**Supplementary Materials: Environmental microplastics in Teleosts: Acute zebrafish larval exposures disrupt swimming activity and chronic exposures reduce growth and reproduction efficiency in F0, and F1 survival in *Danio rerio* and *Oryzias melastigma***

**Supplementary materials and methods**

**Molecular biomarkers**

To perform the measure of ethoxyresorufin O-deethylase activity (EROD) on liver samples, Burke and Mayer method (1974) was used. The reaction mixture consisted of a NaH2PO4/K2HPO4 buffer (0.1 M, pH 7.5), 7-ethoxyresorufin (0.15 mM) and NADPH (5.57 mM). The change in fluorescence was recorded (excitation wavelength 530 nm, emission wavelength 585 nm) and enzyme activity was calculated as pmol/min/mg protein using a resorufin standard.

The amount of reactive oxygen species (ROS) to evaluate the oxidative stress is difficult to measure due to a short half-life, so the measure of thiobarbituric acid reactive substances (TBARS), an oxidative product (Buege and Aust, 1978), was studied. TBARs were measured in fish muscles, and following the colorimetric protocol developed by Buege and Aust (1978) and adapted to a microplate reader as described in Weeks Santos et al. (2019). Results are expressed as nM of TBARs equivalents per mg of protein.

Acetylcholinesterase measurement (AChE) was performed in brain following the Ellman’s protocol (Ellmann et al. 1961), adapted to a microplate reader by Weeks Santos et al., (2021). Each well contained 10 µL of S9, 180 µL of 5,5’-dithiobis 0.5 mM in 0.05 M of Tris Buffer (pH 7.4) and 10 µL of acetylcholine iodide. Results are expressed as nmol/min/mg protein.

The Comet assay was performed on blood cells from medaka. Alkaline comet assay was performed following previously published protocols with minor modifications (Le Bihanic et al., 2014). Briefly, protocol adaptations consisted of sampling 2 µL of blood in anesthetized fish using heparinized needle, transferred in 200 µL of cryoconservation solution (250 mM sucrose, 40 mM citrate trisodique, 5% DMSO, pH 7.6), mixed by manual tube reverse and quickly frozen in liquid nitrogen. Ten µL of cell suspension was added to 140 µL of 1% (w/w) low melting point agarose and laid on slides previously covered with normal melting point agarose (0.8%, w/w). DNA unwind was 20 min long in electrophoresis buffer and DNA electrophoresis was performed at 25 V, 300 mA for 20 min in the dark at 4°C. DNA damage was measured on 100 cells for each sample with Komet 5.5 software (Kinetic Imaging, Liverpool, UK) and expressed as percentage tail DNA.



Figure SM1: Hatching time in hours (A) and hatching rate (B) for zebrafish embryos exposed to 1 or 10 mg/L of MG or PB MPs. (Mean ± SD; Kruskal-Wallis; p>0.05; n=3).



Figure SM2: Head length (A); total length (B) and ratio head/total length (C) were measured for zebrafish embryos at 96 hpf exposed to 1 or 10 mg/L of MG or PB MPs. (Mean ± SD; Kruskal-Wallis; p>0.05; n= 6-10 embryos).



Figure SM3: EROD activity in zebrafish larvae exposed to MG and PB MPs at 1 or 10 mg/L for 96 hpf. EROD activity is given relative to control. (PC: positive control, 70 nM of benzo[a]pyrene; Mean ± SD; ANOVA F(5,13)=6.098 on ranks; \* p<0.05; n=3).



Figure SM4: Larval photomotor response in 5 day-old (Danio rerio) after exposure to control, MG and PB samples at 1 and 10 mg/L. Average swimming velocity over a 5 min period including two light on periods (LON1 and LON2) with one light-off period (LOFF). Mean ± SD; n = 16–24 larvae per treatment; \* p<0.05; \*\* p<0.01, repeated–measure ANOVA.



Figure SM5: Length and weight of zebrafish exposed to MG or PB MPs at 1% in the food. (A) and (B) represent biometry at 2 mpf (n=42-48 per treatment) while (C) and (D) represent length and weight for 4 mpf zebrafish (Mean ± SD; in 4D for females, ANOVA F(2, 40)=7.97, for males ANOVA F(2, 42)=5.56; \*\*p<0.01; n=11 to 18 per sex and per treatment.).

Table SM1: Biomarkers measure in zebrafish after 4 months of exposure to MPs. Mean ± SD; n=3 per fish, treatment and sex. No statistical differences were observed after Kruskal-Wallis test, p>0.05. (AChE: nmol/min/mg protein; EROD: percentage of respective control; TBARS: nmol/mg protein)

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| --- | --- | --- | --- | --- | --- | --- |
| *D.rerio* | AChE | | EROD | | TBARS | |
| Female | Male | Female | Male | Female | Male |
| Control | 263.3 ± 115.4 | 252.2 ± 109.7 | 100 ± 59.3 | 100 ± 25.6 | 53.5 ± 18.5 | 39.5 ± 10.7 |
| MG | 220.8 ± 96.2 | 259.1 ± 132.9 | 47.5 ± 28.6 | 40.5 ± 9.3 | 43.2 ± 16.9 | 41.8 ± 17.0 |
| PB | 279.8 ± 50.6 | 227.0 ± 108.4 | 74.3 ± 16.1 | 73.5 ± 85.6 | 90.9 ± 67.7 | 49.3 ± 28.0 |

Table SM2: Biomarkers measure in medaka after 4 months of exposure to MPs. (Mean ± SD; Kruskal-Wallis test, \*, p<0.05; n=3 per fish, treatment and sex). (AChE: nmol/min/mg protein; EROD: percentage of respective control; TBARS: µmol/mg protein; Comet assay: % tail intensity)

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| *O.melastigma* | AChE | | EROD | | TBARS | | Comet Assay | |
| Female | Male | Female | Male | Female | Male | Female | Male |  |
| Control | 124.5 ± 9.1 | 146.5 ± 27.0 | 100 ± 43.9 | 100 ± 75.1 | 54.5 ± 12.8 | 55.6 ± 8.4 | 9.6 ± 1.9 | 12.7 ± 2.1 |  |
| MG | **195.8 ± 37.7 \*** | 148.2 ± 35.7 | 38.7 ± 31.1 | 110.8 ± 76.2 | 54.6 ± 1.6 | 81.4 ± 29.0 | 9.2 ± 1.6 | 7.9 ± 4.1 |  |
| PB | **183.9 ±11.4 \*** | 153.1 ± 24.9 | **20.0 ± 6.1 \*** | 74.8 ± 32.8 | 47.5 ± 5.9 | 58.0 ± 9.1 | 9.2 ± 1.5 | 10.8 ± 0.9 |  |



Figure SM6: Monitoring of anxiety level in zebrafish (A and B) and medaka (C and D) after MG and PB exposures. Graphics represent the distance travelled (cm/5 min) using zebrafish (A) or medaka (C), while (B) and (D) are the time spent in top zone for zebrafish and medaka, respectively. (Mean ± SD; n= [12-21] from three replicates)



Figure SM7: Different spawning parameters measured for zebrafish, (A) Total amount of eggs collected after pair spawning, (B) Number of fertilized eggs and the ratio of fertilized eggs (C). (Mean ± SD; Attempts n= 56-66 from three replicates)



Figure SM8: Fertilization rate (%) of marine medaka exposed to Control, MG or PB MPs. (Mean ± SD; Attempts n= 18-38 from three replicates)

**Reference**

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