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Environmental samples of microplastics induce significant toxic effects in fish larvae



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ABSTRACT

Microplastics (MPs) are present throughout aquatic ecosystems, and can be ingested by a wide variety of organisms. At present, the physical and chemical effects of environmental MPs on aquatic organisms are poorly documented. This study aims to examine the physiological and behavioral effects caused by fish consuming environmental microplastics at different life stages. MP samples were collected from beaches on three islands (Easter Island, Guam and Hawaii) located near the North and South gyres of the Pacific Ocean. Larvae and juveniles of Japanese Medaka were fed for 30 days with three doses of MPs (0.01, 0.1 and 1% w/w in fish food) approximate to the concentrations measured in moderately and heavily contaminated ocean areas. Ingestion of MPs by medaka larvae caused (variously) death, decreased head/body ratios, increased EROD activity and DNA breaks and, alterations to swimming behavior. A diet of 0.1% MPs was the most toxic. Two-month-old juveniles fed with 0.01% MPs did not exhibit any symptoms except an increase in DNA breaks. Our results demonstrate ingestion and mainly sublethal effects of environmental MPs in early life stages of fish at realistic MP concentrations. The toxicity of microplastics varies from one sample to another, depending on polymer composition, weathering and pollutant content. This study examines the ecological consequences microplastic build-up in aquatic ecosystems, more particularly in coastal marine areas, which serve as breeding and growing grounds for a number of aquatic species.

1. Introduction

Since the 1950s, plastic has been a major concern, attracting interest from the media, scientists, and the general public (Law, 2017). Marine litter, particularly microplastics, has attracted considerable attention, due to its abundance in the aquatic environment. Plastic production has grown exponentially since the 1950s, with millions of tons of plastic waste being discharged into aquatic systems (Geyer et al., 2017; Jambeck et al., 2015).

Marine environments receive huge volumes of plastic debris. In 2014, the amount of plastic on the ocean surface was estimated at 268,940 tons (Eriksen et al., 2014). Due to its extremely slow rate of biodegradation (Cole et al., 2011), this debris tends to accumulate in coastal areas and oceanic gyres (Law, 2017; Eriksen et al., 2014; Lebreton et al., 2018). UV irradiation can lead to mechanical

fragmentation, hydrolysis, bacterial degradation and other phenomena (Cole et al., 2011). Industrial primary materials and wastewater treatment plants can also be a direct source of microplastics entering the environment.

Microplastics are defined as plastic debris between 1 µm and 5 mm in diameter. They come in a variety of forms, colors and materials. Polypropylene and polyethylene are the two most widespread forms of plastic in the aquatic environment (Law, 2017; Cole et al., 2011). Macroplastics and microplastics can be ingested by various marine organisms, including mollusks (Van Cauwenberghe and Janssen, 2014), crustaceans (Murray and Cowie, 2011; Devriese et al., 2015), zooplankton species (Desforges et al., 2015), marine mammals (Bravo Rebolledo et al., 2013; Fossi et al., 2012), sea birds (Bravo Rebolledo et al., 2013), marine turtles (Tourinho et al., 2010) and fishes at all stages of life (Rummel et al., 2016; Peda et al., 2016; Steer et al., 2017). Due to their

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small size, MPs can be ingested and, under certain conditions, translocated to the circulatory system and accumulated in different types of tissues (Browne et al., 2008).

Transfer of microplastics into food webs has also been reported in laboratory studies (Farrell and Nelson, 2013; Setälä et al., 2014). To date, very few studies have dealt with the toxic effects of environmental MPs on marine organisms. Most of them - if not all - are focused on commercial plastics, which are sometimes artificially spiked with pollutants (Peda et al., 2016; Batel et al., 2016; Oliveira et al., 2013; Carlos de Sa et al., 2015). After ingestion, MPs can have a variety of physical and chemical effects on organisms exposed to them.

Physical effects can include (Wright et al., 2013) clogging of the digestive tract and a feeling of fullness, (Cole et al., 2011; Rochman et al., 2013a) along with internal injuries, such as a perforated gut, ulcerative lesions, or gastric rupture, potentially leading to death (Law, 2017) and intestinal alterations (Peda et al., 2016). Organisms that ingest MPs are exposed to a large variety of chemicals, including additives added to plastic during manufacture and pollutants. Plastic additives are plasticizers (phthalates, bisphenol A, etc.) colorants, UV filters, flame retardants, etc. (Koelmans et al., 2014; Koelmans, 2015; Rochman, 2015). Numerous persistent hydrophobic organic pollutants (PCBs, organochlorine pesticides, PAHs, etc.) and metals can also be sorbed onto the surface of plastic debris during their transfer into aquatic environments (Derraik, 2002; Karapanagioti et al., 2011; Koelmans et al., 2016; Koelmans et al., 2013).

A wide range of toxic effects caused by commercial MPs with or without spiked pollutants have previously been reported, including endocrine perturbation and hepatic stress including CYP1A expression, oxidative stress, changes in metabolic parameters, reduced enzyme activity, and cellular necrosis (Law, 2017; Oliveira et al., 2013; Rochman et al., 2013a; Rochman et al., 2014; Browne et al., 2013; Teuten et al., 2009; Mazurais et al., 2015).

The resulting biological consequences can compromise the survival, growth, reproduction, and development of organisms, particularly in early life stages (Mazurais et al., 2015; Nobre et al., 2015; Sussarellu et al., 2016; Beiras et al., 2018).

Fish larvae play a pivotal role in marine ecosystems. Their health and survival are fundamental to the long-term sustainability of healthy fish populations (Steer et al., 2017). Among the least selective feeders, the fish larvae are particularly vulnerable to MPs when ingested. Microplastic ingestion by fish larvae has recently been reported in laboratory conditions (Mazurais et al., 2015) and in the field (Steer et al., 2017).

In this study, we sought to identify the toxic effects associated with the ingestion of plastic particles by larvae and of fish juveniles. The underlying assumption is that microplastics can be ingested in a non-selective way by fish, and can physically or chemically damage cellular structures, with possible consequences on the physiology or behavior of the whole organism.

Three experiments were carried out. In the first one, fish were exposed to different concentrations of a same sample of MPs to determine the toxicodynamic and the toxicity threshold of this kind of material. In the second experiment, the effects of environmental concentrations of MPs from three Pacific islands were evaluated.

To better identify the possible spectrum of deleterious effects, a large range of endpoints were analyzed including CYP1A activity, DNA damage, growth, developmental defects, swimming behavior and mortality.

2. Material and methods

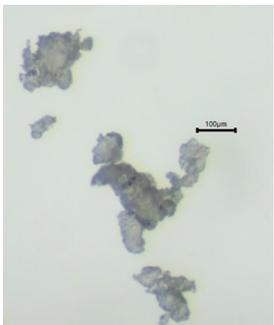
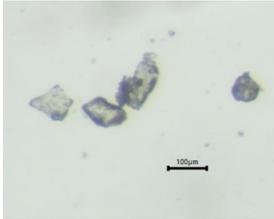
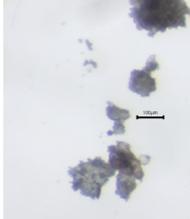
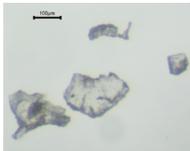
2.1. Microplastic samples

2.1.1. Microplastic sampling and characterization

Environmental microplastics were sampled in 2015, using the microplastic sampling protocol from the National Oceanic and

Table 1

Summary of characteristics of microplastic sampled on three Pacific island beaches.

Sampling place	Polymer composition (% mass) ^a	Particle size (µm) ^b	Photographs of representative MPs
Plastic laboratory containers (Control C-)	PE 65%, PP 25%, PS 10%	d(0.1) 108.2 d(0.5) 408.8 d(0.9) 962.2	
Eastern Island, Anakena Beach (EA)	PE 94.2%, PP 5.8%	d(0.1) 38.9 d(0.5) 316.0 d(0.9) 861.6	
Guam, Pago Bay (Gu)	PE 59.0%, PP 36.5%, NI 4.4%	d(0.1) 16.4 d(0.5) 209.3 d(0.9) 826.8	
Hawaiï, Big Island (Ha)	PE 27.4%, PP 72%, PS 0.7%	d(0.1) 23.6 d(0.5) 305.1 d(0.9) 776.9	

^a NI: none identified, PE: polyethylene; PP: polypropylene; PS: polystyrene,

^b After grinding and sieving at 600 µm d(0.1), d(0.5) and d(0.9) is the size of particle at which 10%, 50% and 90% of the sample is smaller than this size, respectively. d(0.5) is the median size of particles.

Atmospheric Administration (Lippiatt et al., 2013), on beaches from three islands in the Pacific Ocean: Kawa Bay, Big Island, Hawaiï (Ha), Anakena, Easter island (Ea), and Pago Bay,Guam (Gu) (Race for Water Odyssey, 2015). Micro-waste (< 5 mm) was collected from a sandy area of 50 cm² × 10 cm deep by immersing the sand collected in seawater. The floating microparticles on the surface were recovered with a 300 µm sieve and stored in the dark at ambient temperature in 180 mL plastic buckets (PP) (Pannetier et al., 2019a). Microplastics were manually sorted under stereomicroscope, then grinded and sieved at 600 µm to prepare the MP component to be included in the fish food used for the experiment.

Particles from the Ha sample were counted after resuspension of MP samples in 0.22 µm-filtered water (0.5 mg/mL) under a light microscope at x400 magnification using a Malassez counting cell. The concentration of MPs ≥ 10 µm was estimated at about 23,500 particles per mg of powder.

The particle-size distribution of crushed microplastic samples was obtained using a Malvern laser diffraction particle size analyzer. Polymer composition, and to a lesser extent particle size, differ between MP fractions (Table 1). The Ea sample was composed mainly of PE

(94%) and PP (6%), the Ha sample contained primarily PE (27%), PP (77%) and PS (1%), while the Gu sample was made up of PE (59%) and PP (37%). As recently reported (Pannetier et al., 2019a), organic pollutants detected on beach macro and microplastic were mostly PAHs with highest concentration in Ha sample and lowest on Ea.

2.1.2. Control MPs

A control microplastic sample was prepared with clean commercial laboratory plastic grinded with a mortar or electric crusher and sieved at 600 µm. This control MP sample was composed of Low Density Polyethylene (LDPE, 40%), High Density Polyethylene (HDPE, 25%), Polypropylene (PP, 25%) and Polystyrene (PS, 10%) according to the composition of environmental microplastic samples from lakes in Switzerland (Faure et al., 2015). This MP mixture was used as a negative control. A fraction was coated with Benzo[*a*]pyrene to serve as a positive control (B[*a*]P, CAS Number: 50-32-8). In an amber flask, 100 mg of control MPs were mixed with 1 mL of 100 µM (0.25 mg·mL⁻¹ in methanol) of B[*a*]P solution (16 h, 175 rpm, room temperature). The measured BaP concentration was 172 µg·g⁻¹ of MPs. Methanol was evaporated using azote flux (2 h, 37 °C). Positive and negative controls were stored at -20 °C in amber flasks until analysis.

2.1.3. Food preparation

For the first experimentation on larvae, Hawaii MPs were mixed with fish food flakes, tetraMin®Baby (Tetra GmbH, Germany) at 0.01%, 0.1% and 1% (w/w). For the second experimentation on larvae, the food was spiked with 0.1% of Ha, Gu or Ea. A third experiment was carried out using juveniles, wherein MP particles of Ea were mixed with fish food flakes tetraMin® (Tetra GmbH, Germany) at 0.012%. The food mixture dose for one feeding for each batch was 1.5 ± 0.25 mg (Experiments 1 and 2) for larvae and 125 ± 6.5 mg (Exp 3) for juveniles. Depending on the volume of water used in each assay (50 mL for the assay on larvae and 10 L for the assay on juveniles), the targeted concentrations for larvae were 3 µg·L⁻¹ e.g. 70 MPs·L⁻¹ (0.01%), 30 µg·L⁻¹ e.g. 700 MPs·L⁻¹ (0.1%) and 300 µg·L⁻¹ e.g. 7000 MPs·L⁻¹ (1%) of MPs and for juveniles, 1.5 µg·L⁻¹ e.g. 35 MPs·L⁻¹ (0.012%). Besides, Rochman et al., reported 300 µg·L⁻¹ as the maximum MPs concentration in the North Pacific Gyre (Rochman et al., 2013a).

2.2. Fish exposure

2.2.1. Larvae

Japanese medaka embryos were provided by UMS-Amagen (CNRS, Gif-sur-Yvette, France) at early gastrula stage 14–15 according to Iwamatsu (2004). After 24 h of acclimatization at 20 °C, 150 embryos were transferred into plastic petri dishes (Ø9 cm, Grenier, France) in ERS (1 g NaCl, 0.03 g KCl, 4.04 g CaCl₂ and 0.163 g MgSO₄ in 1 L Milli-Q autoclaved water) at 26 °C photoperiod 12:12 5000-lx white light (Snijders Scientific, Tilburg, The Netherlands). Medium (ERS) was renewed every 48 h and the daily dissolved oxygen concentration was checked. Petri dishes were gently shaken (30 rpm) to synchronize hatching. On peak hatching day for each replicate, a batch of 25 pro-larvae was distributed in a glass beaker containing 50 mL of non-contaminated mixing water (1/3 tap water + 2/3 distilled water v/v) and incubated in thermostatic chamber at 26 °C with same photoperiod and light conditions that for embryos. Three days after hatching, trophic contamination started according to the following experimental protocol.

2.2.1.1. Experiment 1. Toxic effects in medaka larvae fed with different concentrations of MPs. Six exposure conditions were tested in triplicate. Control (C) larvae fed only with Tetramin®Baby. Negative plastic control (C-): 1% of commercial microplastic mixture in Tetramin®Baby. Positive plastic control (C+): 1% of commercial microplastic mixture coated with 100 µM of B[*a*]P in Tetramin®Baby. Finally, three treatments called Ha0.01, Ha0.1, and Ha1 with

respectively 0.01, 0.1 and 1% of Hawaii MPs in Tetramin®Baby.

Larvae were fed twice a day, once in the morning (9:00 am) with the MP mixture, and once in the evening (5:00 pm) live brine shrimp, *Artemia salina*. Mixing water was changed every 48 h and dissolved oxygen was measured before water change. To limit the accumulation of MPs in beakers, sidewalls were cleaned and the faeces were removed each time the water was changed. Trophic contamination was carried out for 14 days.

2.2.1.2. Experiment 2. Toxic effects of three environmental samples of MP in medaka larvae. Five treatments were tested in triplicate: Control (C), embryos fed only Tetramin®Baby flakes; negative plastic control (C-), 0.1% of clean microplastics in Tetramin®Baby; Easter Island (Ea), 0.1% of Ea microplastics in Tetramin®Baby; Guam (Gu), 0.1% of Gu microplastics in Tetramin®Baby; Hawaii (Ha), 0.1% of Ha microplastics in Tetramin®Baby. Larvae were fed 3 times a day, morning (9:00 am) and evening (5:00 pm) with MPs mixture and at midday (1:00 pm) with nauplii of brine shrimp. Mixing water was changed every 48 h and dissolved oxygen was measured before changing water. Trophic contamination was carried out for 30 days with 2 sampling points at 14 and 30 days.

2.2.2. Juveniles

The third experiment was carried out on juveniles. Japanese medaka embryos were provided by UMS-Amagen (CNRS, Gif-sur-Yvette, France). On peak hatching day, pro-larvae were placed in batches of 150 in glass beakers containing 500 mL of no-contaminated mixing water (1/3 tap water + 2/3 distilled water) in triplicate. One month after hatching, larvae were transferred in ZebTec system (ZebTec Active Blue Stand Alone, Tecniplast) in 3.5 L tank, 26 °C, 12 h:12 h photoperiod. Temperature, pH and conductivity of water were continuously measured and 10% of water was automatically renewed every day. Larvae were fed 2 times a day with Tetramin®Baby and once a day with nauplii of brine shrimp.

At 2 months post hatching, for each replicate, 50 juveniles of medaka were transferred into a 10 L exposure aquarium at 25 °C, with constant flow and natural photoperiod. Fish were fed 2 times a day for 30 days with 125 mg (250 mg by day) of Tetramin® spiked with 0.012% Ea MPs (Ea) or Tetramin® alone (C). Three replicates were performed for each treatment.

2.3. Toxicity endpoints

2.3.1. Mortality

Mortality was checked daily and dead larvae or juveniles were immediately removed to avoid medium degradation. The mortality rate was determined according to the total number of dead individuals at the end of the experiment compared to the total number of individuals at the beginning of the experiment.

2.3.2. Biometry/malformations

2.3.2.1. Experiments 1 and 2. After 14 and/or 30 days of exposure, 10 larvae per replicate were anesthetized with cold sparkling water. Larvae were individually examined under stereomicroscope (Leica MZ7.5, Nanterre France) at 25× magnification to record morphological abnormalities and lesions (edema, spinal, craniofacial, ocular, cardiac and yolk sac malformations) then photographed with a CCD camera DFP420C Leica. Leica Application Suite V3.8 was used to determine the total body length (from terminal point of the mouth to the end of caudal fin) and the head length (from terminal point of the mouth to the rear of operculum) from pictures of individual larvae. The ratios between head length and total body length were also calculated.

2.3.2.2. Experiment 3. The total body length from the terminal point of the mouth to the end of caudal fin was measured for 10 juveniles. The same fish were also weighed and Fulton's condition factor was

calculated ($FCF = \text{Weight} / \text{Length}^3$).

2.3.3. EROD activity

EROD activity was analyzed in vivo in medaka larvae. The protocol for CYP4501A EROD activity measurement was adapted from Le Bihanic et al. (2013). Briefly, for each replicate, 5 prolarvae per well were transferred into a 48-wells microplate. Mixing water was replaced by 600 μL of a 1 μM ethoxyresorufin solution (ER). After 1 h incubation at 26 °C, the solution was renewed with 600 μL of fresh medium containing 1 μM of ER. Fluorescence of 100 μL solution per well in duplicate was directly measured immediately in microplate reader (Fluostar optima, BMG LABTECH) at 540 nm/580 nm (λ excitation/ λ emission). After 4 h of dark incubation, a new fluorescence measurement was performed from 100 μL /well in duplicate. A resorufin standard curve was used to determine average resorufin production per well. EROD activity was calculated in pM of resorufin. larva⁻¹.min⁻¹. EROD activity was also measured in the S9 fraction extracted from the liver of juveniles. At the end of the exposure, 3 fish livers per replicate were collected, pooled and stored at -80 °C until analysis. On the day of analysis, the three livers (≈ 20 mg) were ground in 150 μL of 10 mM phosphate buffer (KH_2PO_4 , K_2HPO_4 , pH 7.4). The mixture was centrifuged (20 min, 4 °C, 10000 rpm) and the supernatant (S9) was stored in ice at 4 °C. Protein content was evaluated using the colorimetric method according to Lowry et al. (1951) on S9 fraction. EROD activity was measured on S9 fraction according to the method of Burke and Mayer (1974) with minor adaptations.

2.3.4. DNA damage

For larvae, DNA damage was assessed using the comet assay, based on the original protocol of Singh et al. (1988) and adapted with a cellular dissociation step (Morin et al., 2011). The comet assay was performed using a pool of 5 individual larvae per replicate. Dissociation of cells was carried out with dispase II from *Bacillus polymyxa* (Roche, Meylan, France) at 0.125% for 2×10^5 cell·mL⁻¹. After 1 h of lysis and 20 min of DNA unwind, electrophoresis was carried out at 25 V and 300 mA for 20 min at 4 °C in the dark.

For juveniles, the formamidopyrimidine glycosylase Fpg-modified comet assay was performed in alkaline conditions according to the method proposed by Collins et al., (1996) and improved by Kienzler et al. (2012) on blood cells. Following exposure, the blood from 3 fish per replicate was sampled from the heart after fish decapitation using a pipet tip previously coated with heparin (5000 U/mL). Between 5 and 20 μL of blood were collected per fish and then mixed with 200 μL of cryopreservation medium (250 mM of sucrose; 40 mM citrate trisodique, 5% DMSO; pH 7.6) and stored at -80 °C until analysis. At the time of analysis, blood was thawed and diluted to obtain a concentration of 2×10^5 cell·mL⁻¹. Cells were treated for 35 min at 37 °C with either 60 μL of enzyme buffer containing 12 μL of Fpg solution (New England Biolabs, 8 UI/ μL) or 60 μL of enzyme buffer without Fpg. As for classic comet assay, after 20 min of DNA unwind, electrophoresis was carried out at 25 V and 300 mA for 20 min.

The slides were then stained with 20 μL of ethidium bromide (BET; 20 $\mu\text{g}\cdot\text{mL}^{-1}$) and analyzed at $\times 200$ using an epifluorescence microscope (Olympus BX51, France) coupled with a digital camera (Perceptive Instruments, Germany). Comets were recorded with the Comet assay IV software (Instrument Perspective LtD). For each sample, 100 randomly selected nucleoids were analyzed on two replicated gels. DNA damage was expressed as the percentage of tail DNA, which is the percentage of DNA which has migrated from the head (Collins, 2004).

2.3.5. Swimming behavior

The photomotor assay was intended to evaluate the larval locomotion or swimming capacities under a light/dark stimulation according to the protocol detailed in Le Bihanic et al. (2014) with slight modifications. Twelve larvae per replicate were randomly selected (larvae with gross deformities were left out) and placed into a 48-well

microplate, one larva per well in 500 μL of mixing water. After 2 h (minimum) of larval acclimatization in the dark at 26 °C, the larvae were allowed to rest for an additional 30 min in a Daniovision chamber (Noldus, Wageningen, Netherlands). Larvae movements were 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, distance swam and mobility were calculated for each larva. Velocity data refers to the mean velocity in mm/s. Distance refers to distance swum in mm. Data was calculated for each 10 min period. All microplates were analyzed with the same detection/acquisition settings.

2.3.6. Biphotonic imaging of microplastics in medaka

To confirm ingestion of microplastics by larva, 12 larvae by condition were exposed in the same way as for the experiment with Tetramin®Baby alone and to 1% of Hawaii microplastics in Tetramin®Baby. After 48 h of exposure, larvae were euthanized with an overdose of benzocaine and stored at -80 °C. Microscopical examination was performed on the whole body of freshly thawed larvae without sectioning.

Biphotonic imaging analyses were carried out with a Nikon microscope A1R-MP coupled with an Insight DeepSee laser (Spectra Physics), tunable in the 820–1300 nm range. An auxiliary beam at 1040 nm was used in combination with the tunable output for dual excitation wavelength. Observations were performed with an apochromat 25 \times MP1300 immersion objective (NA 1.10, WD 2.0 mm). Different combinations of excitation wavelength and interferometric filters were used to find the optimum setting to discriminate MPs from Tetramin® fluorescence. The most discriminative method was the dual sequential excitation 820/1040 nm with the fluorescence detection in four channels according to the following settings: signal recovery from 820 nm in blue (446/92 nm) and red channel (629/56 nm), signal recovery from 1040 nm in green (525/50 nm) and yellow channel (575/25 nm). The four acquired images were merged showing a blue predominant signal for MPs and a yellow predominant signal for Tetramin®.

2.4. Statistics

For all experiments, each exposure condition was identically replicated 3 times and considered an independent sample. Data is reported 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. Variance homogeneity was evaluated using Levene's test. In case of homogenous variance and normal distribution of data, data was analyzed by a One-way Anova followed by a Tukey post-hoc test. In the other cases, log (number) or Arc sinus (percentage) transformation was applied to data. If the Anova's conditions were not respected, Kruskal-Wallis non-parametric test was performed. Differences were considered significant for $p < 0.05$.

3. Results

3.1. Dose-response effects of environmental mixture of MPs on medaka larvae

No significant effect on mortality was observed following MP contamination at any of the concentrations tested. Mortality rate was between $5.95 \pm 9.45\%$ for control larvae fed only with fish food, and $1.19 \pm 2.06\%$ for larvae exposed to the highest concentration of MPs. After MP contamination, no significant increase in deformities was observed when compared with larvae fed with only fish food (Supplementary data, Fig. S1). No change in head size was observed (Fig. 1A). However, a significant reduction in larvae growth was observed after 14 days of trophic contamination with the lowest MPs concentration at 0.01% (Fig. 1A). Body length of larvae reached

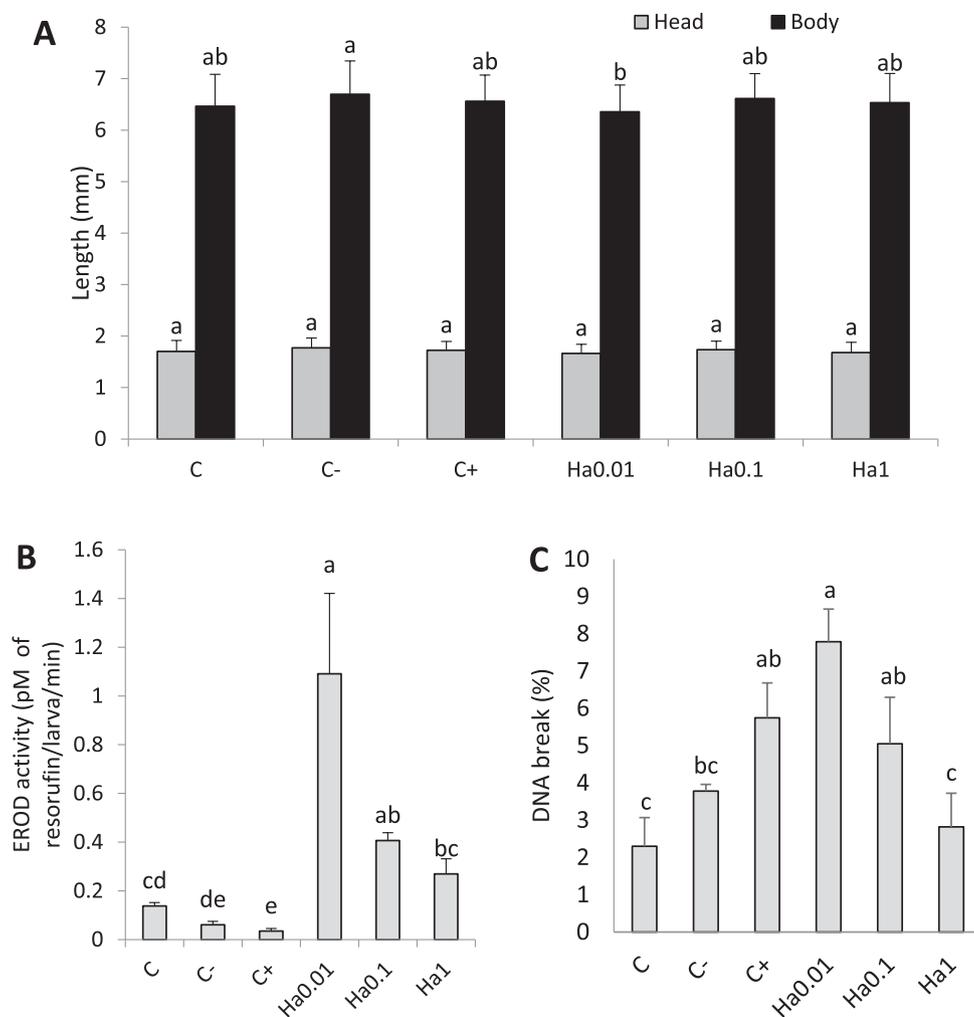


Fig. 1. Head and body length of Japanese medaka larvae (A) (mean \pm SD, n = 10, N = 3), in vivo EROD activity (B) (mean \pm SD, n = 5, N = 3) and DNA breaks (C) (mean \pm SD, n = 5, N = 3) following a 14d-exposure to different concentrations of MP from Hawaii: C: food alone (Tetramin); C-: food + 1% of negative plastic control, C+: food + 1% of B(a)P coated MPs, Ha0.01, Ha0.1 and Ha1: food + 0.01%, 0.1% and 1% of Ha MPs. Different letters at the top of the bars indicate significant differences between treatments (Kruskal-Wallis, $p < 0.05$).

6.70 ± 0.6 mm for negative control and only 6.36 ± 0.523 mm for the Ha 0.01 mixture. No significant change in head/body length ratio was observed (Supplementary data, Fig. S1). Compared to the control food (C), a significant inhibition of CYP1A activity was observed for C+ whereas an induction of CYP1A activity was noted for larvae fed with 0.01% or 0.1% of Ha MPs (Fig. 1B). Compared to the control food (C), a significant induction of DNA breaks was observed for C+, Ha0.01 and Ha0.1 conditions but compared to C- (negative plastic control) significant induction was only observed for C+ and Ha0.01 (Fig. 1C). Behavior of medaka larvae (swimming speed) was not affected by the 14d-exposure to MPs whatever the conditions considered (Supplementary data, Fig. S2).

3.2. Ingestion of MPs by medaka larvae

Isolated MPs and fish food Tetramin were imaged separately using a biphotonic microscope. The dual excitation at 820/1040 nm showed MPs in blue Tetramin in yellow (Fig. 2). The analysis of a mix of MPs and Tetramin was conclusive showing a clear separation between the two mix components. Non-exposed medaka were also observed by using the well-defined optic setting in order to be sure that the blue predominant fluorescence observed from MPs was not generated from auto-fluorescence of medaka. Finally, the blue fluorescent MPs were found in the digestive tract of 25% (3/12) of MP-exposed medaka but

not in the control non-exposed fish.

3.3. Comparing the toxicity of environmental samples of MPs for medaka larvae

At 14 days, exposure to the three samples of MPs did not affect survival, development, growth and DNA integrity of Japanese medaka larvae (Supplementary data, Fig. S3). However, a significant inhibition of EROD activity was observed for C- (7.91 ± 0.26 pM of resorufin/larva/min) and Gu (7.87 ± 0.54 pM of resorufin/larva/min) compared to control food (8.92 ± 0.89 pM of resorufin/larva/min). In addition, no significant variation in mean swimming speed was observed for larvae exposed to MP samples for any of the treatments examined (Supplementary data, Fig. S4).

At 30 days, a significant increase in mortality was noted for larvae exposed to 0.1% MPs from Hawaii ($27.8 \pm 3.9\%$) compared to both controls (Fig. 3). No increase in larval abnormalities was observed in comparison with the control group (Supplementary data, Fig. S5). In addition, smaller head and body lengths were recorded for larvae exposed to Gu as well as a smaller body length for larvae exposed to Ea (Fig. 4A). A significant increase in head/body length ratio for larvae exposed to negative plastic control (C-) and MPs from Ea was also observed (Fig. 4B). Finally, exposure to Ea MPs led to EROD activity inhibition while Ha MPs triggered EROD activity induction in

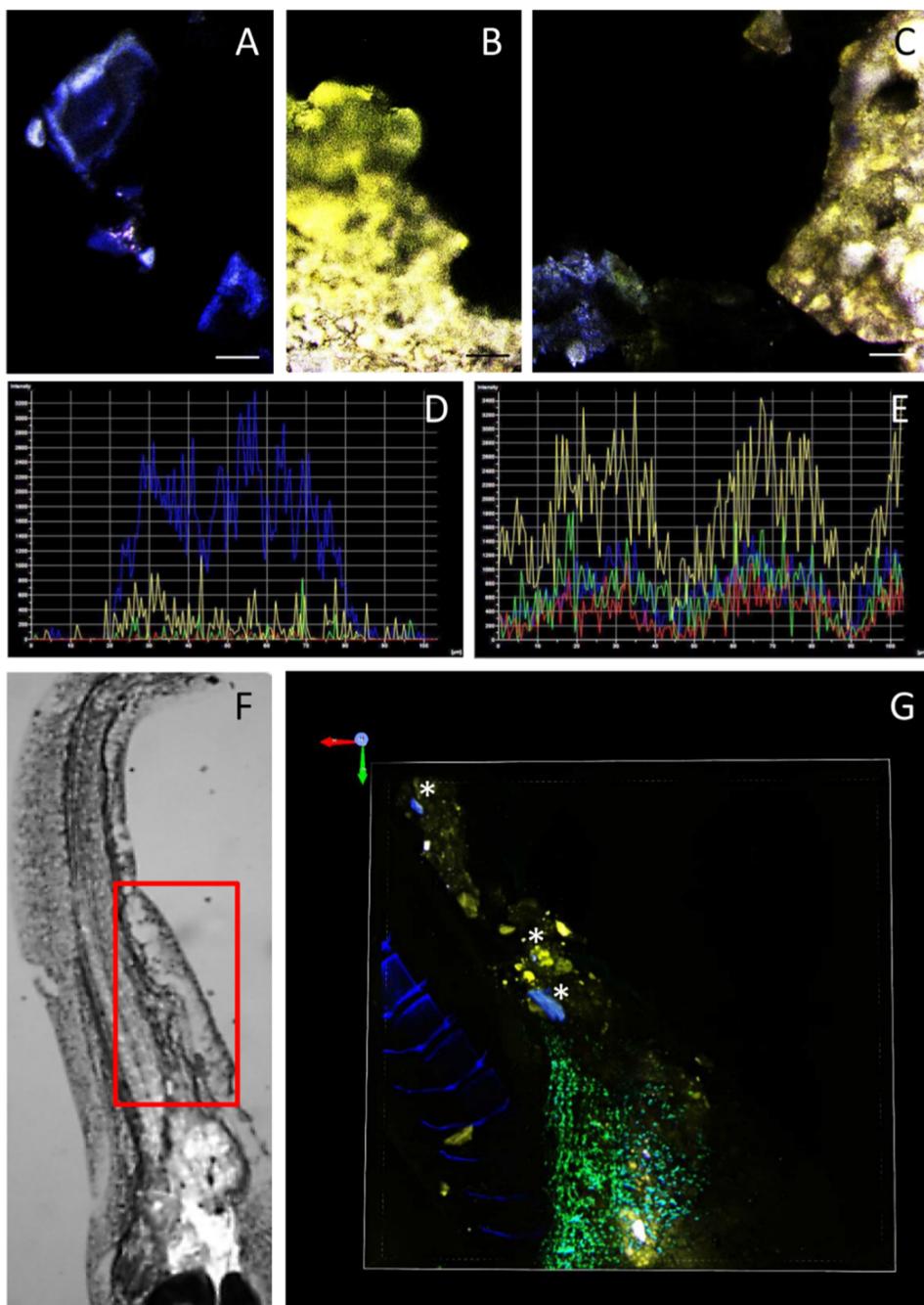


Fig. 2. Biphotonic imaging of MPs in the digestive tract of Japanese medaka larvae. (A) Predominant blue emission of MPs from Hawaii, scale bar 50 μm . (B) Predominant yellow fluorescence from Tetramin, scale bar 50 μm . (C) mix of MPs (blue) and Tetramin (yellow), scale bar 50 μm . (D) Intensity fluorescence profile of MPs from Hawaii, data recorded in 4 channels (blue, green, yellow, red), dual wavelength biphotonic excitation 820/1040. (E) Intensity fluorescence profile from Tetramin, data recorded in 4 channels (blue, green, yellow, red), dual wavelength biphotonic excitation 820/1040. (F) medaka exposed to 1% MPs from Hawaii, observation with macroscope and bright field illumination. (G) Medaka exposed to 1% MPs from Hawaii, observation with biphotonic microscope (*) detection of MPs in the digestive tract, scale 10 $\mu\text{m} \times 999 \mu\text{m}$, 10 $\mu\text{m} \times 162 \mu\text{m}$, xyz. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

comparison to control food C (Fig. 4C). A significant increase in mean swimming speed was observed for larvae exposed to control MPs (C-) and MPs from Ea and Gu in the last dark period (Fig. 5).

3.4. Toxicity of MPs at an older stage of medaka

No mortality was observed in medaka juveniles after 30 days of trophic exposure to Ea MPs. In addition, no effect on fish biometry was observed. The wet weight of control fish was 240.00 ± 13.05 mg compared with 257.33 ± 14.67 mg for MP-contaminated fish. Total body length of control fish (27.8 ± 3.3 mm) was not significantly different from MP-contaminated fish (28.0 ± 0.2 mm). Control and MP-contaminated fish also had similar Fulton's condition Factor 11.10 ± 0.31 and 11.61 ± 0.95 respectively. No change in EROD activity was observed according to the treatment, 0.23 ± 0.48 pmol of resorufin/min/mg of proteins for control and 0.34 ± 0.39 pmol of

resorufin/min/mg of proteins for juveniles exposed to MPs alone. However, the comet assay performed with Fpg treatment highlighted a significant increase of DNA breaks for juveniles contaminated with MPs for 30 days (Fig. 6).

4. Discussion

As mentioned in the introduction, plastic debris could act as a vector for the transfer of chemical contaminants from the living environment into organisms (Rochman et al., 2013a; Koelmans, 2015; Galloway et al., 2017). However, the pollutant bioaccumulation model (OGEMA) suggests that in the case of MP ingestion by fish, chemical transfer would be negligible compared to other routes of uptake (Bakir et al., 2016). Indeed, ingestion of microplastics does not seem to provide a quantitatively important additional pathway for the transfer of adsorbed chemicals from water to biota via the gut (Bakir et al., 2016).

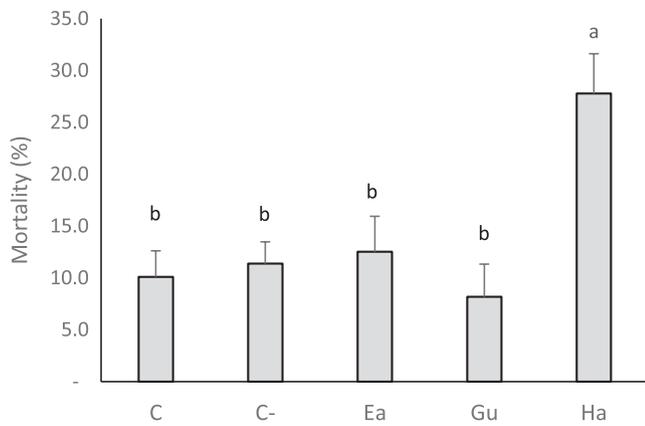


Fig. 3. Mortality of medaka larvae (mean ± SD, N = 3) exposed for 30 days to MPs from three islands. C: only fish food (Tetramin), C-: food + 0.1% of negative plastic control, Ea: food + 0.1% of Ea MPs, Gu: food + 0.1% of Gu MPs, Ha: food + 0.1% of Ha MPs. The letters at the top of the bars indicate significant differences between conditions (Kruskall-Wallis, p < 0.05).

However, several previous experiments in controlled laboratory conditions have suggested that microplastics have a real impact at the cellular level, inducing oxidative stress, changes in metabolic parameters, reduced enzyme activity, and cellular necrosis (Oliveira et al., 2013; Rochman et al., 2013a; Rochman et al., 2014; Browne et al., 2013). To date, very few studies have reported the impacts of MPs at tissue level, including intestinal lesions (Peda et al., 2016), or at individual level including behavioral changes (Carlos de Sa et al., 2015) and reproduction defects (Sussarellu et al., 2016). However, most of these studies focused on commercial microplastics (e.g. unweathered MPs), and often on a single type of microplastics, which does not

represent environmental contamination of aquatic ecosystems. In fact, environmental plastics have very fluctuating physicochemical characteristics from one site or from one sampling period to another with a wide range of size, composition, degree of alteration and chemical impregnation. For these reasons, it is essential to assess the environmental risk of microplastics using environmental samples produced or aged in the environment, taking into account the complexity of their composition, contamination and life history.

The aim of this study was to assess the impact of environmental mixtures of microplastics collected on beaches from different oceanic islands on vulnerable early life stage and juveniles of Japanese medaka.

The results of this study confirm ingestion of microplastics by Japanese medaka larvae in at least 25% of cases. Microplastic ingestion has previously been observed in fish digestive tracts in a few studies (Tourinho et al., 2010; Peda et al., 2016; Lusher et al., 2016; Lusher, 2015; Foekema et al., 2013). The low residence time of MPs in the digestive tract, mostly between 6 and 12 h according to fish species, probably explains the relatively low proportion of contaminated fish. While the residence time of these plastics in the digestive tract of fish is relatively short, recent studies indicate a possible transfer of contaminants from the microplastic surface to the body (Rainieri et al., 2018).

Exposure to virgin microplastics (negative control) induced effects on head/body length ratio and swimming speed of fish larvae, particularly after 30 days of exposure in comparison to control fish exposed to food alone. Our virgin microplastic control is a mixture of four commercial plastics containing PP, PS, HDPE and LDPE. The slight toxicity of virgin MPs observed in our study could be linked to physical but also to chemical properties of MPs. Indeed, plastics contain numerous additives used to improve their properties, and many of these substances are known to negatively impact the health of living organisms (Koelmans, 2015; Rochman, 2015; Lusher, 2015; Oehlmann et al.,

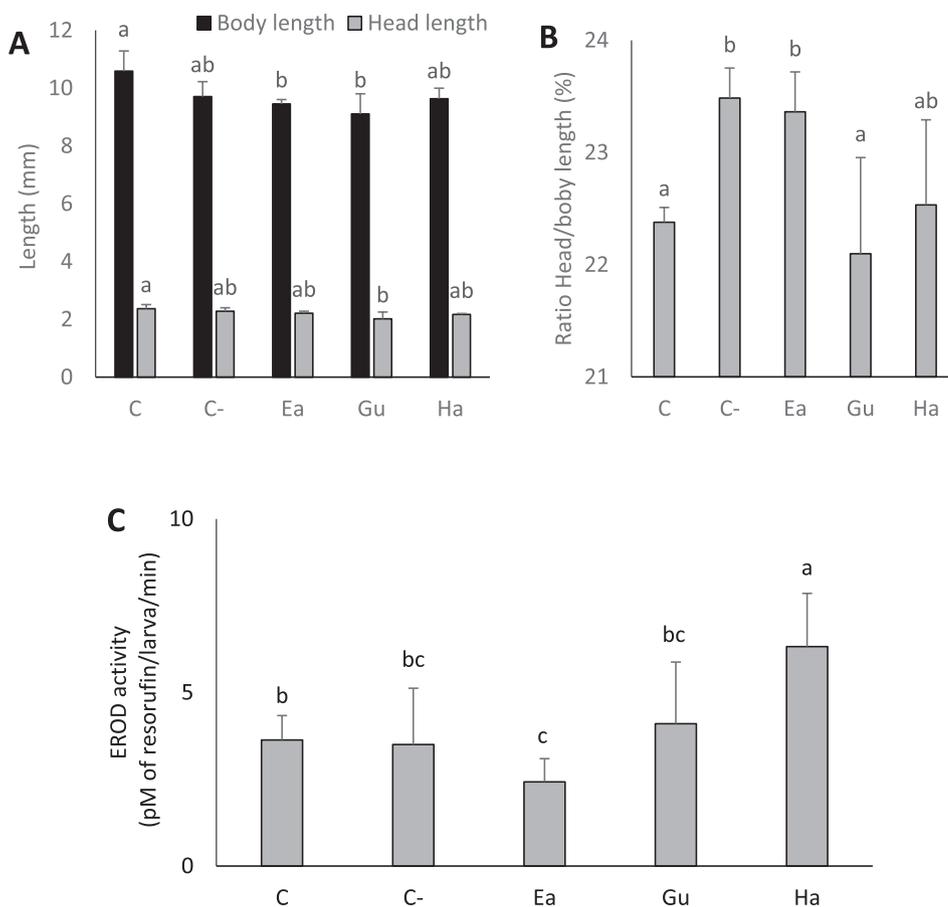


Fig. 4. Body and head lengths (A), Head/body length ratio (B) and EROD activity (C) of Japanese medaka larvae (mean ± SD, n = 10, N = 3) exposed for 30 days to MPs from three islands. C: only fish food (Tetramin), C-: food + 0.1% of negative plastic control, Ea: food + 0.1% of Ea MPs, Gu: food + 0.1% of Gu MPs, Ha: food + 0.1% of Ha MPs. The letters at the top of the bars indicate significant differences between conditions (Kruskall-Wallis, p < 0.05).

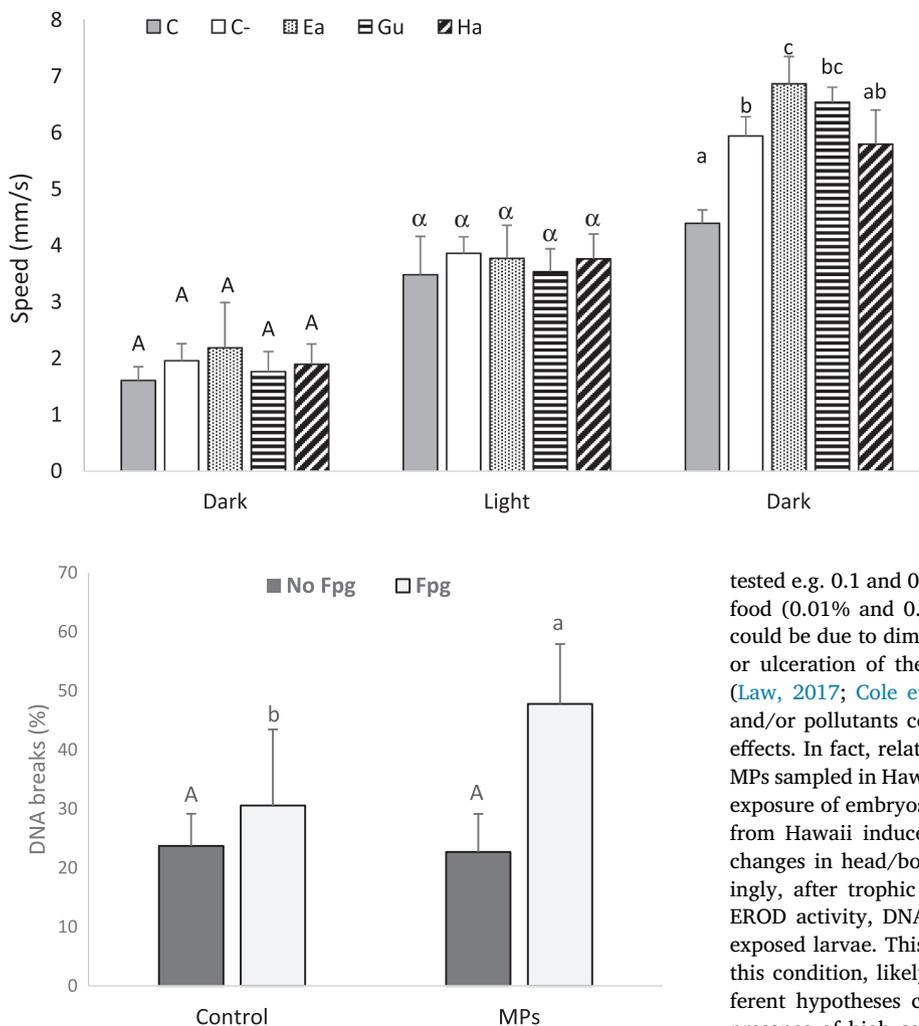


Fig. 6. Level of DNA strand breaks in Japanese medaka juveniles after 30 days of trophic contamination (mean \pm SD, n = 3, N = 3) with food only (C-) or food with Ea MPs (0.1%). The letters indicate significant differences between conditions (Kruskall-Wallis, $p < 0.05$).

2009). Acute toxicity of plastic additives was reported in early life stages of several marine invertebrates (Durán and Beiras, 2017). In addition, delayed hatching, reduced growth, morphological abnormalities, impaired cardiovascular function and cerebrospinal fluid flow were reported in Zebrafish (*Danio rerio*) embryos following exposure to PBDE47, a flame retardant included in some plastics (Lema et al., 2007). In our study, significant modulation of EROD activity after 14 or 30 days of exposure suggests the presence of AhR inhibitors and inducers in MPs. These AhR ligands included at least PAHs, PBDEs, PCBs and other organochlorinated compounds, etc. However, in their study, Koelmans et al. (2013) concluded that concentration of plastic additives found in lugworm or cod tissues stayed below the level of toxicity and consequently did not pose a risk. This is consistent with our results demonstrating few toxicological effects following exposure of larvae to mixtures of commercial MPs.

Exposure to environmental samples of MPs induced an increase of DNA damage and modulation of EROD activity. Increased DNA breaks induced by B(a)P and other PAHs exposure through food uptake have been documented in fish (Wessel et al., 2010). Likewise, numerous studies have shown a significant induction of EROD activity in several fish species after B(a)P exposure (Wessel et al., 2010; Viarengo et al., 1997) or B(a)P-coated MP exposure (Batel et al., 2016). In the present study, significant EROD activity induction was observed in medaka larvae exposed to MPs from Hawaii at the two lowest concentrations

Fig. 5. Swimming speed of Japanese medaka larvae (mean \pm SD, n = 12, N = 3) after 30 days of trophic contamination to MPs from three islands. C: only fish food (Tetramin), C-: food + 0.1% of negative plastic control, Ea: food + 0.1% of Ea MPs, Gu: food + 0.1% of Gu MPs, Ha: food + 0.1% of Ha MPs. The letters at the top of the bars indicate significant differences between conditions for each light condition (Kruskall-Wallis, $p < 0.05$).

tested e.g. 0.1 and 0.01%. It was also observed that low doses of MPs in food (0.01% and 0.1%) induced growth delay. Reduced body length could be due to diminution of food uptake due to clogging, perforation or ulceration of the digestive tract or induction of satiety sensation (Law, 2017; Cole et al., 2011; Rochman et al., 2013a) but additives and/or pollutants coated on MPs are likely involved in these adverse effects. In fact, relatively high concentration of PAHs were detected in MPs sampled in Hawaii (Pannetier et al., 2019a). It has been shown that exposure of embryos and prolarvae of medaka to DMSO-extract of MPs from Hawaii induced increased EROD activity and DNA breaks and changes in head/body length ratio (Pannetier et al., 2019b). Surprisingly, after trophic exposure to 1% MP from Hawaii, no significant EROD activity, DNA breaks, or growth retardation were observed in exposed larvae. This result suggests a lower exposure level to MPs for this condition, likely due to a reduced MP intake by larvae. Two different hypotheses can explain this unexpected result. Firstly, in the presence of high concentrations of MPs, larvae could select fish food rather than MP particles. Secondly, at high concentrations, MPs can probably agglomerate. This has already been documented (Long et al., 2015) but in our experiment no visible aggregates were detected in water.

In our study, toxic effects in medaka larvae were observed from 0.01% of MPs in food corresponding to a concentration of $6 \mu\text{g MPs}\cdot\text{L}^{-1}$. Rochman et al. (2013b) did not observe any effect in Japanese medaka following a 2-months exposure to $8 \mu\text{g LDPE MPs}\cdot\text{L}^{-1}$. These contradictory results can be explained partly by different exposure conditions and developmental stages but more likely by differences in MP ageing and weathering. Based on these initial results, 0.1% of MPs in food ($60 \mu\text{g MPs}\cdot\text{L}^{-1}$) was selected for the two other trophic exposures. Contamination time was extended to one month to enhance the likelihood of detecting induced effects.

Greater toxic effects were observed after 30 days of exposure to microplastic particles than at 14 days. These results could be explained both by the toxico-kinetic of MPs and sorbed pollutants and delayed effects of MPs on survival and growth. According to samples, different severity of effects were observed: lethality for Hawaii sample and sub-lethal effects for Easter Island and Guam MP samples. This increased mortality in larvae exposed to Hawaii samples could be explained by a stop or significant reduction in feeding due to intestinal lesions or obstruction, which could lead to death. Indeed, several studies documented perforated guts, ulcerative lesions, or gastric rupture (Law, 2017) and intestinal alterations (Peda et al., 2016) in different MP-contaminated species. This physical damage could have negative impacts on fish feeding rates, and may compromise intestinal functions (Peda et al., 2016). Sub-lethal effects induced by microplastics from Eastern Island (Ea) and Guam (Gu) were numerous. MP-exposed fish

were significantly smaller than non-exposed fish. Modification of head/body ratio could be related to craniofacial or spinal malformations (not analyzed in this study) and impair feeding efficiency and consequently fish growth retardation (De Meyer et al., 2017). Size modifications and malformation induction may also have a negative impact on swimming capacities, and consequently may alter prey capture and escape capacities. Decreased EROD activity was observed after 14 days of exposure to Gu MPs and after 30 days to Ea MPs. Relatively similar toxicity between Ea and Gu MPs could be linked with the similar composition and concentrations of pollutants in both MPs samples (Pannetier et al., 2019a). After 14 days of MPs exposure, larval swimming speed was not affected after light stimulation. On the other hand, after 30 days of exposure, an increase in swimming speed was observed in all MP-exposed larvae except the group exposed to MPs from Hawaii. Some pollutants including PAHs are known to modify fish behavior in response to light stimulation (Le Bihanic et al., 2014; Vignet et al., 2014). Carlos de Sa et al. (2015), reported a decrease of predatory efficiency, confusion in food uptake and decrease in food intake in the common goby exposed to PAHs. Presence of high number of MP particles in the water column may also affect fish swimming activity and response to light stimulation. Physiological disorders on the metabolism and/or on the neuromuscular system may also explain the abnormal swimming behavior of MP-exposed fish. These effects could lead to decreased fitness in individuals and populations.

No effects of MP contamination were observed in juveniles, with the exception of a marked increase in DNA damage using the Fpg-modified comet assay. The Fpg enzyme treatment specifically induces DNA strand breaks when oxidized bases are present (Kienzler et al., 2012). Oxidative damage has previously been reported in marine lugworms, *Arenicola marina*, after MP exposure (Browne et al., 2013). The absence of other significant toxic effects in juveniles could likely be explained by the lower sensitivity of fish juveniles compared to larvae.

5. Conclusions

This study demonstrates that environmental mixtures of microplastics at realistic environmental concentrations have significant deleterious effects on fish larvae after trophic contamination. EROD activity induction and DNA damage likely indicate that at least some plastic additives and/or sorbed pollutants were directly bioavailable to the fish larvae after microplastic ingestion and can exert toxic effects at environmental concentrations. For some other deleterious effects, such as reduced growth and increase mortality, physical damage induced by MPs cannot be ruled out. Toxicity of microplastics varies from one sample to another, depending on the composition, contamination and life history of the plastics. This study raises the question of the environmental risk of weathered microplastics at sea on living organisms in particular at vulnerable stage, such as fish larvae.

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Authorization to conduct the research and ethical aspects

The experiments on living fish were conducted in accordance with Directive 2010/63/EU for the accommodation and care of animals used

for experiments and other scientific purposes. Experiments on larvae were carried out at the EPOC laboratory in Bordeaux under French ethics committee authorization No. APAFIS # 6952-20 1609281 0228457 v2. Experiments on juveniles were carried out at ANSES in Plouzané, under French ethics committee authorization No. 12/07/16-5.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2019.105047>.

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