus heteroclitus* embryo and larva. *Mar. Environ. Res.* 84, 17–23.
 571 doi:10.1016/j.marenvres.2012.11.003

572 Le Bihanic, F., Clérandeau, C., Le Menach, K., Morin, B., Budzinski, H., Cousin, X., Cachot, J., 2014a.
 573 Developmental toxicity of PAH mixtures in fish early life stages. Part II: adverse effects in
 574 Japanese medaka. *Environ. Sci. Pollut. Res.* 21, 13732–13743. doi:10.1007/s11356-014-2676-3

575 Le Bihanic, F., Morin, B., Cousin, X., Le Menach, K., Budzinski, H., Cachot, J., 2014b. Developmental
 576 toxicity of PAH mixtures in fish early life stages. Part I: adverse effects in rainbow trout. *Environ.*
 577 *Sci. Pollut. Res.* 21, 13720–13731. doi:10.1007/s11356-014-2804-0

578 Le Bihanic, F., Perrichon, P., Landi, L., Clérandeau, C., Le Menach, K., Budzinski, H., Cousin, X., Cachot,
 579 J., 2014c. Development of a reference artificial sediment for chemical testing adapted to the
 580 MELA sediment contact assay. *Environ. Sci. Pollut. Res.* 21, 13689–13702. doi:10.1007/s11356-
 581 014-2607-3

582 Le Bihanic, F., Di Bucchianico, S., Karlsson, H.L., Dreij, K., 2016. *In vivo* micronucleus screening in
 583 zebrafish by flow cytometry. *Mutagenesis* 31, 643–653. doi-org.docelec.u-
 584 bordeaux.fr/10.1093/mutage/gew032

585 Lema, S.C., Schultz, I.R., Scholz, N.L., Incardona, J.P., Swanson, P., 2007. Neural defects and cardiac
586 arrhythmia in fish larvae following embryonic exposure to 2,2',4,4'-tetrabromodiphenyl ether
587 (PBDE 47). *Aquat. Toxicol.* 82, 296–307. doi:10.1016/j.aquatox.2007.03.002

588 Little, E.E., Finge, S.E., 1990. Swimming Behavior as an Indicator of Sublethal Toxicity in Fish. *Environ.*
589 *Toxicol. Chem.* 9, 13–19. doi:10.1002/etc.5620090103

590 Lusher, A., 2015. Microplastics in the Marine Environment: Distribution, Interactions and Effects, in:
591 Bergmann, M., Gutow, L., Klages, M. (Eds.), *Marine Anthropogenic Litter*. Springer International
592 Publishing, Cham, pp. 245–307. doi:10.1007/978-3-319-16510-3_10

593 Lyons, M.C., Wong, D.K.H., Mulder, I., Lee, K., Burridge, L.E., 2011. The influence of water
594 temperature on induced liver EROD activity in Atlantic cod (*Gadus morhua*) exposed to crude oil
595 and oil dispersants. *Ecotoxicol. Environ. Saf.* 74, 904–910. doi:10.1016/j.ecoenv.2010.12.013

596 Mazurais, D., Ernande, B., Quazuguel, P., Severe, A., Huelvan, C., Madec, L., Mouchel, O., Soudant, P.,
597 Robbens, J., Huvet, A., Zambonino-Infante, J., 2015. Evaluation of the impact of polyethylene
598 microbeads ingestion in European sea bass (*Dicentrarchus labrax*) larvae. *Mar. Environ. Res.*
599 112, 78–85. doi:10.1016/j.marenvres.2015.09.009

600 Morin, B., Filatreau, J., Vicquelin, L., Barjhoux, I., Guinel, S., Leray-Forget, J., Cachot, J., 2011.
601 Detection of DNA damage in yolk-sac larvae of the Japanese Medaka, *Oryzias latipes*, by the
602 comet assay. *Anal. Bioanal. Chem.* 399, 2235–2242. doi:10.1007/s00216-010-4602-y

603 Nobre, C.R., Santana, M.F.M., Maluf, A., Cortez, F.S., Cesar, A., Pereira, C.D.S., Turra, A., 2015.
604 Assessment of microplastic toxicity to embryonic development of the sea urchin *Lytechinus*
605 *variegatus* (Echinodermata: Echinoidea). *Mar. Pollut. Bull.* 92, 99–104.
606 doi:10.1016/j.marpolbul.2014.12.050

607 Oliveira, M., Gravato, C., Guilhermino, L., 2012. Acute toxic effects of pyrene on *Pomatoschistus*
608 *microps* (Teleostei, Gobiidae): Mortality, biomarkers and swimming performance. *Ecol. Indic.*

609 19, 206–214. doi:10.1016/j.ecolind.2011.08.006

610 Oliveira, M., Ribeiro, A., Hylland, K., Guilhermino, L., 2013. Single and combined effects of
611 microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps*
612 (Teleostei, Gobiidae). Ecol. Indic. 34, 641–647. doi:10.1016/j.ecolind.2013.06.019

613 Pannetier, P., Cachot, J., Clérandeau, C., Faure, F., Arkel, K. Van, Alencastro, L.F. de, Levasseur, C.,
614 Sciacca, F., Bourgeois, J.-P., Morin, B. Toxicity assessment of pollutants sorbed on
615 environmental sample microplastics collected on beaches: Part I-adverse effects on fish cell
616 line. Submitted to Environ. Pollut. in this issue.

617 Peda, C., Caccamo, L., Fossi, M.C., Gai, F., Andaloro, F., Genovese, L., Perdichizzi, A., Romeo, T.,
618 Maricchiolo, G., 2016. Intestinal alterations in European sea bass *Dicentrarchus labrax*
619 (Linnaeus, 1758) exposed to microplastics: Preliminary results. Environ. Pollut. 212, 251–256.
620 doi:10.1016/j.envpol.2016.01.083

621 Pittura, L., Avio, C.G., Giuliani, M.E., d’Errico, G., Keiter, S.H., Cormier, B., Gorbi, S., Regoli, F., 2018.
622 Microplastics as Vehicles of Environmental PAHs to Marine Organisms: Combined Chemical and
623 Physical Hazards to the Mediterranean Mussels, *Mytilus galloprovincialis*. Front. Mar. Sci. 5,
624 103. doi:10.3389/fmars.2018.00103

625 Rochman, C.M., Hoh, E., Hentschel, B.T., Kaye, S., 2013a. Long-Term Field Measurement of Sorption
626 of Organic Contaminants to Five Types of Plastic Pellets: Implications for Plastic Marine Debris.
627 Environ. Sci. Technol. 47, 1646–1654. doi:10.1021/es303700s

628 Rochman, C.M., Hoh, E., Kurobe, T., Teh, S.J., 2013b. Ingested plastic transfers hazardous chemicals
629 to fish and induces hepatic stress. Sci. Rep. 3, 3263. doi:10.1038/srep03263

630 Rochman, C.M., Kurobe, T., Flores, I., Teh, S.J., 2014. Early warning signs of endocrine disruption in
631 adult fish from the ingestion of polyethylene with and without sorbed chemical pollutants from
632 the marine environment. Sci. Total Environ. 493, 656–661. doi:10.1016/j.scitotenv.2014.06.051

633 Rochman, C.M., 2015. The Complex Mixture, Fate and Toxicity of Chemicals Associated with Plastic
634 Debris in the Marine Environment, in: Marine Anthropogenic Litter. Springer International
635 Publishing, Cham, pp. 117–140. doi:10.1007/978-3-319-16510-3_5

636 Schmidt, K., Staaks, G.B.O., Pflugmacher, S., Steinberg, C.E.W., 2005. Impact of PCB mixture (Aroclor
637 1254) and TBT and a mixture of both on swimming behavior, body growth and enzymatic
638 biotransformation activities (GST) of young carp (*Cyprinus carpio*). Aquat. Toxicol. 71, 49–59.
639 doi:10.1016/j.aquatox.2004.10.012

640 Scott, G.R., Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour:
641 integrating behavioural and physiological indicators of toxicity. Aquat. Toxicol. 68, 369–392.
642 doi:10.1016/j.aquatox.2004.03.016

643 Singh, N.P., McCoy, M.T., Tice, R.R., Schneider, E.L., 1988. A simple technique for quantitation of low
644 levels of DNA damage in individual cells. Exp. Cell Res. 175, 184–191. doi:10.1016/0014-
645 4827(88)90265-0

646 Sogbanmu, T.O., Nagy, E., Phillips, D.H. et al. 2016. Lagos lagoon sediment organic extracts and
647 polycyclic aromatic hydrocarbons induce embryotoxic, teratogenic and genotoxic effects in
648 *Danio rerio* (zebrafish) embryos. Environ. Sci. Pollut. Res. 23, 14489–14501. doi-org.docelec.u-
649 bordeaux.fr/10.1007/s11356-016-6490-y

650 Strähle, U., Scholz, S., Geisler, R., Greiner, P., Hollert, H., Rastegar, S., Schumacher, A., Selderslaghs, I.,
651 Weiss, C., Witters, H., Braunbeck, T., 2012. Zebrafish embryos as an alternative to animal
652 experiments-A commentary on the definition of the onset of protected life stages in animal
653 welfare regulations. Reprod. Toxicol. 33, 128–132. doi:10.1016/j.reprotox.2011.06.121

654 Teuten, E.L., Saquing, J.M., Knappe, D.R.U., Barlaz, M.A., Jonsson, S., Björn, A., Rowland, S.J.,
655 Thompson, R.C., Galloway, T.S., Yamashita, R., Ochi, D., Watanuki, Y., Moore, C., Viet, P.H.,
656 Tana, T.S., Prudente, M., Boonyatumanond, R., Zakaria, M.P., Akkhavong, K., Ogata, Y., Hirai, H.,

657 Iwasa, S., Mizukawa, K., Hagino, Y., Imamura, A., Saha, M., Takada, H., 2009. Transport and
658 release of chemicals from plastics to the environment and to wildlife. *Philos. Trans. R. Soc.*
659 *Lond. B. Biol. Sci.* 364, 2027–45. doi:10.1098/rstb.2008.0284

660 Vicquelin, L., Leray-Forget, J., Peluhet, L., LeMenach, K., Deflandre, B., Anschutz, P., Etcheber, H.,
661 Morin, B., Budzinski, H., Cachot, J., 2011. A new spiked sediment assay using embryos of the
662 Japanese medaka specifically designed for a reliable toxicity assessment of hydrophobic
663 chemicals. *Aquat. Toxicol.* 105, 235–245. doi:10.1016/j.aquatox.2011.06.011

664 Vignet, C., Devier, M.H., Le Menach, K., Lyphout, L., Potier, J., Cachot, J., Budzinski, H., Bégout, M.L.,
665 Cousin, X., 2014. Long-term disruption of growth, reproduction, and behavior after embryonic
666 exposure of zebrafish to PAH-spiked sediment. *Environ. Sci. Pollut. Res.* 21, 13877–13887.
667 doi:10.1007/s11356-014-2585-5

668 Weis, J.S., Smith, G., Zhou, T., Santiago-Bass, C., Weis, P., 2001. Effects of Contaminants on Behavior:
669 Biochemical Mechanisms and Ecological Consequences. *Bioscience* 51, 209. doi:10.1641/0006-
670 3568(2001)051[0209:EOCOBB]2.0.CO;2

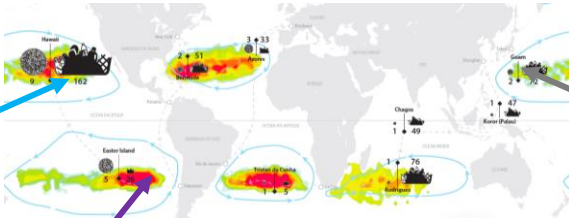
671 Wessel, N., Santos, R., Menard, D., Le Menach, K., Buchet, V., Lebayon, N., Loizeau, V., Burgeot, T.,
672 Budzinski, H., Akcha, F., 2010. Relationship between PAH biotransformation as measured by
673 biliary metabolites and EROD activity, and genotoxicity in juveniles of sole (*Solea solea*). *Mar.*
674 *Environ. Res.* 69, S71–S73. doi:10.1016/j.marenvres.2010.03.004

675 Woo, S., Kim, S., Yum, S., Yim, U.H., Lee, T.K., 2006. Comet assay for the detection of genotoxicity in
676 blood cells of flounder (*Paralichthys olivaceus*) exposed to sediments and polycyclic aromatic
677 hydrocarbons. *Mar. Pollut. Bull.* 52, 1768–1775. doi:10.1016/j.marpolbul.2006.08.027

678

Ha 0.1%

Mortality: \emptyset
Body change: (+)
EROD: +
DNA break: +
Behavior: \emptyset



Ea 0.1%

Mortality: \emptyset
Body change : \emptyset
EROD: \emptyset
DNA break: \emptyset
Behavior: \emptyset

Gu 0.1%

Mortality: \emptyset
Body change : \emptyset
EROD: +
DNA break: \emptyset
Behavior: +



Oryzias latipes larvae and embryos