Aquatic Toxicology 224 (2020) 105521

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox

Microplastics Lead to Hyperactive Swimming Behaviour in Adult Zebrafish

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ARTICLE INFO

Keywords:
microplastics
zebrafish
hyperactivity
energy depletion

ABSTRACT

Microplastic pollution has drawn the attention of both scientists and the public regarding their potential ecotoxicological risks. In the present study, we carried out aqueous exposure experiments to adult zebrafish with polystyrene microplastics (5 μm) at a wide range of concentrations (0.001-20 mg/L, equals to $14.5 \sim 2.9 \times 10^5$ particles/mL). Our results showed the gastrointestinal tract (GIT) was the dominant microplastic accumulation site in zebrafish, followed by the gill, whereas no microplastics were detected in the brain or muscle. Microplastic accumulation in GIT did not cause obvious damages to intestinal villi in general. However, the thickness of muscularis layer in the foregut reduced by 32% after 1 mg/L (1.45×10^4 particles/mL) microplastic exposure. As there were no signs of oxidative stress or other histological changes found in the fish, we further investigated the energy-supplying influential factors. We found that the zebrafish became hyperactive after microplastic exposure, whose swimming distance had increased to 1.3-2.4 folds than that of control, and also stayed at manic and active states much longer. The fish behavioural alteration is probably attributed to the particulate matter stimulation and the up-regulation of estrogen contents. Results also showed that the excessive movements of zebrafish also led to decreased glucose and acetaldehyde metabolite contents and increased amino acid amounts, which further proved the shortage of energy-supplying substances. Therefore, the present study suggests that micro-sized microplastics can induce obvious behavioural abnormality at concentrations that some other toxicological endpoints may not warn effects.

1. Introduction

The wide usage of plastic has led to its ubiquitous presence in the aquatic environment (Gallo et al. 2018). These plastic debris can degrade or fragment gradually into smaller particles, and they are termed as microplastics when the particle size smaller than 5 mm (Anbumani and Kakkar 2018). Recently, a special focus was given to small-sized microplastics (< 10 μm) (Oßmann et al. 2018), and their concentration in plastic mineral water bottles has been reported to reach $5.42 \pm 1.95 \times 10^4$ particles/mL (Zuccarello et al. 2019). Scientists have reported the ingestion of microplastic by plankton (Cole et al. 2013; Sun et al. 2018), mollusks (Li et al. 2018; Li et al. 2015) and various species of fishes (Jabeen et al. 2017), and furthermore be accumulated throughout the food web together with co-existing pollutants (Au et al. 2017; Qu et al. 2020). Moreover, microplastics may even be ingested or inhaled by human beings through seafood or house dust and affect people's health (Chen et al. 2019; Zhang et al. 2020). Therefore, microplastic has drawn the attention of both scientists and the public regarding their toxicity and safety concerns.

Researchers have reported that 9.2-92.3% of fish from aquatic environments had more than one microplastics (Davison and Asch 2011; Jabeen et al. 2017; Neves et al. 2015), which mostly accumulated in the gastrointestinal tract (GIT) (Lusher et al. 2013; Vendel et al. 2017). So far, researchers have mainly focused on the liver damage after microplastic ingestion (Jabeen et al. 2018; Lu et al. 2016). But the investigation of microplastic toxicity to the GIT did not match with its great occurrence. Recently, (Lei et al. 2018) found that microplastic induced gut villi cracking and enterocytes splitting, but there is still a lack of further discussion about the underlying mechanisms. Thus, more investigations are necessary to explore whether microplastic has other

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https://doi.org/10.1016/j.aquatox.2020.105521
Received 10 April 2020; Received in revised form 15 May 2020; Accepted 17 May 2020
Available online 24 May 2020
0166-445X/

potential toxicities to GIT.

Microplastic could impair the feeding ability in aquatic organisms due to a false sense of satiation, which can lead to energy-supply related changes (Barboza et al. 2018; Cole et al. 2015; Watts et al. 2015). After uptake, the longer gut residence time of microplastics and inflammation and depleted energy reserves were observed, which could cause microplastic blockages in the GIT (Wright et al. 2013). Similar phenomena have been observed in field studies. Both macroplastics and microplastics significantly reduced the skeletal growth rates of corals and decreased their prey capture rates (Chapron et al. 2018; Morét-Ferguson et al. 2010). Since microplastics retained in GIT for at least one day, the egestion and counteracting with toxic effects of microplastics require energy cost (Chapron et al. 2018). Along with the energy reserve depletion, inflammatory response, physical injuries, release of residual monomers and additives may also impact the health of biota (Reichert et al. 2018; Rochman et al. 2013; Wright et al. 2013). Disturbance of the energy metabolism in zebrafish by microplastics has been observed (Lu et al. 2016) and nanoplastics can inhibit the locomotion ability of zebrafish larvae (Chen et al. 2017). These studies suggest microplastic exposure may affect the energy supply and motor-related pathways of the muscle tissue. However, there is a need for further investigation as the specific causes are still unclear.

The aims of this study were to better understand the microplastic accumulation and localization in zebrafish, and their downstream effects on the fish physiological state. For this, (i) the uptake of microplastics in different tissues was explored; (ii) the potential threat of microplastic to zebrafish from different histological levels was investigated; (iii) furthermore, due to the decreased thickness of muscularis layers after microplastics exposure, we also explored how the swimming behavior and muscle energy metabolism of zebrafish changed during the exposure.

2. Materials and methods

2.1. Reagents and zebrafish maintenance

Fluorescent microplastics (polystyrene, 5 μm, 10% *m/V* in water suspension) were purchased from the Baseline Chromtech Research Centre, China. The excitation and emission wavelengths are 418 nm and 518 nm, respectively. The plastic composition was identified by Raman microscopy (DXR 2, Thermo Fisher Scientific) with both single point measurement and mapping (**Fig. S1**). Adult zebrafish were fed twice a day with dry flakes and kept at a constant temperature of 26 ± 1 ˚C at pH 7.0 with a 14 h light: 10 h dark photoperiod.

2.2. Microplastic exposure experiment set-up

Zebrafish were exposed to two groups, namely the negative control and the microplastic exposure groups in artificial freshwater. The artificial freshwater was prepared by mixing 960 mL Milli-Q water with 10 mL of 294 mg/L $CaCl_2{\cdot}2H_2O$, 10 mL of 123.3 mg/L $MgSO_4{\cdot}7H_2O$, 10 mL of 63 mg/L $NaHCO_3$, and 10 mL of 5.5 mg/L KCl. Microplastic exposure concentration series consists of 0.001, 0.01, 0.1, 0.25, 0.5, 1, 2, 10, and 20 mg/L (equals to 14.5 to 2.9 × 10^5 particles/mL) microplastics. The exposure lasted for 7 days and the tissue uptake concentrations and histological alterations were measured. To further investigate the uptake kinetics changes, we also exposed zebrafish at the concentration of 1 mg/L (equals to 1.45 × 10^4 particles/mL, which is at the same magnitude of current reported highest microplastic concentrations (Zuccarello et al. 2019)), to test the microplastic accumulation and histological alterations along the 7-d exposure period.

All treatment media were prepared and aerated one day before exposure in order to obtain a complete mixing and oxygen saturation, and the fish exposure density was far below 1 g/L in exposure containers. The fish were fed with 2% of their body weight commercial fish food once per day. The exposure was semi-static with 80% media refreshed daily after half an hour feeding in the morning. To achieve a relatively even distribution of microplastics in the artificial freshwater, we introduced gentle agitations during the exposure period. First, we refreshed the exposure solutions daily to make up the lost and settled microplastics; second, we used a glass rod to stir the exposure solutions gently every 2 h during the 14 h light period.

2.3. Microplastic quantification in different fish tissues

Triplicate glass containers with 12 fish per 4 L exposure media were sampled after exposure. During the sampling, 3 males and 3 females were collected from each group for the uptake quantification in the GIT, gill and muscle tissues, and (1 male + 1 female) zebrafish were pooled together as one replicate for the microplastic uptake quantification in the brain due to the small mass amount of brain from each fish. After sampling, fish were placed in clean artificial freshwater and rinsed thoroughly to remove any attached microplastics at the surface of the skin, fins or gills. Then, fish were anesthetized with 0.4 g/L benzocaine and then rinsed with Milli-Q water twice. In order to avoid contamination, always freshly cleaned pairs of tweezers were used for isolation of different tissues. For the GIT and gill, tissues were separated and transferred to clean centrifuge tubes immediately. For the muscle tissue, the top layer of skins above the muscle was stripped off first. For the brain tissue, the cranium bones above the brain on the fish head were separated first. Then, the muscle or brain tissues were taken out with clean tweezers for further digestion and counting. During the whole process, blank control tissues were also measured in parallel. No fluorescent particles were found in the blank controls.

The zebrafish tissues were digested according to (Jabeen et al. 2018) with slight modifications. Briefly, the tissue samples were placed into 15 mL clean centrifuge tubes (Corning, USA) for digestion and then 2 mL of 30 % H_2O_2 was added, which volume was enough to digest the organic matter of fish tissues. Then, the centrifuge tubes were covered and rotated in an incubator at 65 ˚C with 80 rpm for 24 h. After digestion, tissue samples were filtered through 20 μm nylon membranes (Millipore, USA). The digested debris larger than 20 μm were retained on the membrane and microplastics were in the filtrates. The membranes were rinsed with 0.5 mL of Milli-Q water twice. Then, 50 μL of filtrate was added on a microslide carefully for microplastic quantification under a fluorescent microscope (Olympus, BX53, Japan).

2.4. Histological observation of the gastrointestinal tract

As the GIT accumulated the largest amounts of microplastics than all the other tissues, histological analysis was carried out for the GIT samples. After fixing in 10 % of formalin, GIT samples were embedded in paraffin wax, sectioned into 5 μm thickness, and then stained with hemotoxylin and eosin (HE). The foregut sections were examined on the Olympus microscope for villi and muscularis layer observation. The hindgut sections were examined for goblet cells counting. Morphological photographs were taken, and then the height of villus, the number of the goblet cells, and the depth of muscularis layers were measured using the Image Pro Plus software (version 6.0, Media Cybernetics Inc., USA).

2.5. Oxidative stress responses

To test the oxidative stress responses in the fish, the fish liver samples were separated immediately after dissection and preserved at −80 ˚C until enzyme and antioxidant substance measurements. The analyses of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx), and reduced form of glutathione (GSH) were determined with commercial kits (Jiancheng Biotech, China) at 405 nm, 550 nm, 412 nm, and 420 nm, respectively. All enzymatic activities and GSH content were expressed as specific activity or content per milligram of the total protein.

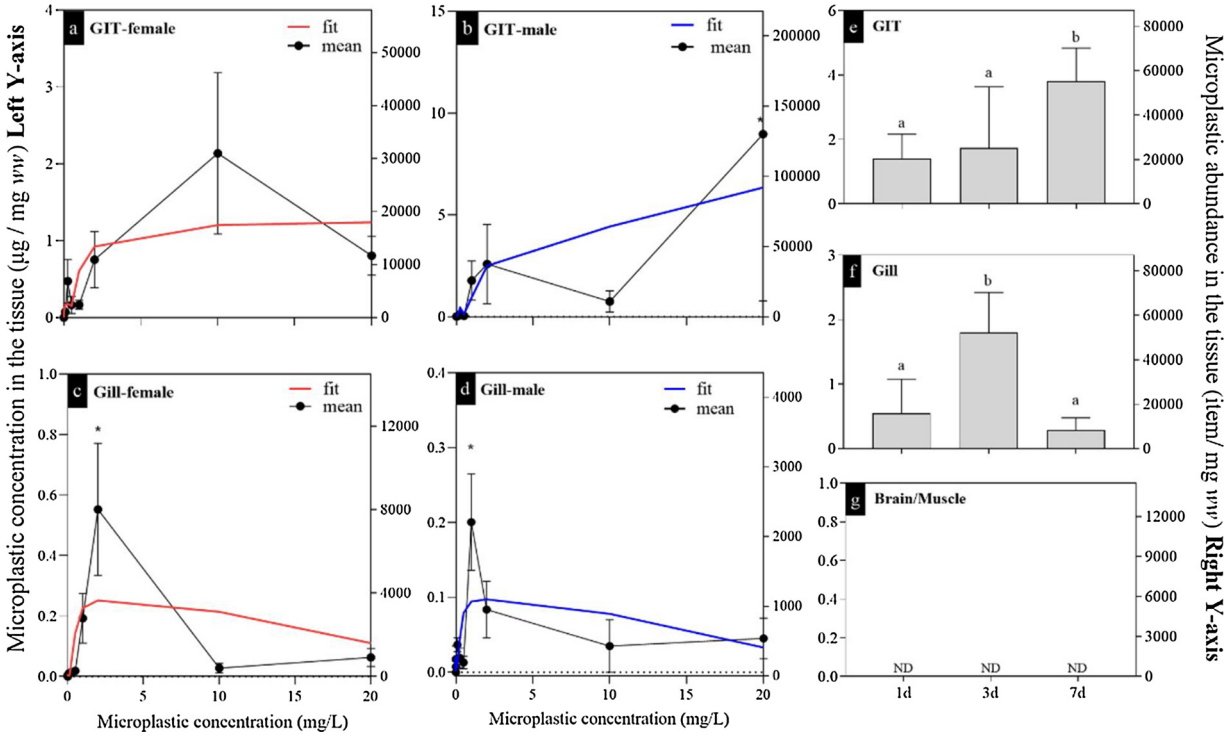

Fig. 1. Microplastic uptake amounts in the GIT (**a**, **b**) and gill (**c**, **d**) of zebrafish along with microplastic exposure concentration increasing after 7-d exposure and the concentration changes during the exposure period the in the (**e**) GIT, (**f**) gill, and (**g**) brain/muscle tissues when exposure concentration is 1 mg/L microplastics (equals to 1.45×10^4 particles/mL). * represents significant differences from that of control ($p < 0.05$), and different alphabet letters represent significant differences between groups ($p < 0.05$). ND means no microplastic particle detected in the tissue.

2.6. Behaviour monitoring

To investigate if the swimming behaviour of zebrafish has been changed, the locomotion monitoring test was carried out for the fish immediately at the end of 7 d exposure in the DanioVision video-track system at 26 ˚C (Noldus, The Netherlands), which has been used to indicate particulate pollutants' effects previously (Chen et al. 2017; Legradi et al. 2018). The EthoVision XT software was applied for the behavioral results analyses. Each experiment was conducted in a 24 × 12 cm (length × width) tank with 400 mL artificial freshwater with female or male fish separately ($N = 3$). Before monitoring, fish were put in the chamber in advance and acclimatized for 5 min. Then, a ten-minute recording of the fish behavior was carried out in a soundproof environment.

2.7. Metabolites analysis of zebrafish muscle tissue

To investigate the energy metabolism status, the fish muscle samples were also used for metabolomics analysis. Thirty mg of muscle were weighed from each sample, 10 μL internal standard (2-chlorophenylalanine) and 250 μL lysis buffer (methanol: acetonitrile: water = 2:2:1) were added. The samples were homogenized by adding two small steel balls on a shaker for 1 min, and then extracted in an ice-water bath for 5 min. Then, the supernatant was obtained after 15 min centrifugation at 13,400 g. The lysis, grind, extraction, and centrifugation processes were repeated again. Three hundred μL supernatant was transferred to a centrifuge tube, 300 μL chloroform added, and then mixed for 20 s using a vortex. After 30 min, the sample was centrifuged at 13,400 g for 5 min. Finally, 180 μL supernatant was quantified by utilizing liquid chromatography- high resolution mass spectrometry (LC-HRMS).

The LC-HRMS was equipped with a binary solvent delivery manager and a sample manager, and a Mass Spectrometer (Waters VION IMS Q-TOF) with an electrospray interface (Waters, USA). The separation column was Acquity BEH C18 column (100 mm × 2.1 mm i.d., 1.7 μm). Solution A was 10 mM ammonium acetate in acetonitrile (pH = 9, v/v = 9:1), and solution B was 10 mM ammonium acetate (pH = 9). Three μL of sample was injected and the column temperature was set at 45 ˚C initially. The column was maintained at 45 ˚C and separation was achieved using the following gradient: 5-25% B over 0-1.5 min, 25-100% B over 1.5-10.0 min, 90% B over 10-13 min, the composition was held at 5% B for 2 min at a flow rate of 0.4 mL/min. The mass spectrometric data was collected using a Waters VION IMS Q-TOF Mass Spectrometer equipped with an electrospray ionization (ESI) source operating in either positive or negative ion mode. The source temperature and desolvation temperature was set at 120 ˚C and 500 ˚C, respectively, with a desolvation gas flow of 900 L/h. The quality control samples were injected at regular intervals (every 6 samples) throughout the analytical run.

2.8. Cryo-Transmission Electron Microscopy

To observe the fine structures alteration in muscularis layers, we applied the cryo-Transmission Electron Microscopy (cryo-TEM) to preserve and observe the sample original biological states. The foregut samples were quickly separated on ice, and then frozen inside thin copper tubes with an inner diameter of 200 μm (Leica EM PACT2). High-pressure freezing accomplished consistent sample vitrification within 5 s. Then, fixation, dehydration, infiltration, embedding and polymerization of samples were performed on a freezer replacement equipment (Leica EM AFS2/FSP). Frozen samples were then stained and transferred to and cut on a frozen ultra-thin slicer (Leica EM UC7/FC7). Grids containing the frozen-hydrated thin sections were mounted into a Gatan cryoholder for direct observation at −170 ˚C in a FEI Tecnai G2 Spirit Twin energy-filtered cryo-TEM.

2.9. Statistical analysis

All the data were confirmed to be normally distributed by utilizing the Shapiro-Wilk test, and *t*-test or ANOVA analysis was applied to compare the differences among two or more than three groups with the SPSS (version 22.0, SPSS Co., USA), respectively. Partial least squares discriminant analysis of metabolomics were analyzed in the MetaboAnalyst 4.0 (Chong et al. 2018).

3. Results

3.1. Uptake of microplastics in different tissues

The uptake abilities for microplastic in zebrafish tissues were fairly different. GIT and gill tissues accumulated microplastics at the magnitude of μg/mg wet weight (*ww*), whereas no microplastics were found in brain or muscle (Fig. 1). For both female and male fish, the microplastic concentrations in the GIT increased rapidly initially from 0.001 to 2 mg/L and then slowly at high concentrations (2-20 mg/L) (Fig. 1a& b). This pattern of accumulation did not show gender differences. At 2 mg/L (equals to 2.9×10^4 particles/mL), which can be deemed as the highest environmental relevant concentration according to our knowledge (Zuccarello et al. 2019), the accumulation amounts in the GIT were 0.8 ± 0.9 and 2.6 ± 1.9 μg/mg for female and male fish, respectively.

The overall microplastic accumulation amounts in the gill was an order of magnitude lower than that of GIT, without showing a cumulative trend with the increase of concentration. Moreover, the highest cumulative microplastic amounts in the gill occurred around 1-2 mg/L for both fish genders with 0.6 ± 0.5 and 0.2 ± 0.2 μg/mg for female and male fish, respectively (Fig. 1**c&d**).

The uptake kinetics results in Fig. 1**e** also showed that the microplastic accumulation amounts in the GIT increased generally during the whole exposure period, with the maximum accumulation concentration occurring on the 7th day after 1 mg/L microplastic exposure. When looking at gill uptake kinetics, the microplastic accumulation amounts in the gill peaked on the 3rd day with 1.8 ± 0.6 μg/mg *ww*, and then decreased by 89% on day 7 (Fig. 1f).

3.2. Histological changes in the gastrointestinal tract

In general, the histological changes of the GIT after microplastic exposure was not significant, but some effects showed up when the concentration larger than 1 mg/L (Fig. 2). As for the height of the foregut villus, it first increased slightly, then decreased and reached the shortest height of villus at 2 mg/L ($p < 0.05$), and then gradually returned to the normal state (Fig. 2b). As for the goblet cells which located in the hindgut, they are responsible for antigen uptake and immune responses (Pelaseyed et al. 2014). The number of goblet cells gradually increased with microplastic exposure concentration increasing, but significant differences only occurred at the highest concentration of 20 mg/L ($p < 0.05$) (Fig. 2c).

Zebrafish have a thick foregut wall, which ensures its digestive function with the muscularis layers (Press and Evensen 1999). The thickness of the muscularis layer in the foregut, has decreased with microplastic concentration increasing at the beginning, and then gradually levels off (Fig. 2d). A significant thickness reduction occurred at the concentration of 1 mg/L (equals to 1.45×10^4 particles/mL), which is an environmental relevant microplastic concentration, attracts our attention. The foregut muscularis layer thickness decreased to 13.8 ± 7.1 μm in the microplastic exposure group, significantly thinner than control (20.2 ± 15.2 μm) after 7-d exposure ($p < 0.05$). According to the kinetics results, this phenomenon appeared on the 3rd day at the latest (Fig. 2**e & f**). Furthermore, the muscle weight changes of zebrafish after exposure would give a direct and visual evidence of muscle content decrease (**Fig. S2**). It showed that the muscle weight decreased gradually with microplastic concentration increase on the whole, and exhibited significant differences when microplastic concentrations higher than 10 mg/L.

3.3. Muscle metabolic analysis

By measuring the relative amounts of metabolites in the muscle tissues, we screened out the metabolites and pathways that showed differential expressions (Fig. 3). We also found that two important metabolites of glucose and acetaldehyde in the Glycolysis pathway were decreased in the microplastic group. As for the glucose content in the microplastic group (relative value = 129.8 ± 38.5) has decreased by 50% compared to control (258.3 ± 15.3) after 7-d exposure ($p = 0.006$) (Fig. 4).

The total amount of amino acids increased by 7% in the microplastic group compared to the control, which is probably because that the catabolism of proteins may have been increased. The detailed changes in the amino acids can be found in **Table S1**.

The ratio of unsaturated fatty acids (UFA) to saturated fatty acids (SFA) has been used as an important indicator for fatty acids composition alteration (Chen et al. 2016). The UFA:SFA ratios were 2.9 ± 0.4 and 2.8 ± 0.9 for the control and microplastic groups respectively, which suggests that the fatty acids composition did not seem to be affected after 1 mg/L microplastic exposure. The detailed changes in the amino acids can be found in **Table S2**.

Moreover, in view of steroids alteration, the contents of estrogen (estradiol), progestogens (11-dehydrocoricosterone), and glucocorticoids (cortisol, aldosterone) increased significantly in the microplastic group ($p < 0.05$) (**Table S3**).

3.4. Microplastic leads to behavioural alteration in zebrafish

We found the locomotion ability of fish after microplastic exposure had increased. For the females, the moved distance was 763 ± 307 cm within 10 min for the control, whereas it significantly increased in the microplastic group with 1801 ± 116 cm by 1.4-fold ($p < 0.05$) (Fig. 5a). Similar phenomenon was observed for the male fish, the swimming distance had increased 1.3-fold to 1896 ± 115 cm (in the microplastic group) than that of control ($p < 0.05$) (Fig. 5b). Furthermore, the swimming status of zebrafish in the microplastic group also changed significantly by prolonging manic and active states significantly to 1.3-2.9 folds and 1.6-2.0 folds compared to the control ($p < 0.05$), respectively. Meanwhile, the static states for zebrafish after microplastic exposure had decreased by 66-83% than that of control significantly ($p < 0.05$) (Fig. 5**c&d**).

3.5. Oxidative stress

All the enzymatic activities of CAT, SOD, and GPx in the liver did not show significant differences after 1 mg/L of microplastic exposure for 7 days (**Fig. S3**). For the reduced form of GSH, though its content was significantly lower than the control group on day 1 which may be due to the transient stress response at the beginning, its content returned to the normal state during the subsequent exposure. GSH is an important redox buffer inside cells, maintaining the normal reduced state (Farooqui and Farooqui 2011). The content of GSH in the muscle also did not show significant differences after 7 d exposure (Fig. 3), which is in accordance with phenomenon that no oxidative damage was found as shown in **Fig. S3**.

4. Discussion

4.1. Uptake of microplastics in different tissues

It is reasonable that microplastic can be accumulated in GIT and gill of the zebrafish, because these two tissues had direct access to

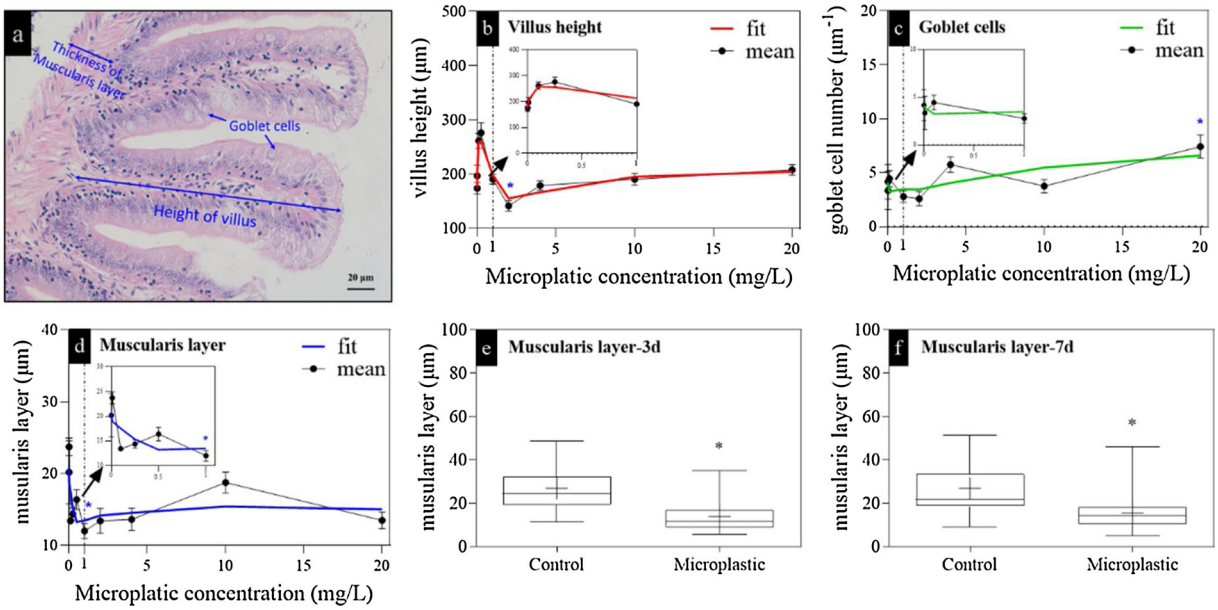

Fig. 2. Histological changes in the gastrointestinal tract (GIT). **(a)** detailed demonstration of histological index; (**b**) villus height (μm), (**c**) number of goblet cells, and (**d**) muscularis mucosal layer thickness (μm) changes with microplastic concentration increasing. Especial focuses on the muscularis layer thickness on day 3 (**e**) and day 7 (**f**) after 1 mg/L microplastics (equals to 1.45×10^4 particles/mL) exposure. * represents significant differences from that of control.

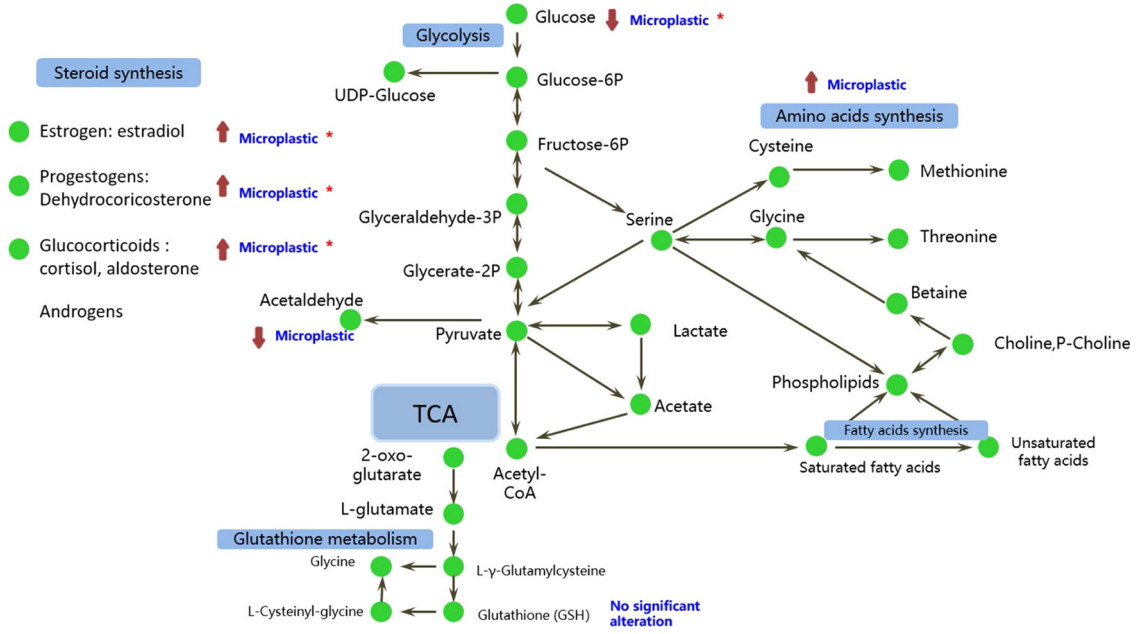

Fig. 3. Schematic illustration of the muscle metabolic pathways related to the energy reserve in the zebrafish muscle tissue. * besides arrows represent significant differences from that of control, otherwise there were no significant differences.

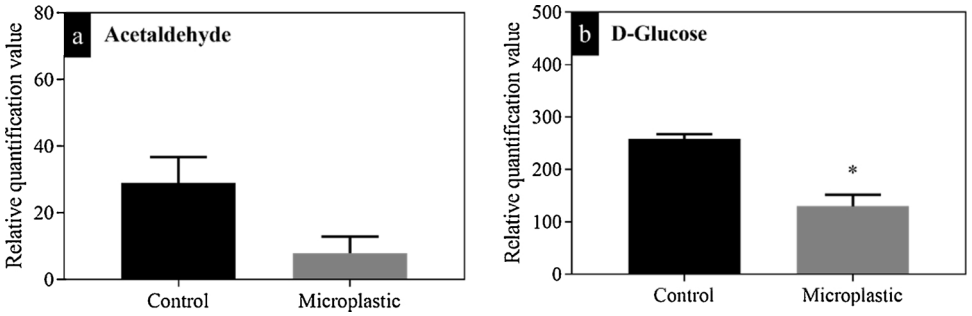

Fig. 4. Relative metabolite values of (**a**) acetaldehyde and (**b**) D-glucose in the Glycolysis/Gluconeogenesis pathway. * represents significant differences from that of control.

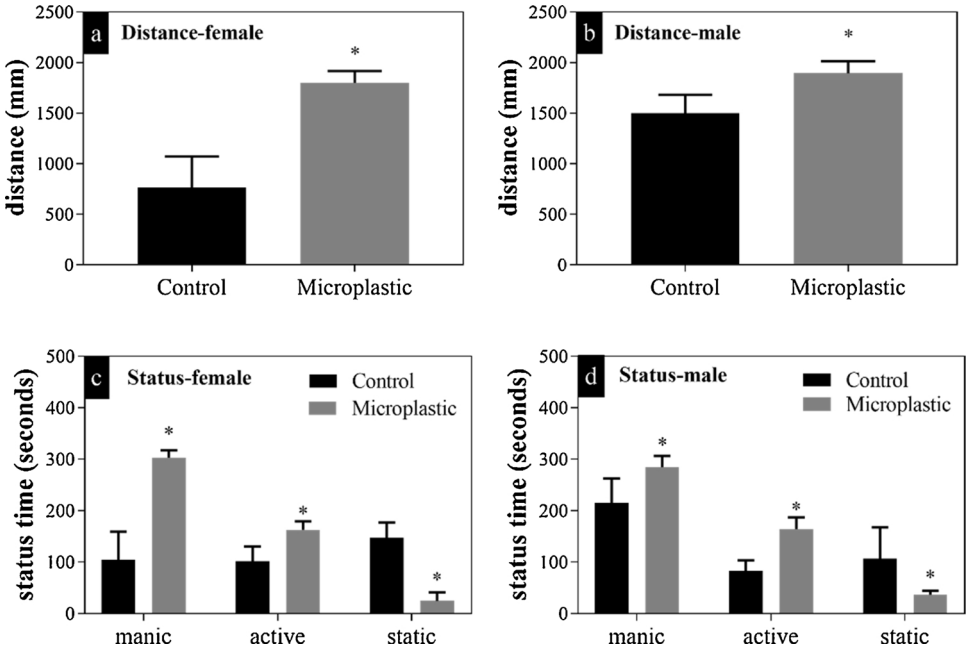

Fig. 5. Behavioural alteration in adult zebrafish after 7 d exposure. **(a)** moved distance within 10 min for the female zebrafish; **(b)** moved distance within 10 min for the male zebrafish; **(c)** time of different swimming status for the female zebrafish; **(d)** time of different swimming status for the male zebrafish. * represents significant differences from that of control.

microplastics during aqueous exposure (Fig. 1). The microplastic uptake concentrations in the GIT increased rapidly initially and then slowed down to approach a relative plateau state with microplastic concentrations increasing. However, there was no significant regularity for the microplastic accumulation in the gill tissue. It is probable that microplastics can adhere to gill tissues but cannot stick firmly. The loosely associated microplastics may fall from and up onto gills due to the variation of organism conditions, such as the respiratory movements of the fish. This way may be similar to the microplastic's adherence to mussel's foot filaments (Kolandhasamy et al. 2018). Similarly, (Watts et al. 2014) have also found that the residence of microplastics in gills can greatly vary among different crabs (*Carcinus maenas*), and even the left and right chamber of gills exhibited different microplastic uptake amounts. Therefore, this indicates that microplastics can be retained or adhered onto gills, but the microplastic residence time in the gill is an order of magnitude lower and relatively variable compared to the GIT.

No access of micro-sized microplastics to the brain and muscle tissues was observed in the present study. Micro-sized plastics have not been found in the muscle tissue, probably because that particles entering muscle tissue often need to penetrate the circulatory system first, which have only been reported for nanoplastics (Browne et al. 2008). This phenomenon has also been corroborated by a field study (Su et al. 2019). As for the brain tissue, the blood-brain barrier (BBB) creates an impenetrable obstacle for most substances to protect the microenvironment of the brain. (Pardridge 2001) stated that only lipophilic chemicals with a molecular weight smaller than 400 Da could easily cross the BBB. Researchers have also stated that fine particles can penetrate through and get into the brain, but usually within the size of 100 nm (Patel et al. 2012; Sela et al. 2015). Therefore, it is very likely that 5-μm microplastics would be blocked outside the brain.

4.2. No obvious villus damage or oxidative stress

We did not observe significant villus damage in the GIT in general, especially when the microplastic exposure concentration lower than 1 mg/L (equals to1.45×10^4 particles/mL) (Fig. 2). This result is different from a previous study which found microplastics could lead to cracking of villus in zebrafish intestines (Lei et al. 2018). Most of the tested microplastics used in that study were manually ground made in a mortar, thus producing sharper edges than the spherical round-shaped microplastics in our study. Besides, no obvious differences were observed for the goblet cell numbers at microplastic environmental relevant exposure concentrations. The main role of the goblet cells is to protect the mucous membranes by secreting mucins and large glycoproteins (Pelaseyed et al. 2014). Thus, this result indicates that microplastic aqueous exposure below 10 mg/L (equals to1.45×10^5 particles/mL) may not affect the immune system.

The oxidative damages have been reported as one of the main effects caused by microplastics (Chen et al. 2017; Jeong et al. 2016; Yu et al. 2018), so we measured several oxidative stress biomarkers, but no obvious effects were found for the enzymatic activities of CAT, SOD, and GPx (**Fig. S3**). In addition, as we know that the oxidative modification of fatty acids is one of the best described effects of reactive oxygen species (ROS) (Pamplona et al. 2002). Thus, the ratio of UFA to SFA in the microplastic group was similar to that of the control, which also implies that the fatty acids composition was largely unchanged (**Table S2**), and provides another evidence that there was no obvious oxidative stress after microplastic exposure.

4.3. Decreased thickness of muscularis layer

What deserves our attention is that the thickness of muscularis layer decreased significantly when the microplastic concentration reached 1 mg/L (equals to1.45×10^4 particles/mL) (Fig. 2d). Further observation revealed that microplastic exposure had led to fine structure alterations in the muscularis layer, including muscle dissociations and muscle morphological changes in the microplastic group. It clearly shows that the thickness of the muscularis layer was thick and uniform, and the myocytes connected with each other firmly in the control group (Fig. 6a &b). However, the muscularis thickness decreased in the microplastic exposure group (Fig. 6c). More interestingly, the intercellular space between myocytes can be clearly observed, which is possibly due to the myocytes became thinner and loosely connected (Fig. 6d). The above evidence suggests that zebrafish were probably under energy reserve depletion after microplastic exposure. The energy depletion phenomenon has also been observed previously in the copepod *Centropages typicus* after 7.3 μm sized microplastics exposure (> 4000 m L^{-1}) (Cole et al. 2015), and the crab (*Carcinus maenas*) after 4-week feeding with 1% plastic (Watts et al. 2015).

One possible explanation is that the decrease of muscularis thickness in the microplastic group is probably due to the microplastics

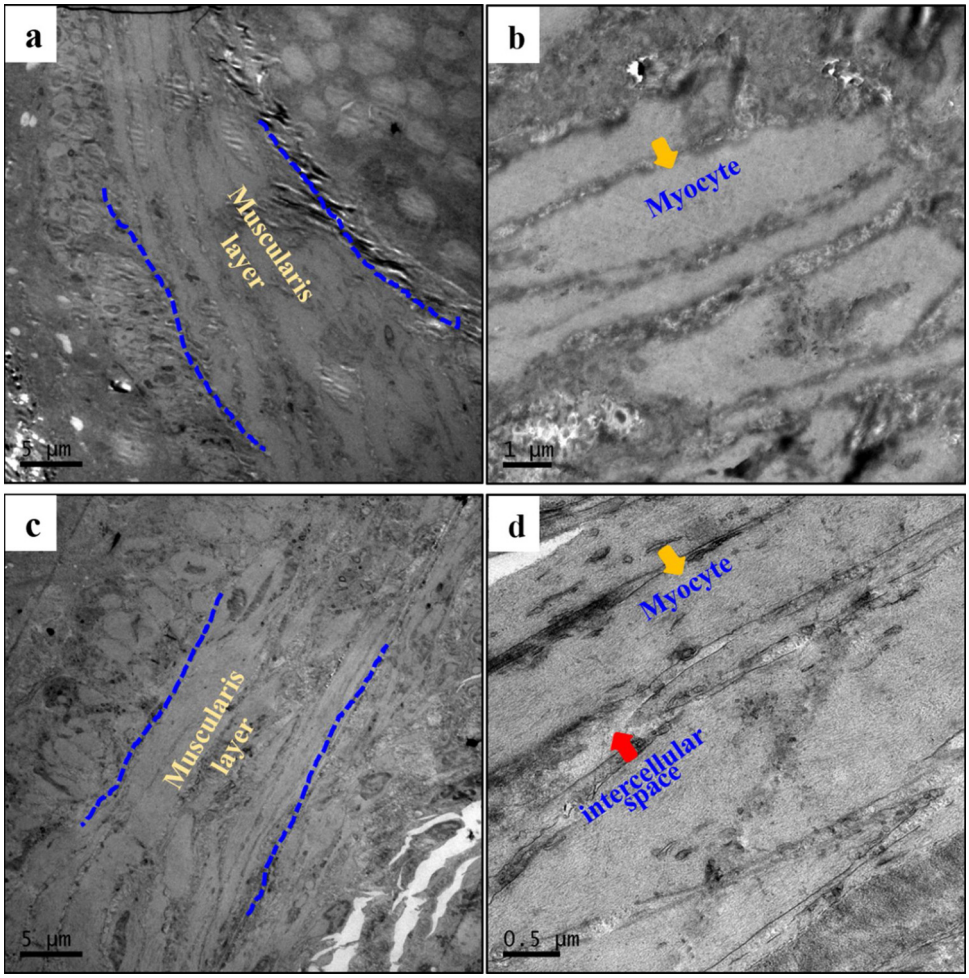

Fig. 6. Fine structures of zebrafish GIT observed under the cryo-TEM. **(a)** the muscularis layer of zebrafish from the control group; **(b)** the myocytes of the muscularis from the control group; **(c)** the muscularis layer of zebrafish from the 1 mg/L microplastic exposure group; **(d)** the myocytes of the muscularis from the 1 mg/L microplastic exposure group.

blockage in the GIT that decreased the food feeding. As microplastics had a longer intestinal residence time than the food (Bellehumeur et al. 2016; Watts et al. 2015), the retained microplastics can occupy a part of the intestinal space, resulting in a decrease in the actual food intake by fish. The digestive capacity of the zebrafish foregut would decline, and then its digestion and nutrient absorption may be affected, which deteriorated the supply of energy from food. Another possible explanation is that the presence of microplastic particles stimulated the hyperactivity of zebrafish, leading to increased swimming movements and energy consumption. In the case of a fixed food supply, the fish muscle amounts may decrease. The first hypothesis has been evidenced in Fig. 1, while the second one still needs to be verified.

4.4. Microplastic leads to hyperactivity in zebrafish

To test the above hypothesis, we carried out the zebrafish locomotion behavioural analysis. It was evidenced that zebrafish did appear hyperactivity after microplastic exposure for 7 days (Fig. 5). The locomotion hyperactivity phenomenon in zebrafish may be attributed to multiple factors. According to previous researches, the hyperactivity behaviour of zebrafish after particulate pollutants exposure was mainly attributed to the oxidative stress effect (Chen et al. 2014; González et al. 2018). However, the microplastics (5 μm) used in this study was much larger than nanoparticles and the oxidative damage phenomenon was not obvious (**Fig. S3**).

A reasonable explanation is that the prolonged manic and active states of fish may be caused by particle stimulation. It has been reported that adult zebrafish became more active after exposure to total particulate matter of cigarette smokes (≥ 0.3 μm), and this kind of effect was not caused by nicotine in tobacco (Massarsky et al. 2015; Massarsky et al. 2018). Furthermore, the metabolomics result also showed that the estrogen 17 β-estradiol (E2) has increased significantly (**Table S3**), which could be another cause of hyperactivity, because E2 has been reported to stimulate the body's ability to accelerate movement due to neurodevelopmental disorder with upregulation of genesis within the hypothalamus brain region (Kinch et al. 2015; Saili et al. 2013). Through the above analysis, it shows that the zebrafish hyperactivity after microplastic exposure was not attributed to oxidative stress, but very likely due to the stimulation of the micro-sized particles.

It is interesting and noteworthy that our former study has found that nanoplastics (50 nm polystyrene) exposure could lead to 5dpf zebrafish larvae's hypoactivity, whereas we found an opposite phenomenon in zebrafish adults after exposure to microplastics (5 μm polystyrene) here. The possible explanations are as follows. First, nanoplastics can enter the circulatory system of organisms, and can subsequently affect the neural system and body development of zebrafish larvae (Browne et al. 2008; Chen et al. 2017). However, microplastics can only be found in the gill and gut tissues of zebrafish adults. Therefore, the toxicological mode of action between micro- and nanoplastics are not the same. Second, the hypoactivity occurred in fish larvae were mainly due to their reduced body length and oxidative damage (Chen et al. 2017). But in the present study, the body lengths of zebrafish adults were

almost not changed as they have already grown up. Besides, no obvious oxidative damage occurred in the adult zebrafish after microplastic exposure.

4.5. Fish energy depletion

In the case of a fixed food supply, excessive movements of zebrafish may lead to energy depletion. Therefore, we further analyzed whether the metabolite contents in the muscle tissue of zebrafish has been changed. We found that two important metabolites of glucose and acetaldehyde decreased after microplastic exposure (Fig. 4). In muscle, the process of glycolysis is the main energy-providing process, in which glucose is broken down into lactic acid and ATP is produced. The decline in glucose, which is a key energetic substance, was likely to be the direct cause of acetaldehyde content decline. Under anaerobic conditions of the muscle, glucose can be metabolized into pyruvate and acetaldehyde with the presence of the pyruvate dehydrogenase complex and alcohol dehydrogenase (van Waarde et al., 1983).

In addition, the slight increase of total amino acid contents (by 7%) may also due to the shortage of energy-supplying substances (Fig. 3, **Table** S1). Increased amounts of amino acids may be attributed to increased proteins catabolism for energy generation. The energy generation process breaks down protein and releases free amino acids (Fu et al. 2019). Other studies have also shown an elevation of amino acids in polystyrene nanoplastics treated bronchus epithelial cells, which was probably because polystyrene particle autophagy process led to protein breakdown to utilize amino acids for cellular fueling (Lim et al. 2019). Therefore, the excessive swimming movement of zebrafish have caused a decrease in the content of energy-supplying substances, suggesting that there can be energy depletion occurred in zebrafish after microplastic exposure.

5. Conclusions

In the present study, we have found that micro-sized microplastics were mainly accumulated in the GIT and gill tissues. At environmentally relevant microplastic exposure concentrations, there was no significant villus damage occurred. However, the thickness of muscularis layers became thinner, which is probably due to the behavioural hyperactivity caused by the stimulation effects of microplastics, together with the lack of energy-supplying substances in the case of a fixed food supply. This study presents new findings about microplastic's effects to aquatic organisms, especially on the behavioural alteration, which is worthy of extensive attention in the future microplastic ecotoxicological studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Qiqing Chen: Data curation, Methodology, Investigation, Writing - original draft. **Carina Lackmann:** Data curation, Investigation, Writing - original draft. **Weiye Wang:** Conceptualization, Investigation. **Thomas-Benjamin Seiler:** Writing - review & editing. **Henner Hollert:** Writing - review & editing. **Huahong Shi:** Writing - review & editing, Supervision.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [21806038], Shanghai Pujiang Talent Program [18PJ1403400], East China Normal University SKLEC Open Research Fund [SKLEC-KF201807], and Shanghai Jiao Tong University Xinhua Hospital Open Research Fund [CEH201807 1].

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105521.

References

Anbumani, S., Kakkar, P., 2018. Ecotoxicological effects of microplastics on biota: a review. Environ. Sci. Pollut. Res. 25, 14373–14396.
Au, S.Y., Lee, C.M., Weinstein, J.E., van den Hurk, P., Klaine, S.J., 2017. Trophic transfer of microplastics in aquatic ecosystems: identifying critical research needs. Integr. Environ. Assess. Manag. 13, 505–509.
Barboza, L.G.A., Vieira, L.R., Branco, V., Figueiredo, N., Carvalho, F., Carvalho, C., Guilhermino, L., 2018. Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, *Dicentrarchus labrax* (Linnaeus, 1758). Aquat. Toxicol. (195), 49–57.
Bellehumeur, K., Lapointe, D., Cooke, S.J., Moon, T.W., 2016. Exposure to sublethal levels of PCB-126 impacts fuel metabolism and swimming performance in rainbow trout. Comp. Biochem. Phys. B 199, 97–104.
Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., Thompson, R.C.I., 2008. ngested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). Environ. Sci. Technol. 42, 5026–5031.
Chapron, L., Peru, E., Engler, A., Ghiglione, J., Meistertzheim, A., Pruski, A., Purser, A., Vétion, G., Galand, P., Lartaud, F., 2018. Macro-and microplastics affect cold-water corals growth, feeding and behaviour. Sci. Rep-UK 8, 1–8.
Chen, Q., Allgeier, A., Yin, D., Hollert, H., 2019. Leaching of endocrine disrupting chemicals from marine microplastics and mesoplastics under common life stress conditions. Environ. Int. 130, 104938.
Chen, Q., Gundlach, M., Yang, S., Jiang, J., Velki, M., Yin, D., Hollert, H., 2017. Quantitative investigation of the mechanisms of microplastics and nanoplastics toward zebrafish larvae locomotor activity. Sci. Total Environ. 584, 1022–1031.
Chen, Q., Hu, X., Wang, R., Yuan, J., Yin, D., 2016. Fullerene inhibits benzo (a) pyrene Efflux from *Cyprinus carpio* hepatocytes by affecting cell membrane fluidity and P-glycoprotein expression. Aquat. Toxicol. 174, 36–45.
Chen, T.-H., Lin, C.-C., Meng, P.-J., 2014. Zinc oxide nanoparticles alter hatching and larval locomotor activity in zebrafish (*Danio rerio*). J. Hazard. Mater 277, 134–140.
Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., Wishart, D.S., Xia, J., 2018. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nucleic Acids Res. 46, W486–W494.
Cole, M., Lindeque, P., Fileman, E., Halsband, C., Galloway, T.S., 2015. The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod *Calanus helgolandicus*. Environ. Sci. Technol. 49, 1130–1137.
Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J., Galloway, T.S., 2013. Microplastic ingestion by zooplankton. Environ. Sci. Technol. 47, 6646–6655.
Davison, P., Asch, R.G., 2011. Plastic ingestion by mesopelagic fishes in the North Pacific Subtropical Gyre. Mar. Ecol. Prog. Ser. 432, 173–180.
Farooqui, T., Farooqui, A.A., 2011. Oxidative Stress in Vertebrates and Invertebrates: Molecular aspects of cell signaling edˆeds. John Wiley & Sons.
Fu, J., Gong, Z., Kelly, B.C., 2019. Metabolomic profiling of zebrafish (*Danio rerio*) embryos exposed to the antibacterial agent triclosan. Environ. Toxicol. Chem. 38, 240–249.
Gallo, F., Fossi, C., Weber, R., Santillo, D., Sousa, J., Ingram, I., Nadal, A., Romano, D., 2018. Marine litter plastics and microplastics and their toxic chemicals components: the need for urgent preventive measures. Environ. Sci. Eur. 30, 13.
González, E.A., Carty, D.R., Tran, F.D., Cole, A.M., Lein, P.J.D., 2018. evelopmental exposure to silver nanoparticles at environmentally relevant concentrations alters swimming behavior in zebrafish (*Danio rerio*). Environ. Toxicol. Chem. 37, 3018–3024.
Jabeen, K., Li, B., Chen, Q., Su, L., Wu, C., Hollert, H., Shi, H., 2018. Effects of virgin microplastics on goldfish (*Carassius auratus*). Chemosphere 213, 323–332.
Jabeen, K., Su, L., Li, J., Yang, D., Tong, C., Mu, J., Shi, H., 2017. Microplastics and mesoplastics in fish from coastal and fresh waters of China. Environ. Pollut. 221, 141–149.
Jeong, C.-B., Won, E.-J., Kang, H.-M., Lee, M.-C., Hwang, D.-S., Hwang, U.-K., Zhou, B., Souissi, S., Lee, S.-J., Lee, J.-S., 2016. Microplastic size-dependent toxicity, oxidative stress induction, and p-JNK and p-p38 activation in the monogonont rotifer (*Brachionus koreanus*). Environ. Sci. Technol. 50, 8849–8857.
Kinch, C.D., Ibhazehiebo, K., Jeong, J.-H., Habibi, H.R., Kurrasch, D.M., 2015. Low-dose exposure to bisphenol A and replacement bisphenol S induces precocious hypothalamic neurogenesis in embryonic zebrafish. P. Natl. Acad. Sci. USA 112, 1475–1480.
Kolandhasamy, P., Su, L., Li, J., Qu, X., Jabeen, K., Shi, H., 2018. Adherence of microplastics to soft tissue of mussels: a novel way to uptake microplastics beyond ingestion. Sci. Total Environ. 610, 635–640.
Legradi, J., Di Paolo, C., Kraak, M., Van Der Geest, H., Schymanski, E., Williams, A., Dingemans, M., Massei, R., Brack, W., Cousin, X., 2018. An ecotoxicological view on neurotoxicity assessment. Environ. Sci. Eur. 30, 46.
Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., Shi, H., Raley-Susman, K.M., He, D., 2018. Microplastic particles cause intestinal damage and other adverse effects in zebrafish *Danio rerio* and nematode *Caenorhabditis elegans*. Sci. Total Environ. 619, 1–8.

Li, H.-X., Ma, L.-S., Lin, L., Ni, Z.-X., Xu, X.-R., Shi, H.-H., Yan, Y., Zheng, G.-M., Rittschof, D., 2018. Microplastics in oysters *Saccostrea cucullata* along the Pearl River estuary, China. Environ. Pollut. 236, 619–625.
Li, J., Yang, D., Li, L., Jabeen, K., Shi, H., 2015. Microplastics in commercial bivalves from China. Environ. Pollut. 207, 190–195.
Lim, S.L., Ng, C.T., Zou, L., Lu, Y., Chen, J., Bay, B.H., Shen, H.-M., Ong, C.N., 2019. Targeted metabolomics reveals differential biological effects of nanoplastics and nanoZnO in human lung cells. Nanotoxicology 13, 1117–1132.
Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., Ding, L., Ren, H., 2016. Uptake and accumulation of polystyrene microplastics in zebrafish (*Danio rerio*) and toxic effects in liver. Environ. Sci. Technol. 50, 4054–4060.
Lusher, A., Mchugh, M., Thompson, R., 2013. Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. Mar. Pollut. Bull. 67, 94–99.
Massarsky, A., Jayasundara, N., Bailey, J.M., Oliveri, A.N., Levin, E.D., Prasad, G., Di Giulio, R.T., 2015. Teratogenic, bioenergetic, and behavioral effects of exposure to total particulate matter on early development of zebrafish (*Danio rerio*) are not mimicked by nicotine. Neurotoxicol. Teratol. 51, 77–88.
Massarsky, A., Jayasundara, N., Glazer, L., Levin, E.D., Prasad, G., Di Giulio, R.T., 2018. Outcomes of developmental exposure to total particulate matter from cigarette smoke in zebrafish (*Danio rerio*). Neurotoxicology 68, 101–114.
Morét-Ferguson, S., Law, K.L., Proskurowski, G., Murphy, E.K., Peacock, E.E., Reddy, C.M., 2010. The size, mass, and composition of plastic debris in the western North Atlantic Ocean. Mar. Pollut. Bull. 60, 1873–1878.
Neves, D., Sobral, P., Ferreira, J.L., Pereira, T., 2015. Ingestion of microplastics by commercial fish off the Portuguese coast. Mar. Pollut. Bull. 101, 119–126.
Oßmann, B.E., Sarau, G., Holtmannspötter, H., Pischetsrieder, M., Christiansen, S.H., Dicke, W., 2018. Small-sized microplastics and pigmented particles in bottled mineral water. Water Res. 141, 307–316.
Pamplona, R., Barja, G., PORTERO-OTÍN, M., 2002. Membrane fatty acid unsaturation, protection against oxidative stress, and maximum life span: a homeoviscous-longevity adaptation? Ann. NY Acad. Sci. 959, 475–490.
Pardridge, W.M., 2001. Crossing the blood–brain barrier: are we getting it right? Drug Discov. Today 6, 1–2.
Patel, T., Zhou, J., Piepmeier, J.M., Saltzman, W.M., 2012. Polymeric nanoparticles for drug delivery to the central nervous system. Adv. Drug Deliver. Rev. 64, 701–705.
Pelaseyed, T., Bergström, J.H., Gustafsson, J.K., Ermund, A., Birchenough, G.M., Schütte, A., van der Post, S., Svensson, F., Rodríguez-Piñeiro, A.M., Nyström, E.E., 2014. The mucus and mucins of the goblet cells and enterocytes provide the first defense line of the gastrointestinal tract and interact with the immune system. Immunol. Rev. 260, 8–20.
Press, C.M., Evensen, Ø, 1999. The morphology of the immune system in teleost fishes. Fish Shellfish Immun. 9, 309–318.
Qu, H., Ma, R., Barrett, H., Wang, B., Han, J., Wang, F., Chen, P., Wang, W., Peng, G., Yu, G., 2020. How microplastics affect chiral illicit drug methamphetamine in aquatic food chain? From green alga (*Chlorella pyrenoidosa*) to freshwater snail (*Cipangopaludian cathayensis*). Environ. Int. 136, 105480.
Reichert, J., Schellenberg, J., Schubert, P., Wilke, T., 2018. Responses of reef building corals to microplastic exposure. Environ. Pollut. 237, 955–960.
Rochman, C.M., Hoh, E., Kurobe, T., Teh, S.J., 2013. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. Sci. Rep-UK 3, 3263.
Saili, K.S., Tilton, S.C., Waters, K.M., Tanguay, R.L., 2013. Global gene expression analysis reveals pathway differences between teratogenic and non-teratogenic exposure concentrations of bisphenol A and 17β-estradiol in embryonic zebrafish. Reprod. Toxicol. 38, 89–101.
Sela, H., Cohen, H., Elia, P., Zach, R., Karpas, Z., Zeiri, Y., 2015. Spontaneous penetration of gold nanoparticles through the blood brain barrier (BBB). J. Nanobiotechnol. 13, 71.
Su, L., Deng, H., Li, B., Chen, Q., Pettigrove, V., Wu, C., Shi, H., 2019. The occurrence of microplastic in specific organs in commercially caught fishes from coast and estuary area of east China. J. Hazard. Mater 365, 716–724.
Sun, X., Liang, J., Zhu, M., Zhao, Y., Zhang, B., 2018. Microplastics in seawater and zooplankton from the Yellow Sea. Environ. Pollut. 242, 585–595.
van Waarde, A., van den Thillart, G., Kesbeke, F., 1983. Anaerobic energy metabolism of the European eel, *Anguilla anguilla* L. Comp. Biochem. Phys. B 149, 469–475.
Vendel, A., Bessa, F., Alves, V., Amorim, A., Patrício, J., Palma, A., 2017. Widespread microplastic ingestion by fish assemblages in tropical estuaries subjected to anthropogenic pressures. Mar. Pollut. Bull. 117, 448–455.
Watts, A.J., Lewis, C., Goodhead, R.M., Beckett, S.J., Moger, J., Tyler, C.R., Galloway, T.S., 2014. Uptake and retention of microplastics by the shore crab *Carcinus maenas*. Environ. Sci. Technol. 48, 8823–8830.
Watts, A.J., Urbina, M.A., Corr, S., Lewis, C., Galloway, T.S.I., 2015. ngestion of plastic microfibers by the crab *Carcinus maenas* and its effect on food consumption and energy balance. Environ. Sci. Technol. 49, 14597–14604.
Wright, S.L., Rowe, D., Thompson, R.C., Galloway, T.S., 2013. Microplastic ingestion decreases energy reserves in marine worms. Curr. Biol. 23, R1031–R1033.
Yu, P., Liu, Z., Wu, D., Chen, M., Lv, W., Zhao, Y., 2018. Accumulation of polystyrene microplastics in juvenile *Eriocheir sinensis* and oxidative stress effects in the liver. Aquat. Toxicol. 200, 28–36.
Zhang, J., Wang, L., Kannan, K., 2020. Microplastics in house dust from 12 countries and associated human exposure. Environ. Int. 134, 105314.
Zuccarello, P., Ferrante, M., Cristaldi, A., Copat, C., Grasso, A., Sangregorio, D., Fiore, M., Conti, G.O., 2019. Exposure to microplastics (< 10 μm) associated to plastic bottles mineral water consumption: the first quantitative study. Water Res. 157, 365–371.