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Abstract

Toxicity of polyethylene (PE) and polyvinyl chloride (PVC) microplastics (MPs), either virgin or spiked with chemicals, was evaluated in two short-lived fish using a freshwater species, zebrafish, and a marine species, marine medaka. Exposures were performed through diet using environmentally relevant concentrations of MPs over 4 months. No modification of classical biomarkers, lipid peroxidation, genotoxicity or F0 behaviour was observed. A significant decrease in growth was reported after at least two months of exposure. This decrease was similar between species, independent from the type of MPs polymer and the presence or not of spiked chemicals, but was much stronger in females. The reproduction was evaluated and it revealed a significant decrease in the reproductive output for both species and in far more serious numbers in medaka. PVC appeared more reprotoxic than PE as were MPs spiked with PFOS and benzophenone-3 compared to MPs spiked with benzo[a]pyrene. Further, PVC-benzophenone-3 produced behavioural disruption in offspring larvae. These results obtained with two species representing different aquatic environments suggest that microplastics exert toxic effects, slightly different according to polymers and the presence or not of sorbed chemicals, which may lead in all cases to serious ecological disruptions.

Keywords: polyethylene, polyvinyl chloride, adsorbed chemicals, growth alteration, reproductive toxicity

1. Introduction

Frequently, manufactured plastic debris end up in the oceans and eventually appear as macroplastics (> 5 mm in size), microplastics (MPs; particles between 1 µm and 5 mm in size) or smaller nanoplastics (< 1 µm in size) [1, 2]. MPs consist of either plastic particles designed and produced for specific purposes [3, 4] or small plastic fragments derived from the fragmentation of larger pieces [5-8]. Given the ubiquity of sources and their persistence in the aquatic environment, MPs are globally distributed in various environments including some areas far from emission sources, i.e. Greenland, Antarctica [9-12]. Consequently, they are found ubiquitously in any water body [13-17] and concentrations in marine ecosystems are still increasing [12].

The ability to ingest MPs has been documented across a wide range of phyla including e.g. zooplankton, molluscs, marine worms, fish, and cetaceans [18-24]. Ingestion and egestion depends to some extent on MPs size and shape [23, 24]. In fish, the ingestion of MPs was demonstrated at all life stages in field sampling [25-30]. The transfer of MPs through food webs was reported in laboratory studies [31-37].

Effects of MPs on marine and freshwater organisms and their potential consequences on ecosystems functioning have been a matter of debate for more than a decade [8, 38-41]. This is related to the fact that the effects of MPs on aquatic organisms are not well understood; particularly, because of the high variety of MPs combined with diverse aging conditions (see [42] for more details). Adverse effects might be attributed to processes, such as the lack of egestion or false satiation after ingestion of MPs devoid of nutritional value [14]. Besides, by interfering with endogenous microbiota, MPs have also been shown to induce a dysbiosis [43-45], which may have consequences on other physiological functions. Some authors reported induction of oxidative stress, inflammatory processes, or physiological stress responses [38, 46-56]. However, used exposure conditions (e.g. duration, routes, and concentrations) often raise the question of ecological relevance. At the same time, a growing number of publications, mainly short-term exposure, report that MPs ingestion induced no or limited toxicity [35, 45, 56-61]. Taken all together, these results also suggest that short-term exposures are not relevant to evaluate MPs toxicity and a fortiori to evaluate toxicity at environmental concentrations.

Plastic polymers are considered biologically inert. The manufacturing of plastic products uses additives, including phthalates, nonylphenol, bisphenol A and brominated flame retardants, to

improve physico-chemical properties. These additives were reported to be transferred to aquatic organisms and to cause toxicity [62-64]. A recent article described a differential toxicity between native particles, “extracted particles”, and additives depending on the type of plastic [65]. In addition to these intrinsic chemicals, it was shown that MP particles sorb significant amounts of organic pollutants from the surrounding environment, such as per- and polyfluoroalkyl substances (PFAS), polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides, and personal care products. This might be of great concern because these compounds are often toxic and can bioaccumulate [39, 63, 66, 67]. While the importance of the role of MPs in the transfer of persistent organic pollutants in biota is still controversial [39, 40, 68], the adsorption of different classes of organic pollutants on the surface of different MP materials was demonstrated under both laboratory and field conditions [52, 69]. In the aquatic environment, organic pollutants are commonly found in the water surface microlayer, where low-density MPs such as polyethylene (PE) are also abundant [55, 67, 70], or in sediments, where high-density MPs such as polyvinyl chloride (PVC) and aged or biofouled low-density MPs accumulate. Perfluorooctane sulfonic acid (PFOS), benzo[a]pyrene (BaP), and benzophenone-3 (BP3) represent different chemical families of organic pollutants frequently detected in aquatic systems. PFOS represents the main perfluorinated alkylated substance that is frequently detected in aquatic environments and is used as a flame retardant in plastic [71]. BaP is a PAH ubiquitously distributed in both coastal and offshore environments. Polycyclic aromatic hydrocarbons are known to adsorb at high rates to different types of MP polymers in seawater [52, 69]. Benzophenone-3 is commonly used as a UV-filter in cosmetics such as sunscreens and is frequently detected in coastal areas. BP3 shows low degradation rates in surface waters [72].

In the present study, we used long-term exposure to evaluate the physiological effects of PE and PVC MPs, with and without the presence of PFOS, BaP, or BP3 on two models fish species, a marine one, marine medaka (*Oryzias melastigma*), and a freshwater one, zebrafish (*Danio rerio*). Studied endpoints included biochemical biomarkers, survival, growth, reproduction, and swimming behaviour of exposed fish along with the survival, growth, and swimming behaviour of unexposed F1 offspring. The aim of this experimental design was to compare the toxicity of two plastic polymer types, PE and PVC, and the effects of adsorbed chemicals. An additional aim was to evaluate the genericity of the effects on teleosts as regards essential life-history traits.

2. Materials and methods

2.1 Husbandry and egg production

Fish brood stocks were routinely maintained in the Laboratoire Ressources Halieutiques, Ifremer (facility authorization A171901; project authorization APAFIS#10883). Brood stocks were reared in isothermal rooms with a 14/10 h light/dark photoperiod in recirculating systems using conditions that guaranteed that ammonia, nitrite, and nitrate levels remain within recommended ranges [73]. Additional details about rearing can be found in Supplementary materials.

Experimental groups were built in similar ways with both species: eggs from several group-spawns (medaka) or pair-spawns (zebrafish) with fertilisation rates above 80% were kept. The day after collection, eggs from different spawns (at least 6 spawns per replicate) were evenly distributed in order to avoid potential biases due to individual spawns. Sixty eggs were transferred to the appropriate number of 10 cm Petri dishes and dishes were incubated at 28 °C under a 14 h/10 h light/dark cycle. Daily care of the Petri dishes consisted of removing dead eggs and changing approximately 20% of water. After hatching, starting at 8 days post-fertilisation (dpf) for medaka and at 72 h post-fertilization (hpf) for zebrafish, chorions were removed from the Petri dishes. Further rearing was performed as described previously [74]. Briefly, hatched larvae were then transferred to 1 L tanks for 1 week and then to 3 L tubes located in independent 10 L tanks in a dedicated flow-through rearing system with a hourly automatic water exchange (total daily exchange: 30% of the volume). Two weeks later, at 1 month, tubes were gently poured in their 10 L tanks. On this occasion, fish were distributed at random to produce per treatment three replicate tanks for medaka and six replicate tanks for zebrafish tanks, containing each 30 individuals to reach the density of 3 fish/L.

2.2 Microplastics, experimental design and diet preparation

Polyethylene MPs were purchased from Micro Powders Inc. (order no. MPP-635 G; New York, USA) for a given size range of 11-13 µm. PVC MPs were purchased from Fainplast (Ascoli Piceno AP, Italia) for a given size range of 125-250 µm. Polymer of MPs were confirmed by FTIR/UATR. PFOS (1,1,2,2,3,3,4,4,5,5,6,6,7,7,8,8,8-heptadecafluorooctane-1-sulfonic acid; CAS 2785-37-3; purity ≥ 98%), BaP (benzo[a]pyrene; CAS 50-32-8; purity ≥ 96%) and BP3 ((2-hydroxy-4-methoxyphenyl)-phenylmethanone; CAS 131-57-7; purity ≥ 98%) were purchased from Sigma Aldrich (Stockholm, SE). Solvent used were deionized water for PFOS and toluene (purity ≥ 96%; Solveco) for BaP and BP3. All other chemicals

and reagents were purchased at the highest purity available from Sigma-Aldrich, unless stated otherwise.

Polyethylene and PVC were spiked with PFOS, BaP and BP3 following protocols described in Cormier et al. [58], with minor modification for the PVC preparation. For the sorption of PFOS, 200 g/L of PVC was mixed with 200 mg/L of PFOS in 1 L polypropylene bottle (Lamaplast; Sesto San Giovanni, Italy), while for BaP and BP3, 125 g/L of PVC was added in 400 mL glass bottles (Thermo Scientific, Lund, SE) mixed with 5000 µg/L and 20 µg/L of BaP and BP3, respectively. Bottles were then placed on a rotary shaker at 20 rpm for 7 days (PFOS) or 2 days (BaP and BP3). After sorption, spiked MPs were filtered with a ceramic funnel equipped with a 1µm Whatman R glass microfiber filter (GE Healthcare Life Sciences; Uppsala, SE), then, filters were rinsed with deionized water and dried by vacuum evaporation. Final concentrations of compounds on MPs are indicated in Table 1.

Fish were exposed to nine different treatment diets: control (Control), virgin polyethylene MPs (PE-MP), virgin polyvinyl chloride MPs (PVC-MP), spiked MPs with perfluorooctane sulfonate (PE-PFOS and PVC-PFOS), benzo[a]pyrene (PE-BaP and PVC-BaP) and benzophenone- 3 (PE-BP3 and PVC-BP3). Diets were prepared with a final ratio of MPs of 1% wet weight (ww). For this purpose, MPs were mixed in a glass tube with pellets of appropriate size following fish growth (see supplementary materials) using a 4 mm bottlebrush fixed on a drill for 3 minutes. Afterward, to reinforce the binding of MPs to pellets, flaxseed oil was added (5 µL per gram of food) and mixed for an additional 3 minutes. The control condition consisted of fish fed with plain food mixed with flaxseed oil as indicated above. Due to MPs size range, exposure to PVC MPs started from 2 months post fertilization, while exposure to PE MPs could start at first feeding (10 dpf in medaka and 5 dpf in zebrafish). Fig. 1 provides a schematic overview of the experimental design of the study.

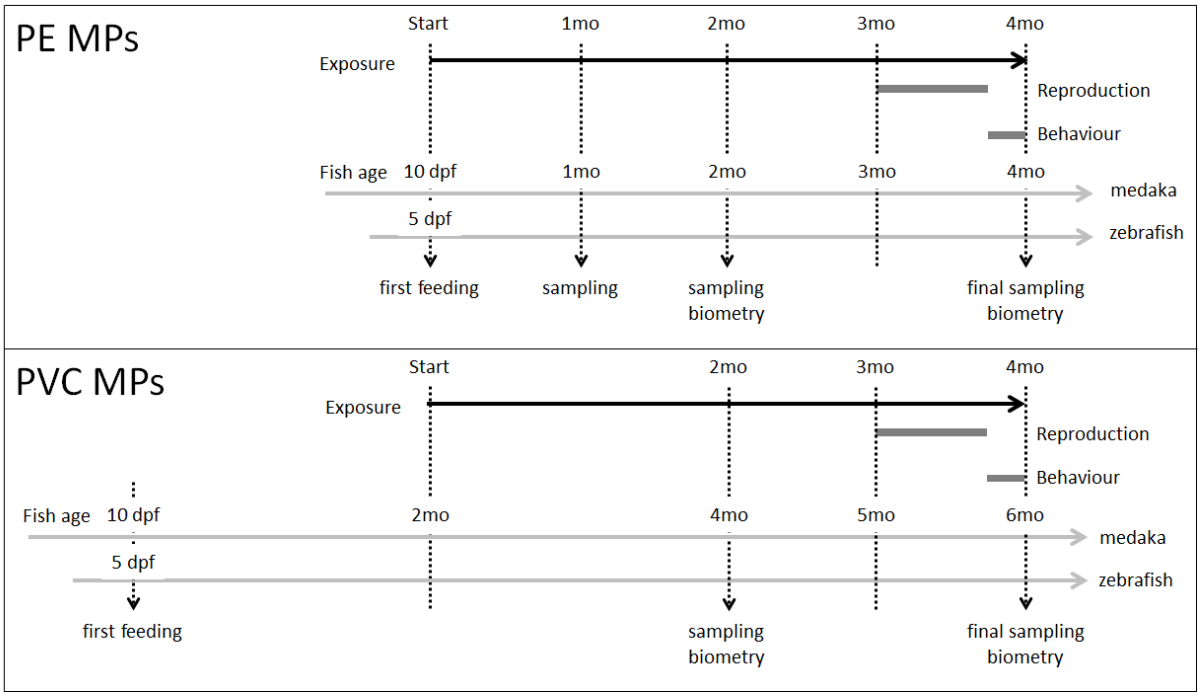

Figure 1. Schematic timing of exposures to PE and PVC MPs.

For practical and exposure management reasons, exposures to PE and PVC MPs of marine medaka and zebrafish were performed sequentially from June 2017 to December 2018, with all sets of treatments including their respective Control replicates.

After four months of exposure, fish were euthanized with benzocaine (500 mg/L, Sigma) for sampling for biomarkers or chemical analyses. For this purpose, and depending on downstream analyses, entire fish or dissected tissues were immediately frozen in liquid nitrogen and stored until analysis.

2.3 Chemical analyses

Chemical load were analysed in fish after 1, 2, and 4 months of exposure to PE MPs and after 4 months of exposure for PVC MPs. At 1 or 2 months pools of 5 fish were sampled to increase the biomass of the sample (n=3), later individual fish were used (n=3-6) to determine PFOS, BaP and BP3 concentrations in whole body. Sampling was performed at the occasion of biometries and euthanatized fish were snap frozen in liquid nitrogen, then stored in amber glass vials (for BaP and BP3 analyses) or micro tubes (for PFOS analyses) and kept at -20°C. Extraction procedures are described below while chemical analyses are described in supplementary materials.

2.3.1 PFOS analysis

To analyse PFOS in fish, the ion pair extraction was performed using alkaline digestion [75, 76]. Frozen fish were grinded in a mortar with the addition of 20 mM of NaOH, sonicated for 30 min and placed on a horizontal shaker overnight (16 h). The pH was neutralized with hydrochloric acid, and 0.5 M of tetrabutylammonium sulphate as well as 10 ng of mass-labelled $^{13}C_4$ PFOS (Wellington Laboratories Inc., Canada) were added. Samples were then sonicated for 15 min. Extraction was performed three times with 2 mL methyl tert-butyl ether and 15 min of sonication, followed by centrifugation 10 min at 8000 rpm. Extracts were evaporated down to dryness using a N_2 stream and then, methanol was used to dissolve them. Moreover, 2 ng of mass-labelled $^{13}C_8$ PFOS (Wellington Laboratories Inc., Canada) was added. Limit of detection (LOD) was 10 pg/g.

2.3.2 BaP analysis

Frozen fish were homogenized in a mortar. Anhydrous sodium sulphate in a 1:5 ratio was added to eliminate water in the sample, as well as 1 ng of BaP d_{12}. Deactivated mini-silica columns were prepared with 10% of deactivated silica and anhydrous sodium sulphate, washed with n-hexane:dichloromethane (3:1, v/v). Samples were eluted with 8 mL of n-hexane:dichloromethane (1:1, v/v), and reduced to 200 µL before a transfer to toluene (reduced to 500 µL), with the addition of 2 ng of perylene d_{12}. LOD was 5 pg/g.

2.3.3 BP3 analysis

Benzophenone-3 is a compound easily metabolized in organisms [77, 78], leading to the formation of metabolites. Benzophenone-1 (BP1) was reported to be the major metabolite of BP3, as detected in the urine of rats and humans [79, 80] as well as in zebrafish [77]. To determine the concentration of BP3 and BP1, frozen fish were ground in a mortar with 1 mL of acetonitrile, followed by 15 min of sonication, 15 min of horizontal shaking and 5 min on a vortex. Samples were then centrifuged for 15 min at 3900 rpm and passed through a filter (GHP acrodisc 13 mm). Filtrates were stored in a freezer (- 20 °C) overnight (16 h) to precipitate proteins. After the precipitation, the samples were centrifuged and the supernatant was evaporated down to 100 µL using a N_2 gas stream. 500 ng of BP3 d_{10} was added as standard and an external quantification was used with a calibration curve of BP3 and BP1 (0.04 to 80 pg/µL). LOD was 0.05 ng/g.

2.4 Molecular and cellular markers

Sampled tissues (liver, muscle and brain) from both medaka and zebrafish were homogenized in 0.1 M phosphate buffer (pH= 7.7; 0.1 M KCl). The homogenates were then centrifuged at 9000 g at 4 °C for 20 min. The supernatant (fraction S9) was then collected in a clean micro

tube and stored at −80 °C before being used for measurement of ethoxyresorufin O-deethylase activity (EROD; liver samples), thiobarbituric acid reactive substances (TBARS; muscle samples), and acetylcholinesterase (AChE; brain samples). The protein concentration in the S9 fraction was measured using Bradford's method with bovine serum albumin (BSA) as a standard [81]. Spectrophotometric measurements were performed in a Biotek Synergy HT microplate reader. To improve detection, tissue pools from 3 males or 3 females were used in triplicates per sex for each treatment. To investigate DNA damage using the Comet assay, blood was collected from anesthetized medaka and cryopreserved until analysis. Detailed methods are given in supplementary materials.

2.5 Individual markers in the F0 generation

2.5.1 Survival, growth and sampling

During exposure, dead larvae or fish were monitored daily. Individual standard length (mm) and body weight (mg) were measured after two and four months of exposure. For this purpose, fish were shortly anesthetized with benzocaine (50 mg/L, Sigma Aldrich, France) as described previously [74] or performed at sampling time (see above). Depending on time point, sex and treatment, between 15 and 50 individuals were monitored per sex per treatment. Adult behaviour (anxiety and activity) was monitored after reproduction assessment and methods are described in supplementary materials.

2.5.2 Reproduction

Reproduction was monitored starting after 3 months of exposure and for approximately one month. Because of biological differences, reproduction was assessed using different methods in medaka (group-spawning) and zebrafish (pair-spawning).

Medaka groups were left undisturbed in their rearing tanks. The day before collection, tanks (all replicates) were siphoned in the afternoon to remove faeces and previous eggs. The next morning, within 4 hours after light onset, tanks were siphoned again and eggs were collected and quickly cleaned. Afterward, they were treated and further reared if needed as described above. This procedure was repeated more than ten times over one month for all replicates of each treatment.

For zebrafish, couples were performed as described above, spawning boxes were inspected on the following morning, and the presence of eggs scored as a successful attempt. In this case, eggs were collected, cleaned, sorted, and transferred in Petri dishes filled with E3 as described above. The general rule was to set up five pairs per condition per attempt, using fish from

each replicate sequentially. For exposure to PE MPs, the number of attempts ranged from 23 for Control to 30 for all other treatments. For exposure to PVC MPs, 53 attempts were performed for Control and 60 for each other treatments.

When eggs were obtained, they were counted and sorted to determine the total number of eggs and to calculate fertilization rates. Then 30 eggs from each spawn were further reared for downstream monitoring.

2.6 Individual markers in the F1 generation

2.6.1 Larval behaviour

Larval behaviour was monitored at 12 dpf for medaka and 5 dpf for zebrafish (which corresponds to approximately 2 days after hatching in both cases) using the larval photomotor response (LPMR) test as described in [82]. Briefly and for both species, larvae were transferred individually into one well of a 24-well plate (Krystal 24, opaque wall and clear bottom microplate, Dutscher), and plates were transferred to an enlighten incubator at 28°C in the behaviour room. Plates were then successively placed into DanioVisionTM (Noldus, NL) in the dark for 10 min of acclimation before the LPMR test, which included the following 5-min steps: Light on–1 (LON1, 70 lux), Light off (LOFF, <1 lux) and Light on–2 (LON2, 70 lux), with constant infra-red light maintained during video recording. Distance travelled (cm) over the 5 min of each period was then calculated using Ethovision XT (Noldus, NL). Larvae with tracking issues were removed resulting in 50 to 100 larvae analysed per treatment.

2.6.2 Embryonic and larval survival

For medaka, embryonic survival and hatching of collected eggs (subsample of 30 eggs par treatment and dates) were monitored over 14 days. For zebrafish, subsamples of 30 eggs from single spawns collected throughout the attempts were monitored over 5 days. For both species, there was a daily monitoring of F1 survival and hatching and a daily exchange of 20% of the medium. In addition, for zebrafish, a larval survival experiment was performed, without feeding, by monitoring over time the natural death of larvae. This aimed at evaluating their available energy resources.

2.7 Statistical analyses

Normality of the data and variance homogeneity were checked using the Shapiro-Wilk and Levene tests respectively. Analysis of variance was performed for normal data and followed by post-hoc Fisher's or Tukey HSD tests. For data that did not fulfil normal distribution, the Kruskal-Wallis non-parametric test was performed followed by multiple comparison post-hoc

tests to identify differences between treatments using Statistica (Tibco). For medaka, the kinetic of number of eggs produced was compared between treatments by using slope comparison methods (Zar (1984) in Prism software, Graphpad). Differences in F1 larval survival were assessed using Log-rank (Mantel-Cox) test, and time at which 50% mortality is reached was calculated using four-parameter logistic regression. Akaike's information criterion was used for comparison with Control and relative virgin MP counterpart, both using Prism software. All statistical analyses were carried out at a 95% level of significance and values are represented as mean ± SD.

3. Results

3.1 Characterization of MPs and monitoring of exposure

Mean particle size was 5 and 105 µm for PE and PVC MPs and their chemical compositions were confirmed (Fig. S1-S2). Virgin MPs were devoid of a significant amount of the three chemicals used in this study (concentration below LOD) while spiking was efficient as shown in Table 1.

Table 1. Concentrations of PFOS, BaP (µg/g) and BP3 (ng/g) measured on virgin and spiked PE and PVC MPs. (LOD were 10 pg/g, 5 pg/g and 5 ng/g for PFOS, BaP and BP3 respectively; Mean ± SD; n=3).

	PFOS	**BaP**	**BP3**
Virgin PE-MP	<LOD	<LOD	<LOD
Spiked PE MPs	70.22 ± 12.41	16.87 ± 0.22	106.00 ± 6.50
Virgin PVC-MP	<LOD	<LOD	<LOD
Spiked PVC MPs	159.54 ± 40.84	11.50 ± 1.35	107.00 ± 1.50

Concentrations of BaP and BP3 measured in fish exposed to MPs spiked with either BaP or BP3 were below detection limits. BP1 was reported to be the major metabolite of BP3, as detected in the urine of rats and humans [79, 80] as well as in zebrafish [77]. For fish exposed to MPs spiked with BP3, BP1 was also monitored and was also below the detection limit. Therefore, no sign of accumulation of these compounds was demonstrated. For PFOS, its concentration in Control fish or fish exposed to PE-MP or PVC-MP was similar in the range of 0.19-0.31 µg/g. PFOS concentration in fish exposed to PE-PFOS was more than 20-fold higher at 7.46 ± 3.21 µg/g 8.86 ± 2.41 µg/g ww after 1 or 2 months respectively. After 4 months of exposure, PFOS concentration was 25 to 60-fold higher in fish exposed compared to Control or virgin MPs (Fig. 2).

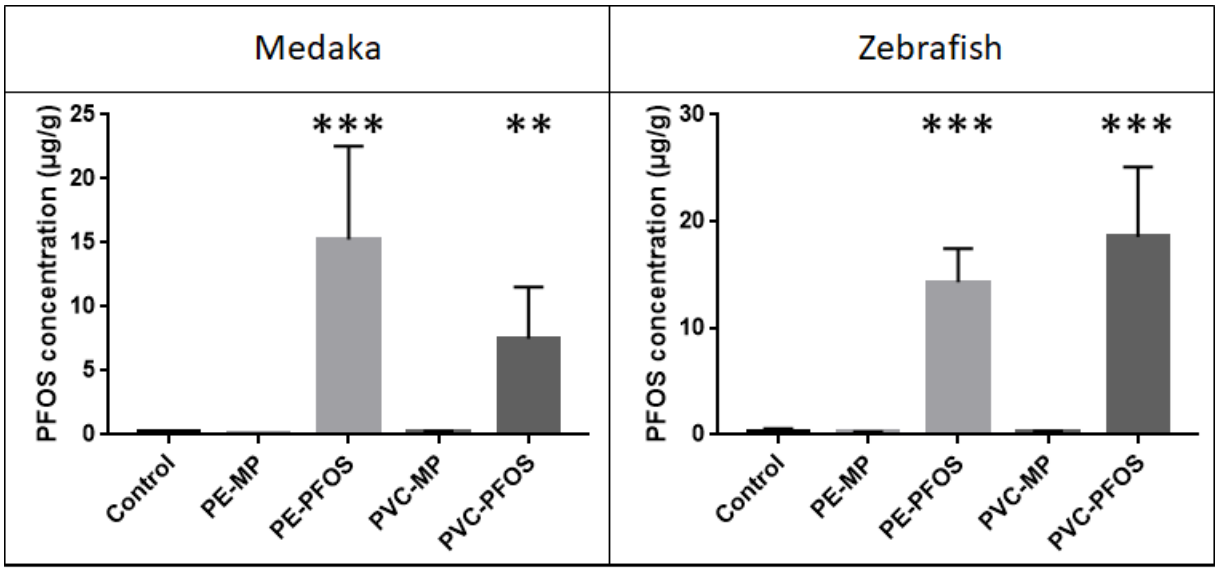

Figure 2. PFOS concentration in fish exposed for 4 months to virgin MPs (PE, PVC) or to spiked MPs (PE-PFOS, PVC-PFOS). Stars indicate significant differences from Control and their respective virgin counterpart. (Mean ± SD; Tukey HSD; **: p<0.01; ***: p<0.001; Fish n=[3-6]).

3.2 Toxicity in the F0 generation

3.2.1 Molecular and cellular markers

EROD, AChE, and TBARS activities were monitored in the liver, brain, and muscle, respectively. There was no significant difference in EROD, AChE, or TBARS activity whatever the species, sex, or MPs either virgin or spiked (Table S1A-S1D). The only exception was for EROD activity measured in zebrafish males exposed to PE MPs for which there was a significant effect of treatment (Kruskal-Wallis$_{(4,15)}$=10.23, p=0.037) but post-hoc test revealed no difference with Control.

In medaka, the Comet assay performed on blood cells revealed no evidence for genotoxicity whatever sex or treatment.

3.2.2 Survival and growth

No significant mortality was recorded whatever the treatment and no deviation from the control was observed. Biometries were performed in both fish species after approximately 2 and 4 months of exposure, i.e. when PE MPs exposed fish were 2 and 4 mpf and PVC MPs exposed fish were 4 and 6 mpf (for medaka, fish exposed to PVC MPs were only measured once at 6 mpf). For the sake of clarity, the duration of exposure will be used in the text hereafter rather than age.

After 2 months of exposure to PE MPs, no significant difference between Control and exposed fish was observed for standard length or body weight whatever the treatment (Fig. S3). For PVC MPs exposures, a reduction in growth was observed in some cases. Body length was not modified in males whatever the species, except for a slight decrease (-6%) in male zebrafish exposed to PVC-MP. In female zebrafish, a slight decrease (less than -15%) was observed for PVC-MP. Regarding body weight, a decrease (-15%) was observed in male zebrafish exposed to PVC-MP and a decrease in body weight was observed in female zebrafish exposed to all PVC MPs except PVC-PFOS (Fig. S4).

After 4 months of exposure, a significant decrease in growth was observed for males exposed to some MPs and for all exposed females whatever the species and treatment (Fig. 3-4 and S5-S6). In further detail, body length was not modified by PE MPs in males in medaka (p=0.18) or zebrafish (p=0.09). In females, significant effects of PE MPs were observed in both species (p<0.001 in medaka and p=0.026 in zebrafish). Post-hoc tests revealed a significant decrease in body length of medaka exposed to PE-MP, PE-BaP, and PE-BP3 as well as for zebrafish exposed to PE-BaP and PE-BP3 (-4 to -11%; Fig. S5-6). Regarding body weight (Fig. 3-4), the significance of effects for the exposure to PE MPs was just above the threshold (p=0.052) in medaka males but post-hoc tests revealed no significant differences. In zebrafish males, there was a significant effect of exposure to PE MPs (p=0.008); post-hoc tests indicated a significant decrease for PE-MP and PE-PFOS exposed males. In females, the effect was greater with a decrease in body weight by 20 to 35% for all MPs, both in medaka and zebrafish (p<0.001).

For PVC MPs, body length (Fig. S5-6) was altered in medaka males (p<0.005) with a weak (<10%) but significant decrease in length for males exposed to PVC-MP and PVC-PFOS. In zebrafish males, significance was just above the threshold (p=0.057) but post-hoc tests revealed no significant differences. In females, there was a strong significant exposure effect by PVC MPs in both medaka and zebrafish (p<0.001). Post-hoc tests revealed a significant reduction in body length for all PVC MPs except PVC-BaP in medaka and all PVC-MPs in zebrafish. Similar effects were observed regarding the body weight. For body weight, post-hoc tests revealed a moderate but significant decrease in medaka males exposed to PVC-MP (-14%) and PVC-PFOS (-16%) but no difference in zebrafish. In females, a stronger decrease was observed for all PVC MPs in medaka (up to -22% for PVC-MP and PVC-PFOS) and in zebrafish (up to -30% for PVC-PFOS, PVC-BaP, and PVC-BP3).

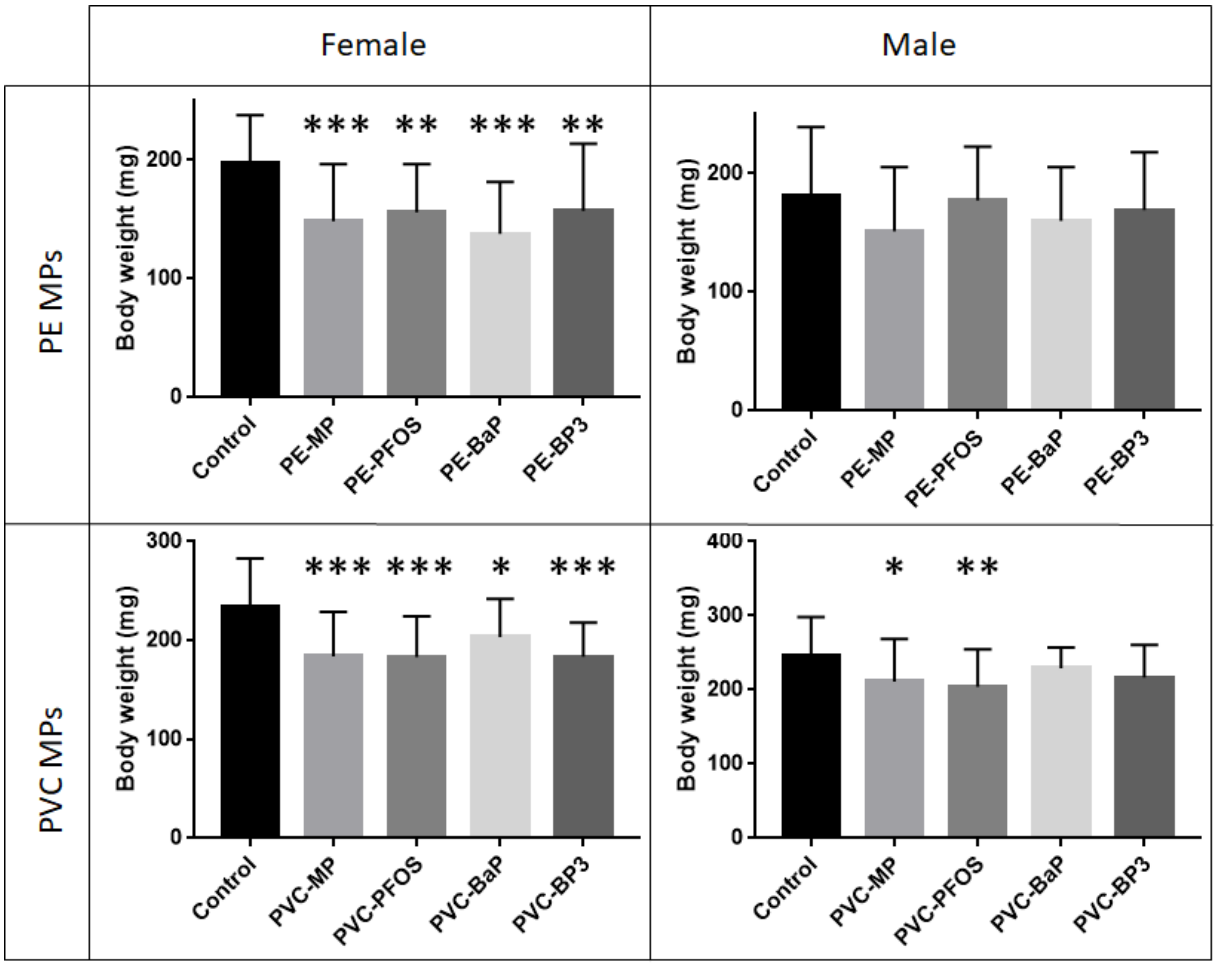

Figure 3. Medaka body weight after 4 months of exposure to PE and PVC, either virgin or spiked. (Mean ± SD; Tukey HSD; *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$; Fish n=[26-51] per sex per treatment from three replicates).

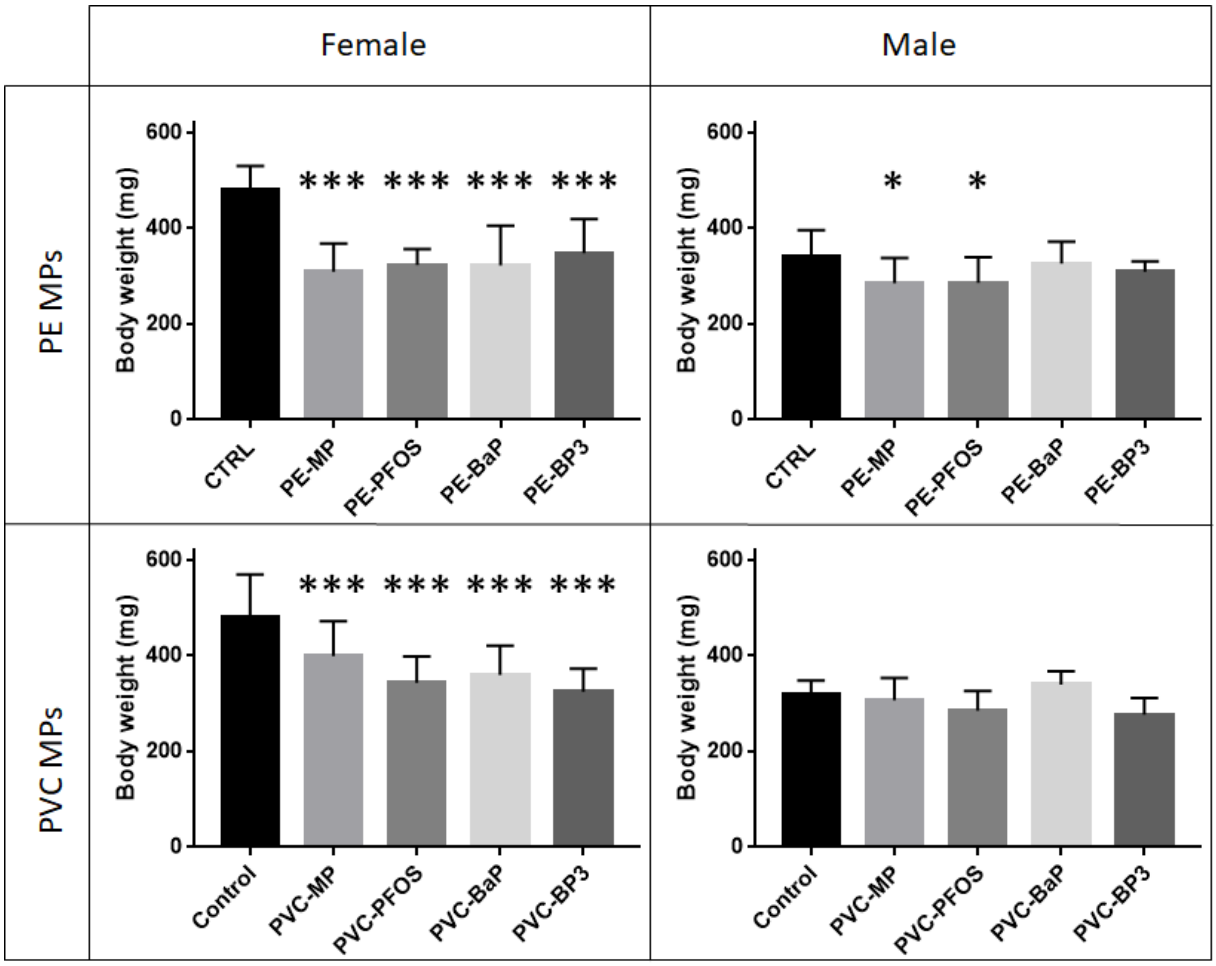

Figure 4. Zebrafish body weight after 4 months of exposure to PE and PVC, either virgin or spiked. (Mean ± SD; Tukey HSD; *: $p<0.05$; ***: $p<0.001$; Fish n=[25-49] per sex per treatment from three replicates).

3.2.3 Adult behaviour

Swimming activity and anxiety level were monitored in adults. None of the treatment (either PE or PVC, virgin or spiked) modified activity or anxiety levels of fish when compared to Control fish (Fig. S7-S8).

3.2.4 Reproduction

For biological reasons, the reproduction was assessed using different mating methods for medaka (group-spawning) and zebrafish (pair-spawning); hence, reproduction variables are different but spawns were obtained for both species and all treatments.

For medaka PE MPs exposures, we observed a significant delay in spawning onset. Fish exposed to PE-MP, PE-PFOS and PE-BP3 started to spawn 16 to 18 days later than the

386 respective Controls, while PE-BaP fish showed a 10 days delay. For PVC MPs exposed
387 medaka, such a delay was not observed but it is noteworthy that these fish (both PVC MPs
388 exposed and respective Control) were bigger than the PE MPs fish (exposed and respective
389 Control; +15-20% depending on the treatment), which may explain an earlier onset of
390 spawning and the fact that this time point was missed. A delay of 3-4 days can, however, be
391 noted when considering the number of eggs spawned (Fig 5).

392 Slopes of reproductive output over time normalised to the number of females in the tank were
393 compared (Fig 5). Slopes of reproductive output of all PE MPs were significantly reduced
394 compared to the one of Control (p<0.01). For Control fish of PE MPs exposure, the slope was
395 1.03 egg per day per female (95% confidence interval: [0.77-1.29]). The slope for PE-MP was
396 43% of Control (p<0.01). For spiked PE MPs, slopes were all below that of the Control
397 (p<0.01) with PE-PFOS being 31%, PE-BaP 56%, and PE-BP3 40%. When comparing with
398 the slope of reproductive output of PE-MP, an increase of 130% was measured for PE-BaP
399 (p=0.017) while there was a significant decrease for PE-PFOS (71%; p=0.013) and no
400 difference for PE-BP3 (93%; p=0.32).

401 For PVC MPs, there was no significant difference between Control (2.83 [2.47-3.20]) and
402 PVC-MP, albeit the slope was at 86% (2.44 [2.07-2.81]; p=0.08). A strong increase in the
403 reproductive output slope was observed for fish exposed to PVC-BaP (148%; p<0.01). For the
404 other two conditions, a significant and similar decrease in slope was observed (PVC-PFOS:
405 63%; p<0.01 and PVC-BP3: 67%; p<0.01). As for PE MPs, comparison of these slopes with
406 the one of PVC-MP confirmed the increase in slope of PVC-BaP exposed fish compared to
407 PVC-MP (172%; p<0.0001), and indicated a decrease of PVC-PFOS (73%; p=0.009) and no
408 difference for PVC-BP3 (77%; p=0.071).

409

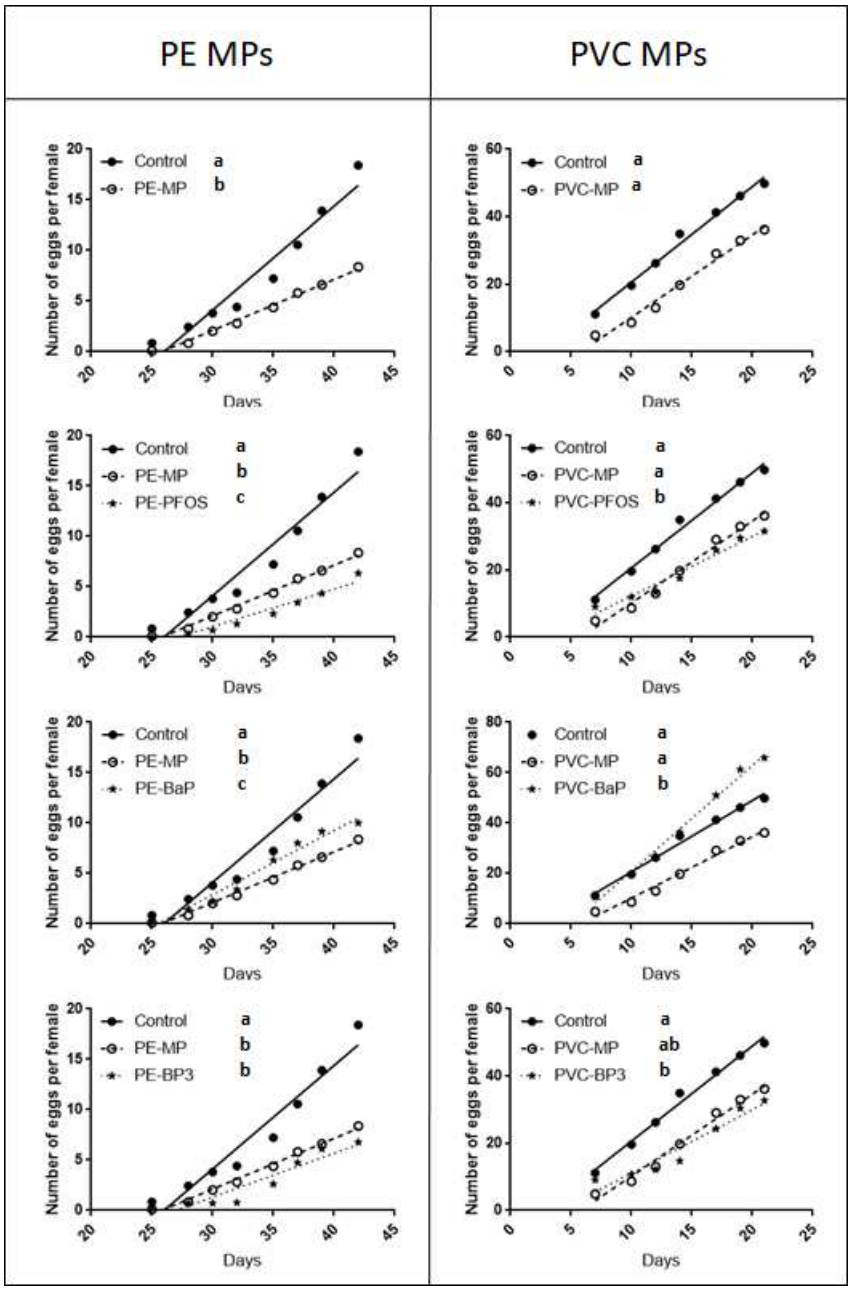

Figure 5. Reproductive output of medaka exposed to PE and PVC, either virgin or spiked. Top panels allow comparison of output from medaka exposed to PE or PVC MPs versus their respective Control. Further panels include comparisons with spiked MPs compared to both Control and virgin MPs. The number of eggs obtained is normalised according to the number of females present in the tank. R square of linear regressions are all above 0.9. Different letters indicate different slopes between treatments.

Fertilisation rate was monitored for each collection of medaka eggs (Fig. S9) and there was no difference over the period of collection ranging between 71 and 80% for PE MPs (ANOVA $F_{(4, 114)} = 0.5707$; p=0.684) and ranging between 90 and 96% for PVC MPs (ANOVA $F_{(4, 108)} = 0.6257$; p=0.645). No change was observed in hatching rates ranging between 44 and 65%

according to treatment (RM-ANOVA; $F_{(28,\ 221.36)}$=0.509, p=0.982 for PE MPs and $F_{(24,\ 172.15)}$=1.152, p=0.293 for PVC MPs).

For zebrafish, individual spawns were monitored. As for medaka, a delay of 2-3 weeks in the onset of spawning was observed for PVC-BaP and PVC-BP3. Reproduction was also monitored by comparing the proportion of successful spawning attempts (*i.e.* when eggs were obtained, independently of spawn size or quality). The proportion of successful spawning attempts was only significantly reduced for PE-BP3 (78.6% for Control vs. 47.5% for PE-BP3; Fisher's exact test, p=0.01; Fig. 6). For all PVC MPs-exposed zebrafish, spawning success was significantly reduced (50.0% for Control vs. 12.1 to 24.2% for virgin and spiked PVC; Fisher's exact test, all p<0.01). There was no difference between virgin MPs (both PE and PVC) and their respective spiked counterparts (Fisher's exact test, p>0.05).

There was no difference between Control and exposed treatment concerning either fertilisation rates ($F_{(5,71)}$=0.794, p=0.56 for PE MPs and $F_{(5,72)}$=2.169, p=0.07 for PVC MPs; Fig. S9) or spawn size ($F_{(5,71)}$=1.404, p=0.23 for PE MPs and $F_{(5,72)}$=1.804, p=0.12 for PVC MPs; Fig. S10).

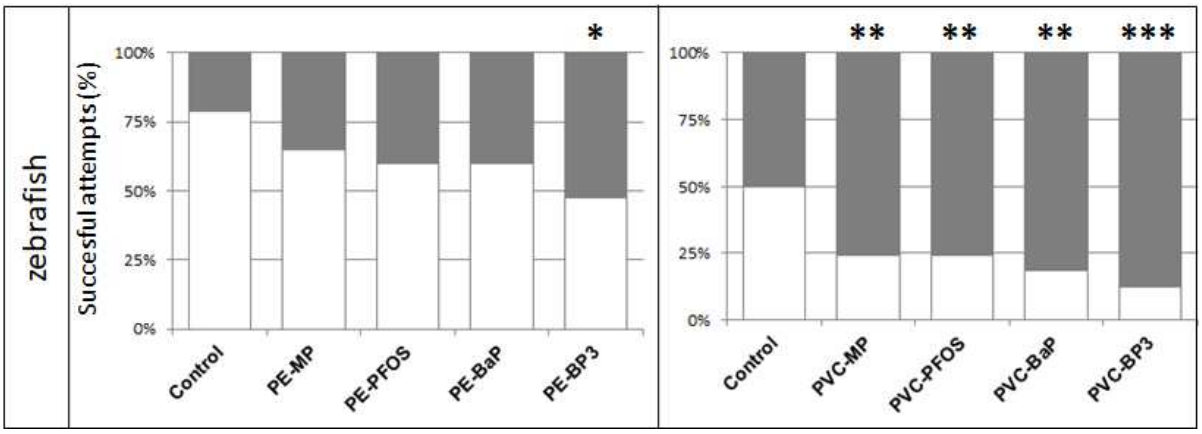

Figure 6. Relative proportions of successful (white) and failed (grey) attempts to obtain eggs from zebrafish exposed to PE and PVC MPs, either virgin or spiked. (Fisher's exact test; *: p<0.05; **: p<0.01; ***: p<0.0001; Attempts n=[23-60] from three replicates).

3.3 Toxicity in the F1 generation

3.3.1 Larval survival

Larval survival was monitored on unfed larvae in zebrafish only. Survival was first overall analysed, then the age at which 50% of mortality was reached was calculated. All treatments affected larval survival with the exception of PE-BaP (Chi^2 value=2.98; p=0.08) and PVC-BaP (Chi^2 value<0.01; p=0.98) (Table S2). Because the Log-rank test appeared quite permissive, we further characterized survival curves by calculating the age at which 50% of

mortality occurred (Fig. S11). This was 11.37 dpf [95% CI: 11.35-11.39] for Control larvae. Values for PE-BaP, PVC-BaP and PE-BP3 did not differ significantly from Controls. Values for PE-PFOS and PVC-MP were significantly reduced by 12.0 and 13.2 hours, respectively. Values for PVC-PFOS and PVC-BP3 were significantly but marginally reduced by 4.08 and 3.6 hours respectively. On the other hand, survival of PE-MP larvae was significantly prolonged with 50% of mortality occurring 13.2 hours later than Control larvae.

3.3.2 Larval growth

Larval growth was evaluated at 14 dpf in medaka and at 5 dpf in zebrafish (Fig. S12). In medaka, there were some differences in standard length of offspring of fish exposed to PE MPs ($F_{(4, 228)} = 7.261$; $p<0.0001$) and PVC MPs ($F_{(4, 158)} = 4.151$; $p=0.0032$), but post-hoc revealed only few small differences. Indeed, PE-MP F1 larvae were slightly shorter (-4%) than Control larvae, while PVC-BaP F1 larvae were slightly longer (+5%). In zebrafish F1 larvae, there was no significant difference between treatments (Kruskal-Wallis$_{(12, 135)}$=16.47, p=0.170).

3.3.3 Larval behaviour

The behaviour of offspring was evaluated at 14 dpf in medaka and 5 dpf in zebrafish using the LPMR assay. Control larvae displayed a regular activity pattern, with an increase in activity during the light off (LOFF) separating the two light on periods (LON1 and LON2) (Fig. 7).

For medaka, there was no significant difference between PE or PVC MPs and their respective controls (RM-ANOVA $F_{(12, 1492.5)}$=0.968, p=0.478, and $F_{(12, 778.14)}$=1.620, p=0.081). In zebrafish exposed to PE MPs, there was no difference in activity pattern when compared to Control within each period (RM ANOVA $F_{(12, 817.83)}$=0.757, p=0.695). For PVC MPs, there was a significant difference between treatments (RM ANOVA $F_{(12, 934.24)}$=3.136, $p<0.001$) and the post-hoc analyses revealed that there was an increase in activity of PVC-BP3 compared to their Control during the LOFF period ($p< 0.005$).

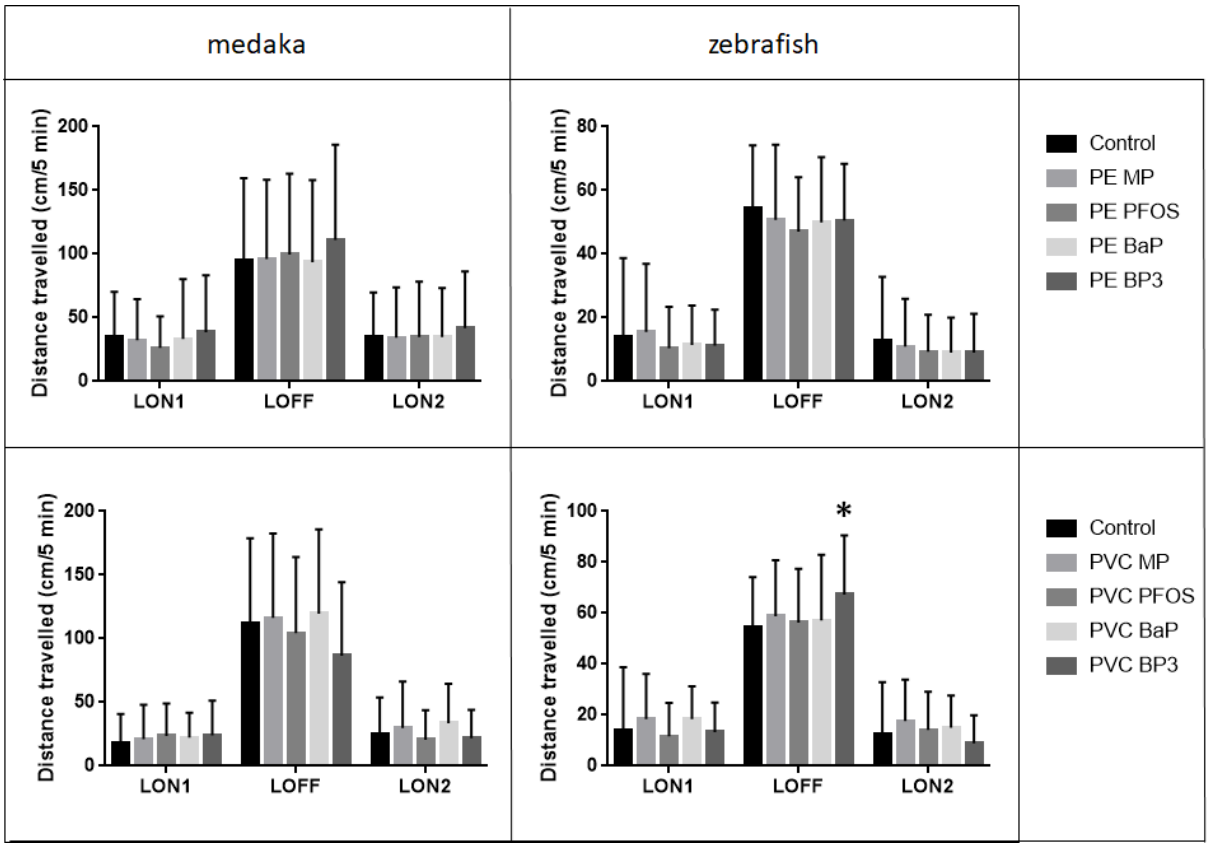

Figure 7. LPMR in F1 larvae issued from fish exposed to PE and PVC, virgin and spiked PVC. Distance travelled over 5 min periods. Data are given as means ± SD (n = 50 to 100 larvae per treatment). (RM-ANOVA; *: $p<0.05$).

4. Discussion

In general, particles that are identified in the lumen of the digestive tract of marine biota are in the higher range of MPs size, smaller ones often being ignored for technical reasons, and because their number is low, e.g. 1 to 5 particles per fish [4, 48]. Egestion of MPs by animals in general, and fish in particular, is quite fast [34]; nevertheless, the presence of MPs has been observed in a large proportion of sampled individuals of a large number of fish species captured in the oceans, seas, and freshwaters [48, 83-87]. Altogether, this suggests that in the wild, exposure is almost constant over time and that the number of MPs ingested by fish is largely underestimated. This is particularly true for small MPs. In this experiment, we exposed fish to food pellets spiked with PE or PVC MPs at a 1% ww of diet. To envision ecological relevance, we related the weight of given MPs to that of a cubic particle of 330 µm on each side, which is the lower threshold of particles commonly collected with the manta trawl. For an adult fed at 3% of its body weight this represented between 3.5 and 2.5 particles given per day per individual, for PE and PVC respectively. Therefore, even if it is difficult to compare with an environmental density of MPs, exposure conditions fell within

environmental ranges. These conditions were also comparable with ratios used in previous experiments using a similar exposure route (0.2-2%) [56, 88-90]. It is however important to mention that the actual number of the particles incorporated into the food was approximately 1500-fold greater for PE MPs than for PVC MPs because of the difference in particle size between PE and PVC MPs. Both, the size of particles and their number may influence their toxicity. Albeit quantification of MPs in digestive tract of exposed fish was not performed, it has been repeatedly reported that exposure to fluorescent MPs either trophic [34, 36, 37, 56] or waterborne [91, 92] resulted in actual ingestion of MPs by fish.

The importance of MPs as a vector for organic pollutants is still under discussion and, according to modelling methods this exposure is suggested to be negligible compared to other sources [39, 68]. However, as discussed by Hartmann et al. [93], it is likely that the actual occurrence of MPs in the environment is underestimated due to current technical limitations of the sampling methods and that MPs are not evenly distributed in the environment. In addition, it seems that the ability to act as vector depends on both chemicals and MPs polymer [94, 95]. Therefore it is likely that, at least in some cases, MPs may act as actual vectors for organic pollutants. In addition, many chemicals are used as additives and can therefore be inherently present in plastics since their production, which is the case for PFOS and BP3. Besides, actual transfer of organic pollutants from MPs to biota has been extensively demonstrated [34, 37, 54, 89, 96-100]. Desorption of chemicals from MPs in the digestive tract and chemical transfer to tissues was reported for several chemicals. The use of fluorescent MPs demonstrated that such chemical transfer was independent of MPs translocation into the organism's tissues e.g. for BaP [34, 37].

To the best of our knowledge, no information is available for concentrations of PFOS and BP3 adsorbed to MPs collected in the environment. There are some scarce reports about polycyclic aromatic hydrocarbons (PAHs), with concentrations ranging from less than 0.1 to more than 46 µg/g of MPs [101-106]. Concentrations of individual PAHs can reach almost 2 µg/g, with concentrations of BaP being up to 0.5 µg/g. The concentration of BaP used here is, therefore, higher than environmental concentrations. Our results show that exposure to MPs spiked with PFOS induced the bioaccumulation of the compound in fish body for both polymer types. On the contrary, neither BaP nor BP3 were detected, not surprising since these compounds are known to be metabolized including in zebrafish for BaP [107] and BP3, partly converted to BP1 [77]. In both cases, metabolites are quickly excreted and so do not accumulate.

The toxicity of MPs, either virgin or spiked, has been studied in various organisms, including fish. In most cases, classical ecotoxicology biomarkers are used and outcomes are very variable including modification of these biomarkers with particular activation of detoxication and inflammatory processes, oxidative stress, and induction of cellular necrosis [47, 49, 50, 53 , 54]. A growing number of articles also report that exposure to MPs has no or very limited effects on fish considering the same biomarkers [35, 45, 56-60, 88]. Studies reporting toxic effects of MPs at the tissue level are even scarcer. Exposure to MPs was shown to produce intestinal lesions in fish [90, 108, 109] while in other cases no inflammation or tissue lesions were observed [35, 56, 88]. Variability in these biological responses may be due to the variety of MPs and their weathering status, exposure route, exposure duration, or concentration. Regarding the latter, it is to note that exposure concentrations are often very high, e.g. mg/L or up to 30% of the diet portion, poorly related to environmental situations. Further, in many cases, exposures are short, focused on one developmental stage, and again may not be representative of life-long exposure occurring in the wild. In the study reported here, exposure lasted at least 4 months and included several developmental stages, from larvae to adult for PE and from juvenile to adult for PVC. As far as we know, this is the longest experimental exposure of fish to MPs, but we nevertheless could not observe alterations of the biomarkers monitored (EROD, TBARS, AChE) or induction of genotoxicity.

The present study used lifelong exposure to enable the identification of growth defects in exposed fish. There are several notable elements regarding these defects: 1) 2 months exposure resulted in no or much weaker effects than 4 months; 2) growth reduction was similar for all MPs PE and PVC, virgin or spiked, and so was not related to extrinsic chemicals; 3) growth reduction was mainly visible in females and 4) effects were similar in both species. A long exposure duration was necessary to observe growth defect. This could explain why no effect on growth was reported for shorter exposures to MPs in previous studies [35, 56, 58, 90]. According to their smaller size (and consequently higher number distributed), a higher toxicity of PE MPs would have been expected, which is not the case. This may be due to additives included in PVC particles. The similar effects of PE and PVC MPs suggests that neither particle size (in the studied range) nor particle numbers have influenced growth disruption, or that they are compensated by polymer type-associated toxicity. The similarity between virgin and spiked MPs, either PE or PVC, suggests that the effect of exposure to MPs on growth is not related to chemicals burden either spiked or additives and argues in favour of the hypothesis of energy budget unbalance [110]. This

hypothesis would fit with the major difference observed between effects in females and males in both species. Indeed, in several fish species, mature females have higher food consumption and/or metabolic costs compared to males as this was shown in e.g. northern pike, walleye, yellow perch, catfish, or zebrafish [111-114]. Therefore, disruption of energy budget due to less food consumption and/or assimilation, plus an increase in metabolic costs or dysbiosis reported for numerous organisms [29, 38, 44, 45, 91, 115-118] would have larger consequences in females than in males.

We observed no change in behaviour of directly exposed fish whatever the MPs and the species. Comparisons with previous publications are not easy since contradictory effects have been reported as decrease [91, 119] or increase [92], or no change in swimming activity [120].

Studies of the effects of MPs exposures on reproduction are scarce. Such effects on reproduction were already reported in Pacific and Pearl oysters after more than 2 months of exposure [118, 121]. In fish, there are only a few reports pointing towards disruption of reproduction [90, 122, 123]. As for growth, there are several notable elements regarding reproduction defects: 1) reproduction output was reduced in most cases upon exposure to MPs but no change was observed in spawn quality; 2) there were differences between polymers and virgin or spiked MPs and 3) there were differences between marine medaka and zebrafish. Regarding reproductive output, in medaka there was a decrease in the number of eggs produced per female per day upon exposure to almost all MPs. This situation contrasts with zebrafish for which a decrease in reproduction success was observed for PE-BP3 only, and for all PVC MPs. When compared to previous reports, these results differ with equal number of eggs spawned after a 21–day long waterborne exposure of Japanese medaka adults to polyester or polypropylene microfibers [123], but are in agreement with the decrease in spawned eggs number after waterborne exposure of marine medaka or Japanese medaka to 10 µm fluorescent polystyrene [90, 122]. It is to note, however, that in both cases, this decrease corresponds to only one collection time point after 2 or 2.5 months of exposure. Spawn quality reported in these articles was mostly unchanged, as we also observed.

In medaka, a delay in spawning onset was observed for almost all MPs treatments while in zebrafish this was only the case for PVC-BaP and PVC-BP3. Along with this delay in spawning onset, co-occurs the decrease in reproduction output which was almost general in medaka while occurring mainly with PVC MPs in zebrafish. These results point out sensitivity differences between species responses, which appeared to be stronger in marine medaka compared to zebrafish. This is not supported by the previous comparison of

590 sensitivity between both species for reproduction traits, so it is not possible to say that this is
591 related to a higher sensitivity of marine medaka to reprotoxic compounds in general, or to
592 MPs in particular. Beyond species differences, it should be noted that another major
593 difference is the salinity of the environment. Comparison of sensitivity according to salinity
594 was made using species sensitivity distributions and differences vary according to chemical
595 groups [124, 125]. In general, organic chemicals appear more toxic to saltwater species,
596 however, the underlying reasons are not clear.

597 Considering only zebrafish leads to the conclusion that PVC MPs are more reprotoxic than PE
598 MPs and that PVC is the major driver for toxicity, while for PE, BP3 is responsible for
599 reprotoxicity. The same hypothesis is not true when considering medaka for which exposures
600 to PE MPs had more severe effects than PVC MPs, effects of both plastic types being partially
601 modified by spiked chemicals. In both cases, these modifications can be an increase (BaP) or
602 a decrease (BP3 or PFOS) in reproduction output. Dietary exposure to BaP, albeit at much
603 higher concentration, resulted in no modification of reproduction output [126, 127] which is
604 in agreement with the absence of specific reprotoxicity of BaP after exposure to either PE-
605 BaP or PVC-BaP. It is noteworthy that chronic waterborne exposure of fish to PFOS with
606 PFOS body burden similar to the one obtained in the present work was reported to result in
607 physiological disruption indicative of endocrine disruption [128, 129]. This may provide a
608 mechanism underlying effects of PFOS spiked MPs on reproduction, even if the lowest
609 concentration used in these studies (0.6 µg/L) is at the level of the highest monitored
610 concentrations for PFOS. Endocrine disruption was also observed in fish waterborne-exposed
611 to BP3 but at concentrations three- to tenfold higher than the highest environmental
612 concentrations [78, 130].

613 PE and PVC MPs (both virgin and spiked) were equally able to decrease growth after
614 approximately 4 months of exposure, while it appeared that reprotoxicity of PVC was higher.
615 This difference in response between polymers types and depending on endpoint has recently
616 been shown in *Daphnia sp.* [65]. In this latter study, polyurethane and polylactic acid MPs
617 (native and from which chemicals were extracted using methanol) are toxic for some
618 measured endpoints while the extracted chemicals are not, contrary to PVC for which
619 extracted chemicals are toxic but not the MPs (native or extracted), and all MPs being more
620 toxic than kaolin particles [65]. Since there were differences between PE and PVC MPs (both
621 virgin and spiked) it is difficult to definitely assign reprotoxicity to either MPs or spiked

chemicals, and the interference between both. Dedicated experiments are needed for this purpose.

In order to fully describe the consequences of a long-term exposure on population recruitment, some offspring traits were monitored. Some disruptions were observed in F1 from exposed fish but they were not major. The slight reduction in time needed to reach 50% mortality may be related to a decrease in the energy initially stored/provided to eggs. This was however not translated in early developmental defects since larval growth and behaviour were mostly not altered. Regarding this latter trait, the increase in response to LOFF period observed in F1 from PVC-BP3 exposed zebrafish is quite important with an increase of 24% compared to control larvae which may be detrimental for their survival. Disruption in behaviour during LOFF has also been described as an indicator of increased anxiety and/or neurotoxicity [131]. It is noteworthy that no such difference was observed in marine medaka but because of a later hatch in medaka, larval behaviour assessment is done much later (14 dpf) compared to zebrafish (5 dpf). We can therefore make the hypothesis that this effect, observed only in PVC-BP3, is related to maternal transfer of BP3, which may be eliminated in medaka larvae. This would be in agreement with a previous report which has shown that BP3 disrupt marine medaka larval behaviour [59].

In other experiments, we have used the same PE MPs, virgin or spiked, to evaluate acute toxicity using regulatory tests in zebrafish and medaka embryos [57-59]. The conclusion is that no acute toxicity was observed at environmentally relevant concentrations. This indicates that the toxicity of MPs comes from chronic exposure and so that long exposures are necessary to properly assess MPs toxicity which cannot be evaluated using regulatory tests using acute toxicity endpoints.

In conclusion, our study presents clear evidence that a reduction in growth for females and a decrease in reproduction output co-occur upon chronic exposure to MPs, and that combination may have important negative consequences on individual abilities to contribute to recruitment, which may lead to adverse effects at a population level. Our results fit well with the tentative adverse outcome pathway proposed recently to link exposure to MPs to population consequences involving disruption of energy budget [110] and further work is needed to evaluate this in detail. It is important to note that these effects depend on polymers type (PVC being more toxic than PE), the presence or not of chemicals (BP3 being more toxic than PFOS or BaP) and the duration of exposure. Regarding this last point, our result clearly advocate long-term, chronic exposures as reliable for MPs toxicity evaluation in fish. In

addition, further studies are necessary to better understand the influence of size and polymer type on MPs toxicity, as well as the role of additives. Globally, we observed no major difference between both studied species but more similar comparative studies like this one are necessary to conclude on the genericity of our findings.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CRediT authorship contribution statement

Bettie Cormier: Data curation; Formal analysis; Investigation; Roles/Writing - original draft. **Florane Le Bihanic:** Data curation; Formal analysis; Investigation; Roles/Writing - original draft. **Jean-Claude Crebassa:** Investigation. **Mathieu Cabar:** Data curation; Investigation. **Mélanie Blanc:** Investigation. **Maria Larsson:** Investigation. **Florian Dubocq:** Investigation. **Leo Yeung:** Investigation. **Christelle Clérandeau:** Data curation; Investigation. **Steffen H Keiter:** Conceptualization; Funding acquisition; Supervision; Writing - review & editing. **Jérôme Cachot:** Conceptualization; Funding acquisition; Project administration; Supervision; Writing - review & editing. **Marie-Laure Bégout:** Conceptualization; Data curation; Funding acquisition; Investigation; Project administration; Supervision; Validation; Roles/Writing - original draft; Writing - review & editing. **Xavier Cousin:** Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Supervision; Validation; Visualization; Roles/Writing - original draft; Writing - review & editing.

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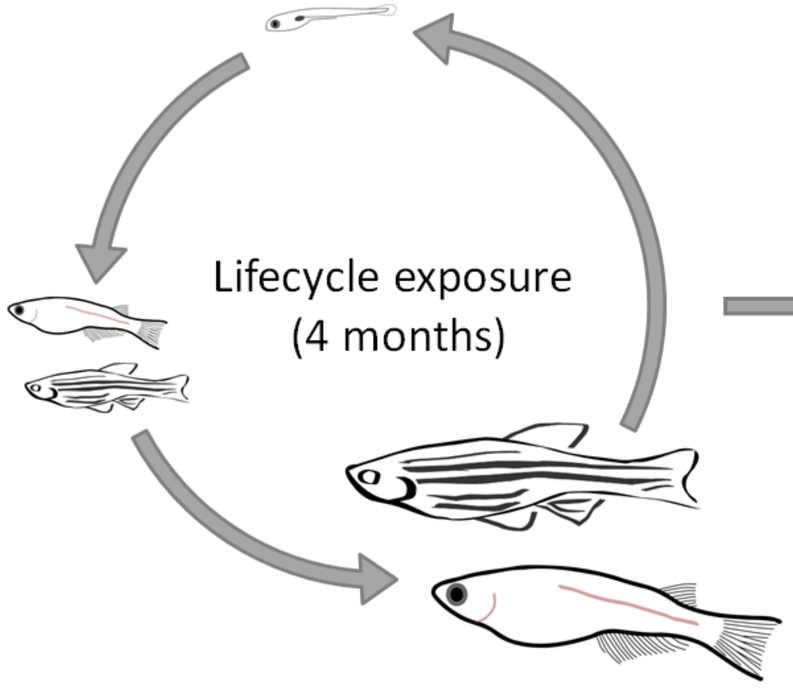
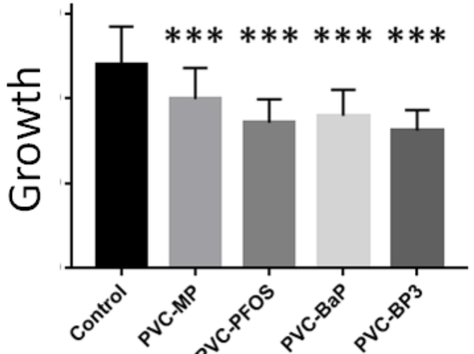
Microplastics = PE or PVC virgin or spiked with PFOS or BaP or BP3

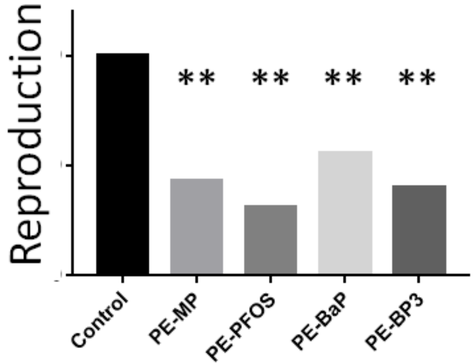
Fish= zebrafish and marine medaka

Biological disruptions

Dietary exposure
1% ww diet







Severity varies with

- Plastic types
- Presence or absence of chemicals
- Nature of chemicals