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

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Abstract

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

Capsule

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

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 (*Oryziaz 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 zooplancton

(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 $\mu g.g^{-1}$. 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),

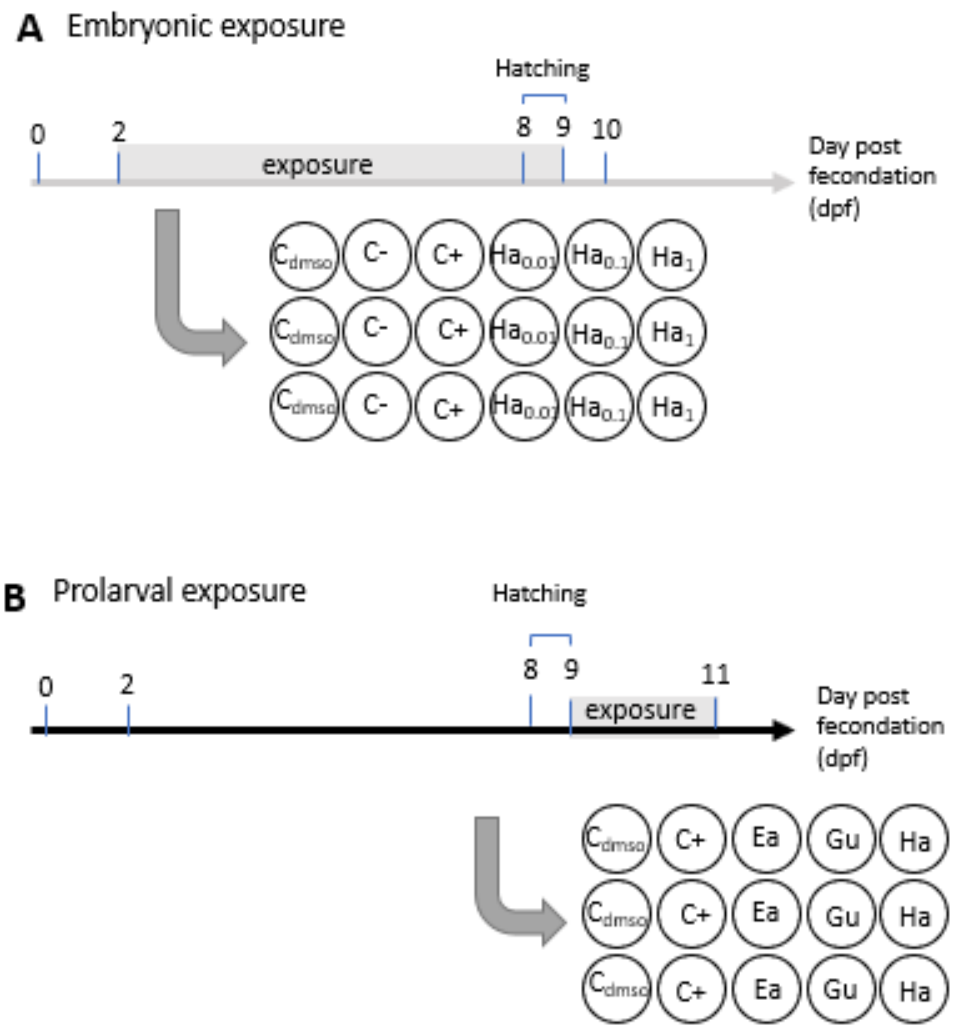
polychlorinated biphenyls (PCBs) and dichlorodiphenyltrichloroethane (DDT). The concentration of total PCBs (<5ng.g^{-1}) and DDT (<4 ng.g^{1}) was under quantification limits for all samples. Concentration of totals PAHs was 18 ng.g^{-1} for Ha, 6 ng.g^{-1} for Gu and 2 ng.g^{-1} for Ea. More information 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 600µm. 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 µM of B[a]P; (4) 0.01% of Ha microplastic extract in ERS (Ha0.01); (5) 0.1% of Ha microplastic extract in ERS (Ha0.1); (6) 1% of Ha microplastic extract in ERS (Ha1). 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 µM 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) Hawai (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 $CaCl_2$ and 0.163 g $MgSO_4$ 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, Regensbourg, 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 ∅ 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 fine), 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.larva^{1}.minute^{-1}.

2.3.4 Comet assay

187 The Comet assay was performed according to the original protocol of Singh et al. 1988 and adapted
188 to whole medaka larvae with enzymatic dissociation step (Morin et al., 2011). The comet assay is
189 carried out with 5 larvae by replicate. After being anesthetized in benzocaine (200 $mg.L^{-1}$), prolarvae
190 were crushed and cells dissociated through 45 minutes of shaking in MEM-dispase solution (0.125%)
191 at 37°C. After dissociation, cells were rinsed clean, centrifuged, and fixed between two layers of
192 LMPA (Low melting point agarose, Sigma-Aldrich) on microscope slides pre-coated with of NMPA
193 (Normal melting point agarose) at 200 000 $cell.mL^{-1}$. Agarose polymerization (2x15 min, 4 °C, dark
194 condition) was followed by lysis at 4 °C in the dark for 1h in lysis solution (2.5 mM NaCl, 100 mM
195 EDTA, 10 mM Tris-HCl, 1% Tritonx100, pH 10). After lysis, slides were placed at 4 °C in an
196 electrophoresis tank containing an alkaline solution (0.3 M NaOH, 1 mM EDTA, pH>13) for 20 min in
197 the dark before electrophoresis at 25 V and 300 mA for 20 min. Electrophoresis was stopped by
198 washing three times in a neutralizing solution (Tris-HCl 0.4 M; pH 7.5) and slides were then
199 transferred into absolute ethanol for 20 min and dried overnight. The prepared slides were stained
200 with 20 µL of ethidium bromide (BET; 20 $µg.mL^{-1}$) and analyzed at x200 using an epifluorescence
201 microscope (Olympus BX51, Rungis, France) coupled with CCD camera (Zeiss, Germany) and the
202 Comet assay IV software (Instrument Perspective LtD, Bury St Edmunds, UK). For each sample, 100
203 randomly selected nucleoids are analyzed on two replicated gels. DNA damage are expressed as
204 percentage tail DNA, which is the percentage of DNA which has migrated from the head (Collins,
205 2004).

206

207 *2.3.5 Photomotor assay*

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

with 500 µL 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 ± SD for each 10-min period. Velocity was expressed as mean ± 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 ± 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.

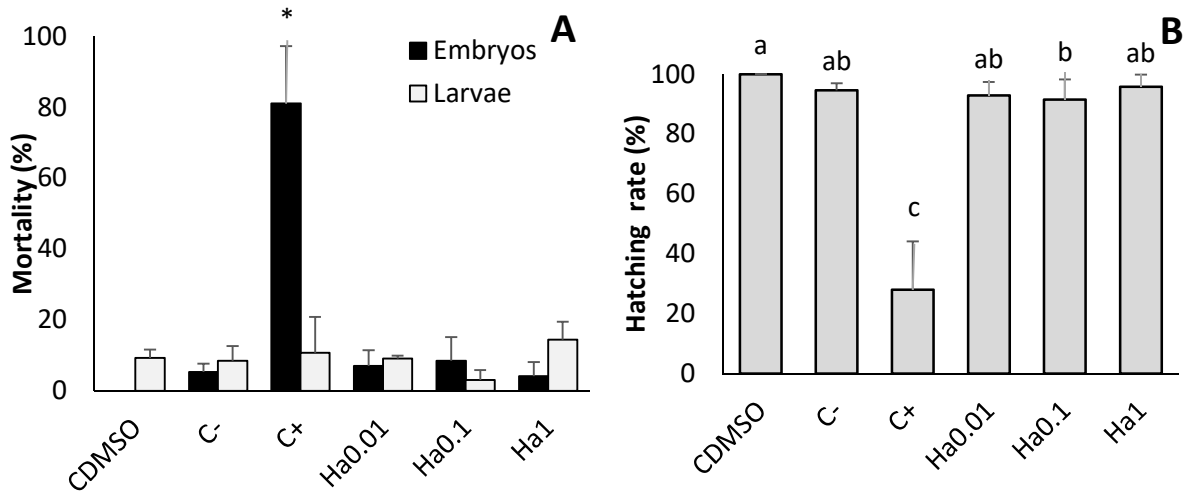


*Figure 2: Embryonic and larval mortality rates (A) and hatching rate (B) (%, mean±SD, n=25, N=3) following exposure for 8 days to different concentrations of organic extracts of microplastic from Hawaï: CDMSO: 1% of DMSO; C-: 1% of virgin MPs extract, C+, 1% of B(a)P coated MPs extract, Ha0.01, Ha0.1 and Ha1: 0.01%, 0.1% and 1% of Ha MPs extract. * and letters indicate significant differences between treatments (Kruskall-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 prolarvae 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±1.2% for Ha1 and 7.5±3.5% for control.

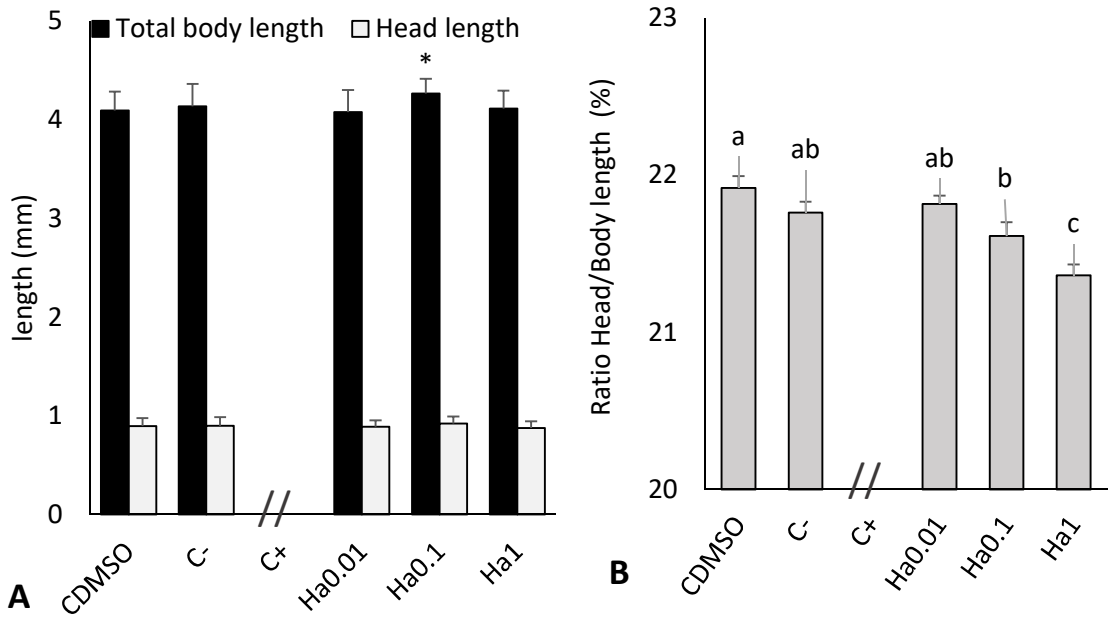


*Figure 3: Total body length and head length (A) and ratio head/body length (B) of prolarvae at hatching (mean±SD, n=10, N=3) after 8 days of exposure to different concentrations of MP extracts. C_{DMSO}: 1% of DMSO; C-: 1% of virgin extract, C+, 1% of B(a)P coated MPs extract, Ha0.01, Ha0.1 and Ha1: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

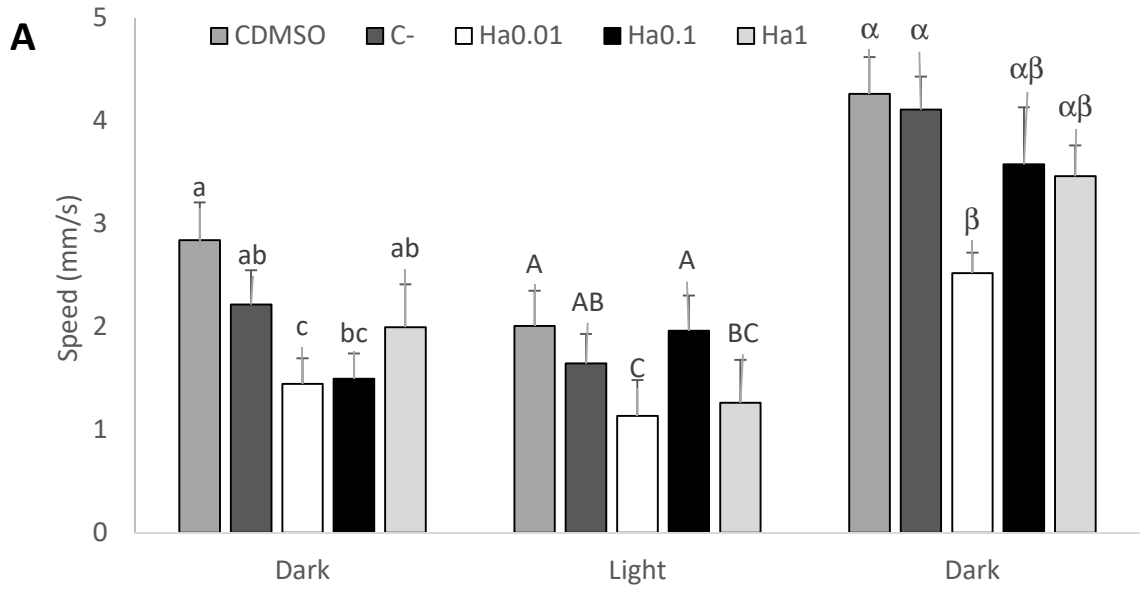
3.1.3 EROD and COMET

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± 2.03% for control and 2.84± 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-).



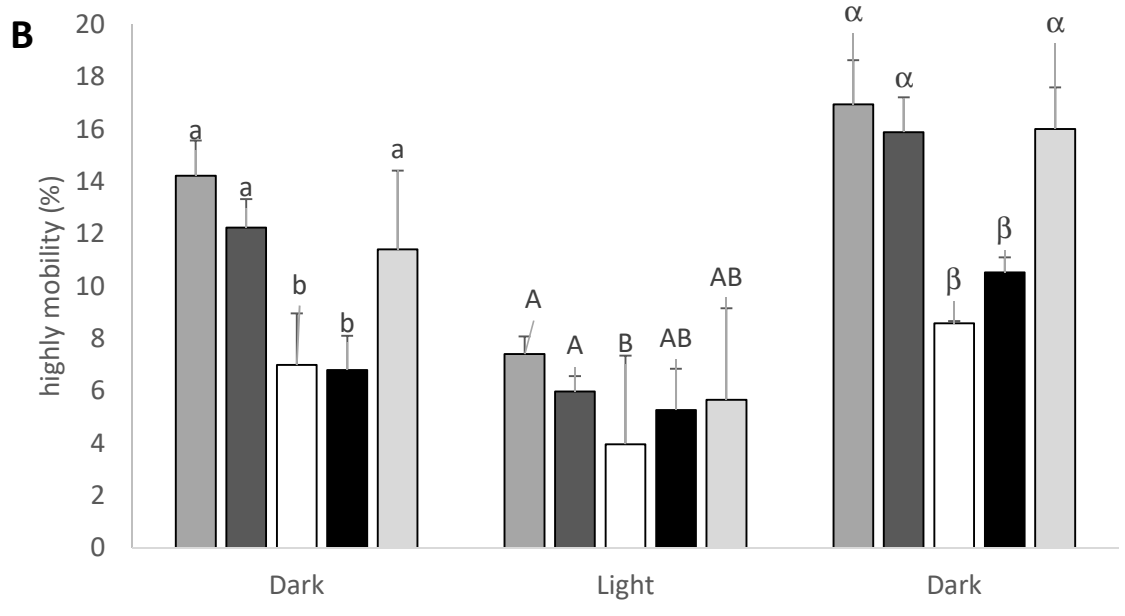


Figure 4: Swimming speed (A) and high mobility (B) variations of Japanese medaka prolarvae during dark/light stimulation (mean±SE, n=12, N=3) after exposure to MPs organic extracts. CDMSO: 1% of DMSO; C-: 1% of virgin extract, Ha0.01, Ha0.1 and Ha1: 0.01%, 0.1% and 1% of Ha MPs extract. Letters indicate significant differences between treatments (Kruskall-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± 3.33%, C+ 2.00±1.96%, Ea 3.00± 3.03%, Gu 4.20± 4.17% and Ha 7.90± 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± 1.37%.

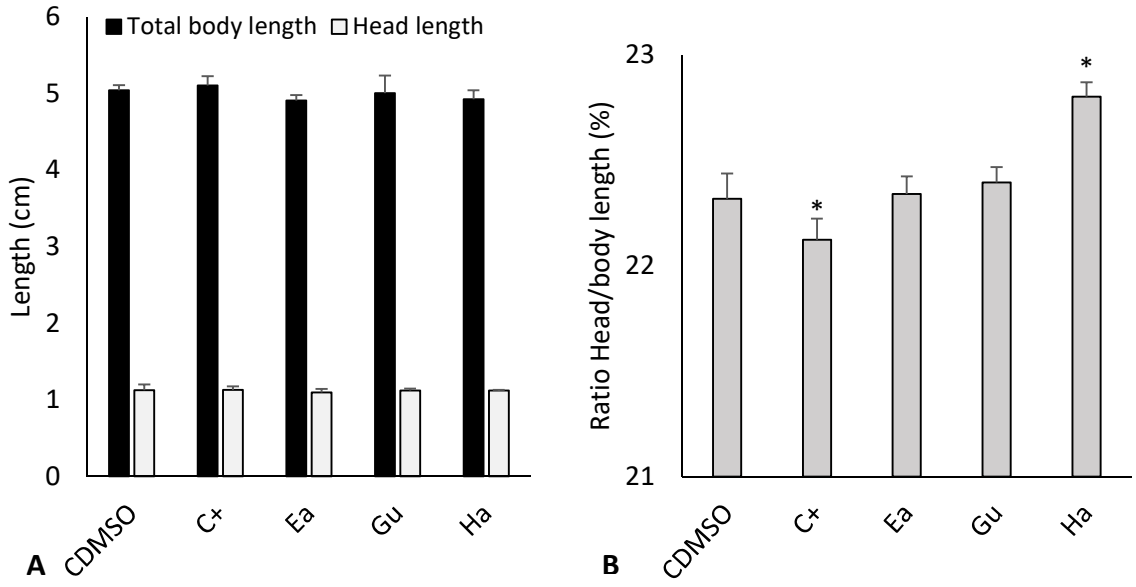


*Figure 5: Total body and head length (A) and Ratio head/body length (B) of Japanese medaka prolarvae after 48h of exposure to different MPs extractss (mean±SD, n=10, N=3). C_{DMSO}: 0.1% of DMSO; C+, 0.1% of B(a)P coated MPs extract, Ea, Gu and Ha: 0.1% of Ea, Gu and Ha MPs extract. * indicate significant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

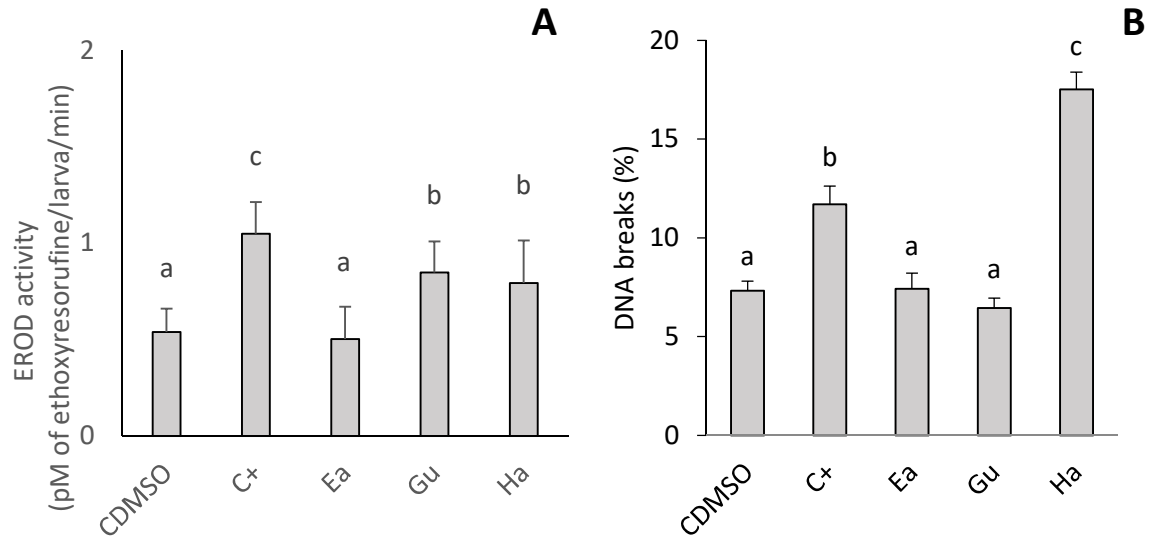


Figure 6: EROD activity (A) and DNA strand breaks (B) (mean±SD, N=3) in Japanese medaka prolarvae after 48h of exposure to different MPs extracts. C_{DMSO}: 0.1% of DMSO; C+, 0.1% of B(a)P coated MPs extract, Ea, Gu and Ha: 0.1% of Ea, Gu and Ha MPs extract. Letters indicate significant differences (Kruskall-Wallis,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

3.2.4 Behavior

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

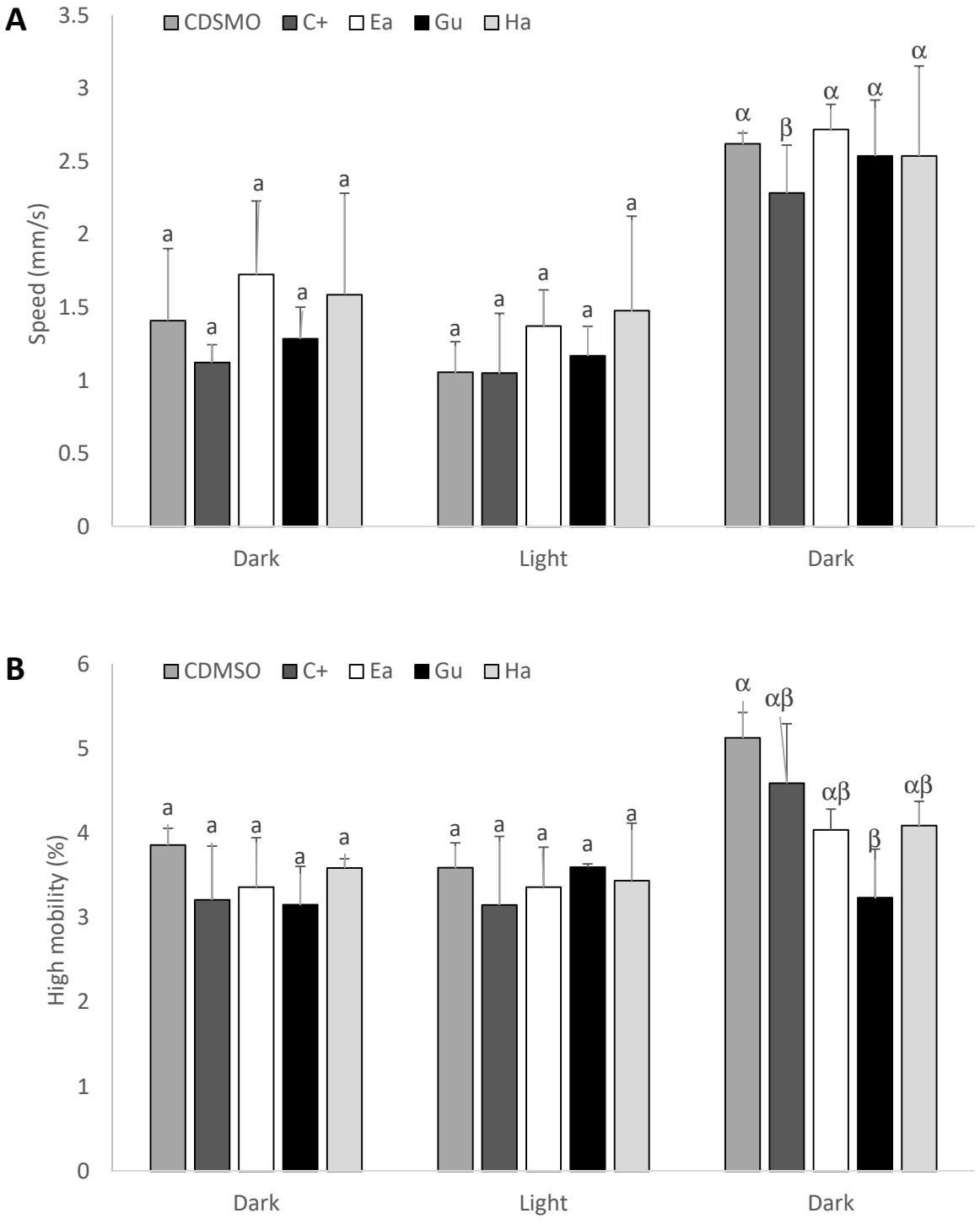


Figure 7: Swimming speed (A) and high mobility (B) variations during dark and light stimulation (mean±SE, n=12, N=3) in Japanese medaka prolarvae after 48h of exposure to different extracts of MPs. C_{DMSO}: 0.1% of DMSO; C+: 0.1% of B(a)P coated MPs extract, Ea, Gu and Ha: 0.1% of Ea, Gu and Ha MPs extract. Letters indicate significant differences between conditions by period (Kruskall-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 µM) 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 inductors 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 inductors 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 $\mu g.L^{-1}$) 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

393 chemical and concentration tested (Irons et al., 2013). Decreases in speed and mobility were
394 reported in literature after exposure of fish embryos or larvae to a large variety of organic pollutants
395 such as PAHs, pesticides and psychotropic drugs (Oliveira et al., 2012; Le Bihanic et al., 2014a; Faria
396 et al., 2015; Chiffre et al., 2016). A study have also reported an increase in fish swimming activity
397 after exposure to environmental concentrations of PAHs (Le Bihanic et al., 2014c). In the present
398 study, prolarvae were exposed to a cocktail of contaminants, and effects on behavior were low and
399 not dose-dependent. These could suggest that the different chemicals in the MP extracts had an
400 antagonistic effect on behavior or that the chemical concentrations were too low. In the natural
401 environment, locomotion impairment can have a significant impact on fish survival (higher
402 predation) or growth rate (feeding efficiency) and can lead to reduced populations (Little and Finge,
403 1990). Conversely, an increase in swimming activity can lead to high energy consumption, which can
404 in turn impair other physiological pathways. Since in this study no attempt was made to measure
405 energy reserves, the ecological relevance of the behavioral endpoints measured for further
406 development of the larvae is not known. Persistent organic pollutants are known to have impacts on
407 fish and other species' behavior (Scott and Sloman, 2004; Weis et al., 2001). For example, DDT at
408 sublethal concentrations is known to have negative impacts on fish's central nervous system, thus
409 also on their locomotion (Davy et al., 1972). MP extracts from Easter Island did not show any toxic
410 effects on medaka prolarvae, although the chemical contamination profile of Easter Island's plastics
411 was similar to Guam beach at least for the chemicals analyzed. Different types of plastic have shown
412 different contaminant sorption properties or capacities to release harmful additives (Rochman et
413 al., 2013a). Plastic life history, including discharge area and time of transportation, could influence
414 degradation, chemical contamination and ageing of MPs (Rochman, 2015). Ageing of polymer could
415 also have an influence on chemical sorption and desorption (Rochman, 2013a) and could somewhat
416 explain why Ea extract was less toxic than Gu extract.

417 Results obtained here with embryos or larvae of medaka confirm the toxicity of MP extracts
418 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.

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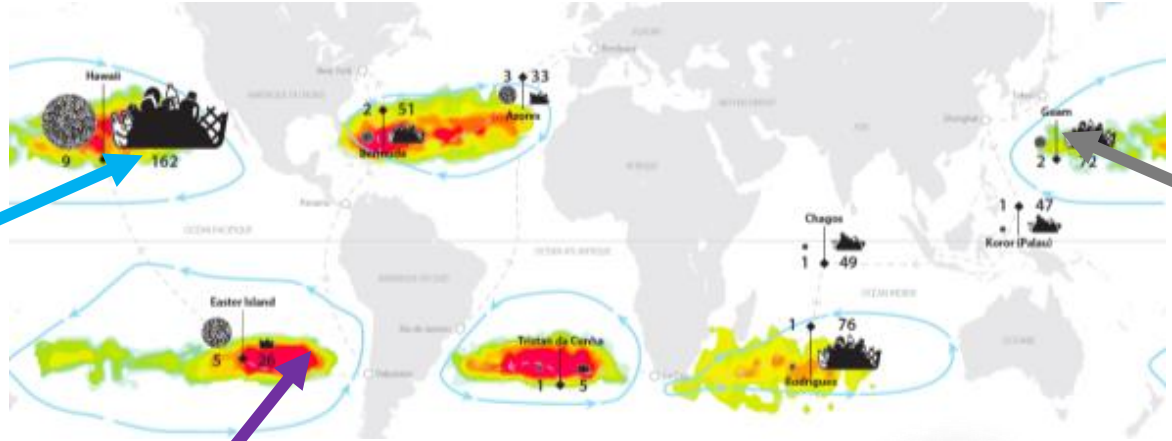
Ha 0.1%

Mortality: ⌀
Body change: (+)
EROD: +
DNA break: +
Behavior: ⌀



Gu 0.1%

Mortality: ⌀
Body change : ⌀
EROD: +
DNA break: ⌀
Behavior: +

Ea 0.1%

Mortality: ⌀
Body change : ⌀
EROD: ⌀
DNA break: ⌀
Behavior: ⌀

Oryzias latipes larvae and embryos