



## Polystyrene microplastics alter the trophic transfer and biotoxicity of fluoxetine in an aquatic food chain

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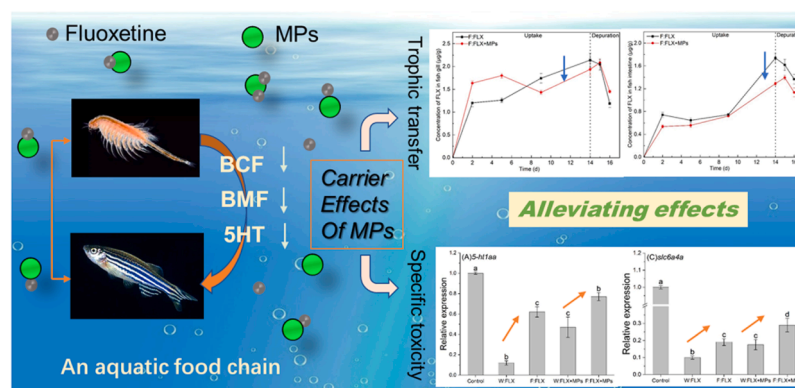
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### HIGHLIGHTS

- MPs altered the accumulation of fluoxetine in different trophic organisms.
- Fluoxetine exhibited transfer in the food chain but did not undergo biomagnification.
- MPs inhibited the trophic transfer of fluoxetine in the food chain.
- The specific biotoxicity induced by fluoxetine in fish was alleviated by MPs.

### GRAPHICAL ABSTRACT



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### ABSTRACT

Microplastics (MPs) and fluoxetine are ubiquitous emerging pollutants in aquatic environments that may interact with each other due to the carrier effects of MPs, posing unpredictable risks to non-target organisms. However, limited studies have focused on the carrier effects of MPs in the aquatic food chain. This study evaluated the influences of polystyrene MPs on the trophic transfer and biotoxicity of fluoxetine in a simple food chain composed of brine shrimp (*Artemia nauplii*) and zebrafish (*Danio rerio*). The finding reveals that carrier effects of MPs enhanced the accumulation of waterborne fluoxetine in brine shrimp, but suppressed that in zebrafish due to the distinct retention times. The accumulated fluoxetine in shrimp was further transferred to fish through the food chain, which was alleviated by MPs due to their cleaning effects. In addition, the specific neurotransmission biotoxicity in fish induced by fluoxetine was mitigated by MPs, whilst the oxidative damage, apoptosis, and immune responses in zebrafish were reversely enhanced by MPs due to the stimulating effect. These findings highlight the alleviating effects of MPs on the trophic transfer and specific biotoxicity of fluoxetine in the food

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chain, providing new insights into the carrier effects of MPs in aquatic environments in the context of increasing global MP pollution.

## 1. Introduction

The mass production and extensive use of plastic products have resulted in the 21st century being referred to as the “plastic century” [1]. Due to the increasing application and inappropriate disposal of plastic products, their wastes were continuously released into aquatic environments with detected concentrations ranging from  $\mu\text{g/L}$  to  $\text{mg/L}$  [2, 3]. Under various biological, chemical, and/or mechanical forces, these plastic wastes were gradually broken down and fragmented into microplastics (MPs) with a particle size smaller than 5 mm [4].

In aquatic environments, MPs are readily ingested by various aquatic organisms due to their small sizes. This ingestion may hinder growth, disrupt predation behavior, and induce histopathological damage in non-target organisms [5,6]. In addition, the complex food webs may further transfer the accumulated MPs from organisms at lower trophic levels to top predators, finally entering the human body [7]. In fact, MPs have been detected in different human bodies, including lungs, breast milk, blood, and placenta [8]. Hence, a deeper understanding of MP transfer in the food chain is required. Sarker et al. [9] found that the accumulation of MPs in organisms in the Sundarbans mangrove forest increased with trophic levels, indicating a transfer and biomagnification of MPs in successive trophic levels. Li et al. [10] also confirmed the transfer of three polystyrene MPs in the food chain including *Chlorella pyrenoidosa*, *Daphnia magna*, and zebrafish (*Danio rerio*). Moreover, MPs have been regarded as the carriers of different pollutants due to their small sizes and large specific surface areas, capable of adsorbing pollutants from surrounding environments, thus facilitating or alleviating the accumulation of co-existing pollutants in living organisms [11].

As a result, there is a need for a thorough understanding of the carrier effects of MPs on co-existing pollutants in aquatic environments, particularly on the trophic transfer in the aquatic food chain. Batel et al. [12] first reported that the presence of polyethylene (PE) MPs could increase the transfer of the co-existing benzo(a)pyrene (BaP) in the food chain between brine shrimp (*Artemia nauplii*) and zebrafish, which did not activate the activity of cytochrome P4501A in zebrafish. Subsequently, Cousin et al. [13] reported that the *Paramecium* and brine shrimp could ingest BaP via the spiked MPs, and effectively transfer it to the zebrafish and marine medaka (*Oryzias melastigma*) larvae, giving rise to whole-life exposure. In a food chain with green algae (*Chlorella pyrenoidosa*) and snails (*Cipangopaludian cathayensis*), Qu et al. [14] found that PE MPs significantly enhanced the transfer of the co-existing methamphetamine to snails, with a 1.42-fold increase in the biomagnification factor (BMF). Furthermore, the carrier effects of MPs facilitated the transfer of polycyclic aromatic hydrocarbons in a food chain with *Daphnia magna* and zebrafish but failed in the food chain with *Chironomus riparius* larvae and zebrafish [15]. These different results highlight that the knowledge regarding the trophic transfer of MPs and associated pollutants in the food chain is still poorly understood.

Among the co-existing pollutants in aquatic environments, the presence of pharmaceuticals constitutes a cause for great public concern due to their specific design for humans. Antidepressants, as one of the most widely prescribed pharmaceuticals, have been largely released into various aquatic systems due to the huge consumption to treat depression over the past decades, thus threatening the reproduction, development, behavior, and survival of different aquatic organisms [16]. Hence, antidepressants and MPs have been simultaneously detected in the same aquatic environments [17,18]. In addition, recent studies suggested that the presence of MPs could interact with different antidepressants in aquatic environments, thereby altering the accumulation, biotransformation, and toxicity of the co-existing antidepressants in aquatic ecosystems [19–22]. However, relatively little attention has been paid to

the influences of MPs on the trophic transfer of the interacted antidepressants in the aquatic food chain.

Fluoxetine, an antidepressant, has been widely used in clinical and daily treatments to alleviate depression by selectively inhibiting the reuptake of serotonin (5-HT). Since 5-HT plays a crucial role in regulating neuroendocrine pathways and hormonal levels in aquatic organisms, waterborne fluoxetine has been shown to alter the feeding and swimming behavior of aquatic organisms [23], leading to disruption in physiological and reproductive development [24]. In addition, a trophic transfer of fluoxetine has been observed in a field study, implying an increasing threat to higher trophic levels and even humans [17]. Hence, fluoxetine has been referred to as one of the most toxic pollutants in aquatic environments [25]. Owing to the increasing release, both fluoxetine and MPs have been demonstrated presence in the same river waters [17,18]. Since the presence of MPs could rapidly adsorb and desorb the co-existing fluoxetine in different aquatic environments [22], their interaction may pose unpredictable risks to different aquatic organisms. Nevertheless, to the best of our knowledge, there is still a knowledge gap concerning the transfer process of MPs and associated fluoxetine in the aquatic food chain.

Therefore, this study focused on the influences of MPs on the trophic transfer of fluoxetine in a simple aquatic food chain composed of brine shrimp (*Artemia nauplii*) and zebrafish and the related alterations in the biotoxicity for zebrafish after waterborne and foodborne fluoxetine exposure in the presence of MPs. Both brine shrimp and zebrafish are widely used as model organisms for toxicology research. Among the MPs, polystyrene MPs were selected as target MPs, which is one of the five primary high-production plastics in the world [26] and is also one of the components of plastic debris commonly observed in aquatic environments [27]. The main aims of this study are to investigate (1) the accumulation and clearance of fluoxetine in brine shrimp and zebrafish following waterborne exposure with and without polystyrene MPs; (2) the trophic transfer of fluoxetine in the aquatic food chain in the presence of polystyrene MPs; and (3) the alterations in the biotoxicity of high-trophic level fish, including the serotonergic neurotransmission, oxidative stress, apoptosis, and immunity. The results will expand our understanding of the carrier effects of MPs in aquatic food chains with co-existing pollutants.

## 2. Materials and methods

### 2.1. Materials

Green fluorescent PS MPs (1  $\mu\text{m}$ ) with an excitation of 488 nm and an emission of 518 nm were obtained from Da'E Scientific (Tianjin, China). The stocks of MPs were prepared in deionized water (10 mg/mL,  $4.5 \times 10^{10}$  items/mL). The working solution of MPs (10 mg/L) was prepared in different waters by sequential dilution with ultrasonication for 30 min. Following the manufacturer's instructions, the MPs were stained with the dye of 4-chloro-7-nitrobenzofurazan, which was contained within the polymer using the swelling method. The fluorescence intensity of MPs solution filtrate was similar to that of the clean solution. Hence, the leaching of dye from MPs during the exposure periods could be ignored, as well as any related toxicity caused by the dye. The MPs were stored at 4 °C in the dark and sonicated for 30 min before each use. The images of MPs were obtained with scanning electron microscopy (Hitachi, Tokyo, Japan) and are listed in Fig. S1. The hydrodynamic diameter and zeta potential of MPs in different test waters were measured using a Zetasizer Nano ZS (Malvern, UK), and the results are listed in Fig. S1. The measured diameter of MPs was 995 nm and 1280 nm in deionized water and artificial seawater, respectively (Fig. S1).

Fluoxetine hydrochloride (CAS 56296–78-7, purity >98%) was purchased from Sigma-Aldrich (Shanghai, China) and used in this study. Fluoxetine-d5 hydrochloride (CAS 1173020–43-3) served as an internal standard and was obtained from Toronto Research Chemicals (Toronto, Canada). All chemicals were prepared with ultrapure water and stored at 4 °C in the dark. All solutions were prepared for 12 h before each exposure.

## 2.2. Test organisms

Two species, the brine shrimp and zebrafish, were used as the test organisms in this study. In previous studies, both brine shrimp and zebrafish are widely used as model species in laboratory toxicity to represent the basic consumers of invertebrates and the high consumers of fish in aquatic environments [13,28]. In addition, their predator-prey process also reflected the basic trophic transfer relationship for a pollutant from primary consumer to secondary consumer and has been used as a simple food chain in different research [12,29,30].

The brine shrimp cysts were obtained from Nanjing Ezerinka Biotechnology Co., Ltd, China, and stored at –20 °C until use. One gram of cysts was incubated with vigorous aeration in an incubator with 1 L artificial seawater at 26 °C under continuous illumination. The artificial seawater (30 ± 1‰ salinity) was prepared using commercial sea salt obtained from Ezerinka Biotechnology, Nanjing, China. Hatched larvae were harvested and then maintained in fresh mediums for 24 h before each exposure. The temperature was kept at 26 ± 0.5 °C with a photoperiod of 12/12 h (light/dark). The pH value was kept at 7.5.

Adult male zebrafish of the AB strain (six months old, 4.4 ± 0.2 cm, 0.35 ± 0.05 g) were obtained from Nanjing Ezerinka Biotechnology Co., Ltd, China, and acclimated in the laboratory for at least two weeks. They were housed in a water circulation system with a temperature of 28 ± 0.5 °C and a photoperiod of 14/10 h (light/dark). The pH value was kept at 7.5. The dissolved oxygen (DO) level was 8.0 mg/L. Fish were fed twice a day at the rate of 6% of body weight with live brine shrimp. Feces and uneaten food were promptly removed.

## 2.3. Experimental design

### 2.3.1. Exposure conditions

In each exposure experiment, three treatments were included: control (test water), fluoxetine alone (100 µg/L FLX), and fluoxetine + MPs (100 µg/L FLX + 10 mg/L MPs). Each treatment was conducted with three replicates. The concentration of MPs was selected based on the maximum concentrations of MPs detected in the Yellow River estuary, China (6.96 mg/L), as well as the environmentally relevant threshold [3, 31]. The concentration of fluoxetine was selected based on the human therapeutic plasma concentration of fluoxetine (91 µg/L) [32] and our previous study [16]. All treatments were conducted under semi-static conditions, in which all exposure solutions were replaced daily at a replacement rate of 90% v/v to maintain a stable exposure concentration. The actual concentration of fluoxetine in each treatment was verified every day before and after each water change. Given that the bioavailability and bioaccumulation dynamics of fluoxetine were strongly influenced by water parameters, such as pH [16], the parameters of the exposure water in each tank were kept the same as those during the acclimatization period and checked every day before and after each water replacement. The water parameters were maintained as follows: pH, 7.5; DO, 8.0 mg/L; and hardness (CaCO<sub>3</sub>), 124.7 ± 5.2 mg/L. The dead organisms were removed promptly. All exposure experiments were approved by the Animal Ethics Committee of Hohai University.

### 2.3.2. Waterborne exposure experiment

At 24 h post-hatch, 500 brine shrimps (at 1 and 2 d stage) were selected under a stereoscopic microscope (Olympus SZX16, Japan) and exposed in a 2 L glass beaker with 1.5 L of exposure solutions (control,

fluoxetine alone, or in combination with MPs) prepared in artificial seawater (30 ± 1‰ salinity). Each treatment contained 1500 larvae. The exposure time was 24 h, followed by 12 h of clearance. All experiments were conducted in an incubator with a temperature of 26 °C, an illumination of 2000 Lux, and a photoperiod of 12/12 h (light/dark). The pH value was kept at 7.5. At the time of 4, 8, 12, and 24 h during exposure periods, and 2, 4, 8, and 12 h during clearance periods, 50 shrimps were sampled from each tank. After rinsing with ultrapure water for 30 s, all sampled shrimps were weighed and frozen in liquid nitrogen for further treatment. No food was provided for brine shrimp during waterborne exposure periods.

For zebrafish, approximately 60 healthy individuals were randomly exposed to different treatments (control, fluoxetine alone, or in combination with MPs) with three replicates. Each test vessel was a 50-L glass tank filled with 30 L of test solution. Based on the mean wet weight of the zebrafish, the biomass loading rate of 0.8 g/L was below the OECD 230 guideline recommendation (5 g/L). All zebrafish were maintained at 28 ± 0.5 °C with a 14 h light/10 h dark cycle. The pH value was kept at 7.5. The DO level was 8.0 mg/L. The exposure time was 14 d, followed by 48 h of clearance. At the time of 2, 5, 9, and 14 d during exposure periods, and 24 and 48 h during clearance periods, 8 fish were sampled from each tank. After rinsing for 30 s with ultrapure water, all sampled fish were disposed of on crushed ice. The brain, intestine, and gill tissues were collected and stored in liquid nitrogen for further treatment. During the exposure and clearance periods, zebrafish were moderately fed with brine shrimp before each water replacement.

### 2.3.3. Transfer experiment in the food chain

To investigate the transfer of fluoxetine in the aquatic food chain, the 24 h post-hatched brine shrimp were exposed to different solutions (control, fluoxetine alone, or in combination with MPs) for 24 h. After rinsing with ultrapure water for 30 s to clean the exoskeleton surface, the treated brine shrimp were fed to zebrafish. During exposure periods, 8 fish were collected on days 2, 5, 9, and 14. The remaining fish were fed with untreated brine shrimp for a 48-hour clearance exposure. Then, 8 fish were collected at the clearance times of 24 and 48 h. During the exposure and clearance periods, the pH value of different solutions was kept at 7.5. After rinsing, all collected fish were disposed of. The brain, intestine, and gill tissues of zebrafish were removed and stored in liquid nitrogen for further treatment.

## 2.4. Chemical analyses

The concentrations of fluoxetine in exposure solutions and biological samples were treated and determined according to the methods described by Yan et al. [16]. In brief, exposure solutions were extracted with a preprocessed Oasis MCX cartridge (150 mg, 6 cc, Waters, USA) and then eluted with 6 mL of methanol. The biological samples were extracted using acetonitrile and formic acid by sonication and further purified with the same MCX cartridge as water samples. The extracts were reconstituted with 1 mL methanol for further chemical analyses. Fluoxetine-d5 was used as the surrogate standard during chemical analyses.

Subsequently, the concentrations of fluoxetine in different samples were determined according to our previous study with a Waters Acquity ultra-high-performance liquid chromatography-tandem mass spectrometer (ACQUITY UPLC Xevo TQ, Waters, USA) with positive electrospray ionization [16]. The multiple response monitoring mode was used in fluoxetine detection. The limits of quantitation (LOQs) of fluoxetine in water was 10 ng/L, and those in biological samples were 0.05 µg/g. The recoveries of fluoxetine in different matrices ranged from 88.7 to 105.4%. Details can be found in the [Supplemental Materials](#).

In addition, the concentrations of MPs in brine shrimp and zebrafish intestine and gill tissues were determined according to the methods reported by Ding et al. [33] with some modifications. In brief, the biological samples were cleaned and digested with 10% KOH solutions in a

constant temperature oscillator for 24 h at 60 °C until the solution was clarified. Then, the quantification of MPs in the digested solution was performed using a fluorescence spectrophotometer (Hitachi F-7000, Japan), with excitation at 488 nm and emission at 518 nm. The external calibration curve was prepared using the stocks of fluorescent MPs.

### 2.5. Expression of functional genes

Given that exposure to fluoxetine may alter the neurotransmission, oxidative stress, apoptosis, and immunity in different aquatic organisms [16,34], genes related to these functions in zebrafish were tested, including serotonergic neurotransmission process in the brain (*5-ht1aa*, *5-ht2c*, *slc6a4a*), oxidative stress (*sod1*, *hsp70*), apoptosis (*bcl-2*, *bax*, and *caspase-3*), and immunity (*cox2*, *TNF- $\alpha$* , *il-10*, and *NF- $\kappa$ B*) in the intestine. The  $\beta$ -actin gene expression in different tissues was selected as the reference gene, as reported in our previous study [16]. Target gene expression was quantified using the quantitative real-time Polymerase Chain Reaction (qPCR).

Total RNA obtained from the brain and intestine tissues was isolated with a MiniBEST universal RNA extraction kit (TaKaRa, Dalian, China). After verifying the quality, the RNA was reversely transcribed to obtain the cDNA using an iScript cDNA synthesis kit (Bio-Rad, USA). The primer sequences for target genes are listed in the [Supplemental Materials](#). All qRT-PCR amplifications based on the SYBR Green reaction were conducted by Biozeron Biotechnology (Shanghai, China) using a Bio-Rad qRT-PCR system (CFX96 Touch, Bio-Rad, USA). The thermal cycling conditions were as follows: one cycle of 3 min at 95 °C; followed by 40 cycles of 15 s at 94 °C, 20 s at 65 °C, and 20 s at 72 °C. Each sample was run in triplicate. After completion of the qPCR amplification, the relative fold change of each gene was calculated based on the Ct method and presented as normalized values against the reference gene and the control treatment.

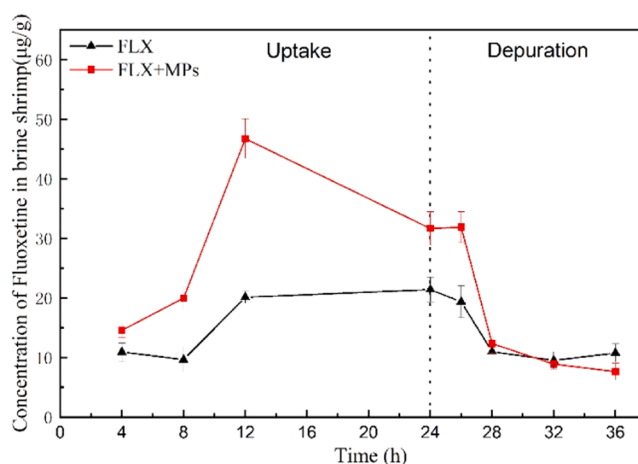
### 2.6. Statistical analyses

The kinetic parameters (uptake rate constant  $k_1$ , elimination rate constant  $k_2$ , bioconcentration factors BCF, and half-lives  $t_{1/2}$ ) of fluoxetine in brine shrimp and zebrafish following waterborne exposure were calculated based on a two-box kinetic model [35]. The kinetic parameters (uptake rate constant through food  $k_d$ , elimination rate constant through fecal excretion  $k_e$ , biomagnification factors BMF, and half-lives  $t_{1/2}$ ) of fluoxetine in brine shrimp and zebrafish following foodborne exposure were calculated following previous methods with some modifications [36,37]. Details are listed in the [Supplemental Materials](#).

Fluoxetine concentrations in different biological samples were calculated based on wet weight (ww). Statistical analyses were performed using SPSS 22.0 (IBM SPSS Statistics). Data are presented as the mean  $\pm$  standard deviation. After verifying normality and variance homogeneity using the Kolmogorov-Smirnov and Levene's test, data collected from different treatments were analyzed using one-way analysis of variance (ANOVA) and Dunnett's test.  $P < 0.05$  was defined as significant for all treatments.

## 3. Results and discussion

No fluoxetine was detected in the control treatment. In the fluoxetine-only treatment, the actual concentrations of fluoxetine were  $95 \pm 4$   $\mu$ g/L and reduced to  $85 \pm 3$   $\mu$ g/L in the presence of MPs. Since fluoxetine is recalcitrant to degradation over a short time [38], the decreased fluoxetine concentrations may be partially attributed to the absorption by MPs ([Fig. S2](#)). No mortality and abnormality were observed in brine shrimp and fish, indicating that no acute toxicity occurred in any treatments.



**Fig. 1.** Accumulation and clearance of fluoxetine in brine shrimp with/without MPs following waterborne exposure (W: waterborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).

### 3.1. Accumulation and elimination of fluoxetine in brine shrimp

The accumulation of and clearance of fluoxetine in brine shrimp are presented in [Fig. 1](#). After 24 h of accumulation, the fluoxetine concentration in brine shrimp reached 21.4  $\mu$ g/g with an equilibrium. In the presence of MPs, the equilibrium concentration was further enhanced by more than 2-fold, reaching 46.7  $\mu$ g/g. It indicates that the presence of MPs plays a positive carrier effect on the accumulation of fluoxetine in brine shrimp. During the clearance periods, more than 80% of the accumulated fluoxetine in brine shrimp was cleared within 4 h. When MPs were co-added, the high accumulation of fluoxetine in brine shrimp was rapidly eliminated to 7.61  $\mu$ g/g, which is consistent with the changes in the fluoxetine alone treatment. This finding suggests that the presence of MPs may not only enhance the uptake of fluoxetine by brine shrimp but also greatly enhance their clearance ability. Since MPs could absorb the surrounding fluoxetine in waters ([Fig S2](#)), the uptake of MPs may introduce more fluoxetine into brine shrimp. As the adsorbed fluoxetine could be largely and rapidly released from MPs in seawater and digestive fluid [22], the enhanced uptake and accumulation of fluoxetine in brine shrimp was expected, which is consistent with the increased accumulation of MPs in brine shrimp ([Fig S3](#)). In addition, the rapid clearance of MPs from brine shrimp ( $t_{1/2} = 0.15$  h) with high efficiency (93%) may also lead to rapid excretion of the adsorbed fluoxetine without time to desorption ([Fig S3](#)), thus resulting in a high clearance of fluoxetine from brine shrimp. Hence, the enhanced fluoxetine accumulation into and clearance from brine shrimp by the presence of MPs may be partially attributed to the rapid absorption and clearance of MPs from brine shrimp. The results of kinetic parameters also indicate that the co-occurrence of MPs significantly enhanced the rate constants of accumulation and clearance of fluoxetine in brine shrimp by 3.3 times and 2.1 times, respectively ([Table 1](#)). Ultimately, these enhancements led to an increase in the bioconcentration factor (BCF) of fluoxetine from 0.24 to 0.38. Similar carrier effects of MPs have been also found in previous studies, in which the concentrations and bioavailability of co-existing pollutants roxithromycin and benzophenone 3 in *Daphnia magna* were significantly enhanced by different MPs

**Table 1**

Kinetic parameters of fluoxetine in brine shrimp with/without MPs following waterborne exposure (W: waterborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).

Treatments	$k_1$ ( $\text{h}^{-1}$ )	$k_2$ ( $\text{h}^{-1}$ )	BCF (L/kg)	$T_{1/2}$ (h)	Clearance rate
FLX	0.015	0.063	0.238	11.00	83.7%
FLX + MPs	0.050	0.131	0.382	5.29	49.9%

[39,40]. Given that the MPs ingestion by zooplankton is rare [41], this enhancement may be attributed to the rapid release of the absorbed pollutants from MPs in zooplankton.

### 3.2. Accumulation of waterborne fluoxetine in zebrafish

Changes in fluoxetine accumulation and clearance in zebrafish intestine and gill tissues are listed in Fig. 2. In general, fluoxetine concentrations increased over time in zebrafish, which were slightly altered in gills but significantly reduced in intestines by the presence of MPs. In the presence of MPs, the fluoxetine concentrations in waters were decreased through absorption, which further lowered the fluoxetine accumulation in fish gills and intestines. Meanwhile, given that the desorption of fluoxetine from MPs was rare in fresh water and intestinal fluid [22], the fluoxetine desorption from MPs in gill and intestinal tissues may be negligible, suggesting that the absorbed fluoxetine may further eliminate with MPs and make little contribution to the fluoxetine accumulation in fish. Kinetics parameters also revealed that MPs markedly decreased the uptake rate constant of fluoxetine in intestines and gills when compared to the fluoxetine-alone treatment (Table 2). After 2 days of clearance, the accumulated concentrations of fluoxetine in zebrafish gills and intestines were significantly eliminated, but this decline was attenuated by the presence of MPs. Similarly, the elimination rate constant of fluoxetine in zebrafish gills and intestines was decreased by 98% and 65%, respectively, by the presence of MPs.

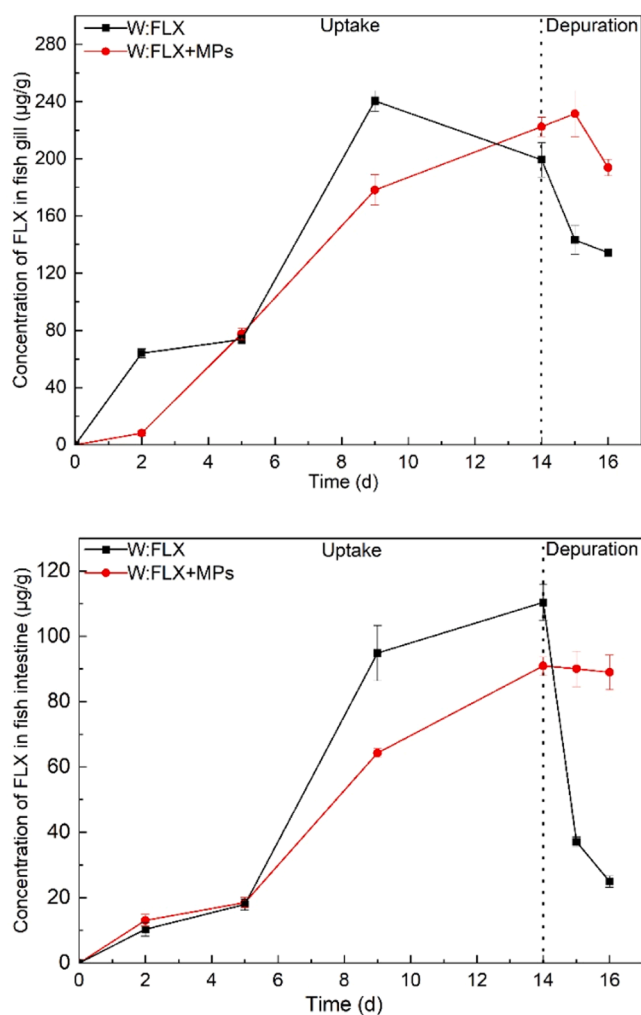


Fig. 2. Accumulation and clearance of fluoxetine in the zebrafish gill and intestine tissues with/without MPs following waterborne exposure (W: waterborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).

Table 2

Kinetic parameters of fluoxetine in different zebrafish tissues with/without MPs following waterborne exposure (W: waterborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).

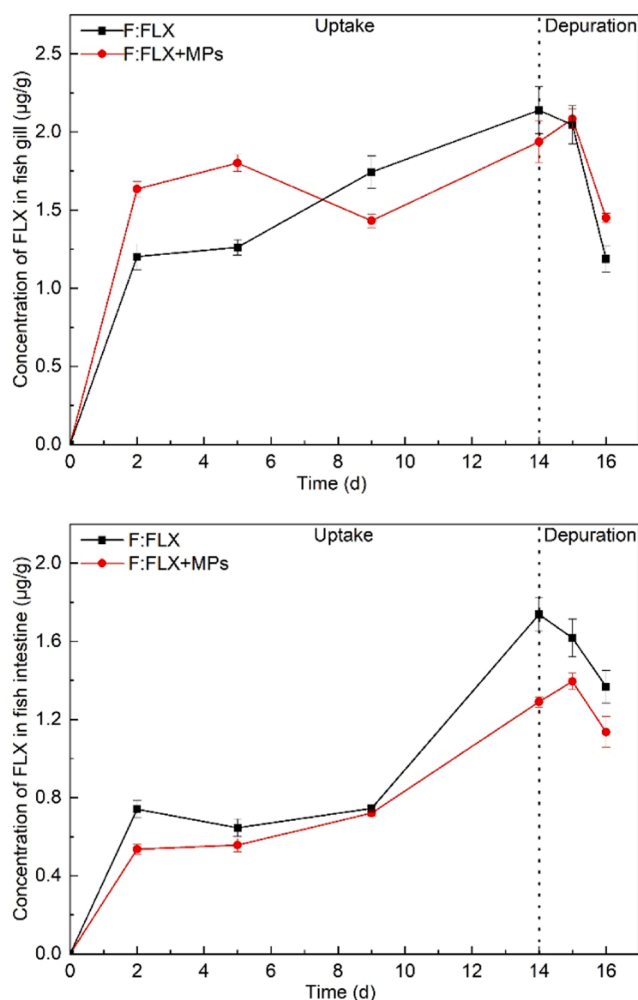
Treatments	Tissues	$k_1$ ( $d^{-1}$ )	$k_2$ ( $d^{-1}$ )	BCF (L/kg)	$T_{1/2}$ (d)	Clearance rate
FLX	intestine	3.7489	0.7439	5.04	0.93	83.7%
	gill	6.4089	0.1969	32.54	3.52	35.5%
FLX+MPs	intestine	0.0199	0.0107	1.86	64.78	2.1%
	gill	0.1038	0.0690	1.50	10.05	12.8%

Meanwhile, the clearance rate of MPs from zebrafish gills and intestines was 95% and 75% (Fig. S4), respectively, which is consistent with the clearance of fluoxetine from zebrafish. These results indicate that the presence of MPs may hinder both the accumulation and clearance of fluoxetine in zebrafish, leading to a significant reduction in fluoxetine bioaccumulation, especially in the intestines. This finding is supported by changes in the bioaccumulation factor (BCF) and the half-life of fluoxetine in zebrafish, in which the presence of MPs extended the half-life to approximately 60 days and decreased the BCF by nearly 4-fold. Inconsistent with this finding, Schell et al. [42] found that MPs reduced the bioconcentration of chlorpyrifos and hexachlorobenzene in zebrafish compared to the absence of MPs, suggesting a cleaning effect of MPs. This effect has been also proposed by modeling studies [43,44], emphasizing the different influences of MPs on the bioaccumulation of coexisting pollutants in aquatic organisms. The carrier effect of MPs could introduce a large number of pollutants to the fish gill and intestine tissues [45], but the short retention time of MPs in these tissues may lower the desorption of pollutants from MPs, and even inhibit the uptake and clearance of co-existing pollutants by affecting the physiological structure and metabolic function of fish tissues [46,47].

Compared with brine shrimp, zebrafish exhibited a higher uptake and a lower clearance of fluoxetine, leading to a stronger accumulation potential. The uptake pathway of waterborne pollutants by aquatic organisms partially occurs via gills and skin, which could directly go into the bloodstream [42]. The abundant capillaries of fish gills would therefore have a higher pollutant burden than that in brine shrimp. In this study, higher concentrations of fluoxetine were observed in the gill tissues of zebrafish than in the intestines. In addition, the uptake of waterborne pollutants from the intestine tissues could not be ignored, especially in the presence of particulate matter. Gouin et al. [44] suggested that the ingestion of MPs from the gastrointestinal tract of fish could decrease the body burden of waterborne pollutants by 20%. In the presence of MPs, the bioaccumulation of fluoxetine in brine shrimp was strongly enhanced but relieved in zebrafish. These inconsistent findings may be partially attributed to the different intestinal environments of the two organisms, including intestinal length, gastrointestinal fluids, and microbial environments. Kim et al. [48] confirmed that the gastrointestinal structure of organisms is a crucial factor influencing the clearance of MPs from organisms, thereby affecting the accumulation of co-existing pollutants. Our previous research also found that fish have a stronger clearance ability for MPs than shrimp [49], and the co-existing pollutants may not have sufficient time to be desorbed in fish intestines, weakening the accumulation of fluoxetine in fish. In addition, a higher desorption of fluoxetine from MPs was observed in the seawater than in freshwater [22], which may be partially attributed to the different accumulation of fluoxetine in brine shrimp and zebrafish caused by MPs.

### 3.3. Food chain transfer of fluoxetine

To investigate the trophic transfer of fluoxetine from brine shrimp to zebrafish, the fluoxetine concentrations in zebrafish after foodborne exposure and clearance were studied, and the changes in the gill and intestine tissues of fish are listed in Fig. 3. The concentration of fluoxetine markedly increased in different zebrafish tissues, both in the



**Fig. 3.** Accumulation and clearance of fluoxetine in the zebrafish gill and intestine tissues with/without MPs following foodborne exposure (F: foodborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).

**Table 3**

Kinetic parameters of fluoxetine in different zebrafish tissues with/without MPs following foodborne exposure (F: foodborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).

Treatments	Tissues	$k_d$ ( $d^{-1}$ )	$k_e$ ( $d^{-1}$ )	BMF	$T_{1/2}$ (d)	Clearance rate
FLX	intestine	0.0156	0.1226	0.064	5.65	21.3%
	gill	0.0063	0.2947	0.055	2.35	44.5%
FLX+MPs	intestine	0.0002	0.0640	0.028	10.83	12.0%
	gill	0.0024	0.1445	0.041	4.80	25.1%

fluoxetine alone and in combination with MPs treatments, when compared to the control. Kinetics parameters of fluoxetine in the food chain were subsequently calculated (Table 3). The results showed that the BMF values of fluoxetine in different tissues were consistently lower than 1 in all treatments, indicating the biomagnification of fluoxetine was not evident in this food chain. Similarly, Liao et al. [50] found that the emerging pollutant of di(2-ethylhexyl) phthalate was not biomagnified in the food chain from *Chlorella pyrenoidosa* or *Daphnia magna* to *Micropterus salmoides*. Our field study also confirmed that most pharmaceuticals present in river waters did not exhibit biomagnification along the aquatic trophic transfer, and the hydrophobicity of pharmaceuticals emerged as a key factor influencing their bioaccumulation and biomagnification [17].

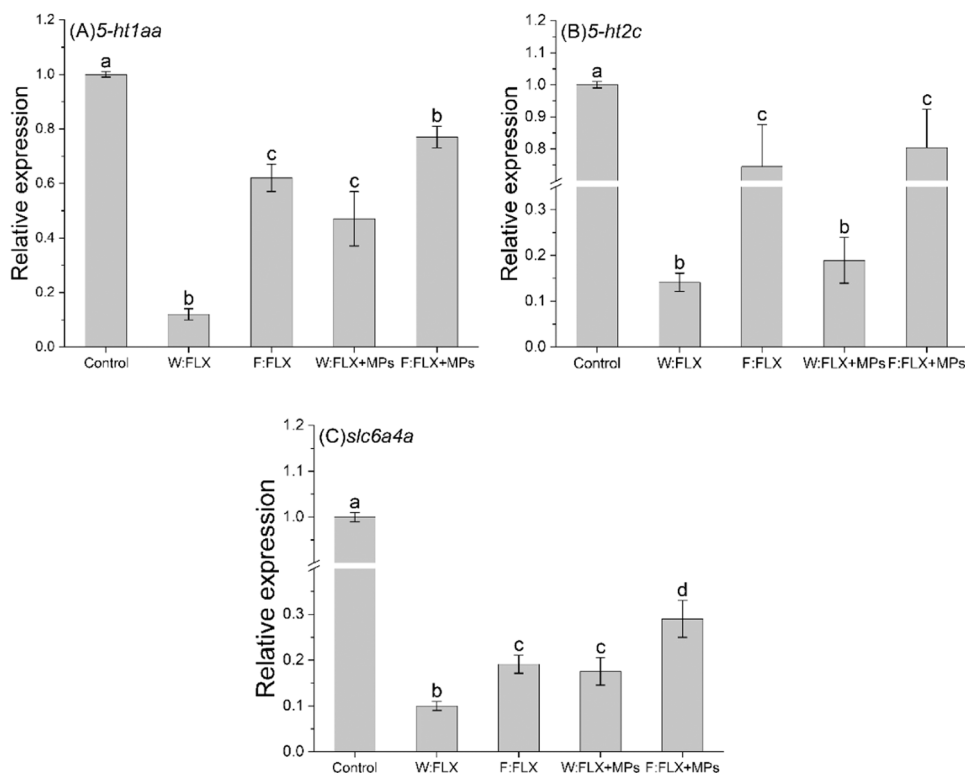
Interestingly, the presence of MPs lowered the uptake and transfer of fluoxetine by zebrafish through trophic transfer, leading to a 56% and 25% decrease in the BMF of fluoxetine in the intestines and gills, respectively. It has been reported that the presence of MPs could induce serious intestinal damage and false satiety in aquatic organisms [51], thereby lowering the absorption function of zebrafish intestines. In this study, the high accumulation of MPs in fish intestines (Fig. S5) may also reduce the uptake of nutrients and pollutants due to the intestinal damage and false satiety caused by MPs. The high accumulation of fluoxetine in harvest shrimp caused by MPs may be excreted in feces along with MPs without adequate absorption, thus alleviating the transfer of fluoxetine from brine shrimp to zebrafish. Hence, further study should focus on the residue of MPs and fluoxetine in fish feces. This finding is consistent with the influence of MPs on the fluoxetine BCF in zebrafish following waterborne exposure, suggesting a cleaning effect of MPs on the transfer of fluoxetine from brine shrimp to zebrafish. Similarly, Thiagarajan et al. [52] found that the presence of polystyrene MPs significantly reduced the BMF of co-existing titanium dioxide nanoparticles in the food chain. This reduction may be attributed to the fast clearance of MPs from the zebrafish intestines, resulting in insufficient release of co-existing pollutants from MPs and quick excretion with feces [12]. Since the accumulated concentrations of fluoxetine in fish intestines were comparable to or even higher than those in the gills after foodborne exposure, the intestine tissue seems to be a key route for fluoxetine to enter zebrafish from the food chain. However, a previous study has reported that the presence of polyethylene MPs significantly increased the accumulation of co-existing methamphetamine in aquatic organisms by 1.42-fold compared to methamphetamine alone, demonstrating a promoting effect of MPs on the trophic transfer of co-existing pollutants [13]. The binding properties between MPs and pollutants, as well as the retention time in the intestine of aquatic organisms, appear to be crucial factors affecting their trophic transfer in the food chain.

In addition, the bioaccumulation of fluoxetine in zebrafish under different exposure routes was compared. It finds that the concentrations of fluoxetine transferred in the food chain were only 10% of those from waterborne exposure. This result implies that, in natural environments, waterborne exposure is the crucial pathway for fluoxetine uptake by zebrafish, while the transfer in the food chain should not be overlooked. In the presence of MPs, the accumulation and magnification of fluoxetine in different fish tissues were decreased by 63%–95% and 25%–56% after waterborne and foodborne exposure, respectively. This finding suggests that the cleaning effect of MPs on the fluoxetine accumulation in zebrafish was suppressed in the food chain.

### 3.4. Biototoxicity responses in zebrafish

#### 3.4.1. Serotonergic response

It is known that fluoxetine is designed to treat depression by selectively inhibiting the reuptake of 5-HT at the presynaptic membrane in the central nervous system, thereby increasing 5-HT levels in the synaptic cleft and exerting the antidepressant effect [53]. Therefore, we initially focused on the alterations in serotonergic neurotransmission processes in zebrafish brains, including the gene expression of *5-ht1aa*, *5-ht2c*, and *slc6a4a* (Fig. 4). Exposure to fluoxetine significantly inhibited the gene expression of the 5-HT receptor (*5-ht1aa* and *5-ht2c*) by 23%–88% compared to the control. The inhibition may further lead to a relatively higher level of free 5-HT in the synapse, exhibiting an antidepressant effect. Similarly, Cunha et al. [54] found that exposure to waterborne fluoxetine inhibited the gene expression of 5-HT receptors in zebrafish. However, the inhibitory effects of fluoxetine on the 5-HT receptors were alleviated in the food chain, which is consistent with the alterations in the fluoxetine accumulation in zebrafish in the same exposure scenario. Notably, the presence of MPs significantly attenuated the inhibitory effects of fluoxetine on the 5-HT receptors in zebrafish, with a 1.3–3.9 times reduction after waterborne exposure and a 1.1–1.2 times reduction after foodborne exposure. It suggests that MPs may



**Fig. 4.** Expression of genes related to 5-HT neuromass transfer function (*5-ht1aa*, *5ht2c* and *slc6a4a*) in zebrafish. Different lowercase letters indicate significant differences between different treatments ( $P < 0.05$ ), (C: the control, W: waterborne exposure, F: foodborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).

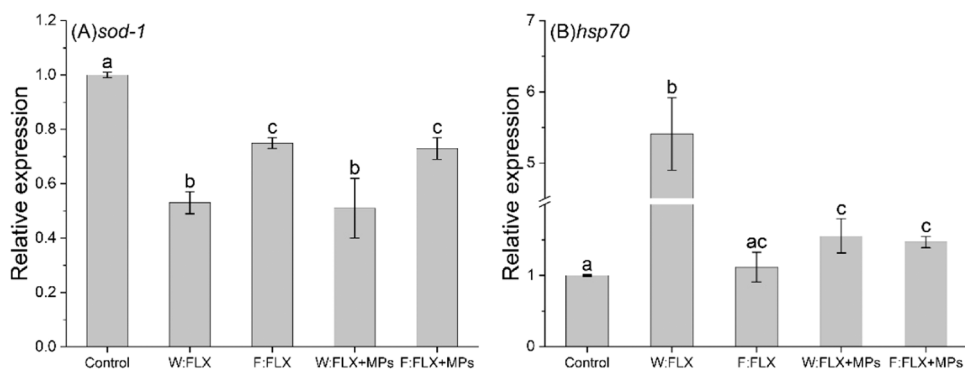
strongly interact with the co-existing fluoxetine in waters, while the trophic transfer process may further weaken the influences of MPs.

As a specialized transmembrane transport protein responsible for the reuptake of 5-HT from the synaptic cleft, serotonin transporter (*slc6a4a*) plays a crucial role in reducing 5-HT levels in the synaptic cleft [34]. In this study, the expression of the *slc6a4a* gene was significantly inhibited by fluoxetine in all treatments, decreasing to 10%–29% of the control. It is consistent with our previous studies, in which the gene expression of the *slc6a4a* in the brain of crucian carp and zebrafish was significantly reduced by the presence of fluoxetine, demonstrating a consistent mechanism of antidepressant action [16,55]. Similar to the effects on 5-HT receptors, the presence of MPs weakened the inhibitory effects of fluoxetine on the serotonin transporter in zebrafish, especially following waterborne exposure. The exposure route emerges as a crucial factor in assessing the influences of MPs on the effects of fluoxetine on the serotonin transporter in zebrafish.

### 3.4.2. Oxidative stress

Oxidative stress is caused by the imbalance between oxidation and antioxidant activities in the body, playing a crucial role in aging and diseases. Previous studies have demonstrated that exposure to MPs and fluoxetine could induce oxidative stress in different organisms [39,56]. In this study, exposure to fluoxetine alone significantly inhibited the expression of the *sod1* gene in the intestines compared to the control (Fig. 5), suggesting that the antioxidant system of zebrafish may inadequately counteract the oxidative stress induced by fluoxetine, leading to a decrease in the gene expression of *sod1* [34]. When the MPs were co-added, the inhibitory effect of fluoxetine on the *sod1* gene expression was slightly increased, potentially attributing to the compensation induced by MPs, which has been reported to cause strong oxidative damage in zebrafish [57].

Heat shock protein (*hsp*) 70 also plays a significant role in the organismal antioxidant defense. In this study, the *hsp70* gene was markedly upregulated by 5.41-fold in zebrafish following waterborne



**Fig. 5.** Expression of oxidative stress-related gene (*sod-1* and *hsp70*) in zebrafish. Different lowercase letters indicate significant differences between different treatments ( $P < 0.05$ ), (C: the control, W: waterborne exposure, F: foodborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).

exposure to fluoxetine (Fig. 5). In general, the upregulation of the *hsp70* gene in fish has been considered a stress response of organisms treated with different pollutants [58]. The upregulation of *hsp70* gene, coupling with the downregulation of *sod1* gene, implies an insufficient antioxidant function in zebrafish to eliminate oxidative free radicals, potentially resulting in oxidative damage. The presence of MPs reduced the upregulation of the *hsp70* gene caused by fluoxetine. The modulation of MPs on the oxidative damage induced by fluoxetine was more pronounced in fish following waterborne exposure than foodborne exposure, highlighting the crucial role of food in influencing the carrier effect of MPs.

### 3.4.3. Apoptosis response

Given that the antioxidant defense system in zebrafish intestines was damaged by fluoxetine alone or in combination with MPs, programmed cell apoptosis may appear. The expression of apoptosis-related genes, including *bcl-2*, *bax*, and *caspase-3*, were studied (Fig. 6). Compared to the control, the expression of all apoptosis genes in zebrafish intestines was enhanced by fluoxetine, confirming the oxidative damage caused by fluoxetine. *Bcl-2* is an anti-apoptotic member in organisms, and *bax* plays a pro-apoptotic role. The over-expression of *bax* in fish may lead to a significant increase in *bax/bax* homodimer formation, thus initiating cell apoptosis. For the over-expression of *bcl-2*, the *bax/bax* homodimer may be further dissociated, thus counteracting apoptosis in cells [59]. Although fluoxetine upregulated the expression of both *bcl-2* and *bax* genes simultaneously, the *bcl-2/bax* ratio was consistently less than 1, indicating enhanced pro-apoptosis in fish intestine cells. Caspase-3, an executor in apoptosis downstream of the caspase cascade, is regulated by the released cytochrome c (Cyt-c) in cells. Since the released Cyt-c is regulated by the *bcl-2* and *bax* through mitochondrial pathways, an upregulation in the gene expression of *caspase-3* was expected [60]. Thus, the gene expression of *caspase-3* activated by fluoxetine in this study suggests that the activation of fluoxetine in apoptosis pathways in zebrafish intestines may be through regulating the gene of *bcl-2* and *bax*.

In addition, co-exposure to MPs further enhanced the apoptosis in fish caused by fluoxetine, contrary to the accumulation of fluoxetine in zebrafish. This enhancement may be attributed to the presence of MPs, which has been reported to significantly increase cell apoptosis in various aquatic organisms [61]. Compared to waterborne exposure, the apoptosis in zebrafish intestines was significantly reduced by fluoxetine in most cases in the food chain, indicating the effectiveness of foodborne exposure in alleviating the cell apoptosis.

### 3.4.4. Immune response

The immune responses in zebrafish intestines were studied in different treatments, mainly focusing on the gene expression of *NF-κB*, *TNF-α*, *cox2*, and *il-10* (Fig. 7). Given the crucial role of *NF-κB* in cellular inflammatory responses [62], it is not surprising that fluoxetine promoted the gene expression of *NF-κB* in zebrafish intestines, thus facilitating the transcription of inflammatory factors, including the pro-inflammatory factor of *cox2*. Given that exposure to MPs alone could enhance the cellular inflammatory response in different aquatic organisms [62], it is not surprising that co-exposure to MPs rapidly enhanced the immune responses in zebrafish intestines caused by fluoxetine, resulting in a 1.6 and 1.2-fold increase in the gene expression of *NF-κB* and *cox2*, respectively. This enhancement in immune responses is consistent with the observed apoptosis in zebrafish intestines. Furthermore, the inflammatory response was effectively alleviated in fish following foodborne exposure. As an anti-tumor and anti-inflammatory factor, the upregulation of *TNF-α* and *il-10* gene expression in zebrafish implies an activation in the self-regulation against inflammation [63]. However, when MPs were introduced, the self-regulation induced by fluoxetine in zebrafish was slightly altered, which may be linked to the significant induction of inflammation caused by MPs, potentially surpassing and disrupting the self-regulation function in fish.

Hence, the presence of MPs significantly alleviated the influences of fluoxetine on the serotonergic neurotransmission processes in zebrafish

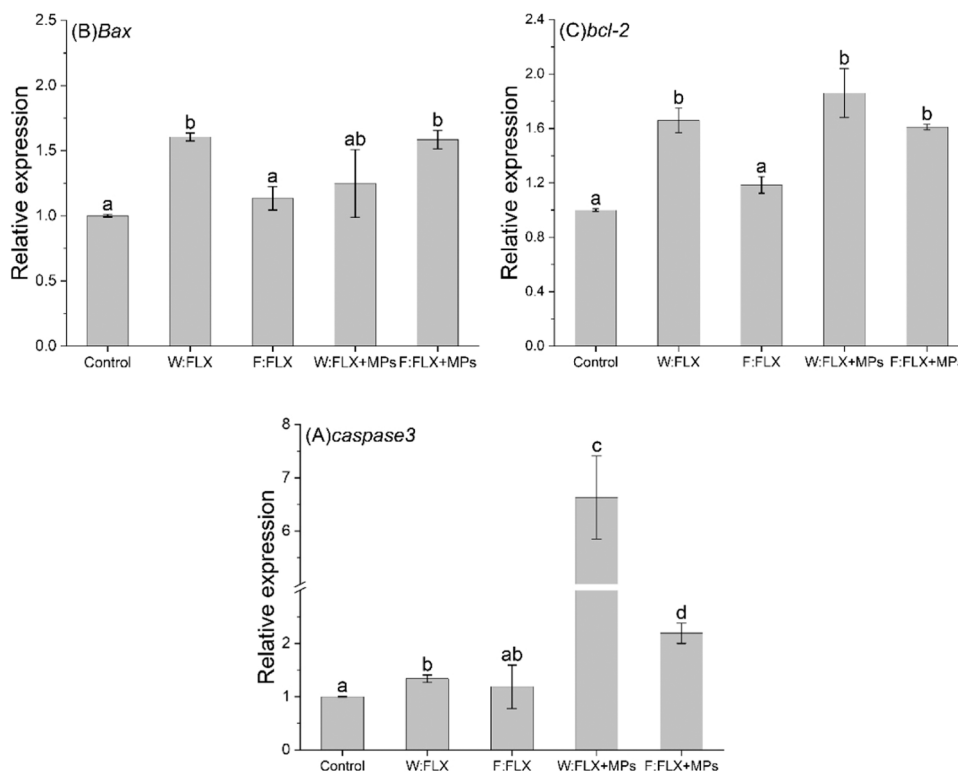
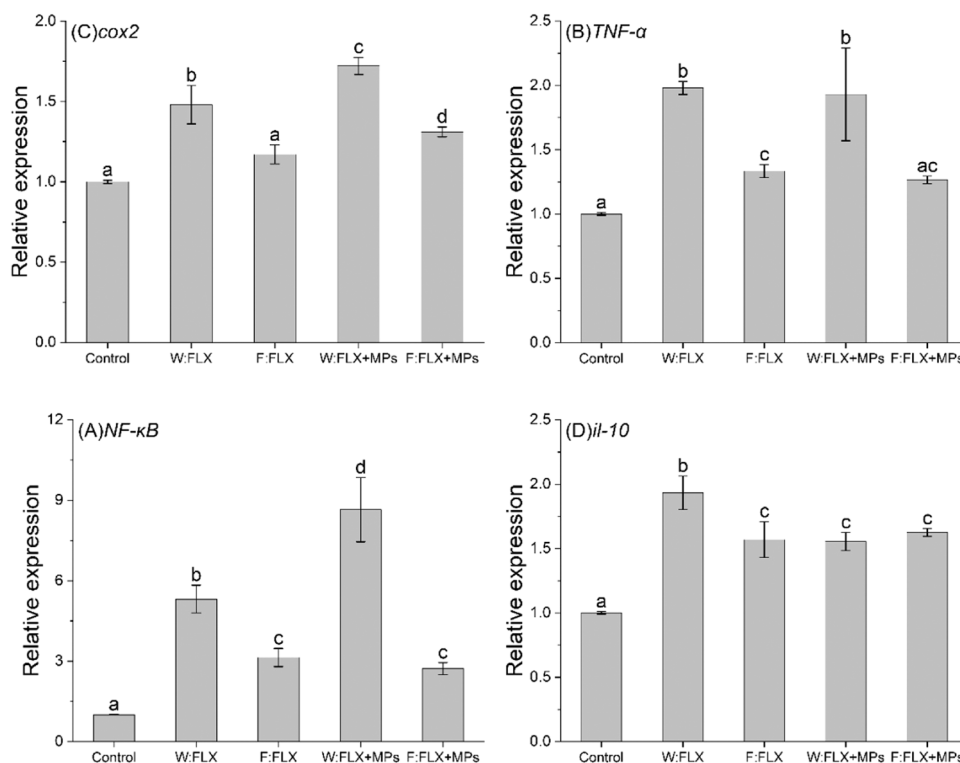


Fig. 6. Expression of apoptosis-related genes (*bax*, *bcl-2*, and *caspase3*) in zebrafish. Different lowercase letters indicate significant differences between different treatments ( $P < 0.05$ ), (C: the control, W: waterborne exposure, F: foodborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).



**Fig. 7.** Expression of immune response-related genes (*cox2*, *TNF-α*, *NF-κB*, and *il-10*) in zebrafish. Different lowercase letters indicate significant differences between different treatments ( $P < 0.05$ ), (C: the control, W: waterborne exposure, F: foodborne exposure, FLX: fluoxetine, and MPs: polystyrene MPs).

brains. However, due to the stimulation of MPs on oxidative stress, apoptosis, and inflammation in zebrafish, the related biological responses induced by fluoxetine were enhanced. Therefore, employing specific bio-endpoints, such as serotonergic response for fluoxetine, is crucial to explore the carrier effects of MPs on the co-existing pollutants. Additionally, exposure routes are essential factors influencing the carrier effects of MPs, and these effects could be alleviated in the food chain.

In summary, the presence of MPs alleviated the trophic transfer of fluoxetine in an aquatic food chain, as well as the specific bio-endpoints in the high consumer. In the context of global MP and pharmaceutical pollution with rapid increase, they may inevitably meet and interact with each other in different aquatic environments, thereby altering the transfer of pharmaceuticals in waters and the food chain, as well as the related bioaccumulation and toxicity. This finding suggests that the ecological risk of fluoxetine or other pharmaceuticals in aquatic systems may be overestimated or underestimated in the current environmental risk assessment and management strategies due to the neglect of the carrier effects of MPs. Therefore, in future assessments, the combined effects of MPs and pharmaceuticals on different aquatic organisms should be strengthened, especially in aquatic food chains. Furthermore, there are also some limitations in this study. Considering that the present study was conducted in a simple food chain including brine shrimp and zebrafish, it could not completely reflect the complex food web in actual aquatic environments. Thus, more aquatic organisms should be included in the aquatic food chain in future studies, including aquatic organisms with different dietary habits and living habits. Since the bioaccumulation and metabolism of fluoxetine antidepressants in waters were strongly influenced by water quality parameters (e.g. pH) [16], future studies should be also conducted with different water quality parameters to map different natural aquatic environments.

#### 4. Conclusion

This study investigated the influences of MPs on the trophic transfer

and biotoxicity of fluoxetine in the food chain composed of brine shrimp and zebrafish. It reveals that the co-existing MPs accelerated the dynamic accumulation of waterborne fluoxetine in brine shrimp but remarkably weakened in zebrafish. This discrepancy may be attributed to variations in the residence time of MPs in the two organisms. Fluoxetine could transfer from brine shrimp to zebrafish in the food chain, which was further alleviated by the presence of MPs. The uptake and excretion pathways of fluoxetine in the food chain shifted from gills to intestines when compared to waterborne exposure, implying the pivotal role of exposure routes in shaping the carrier effects of MPs in aquatic environments. In addition, the co-added MPs alleviated the serotonergic neurotransmission in zebrafish brains induced by fluoxetine. Nevertheless, the induction of oxidative damage, apoptosis, and immune response in zebrafish intestines caused by fluoxetine was remarkably enhanced by the presence of MPs due to their roles in stimulating these related responses. The trophic transfer of fluoxetine in the food chain reduced its biotoxicity in zebrafish, which was further alleviated by the presence of MPs. However, the uptake routes of fluoxetine in zebrafish in the presence of MPs remain unclear, and more attention should be paid to the underlying mechanisms in future studies.

#### Environmental implication

The ubiquitous microplastics in aquatic environments have attracted rapidly increasing attention due to their potential threats to aquatic organisms, particularly on their carrier effects. However, the knowledge regarding the carrier effects is still poorly understood in the food chain. Herein, we investigated the influences of microplastics on the trophic transfer of co-existing fluoxetine in the food chain. The results revealed that the carrier effects of microplastics alleviated the trophic transfer of fluoxetine in the food chain and the specific biotoxicity through cleaning effect. This finding provides new insights into the potential risks of microplastics under the increasing global microplastic pollution.

## CRedit authorship contribution statement

**Peiyuan Zhu:** Methodology, Investigation, Data curation. **Yonghua Wang:** Writing – review & editing, Conceptualization. **Zhenhua Yan:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Conceptualization. **Haizhou Zhao:** Writing – review & editing, Funding acquisition. **Guanghua Lu:** Writing – review & editing. **Chao He:** Writing – review & editing, Supervision. **Jun Hou:** Writing – review & editing.

## 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.

## Data availability

Data will be made available on request.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jhazmat.2024.134179](https://doi.org/10.1016/j.jhazmat.2024.134179).

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