Combined effects of micro-/nano-plastics and oxytetracycline on the intestinal histopathology and microbiome in zebrafish (*Danio rerio*)

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HIGHLIGHTS

- Micro-plastics co-exposure with OTC alleviate the intestinal damage induced by OTC.
- Intestinal epithelial damage increase with the decrease of plastic sizes.
- MOTC and NOTC affect the intestine microbiome diversity adversely.
- Micro-/nano-plastics co-exposure with OTC increased the abundance of ARGs.

GRAPHICAL ABSTRACT
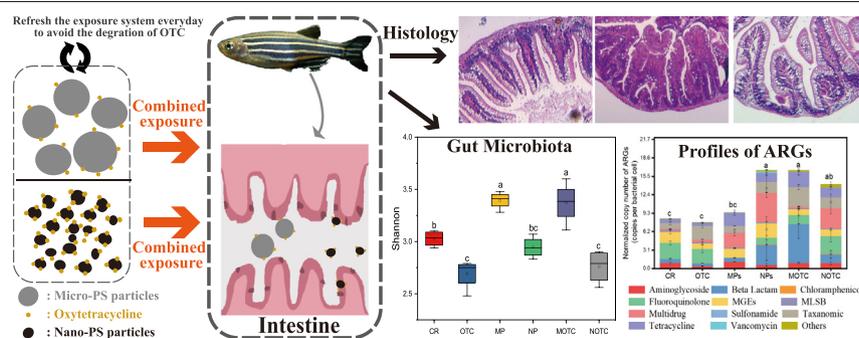
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ABSTRACT

Accumulated evidence has demonstrated that microplastics and oxytetracycline (OTC) affect organisms, but few studies have investigated their combined effects on aquatic organisms. In this study, adult zebrafish (*Danio rerio*) were exposed to single and binary-combined contamination of micro-, nano-sized polystyrene plastics and OTC for 30 days, and the intestinal histopathology, gut microbiota and antibiotic resistance genes (ARGs) of zebrafish were measured. The results showed that the intestinal epithelial damage increase with the decrease of plastic sizes. Nano-sized plastics, OTC and their combined exposure caused intestinal epithelial damage, and co-exposure with micro-sized plastics reduced the intestinal damage caused by single OTC exposure. The gut microbial communities were affected by the combined exposure to microplastics and OTC. Compared with the blank control, the relative abundance of *Fusobacteria* increased 12.7 % and 21.1 % in OTC combined with 45–85 μm micro-plastics (MOTC) and 40–54 nm nano-plastics (NOTC), respectively, and that of *Bacteroidetes* increased 26.2 % and 18.6 % in the MOTC and NOTC treatments, respectively. The effects of MOTC and NOTC on the biodiversity of the zebrafish gut microbiome were different; MOTC increased the biodiversity by 11.3 % compared with the blank control, whereas NOTC decreased the biodiversity by 8.8 % compared with the blank control. Furthermore, the abundance of ARGs in 40–54 nm nano-plastics, MOTC and NOTC treatments was increased 96.9 %, 96.6 % and 68.8 % compared with the control group, respectively. Additionally, significant differences were observed in ARGs characteristics between the micro- and nano-plastics treated groups whether combined with OTC or not. These results are essential to further understand the combined ecotoxicological effects of micro- or nano-plastics and antibiotics on aquatic organisms.

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1. Introduction

Global plastics production reached 368 million tonnes in 2019, which has incredibly increased over 180 times since the 1950s (Association of Plastic Manufacturers, 2020). Due to the insufficient capacity of waste management systems, plastic pollution in the environment is inevitably increasing (Lau et al., 2020). Due to the action of wave, UV radiation, and biological degradation, plastic debris with a size <5 mm is recognized as microplastics (Thompson et al., 2004), which have been frequently detected in the environment (Klein and Fischer, 2019; Yan et al., 2019; Zhang and Liu, 2018), even in the Antarctic and Arctic (Waller et al., 2017).

Several studies have shown that microplastics are readily ingested by organisms and passed on to predators (Diepens and Koelmans, 2018), further accumulating toxic elements due to the Trojan horse effect (Paul-Pont et al., 2016). Some of the ingested microplastics are excreted immediately (Jeong et al., 2016), while a concentration of 500 μg/L can enter the tissues and cause toxic effects at different biological levels (Karami et al., 2016). Microplastic occurrence and toxicological effects in higher trophic levels could be found in many studies (Alomar and Deudero, 2017; Gardon et al., 2018). Related adverse effects include physical organ damage, oxidative stress and metabolic disorders, differential genes expression related to glycolysis and lipid metabolism, and stress responses of the immune system (Wen et al., 2018). In addition, microplastics can also impact the structure of microbial communities in the fish gut (Xu et al., 2021; Yan et al., 2020; Zhang et al., 2021).

Furthermore, several recent studies suggested that the ecotoxicological effects of microplastics are closely related to the size of plastic particles (Sendra et al., 2021; Wang et al., 2020). Since ingestion is the main pathway of micro-sized plastics uptake, the gut is the major accumulating organ (Grigorakis et al., 2017; Lu et al., 2016; Watts et al., 2014). While nano-sized plastics are assumed to be able to accumulate in many other organs due to their ability to cross the cell and blood–brain barrier (Jeong et al., 2018; Shosaku, 2006). However, conflicting results of micro- and nano-particles accumulation and the following effects have been reported. Deng et al. (2017) reported that 20 μm particles were detected among all tissues of mice whereas 5 μm particles accumulated more in the gut. Micro-size plastics were reported to accumulate more in red tilapia and result in more severe intestinal damage to zebrafish than nano-plastics (Ding et al., 2020; Jin et al., 2018). However, the underlying relationship between the physical characteristics of microplastics (plastic type, shape, size) and subsequent effects has not yet been clarified.

Microplastics could also behave as chemical absorbents because of their large specific surface areas and hydrophobicity. While coexisting with pollutants, the ingestion of microplastics with pollutants absorbed by aquatic organisms may increase pollutant accumulation (Chen et al., 2017a; Wang et al., 2020; Zhou et al., 2020b), but also possibly reduce the accumulation of pollutants. The variation mainly depends on the reversibility of sorption and fugacity gradient of pollutants between microplastics and organisms (Koelmans, 2015; Koelmans et al., 2016). Additionally, co-exposure to microplastics and these pollutants may disrupt the detoxification processes of organisms (Rocha et al., 2020; Tang et al., 2018), and various types of pollutant sorption in organisms by plastic particles have been reported (Oliveira et al., 2018; Shi et al., 2020; Zhou et al., 2020b).

As a commonly used antibiotic in veterinary and aquaculture applications, oxytetracycline (OTC) is barely metabolized in vivo and is ultimately discharged bioavailable through faeces and urine ultimately (Cravedi et al., 1987; Zhang et al., 2018). The reported concentration of OTC detected was up to 7028 ng/L in freshwater aquaculture system (Monteiro et al., 2016) and 15,163 ng/L in seawater aquaculture farm (Chen et al., 2015). Moreover, the abuse of antibiotics has led to a significant increase in the abundance of ARGs in the environment (Hu et al., 2013; Rose et al., 2017). Previous research has proven the increased diversity and abundance of ARGs in the gut of mammals under the abuse of antibiotics (Looft et al., 2012; Yin et al., 2015). New evidence of microbiome composition and antibiotic resistance genes (ARGs) abundance changes under OTC exposure has also been reported (Zhu et al., 2018a).

Considering the large amount of OTC used in freshwater and seawater aquaculture systems (Monteiro et al., 2016; Chen et al., 2015) and the large amount of equipment used in fisheries and aquaculture systems (such as fish nets, floats and fish cages) based on PS-plastics (FAO, 2016; UNEP, 2016; Kumar and Karnatak, 2014), the co-occurrence of PS-plastics and OTC in aquaculture environments is inevitable. However, the effects of microplastics on the combined exposure of OTC have rarely been studied. Previous studies have shown that microplastics can increase the accumulation of OTC in aquatic organisms (Han et al., 2021; Zhou et al., 2020a). Nevertheless, to the best of our knowledge, no study has investigated the effects of OTC associated with micro-/nano-plastics on intestinal microbiota and antibiotic resistance genes in aquatic organisms.

In the present study, a continuous 30-day exposure experiment on zebrafish was conducted to simulate the combined aquatic pollution of antibiotics and micro/nano-plastics. Histopathological analysis, intestinal 16S rRNA sequencing and HT-qPCR were performed to investigate the single and combined effects of two normal sized PS-microplastics and OTC on the following aspects: (i) the intestinal damage induced by single and combined exposure of 5 different size microplastics and OTC; (ii) the alteration of the microbial community between micro- and nano-plastics; and (iii) the effects of pollutant exposure on the profile of ARGs in zebrafish, thereby developing the understanding of the possible adverse effects of microplastics and OTC on aquatic organisms.

2. Methods and materials

2.1. Chemicals and zebrafish maintenance

OTC (purity > 95 %, CAS number 2058-46-0) was purchased from Aladdin (Shanghai, China). Micro and nano sized polystyrene sphere were acquired from Narui Materials Ltd. (Shanghai, China), three size ranges of micro-sized plastic particles (158–234 μm, 45–85 μm and 4–8 μm) and two size ranges of nano-sized plastic particles (394–407 nm and 40–54 nm) was prepared for the exposure experiment, scanning electron microscopy image can be found in the Supplemental Material (Fig. S1). Zebrafish were chose as the test organism since they were suggested as biological indicator to evaluate the ecotoxicology of chemicals by the Organization for Economic Cooperation and Development (OECD, 2019, 2004). The adult wild-type zebrafish (*Danio rerio*, six months old) were purchased from the Yudu Aquatic Fishery (Yindou Road, Jimei District, Xiamen) with an average weight of 0.25 ± 0.05 g. All zebrafish were acclimated in the laboratory with aerated tap water at 24 ± 1.5 °C for >2 months at a 14:10 h light-dark cycle, and the dissolved oxygen content was maintained above 7.72 mg/L. During the acclimation period, half of the water was replaced every 3 days to reduce the production of ammonia, nitrite and nitrate. Zebrafish were fed with commercial diet twice a day.

2.2. Exposure experiment and sample collection

After acclimatization, 12 treatment groups were set for the histological analysis: control, single oxytetracycline exposure, three size ranges of micro-sized plastics alone and combined with OTC, two size ranges of nano-sized plastics alone and combined with OTC.

Microplastics suspensions were prepared using UV-sterilized aerated water and sonicated prior to use, and concentrations were selected by reported and predicted environmentally relevant concentrations in water (1770 items/L, approximately 60–70 μg/L in Dubaish and Liebezeit, 2013; 1700–4900 items/L, approximately 60–186 μg/L in Leslie et al., 2017; 8902 items/L, approximately 338 μg/L in Yan et al., 2019). Concentration of OTC was chosen based on previous works that exert significantly effects on biochemical and microbiome level (Keerthisinghe et al., 2020; Li et al., 2020), although it is slightly higher than environmental relevant concentration, much higher OTC concentration was detected in some special scenes such as aquaculture farms (235 μg/L, Li et al., 2008). Furthermore, this concentration was helpful to determine the effecting mechanisms of OTC. Needless to mention that rapid growth of the pharmaceutical industry

may cause increasing release of antibiotics (Godoy et al., 2015). Four replicates were set for each exposure treatment, and eight zebrafish were randomly allocated to each replicate in 5 L exposure solution. During one-months exposure, all tanks were continuously aerated to ensure comparable O_2 saturation levels and to prevent microplastics from aggregating and sinking (Lu et al., 2016; Qiao et al., 2019). The exposure solution was refreshed every day and the chemicals were supplemented to keep the exposure concentrations unchanged. After the exposure, the body weight and length of sampled zebrafish were recorded, and the body mass index (BMI) were calculated by the following method:

$$BMI = W/L^2,$$

where W means the body weight of zebrafish, L means the body length of zebrafish.

2.3. Histological analysis

After 30 days of exposure, 2 fish in each tank, a total of 96 fish were sacrificed and intestine were quickly dissected out. Segments of the middle intestine were fixed in 4 % paraformaldehyde for >24 h, then embedded in paraffin and sliced transversely into 3–4 μm thickness sections, each sample was sliced at least twice. After stained with hematoxylin & eosin (H&E) for histological analysis, all samples were subsequently examined under a microscope (Motic AE31, German).

2.4. Intestine DNA extraction and microbiota analysis

Six treatment groups were set for the intestinal microbiome alteration and ARGs profile analysis: control, single oxytetracycline exposure, 45–85 μm micro-sized plastics alone and combined with OTC (abbreviated as MP and MOTC), 40–54 nm nano-sized plastics alone and combined with OTC (abbreviated as NP and NOTC). These two specific size ranges were chosen for their representative results of intestinal histopathology analysis. Sampled zebrafish were rinsed three times with sterilized water before DNA extraction. The genomic DNA of intestinal contents from zebrafish was extracted using DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. In each glass tank, 6 intestines from fish were pooled together as a replicate sample. After quality determination, the gDNA samples were amplified by specific primers 338F (5-GTGCCAGCMGCCGCGGTAA-3) and 806R (5-GGACTACHVGGGTWTC-TAAT-3) targeting the 16S rRNA (V3-V4) of bacteria. PCR products were purified by AMPure XP (Beckman Coulter, Indianapolis, IN).

The DNA libraries were sequenced on an Illumina MiSeq platform and 253 bp paired end reads were generated. Then high-quality clean tags were obtained by quality filtering of raw tags. The clean tags were further compared with reference database (Silva database) using UCHIME algorithm to detect and remove potential chimera sequences and to obtain effective tags. The effective tags were clustered into operational taxonomic units (OTUs) defined by 97 % similarity. After that, OTUs were grouped and normalized at various levels of taxonomy classification (phylum, class, order, family and genus) to get the relative abundance of each taxon.

Bacterial alpha diversity-indices, including Chao1, Shannon and observed species, were calculated using QIIME (Version 1.7.0) and displayed with RStudio (version 1.3.1093, based on R software version 3.6.3). Beta diversity was assessed with weighted UniFrac and visualized with both Principal Coordinate Analyses (PCoA) and Partial Least Squares Discriminant Analysis (PLS-DA). We also performed the reconstruction of unobserved states (PICRUSt) to predict functional profiles derived from Kyoto Encyclopedia of Gene and Genomes (KEGG) catalog with the 16S rRNA data.

2.5. High-throughput quantitative PCR for antibiotic resistance genes analysis

The Wafergen SmartChip Realtime PCR system (Wafergen, Fremont, CA) was used to perform high-throughput quantitative PCR to evaluate the abundance and diversity of ARGs in samples. A total of 384 primer sets targeting 326 ARGs, 57 mobile genetic elements (MGEs, including 9 insertional MGEs, 3 integrase, 11 plasmid and 11transposases) and a 16S rRNA gene were used in this study, details on the primers used are supplied in Table S1. A protocol for detecting ARGs and processing data described in previous studies (Chen et al., 2017b; Zhu et al., 2018b) were used in this work, the conditions were followed as well. Briefly, each PCR system consisted of LightCycler 480 SYBR Green I Master mix, each primer, nuclease-free PCR grade water and gut DNA template in a 100 nL well. The mixture was heated for 10 min at 95 °C and then 40 cycles of 30s at 95 °C and 30s at 60 °C. Following outcomes were analyzed using SmartChip qPCR software (V 2.7.0.1, Wafergen Biosystems, Inc.; Takara Bio.). A threshold cycle (CT) value below 31 was the standard used to calculate the relative abundance of ARGs (copies/16S rRNA gene), and the normalized copy number of ARGs per cell was calculated based on a previously developed formula (Chen et al., 2017b; Zhu et al., 2018a).

2.6. Statistical analysis

All data were shown as means ± SE (Standard Error of mean). Comparisons among groups were performed using one-way ANOVA followed by turkey post hoc test using SPSS 20.0 (IBM, USA). The data were considered significant when $P < 0.05$ or $P < 0.01$. Permutational Multivariate Analysis of Variance (PERMANOVA, Adonis) based on Bray-Curtis was used to analyse the degree of explanation of different treatments. Procrustes analyses was performed to analyse the relation between ARGs profiles and bacterial composition.

3. Results

3.1. Intestinal histopathological changes in zebrafish induced by microplastics and OTC exposure

After 30 days of exposure, no significant alteration of body weight, body length and BMI was recorded in all treatments compared with the control treatment (Fig. S2, $P > 0.05$).

Histopathological changes in the intestinal sections from the control, OTC, micro- and nano-plastics exposure groups were examined by H&E staining and imaged through microscopy (Fig. 1). No significant change of the intestinal wall thickness was observed after the single or combined chemicals exposure. In the micro-sized plastic exposed treatments, no significant damage was observed in the single exposure of large (158–234 μm) and medium sized (45–85 μm) micro-plastic (Fig. 1B, C). While slight ruptures and lysis of the lining epithelium of intestinal villi were observed after small sized micro-plastic (4–8 μm) single exposure (Fig. 1D). In the nano-sized plastic treated groups, the vacuolation of intestinal epithelial cells was remarkable, the dissolution and shedding were also observed (Fig. 1E, F).

Compared with control, OTC exposure caused fracture of the lining epithelium of intestinal villi and vacuolation of intestinal epithelial cells (Fig. 1G). Meanwhile, in the micro-sized plastics combined with OTC treatments, ruptures and lysis of the lining epithelium of intestinal villi were observed in all three sized micro-plastics treatments (Fig. 1H, I & J). In the nano-sized plastics combined with OTC treatments, the vacuolation, dissolution and shedding of intestinal epithelial cells were observed (Fig. 1K, L).

3.2. Single and combined effects of microplastics and OTC on the microbial community of zebrafish

The effects of micro-/nano-plastics and OTC exposure on the zebrafish intestinal microbiome were investigated by bacterial 16S rRNA sequencing.
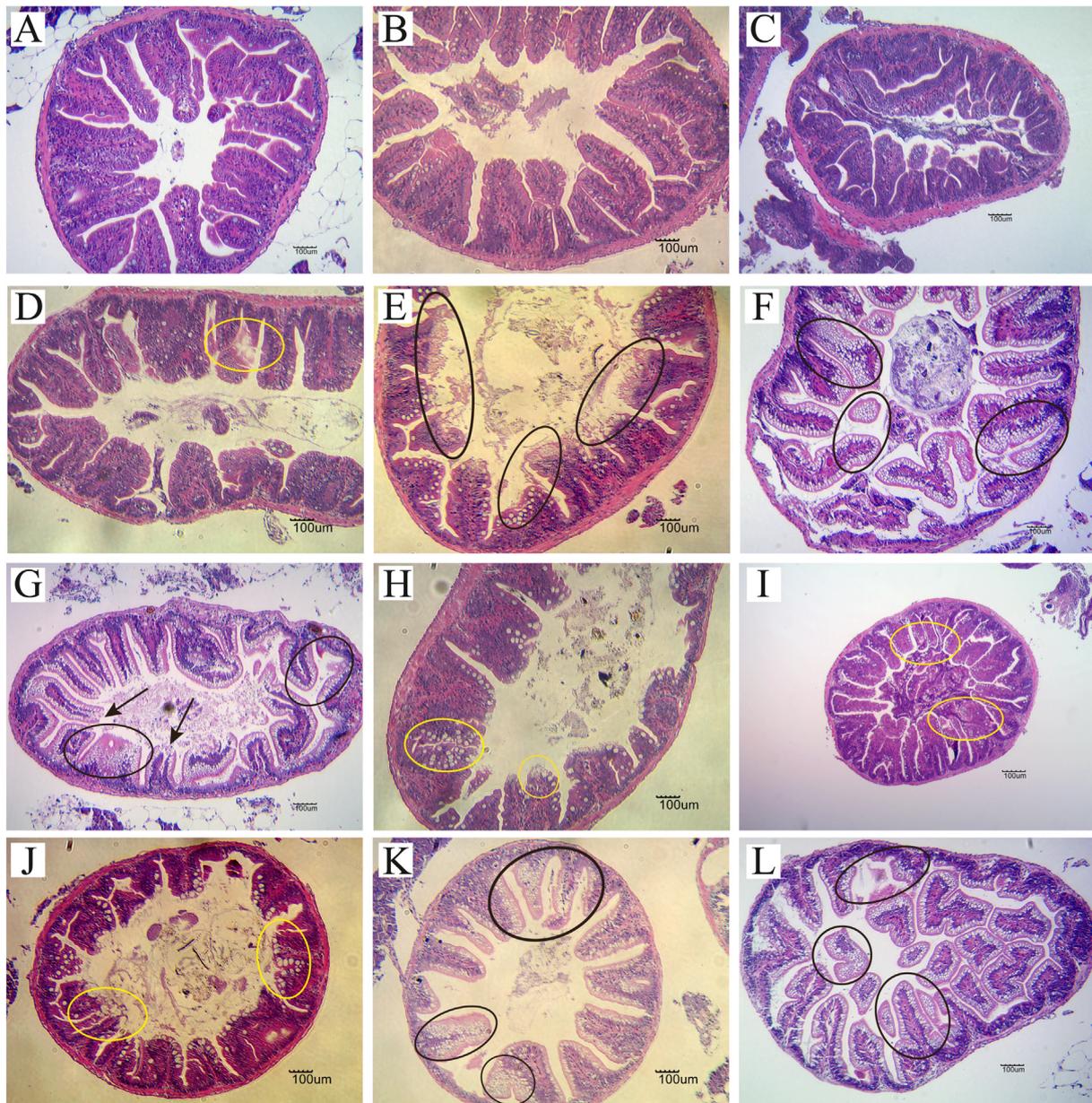
Fig. 1. Representative image of H&E staining of zebrafish intestine. A. Control group exhibited normal structure; B–C. Intestine exposed to 158–234 μm, 45–85 μm micro-plastics exhibited much normal structure; D. Intestine exposed to 4–8 μm micro-plastics exhibited slight fracture of intestinal villus (yellow oval). E–F. The intestine exposed to 394–407 nm and 40–54 nm nano-plastics exhibited severe lysis and vacuolation of epithelial cells (black oval); G. Intestine exposed to OTC exhibited vacuolation of epithelial cells and fracture (black arrow and oval); H–J. Intestine exposed to OTC combined with 158–234 μm, 45–85 μm and 4–8 μm micro-plastics exhibited slight ruptures and lysis of intestinal villus (yellow oval); K–L. Intestine exposed to OTC combined with 394–407 nm and 40-54 nm nano-plastics exhibited severe vacuolation of epithelial cells (black oval).

The composition of the gut microbiota at the phylum level varied in zebrafish intestines exposed to MP/NP and OTC (Fig. 2). The dominant phyla identified in the intestine of zebrafish in all treatments were *Fusobacteria* and *Proteobacteria*. Compared with that in the control, the relative abundance of *Fusobacteria* increased in all treatments except the MP group (Fig. 2B); the increase reached 66.7 % in the OTC treatment, and 12.7 % and 21.1 % increases were also detected in the MOTC and NOTC treatments, respectively. The relative abundance of *Bacteroidetes* decreased in the OTC group and remained comparable in the MP and NP treatments, while it increased 26.2 % and 18.6 % in the MOTC and NOTC treatments, respectively (Fig. 2C, $P < 0.05$). The relative abundance of *Firmicutes*, *Actinobacteria* and *Verrucomicrobia* tended to decrease in all treatments compared with blank treatment (Fig. 2D–F, $P < 0.05$). The relative abundances of *Planctomycetes* and *Cyanobacteria* in the OTC and NOTC

treatments were similar but increased significantly in the MOTC treatments (Fig. 2G–H, $P < 0.05$).

The alteration of richness and diversity of the intestinal microbiome in zebrafish exposed to OTC and micro-/nano-plastics was then measured using Chao1, Shannon and Simpson indices. An increasing tendency of the Chao1 index was observed in all treatments compared with the control, but no significant difference was found between all treatments (Fig. 3A, $P > 0.05$). Compared with the control, the OTC and NOTC treatments decreased the biodiversity by 11.1 % and 8.8 % (based on Shannon index) in the zebrafish microbiome, whereas the MP and MOTC treatments increased the biodiversity by 12.1 % and 11.3 % (Fig. 3B, $P < 0.05$). A similar pattern was also found for the Simpson index (Fig. 3C).

Unsupervised multivariate statistical methods were used to analyse the β diversity of the zebrafish microbiome, including PCoA and PLS-DA. The
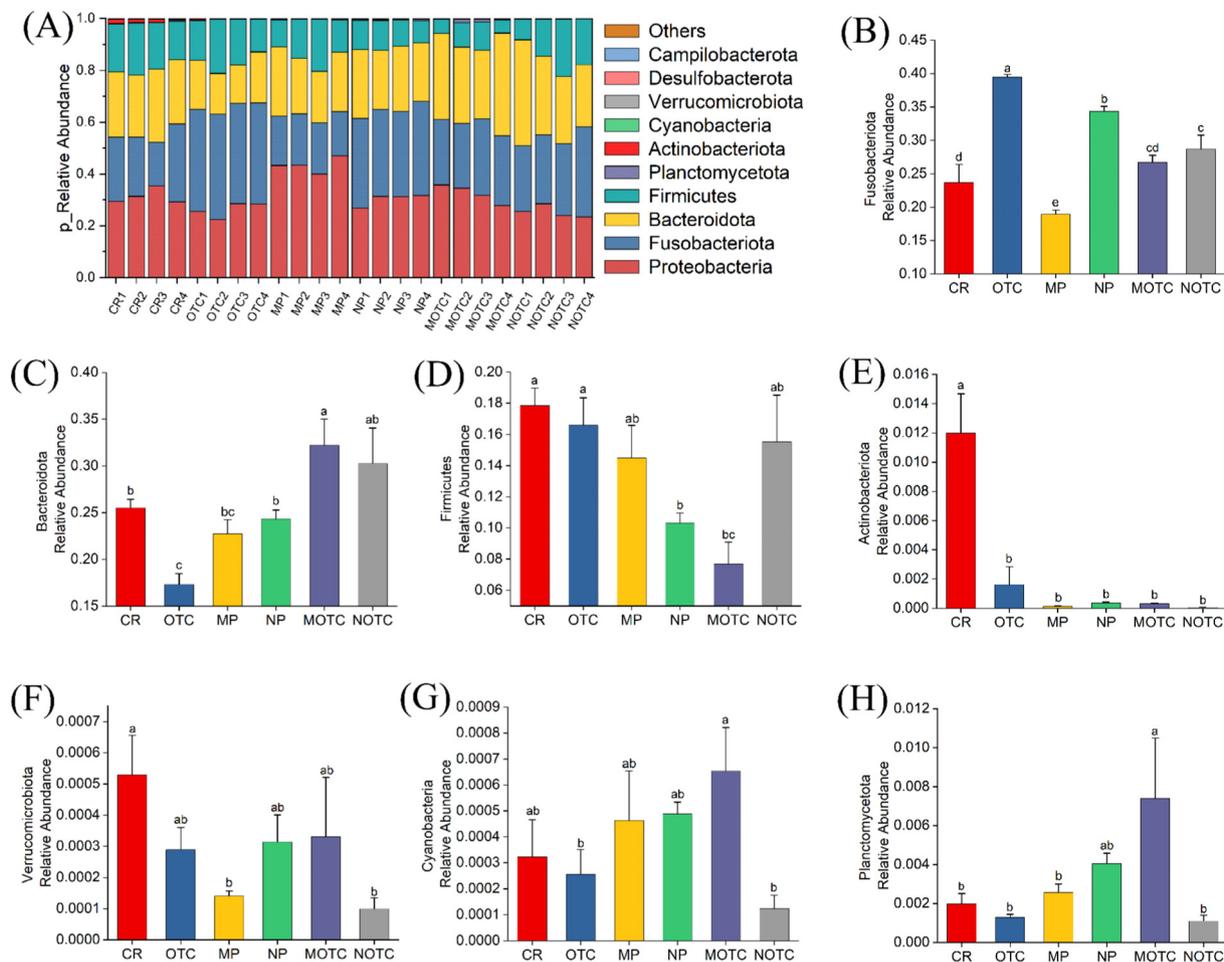
Fig. 2. The composition of the gut microbiota at phylum level. A. Relative abundance of the top 10 microbiota at the phylum level. B–H. Relative abundances of the seven altered bacterial phyla. For A–H, $n = 4$ samples per group. One-way ANOVA was applied to analyse the difference between treatments ($P < 0.05$). All data are presented as the means ± SEM, letters above the bars indicate a significant difference when the bars do not share a same letter, while no significant difference was observed otherwise.

results of PCoA of the microbial community in the zebrafish gut based on Bray-Curtis distance showed a clear separation between different treatments (Fig. 3D, PERMANOVA, $P < 0.01$), with 39.36 % and 25.81 % of the variation explained by the first two (PC1 and PC2) principal components. To exclude the effects of intragroup differences, PLS-DA analysis suggested that the MOTC groups exhibited significant separation from the other groups, whereas the NP and NOTC treatments were slightly clustered (Fig. 4E).

3.3. Predicted functions of gut bacteria in zebrafish under microplastics and OTC exposure

Predicted Kyoto Encyclopedia of Genes and Genomes database (KEGG) analysis associated with gut microbiota was performed to further understand the biological functions of gut bacteria among different treatments. The pathway annotation suggested that pathways related to metabolism, genetic information processing, cellular processes and environmental information processing were the dominant enriched pathways, accounting for 81.33 %, 12.15 %, 3.49 %, and 2.46 % of the total enriched genes, respectively.

Microplastics and OTC treatments significantly affected the function of the zebrafish microbiome (Fig. S3). In total 151 pathways in pollutant treatments were predicted to be significantly regulated in the zebrafish gut microbiota compared with the control (Fig. S4). Moreover, the OTC treatments showed a higher abundance of pathways related to folding, sorting and degradation, replication and repair, translation and nucleotide metabolism than the control, while the abundance decreased in the MP, NP

and MOTC treatments (Fig. 4, $P < 0.05$). An adverse pattern was observed in pathways related to cell motility, xenobiotic biodegradation and metabolism, signal transduction and lipid metabolism.

3.4. Effects of microplastics and OTC on the diversity and abundance of ARGs in the zebrafish gut

A total of 74 ARGs and MGEs were detected in the zebrafish intestines among all the treatments. Specifically, 15 aminoglycoside, 6 β-lactamase, 3 chloramphenicol, 3 fluoroquinolone, 5 MLSB, 12 multidrug ARGs, 2 sulfonamide, 3 taxanomic, 9 tetracycline, 1 vancomycin, 3 other ARGs and 12 MGEs were detected. The numbers of ARGs and MGEs detected in each group are presented in Fig. 5A. The UpSet plot revealed the shared ARGs and MGEs detected in different treatments (Fig. 5B), and 26 ARGs and MGEs were shared among all treatments in total. Notably, the MP and NP treatments had the same proportion of shared ARGs as the other treatments (43 ARGs). While the Shannon indices showed a significant difference between the MP and NP treatments (Fig. 6A, $P < 0.05$), PCoA based on the Bray-Curtis distance also showed a significant separation between different treatments (Fig. 6B, PERMANOVA, $P < 0.01$).

However, the normalized copy numbers of ARGs and MGEs in the NP, MOTC and NOTC treatments were significantly higher than those in the control treatment (Fig. 7, $P < 0.05$), and the increases were up to 96.9 %, 96.6 % and 68.8 %, respectively. Specifically, the abundances of β-lactamase, sulfonamide, taxanomic and tetracycline ARGs in MOTC treatments were significantly higher than those in the control (Table 1, $P < 0.05$). The abundance of multidrug ARGs in the MP and NP treatments
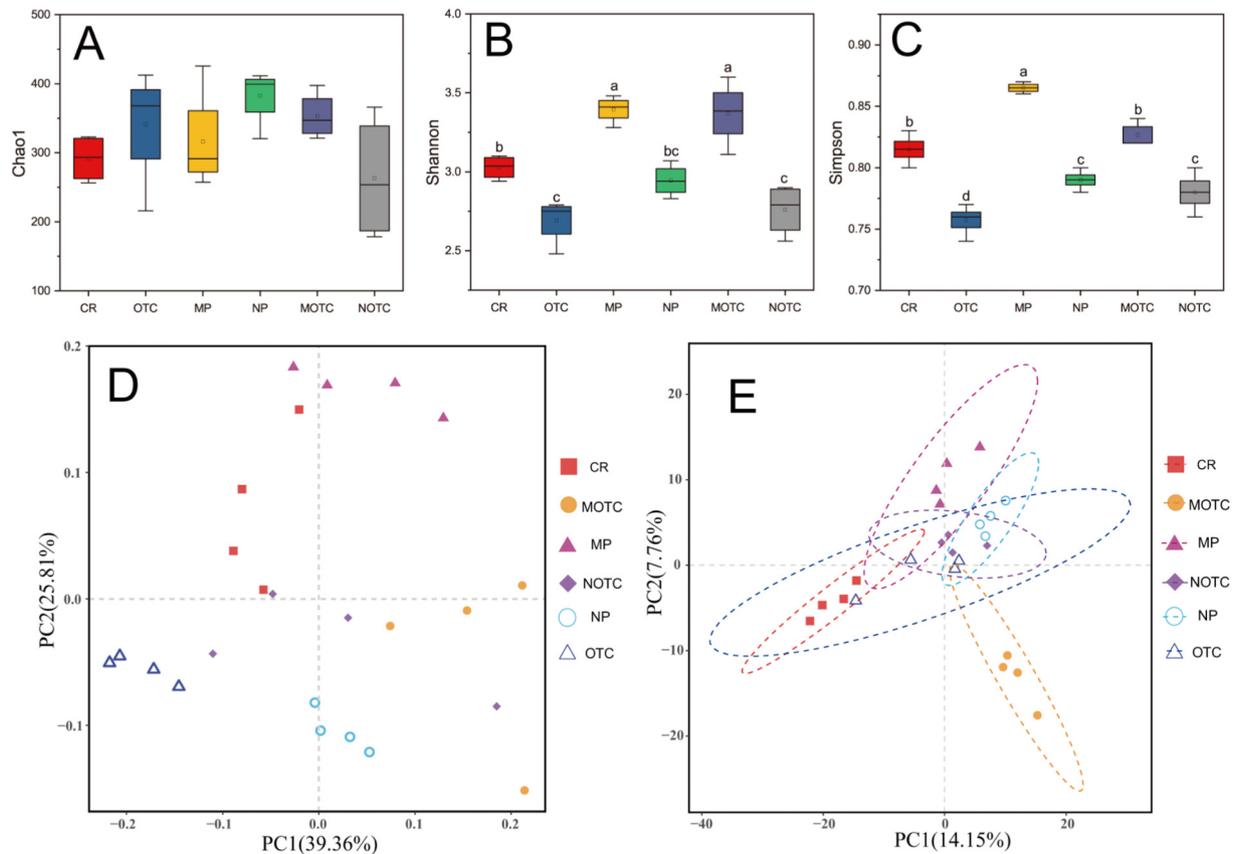
Fig. 3. Gut microbiota diversity of zebrafish. A–C. Different α-diversity indices (Chao1, Shannon and Simpson) of zebrafish gut microbiota. $n = 4$ samples per group, data are presented as the means ± SEM. D–E. PCoA and PLS-DA of bacterial β-diversity based on Bray-Curtis distances. Each node represents a sample. One-way ANOVA was applied to analyse the difference in α-diversity between treatments, and the P value is depicted for each analysis ($P < 0.05$). The letters above the boxes indicate a significant difference when the boxes do not share the same letter, while no significant difference was observed otherwise.

was significantly higher than that in the control and OTC treatments (Table 1, $P < 0.05$).

The heatmap displays the richness and distribution of all ARGs and MGEs detected in zebrafish (Fig. S5). The type of enriched ARGs and MGEs was noticeably distinct between the guts exposed to different treatments, and the most enriched ARGs could be found in the NP treatment.

3.5. Correlation between the zebrafish gut microbiota and antibiotic resistance genes

The correlation between antibiotic resistance genes and the zebrafish microbiome was explored to test whether OTC and micro-/nano-plastics affected the ARGs by altering the phylogenetic structures of bacterial communities. The results showed that the Bray-Curtis distances calculated from ARGs (HT-qPCR data) were significantly correlated with bacterial composition (16S rRNA sequence data), Procrustes analyses (Fig. 8) revealed that the ARGs profiles of zebrafish correlated with bacterial composition ($M^2 = 0.4868$, $P < 0.001$, 9999 permutations). Network analysis of co-occurrence pattern between ARGs and bacterial taxa also suggested a strong (Spearman's correlation coefficient $r > 0.7$) and significant ($P < 0.05$) correlation between the subtype of ARG and bacterial phylum (Fig. S6). Moreover, the heatmap of the correlation between the bacterial abundance at the order level (top 20) and ARGs further revealed that the order of *Pseudomonadales* and *Burkholderials* were correlated with the ARGs (Fig. S7, Spearman correlation analysis, $P < 0.05$).

4. Discussion

Microplastics and antibiotics are two new types of pollutants. This study suggested that OTC and their combined exposure with microplastics can

cause intestinal tissue damage in zebrafish to different extents. We observed severe histopathological damage in the OTC treatment, which might be correlated with the relatively lower body weight and body length in the OTC treatment, although the decrease was not significant. Previous studies also shown that exposure to OTC may lead to weight loss (Yu et al., 2020), and intestinal pathological damage may explain this decrease to some extent, since intestinal damage may affect the digestive function of organisms, reduce energy metabolism and nutrient absorption (Tan et al., 2020).

Histological analysis showed that no significant histological alteration was observed in the large and medium sized micro-plastics, while slight intestinal damage was observed in small size micro-plastics. When the plastic particle size decrease to nanoscale, severe damage of zebrafish intestine was detected. These results suggested that the effect of microplastic on the histological change was size dependent, the intestinal damage becomes more severe as the size of the plastic particles decreases. This size-dependent effect should be critical to their accumulative and ecotoxicological effects. Some previous studies have shown that exposure to nano-sized microplastics can cause more severe intestinal damage than micro-plastics (Kang et al., 2021; Ma et al., 2016). Therefore, we speculated that microplastics with smaller sizes have a greater ability to cause intestinal inflammation and abnormalities. Proposed explanations are discussed as follows.

Microplastics can lead to physical damage to the intestinal epithelium. After plastic particles ingestion in intestinal tissue, plastic ingestion promotes abrasion of the epithelium, specifically villi cells and crypt cells, causing the loss of cells and structural damage to the intestinal epithelium. Then the abrasion damage of the epithelium triggers immune responses that involve increased irrigation in the intestinal vessels (Ahrendt et al., 2020). The retention and depuration time of nano-plastics in organisms
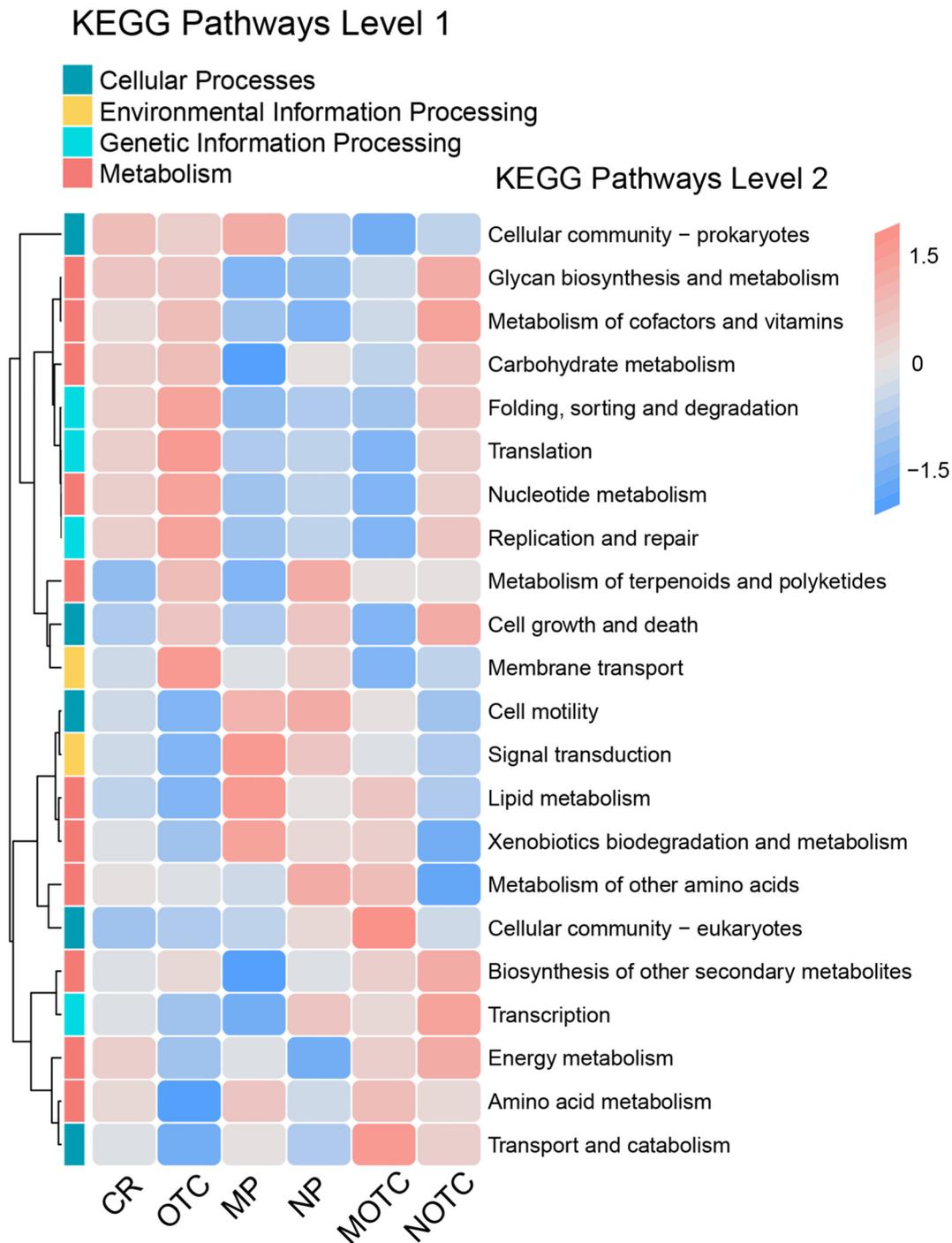
Fig. 4. Heatmap of predicted biological functions of zebrafish across different treatments. PICRUSt was used to predict and compare the functions of bacterial microbiota using KEGG database at levels 1 and 2.

are longer than those of micro-plastics (Jeong et al., 2016; Rosenkranz and Davidson, 2009), and they are more easily internalized into organisms (Collard et al., 2017; Moos et al., 2012). This means that the bioaccumulation of nano-plastics is higher than that of micro-plastics, which might eventually lead to the more severe damage mentioned above.

Moreover, nano-plastics might further cause intracellular damage and make the intestinal tissue more vulnerable. Nano-plastics can format protein coronas on the surface by interacting with biomolecules due to their large specific surface area and complex surface structure (Kelly et al., 2015; Lundqvist et al., 2008; Ramsperger et al., 2020), which might easily be ignored by the immune system and internalized into cells (Ramsperger et al., 2020). After internalization, inflammasome activation and cellular

phenotypes such as proliferation, apoptosis, differentiation, and migration induced by pollutant exposure might cause further damage to cells and tissue (Jiang et al., 2011; Shang et al., 2014). Furthermore, processes related to cellular components, including the intrinsic component and the integral component of the membrane, were significantly induced, and the enhanced permeability of the barrier function in intestinal epithelial cells might make the intestine more susceptible (Li et al., 2021).

Another intriguing finding is that in the micro-plastic combined with OTC treatments, slight physical damages of the intestines were observed compared with single micro-plastic exposed treatments, which may be due to the adsorption abilities of microplastics to OTC. While the degree of intestinal damage decreased compared with that of the OTC treatment,
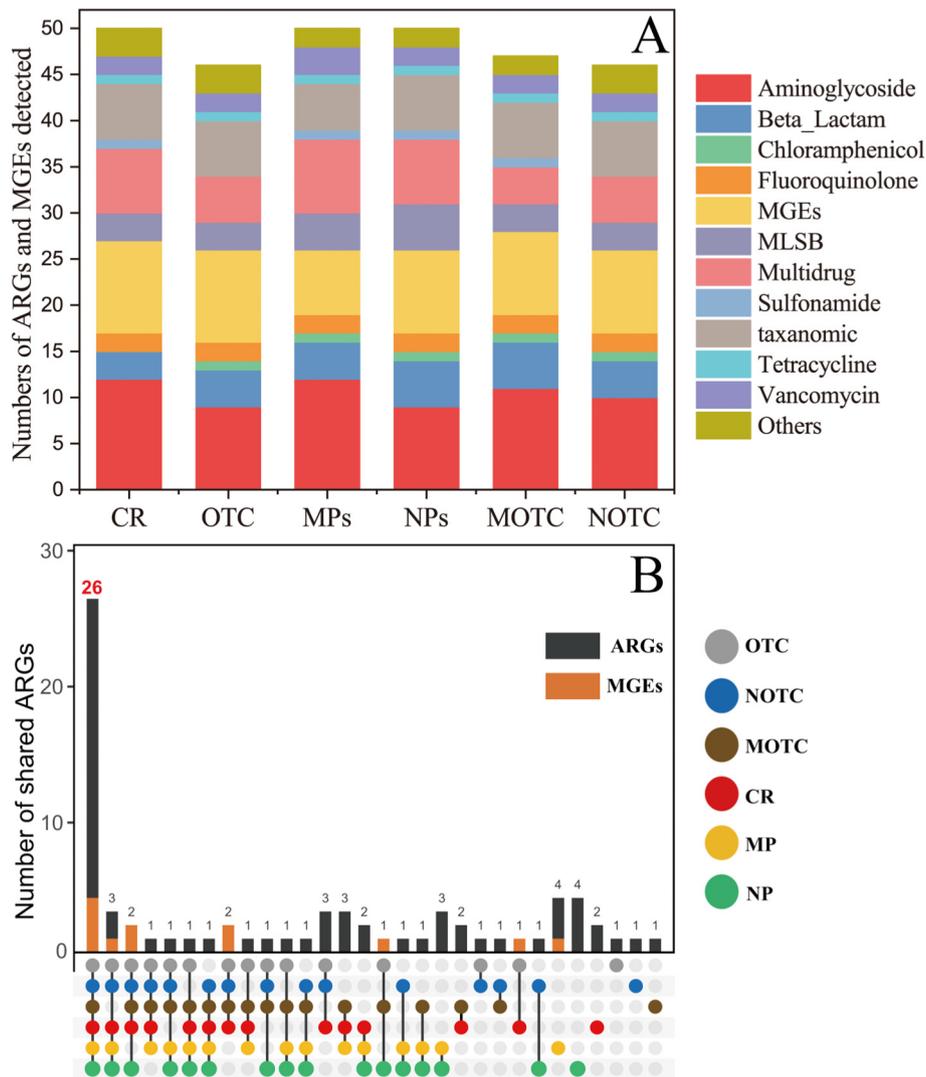
Fig. 5. A. The numbers of ARGs and MGEs detected in each treatment. B. UpSet plot showing the overlap of ARGs among different treatments. The bar chart on the bottom left indicates the total number of ARGs for each treatment. The number of ARGs shared among different groups is indicated in the upper bar chart. Circles in the matrix on the bottom chart indicate sets of groups that intersect.

which might be related to the reduction of OTC accumulation in the intestines, since the retention time of large size microplastics is limited. These results further confirmed the carrier effect of microplastics on antibiotics (Zhang et al., 2018). However, such alleviation was not observed in nano-sized plastic treatments, that might be related to the damage caused by nano-plastics.
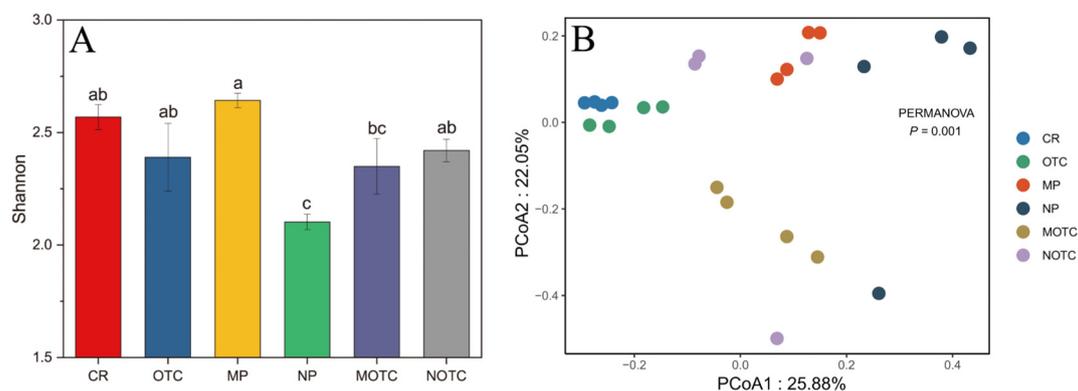

Fig. 6. Diversity of ARGs in zebrafish intestine. A. Shannon index of ARGs in zebrafish intestine. Data are presented as the means ± SEM ($n = 4$). One-way ANOVA was applied to analyse the difference between treatments ($P < 0.05$). B. PCoA based on the Bray-Curtis distance showed that ARGs and MGEs of zebrafish intestine present significant separation (n = 4, PERMANOVA, $P < 0.01$) in different treatments; letters above the bars indicate a significant difference when the bars do not share the same letter, while no significant difference was observed otherwise.
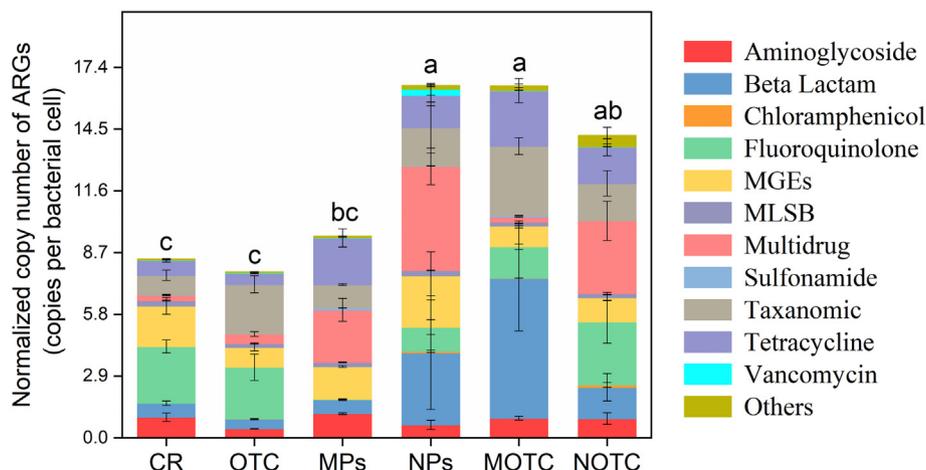
Fig. 7. Normalized copies per bacterial cell in each treatment, presenting the enrichment of ARGs after exposure for 30 days. One-way ANOVA was applied to analyse the difference in the total normalized copy number between all treatments ($P < 0.05$). Data are presented as the means ± SEM ($n = 4$); letters above the bars indicate a significant difference when the bars do not share the same letter, while no significant difference was observed otherwise.

Microplastics and OTC have been proven to affect the composition and structure of intestinal microbiota in many organisms (Li et al., 2020; Liu et al., 2019; Zhu et al., 2018b). However, to our knowledge, no previous study has compared the effects of micro- or nano-plastics co-exposure with antibiotics on the gut microbiome. Our results showed that the combined effect of microplastics and OTC on the composition alteration was conspicuously different from that of the single pollutant exposure. The abundance of *Fusobacteriota* in the OTC and NP treatments might be the response to the severe intestinal damage in those two treatments, since some members of *Fusobacteriota* metabolize carbohydrates (including mucins) into a short-chain fatty butyrate, acting as a strategy to defend against carcinogens and inflammatory (Andoh et al., 1999; McBain et al., 1997). Furthermore, compared with the control, the relative abundance of *Bacteroidetes* decreased significantly in the OTC treatment and slightly decreased in the MP or NP treatments, which is similar with previous studies (Liu et al., 2019; Lu et al., 2018). The increased abundance of *Bacteroidetes* in two combined exposure treatments was clearly distinguished from that in the other treatments, which further demonstrated that the combined exposure of microplastics and OTC exhibited a different influence on the gut microbiota of zebrafish, since the abundance of *Bacteroidetes* might related to body weight fluctuations (Lu et al., 2017; Zhang et al., 2021), and some members of *Bacteroidetes* might cause infections (Liang et al., 2019).

The abundance of *Firmicutes* is related to lipid metabolism, and increased *Firmicutes* abundance might correlate with the development of obesity (De Filippo et al., 2010; Ley et al., 2006). Some groups in *Firmicutes* are able to produce butyrate, which mainly provides nutrition and energy to epithelial and gastrointestinal cells, elevates mucus production, and

functions as an anti-inflammatory agent, and its depletion may impair gut barrier integrity (Collinder et al., 2003; Marx, 2015). Therefore, decreased *Firmicutes* may suggest the defection of fish to defend against intestinal damage. *Actinobacteria* presents a global interest in biotechnological application due to its capacity to synthetize secondary metabolites capable of removing xenobiotics (Alvarez et al., 2017), and decreased *Actinobacteria* might reflect the damage caused by contaminants. Studies have also suggested that the abundance of *Verrucomicrobia* is positively correlated with body weight (Zhang et al., 2021). These results demonstrated that the effect of combined exposure varies with that of single exposures.

We also found that micro- and nano-sized plastics had opposite effects on Shannon and Simpson indices of diversity regardless of independent exposure or combined exposure with OTC, indicating that micro- and nano-plastics had different effects on the alpha diversity independently or co-exposure with OTC. Specifically, MOTC exposure significantly increased the diversity of the intestinal microbiome compared with the control, while a converse decrease was observed in the NOTC treatments. The significantly up- and down-regulated third-level pathways in the MOTC and NOTC treatments were also clearly distinguished, which further indicated that micro- and nano-plastics combined with OTC exposure generated varied effects.

The difference in microbial diversity between treatments (including MOTC and NOTC) might be related to the degree of intestinal damage. We can see that the most severe intestinal damage observed in the treatments (OTC, NP, and NOTC, Fig. 1) also detected significantly decreased biodiversity (Fig. 3B and C). Unaffected intestines can secrete glycoproteins from the mucous layer, which compete with pathogenic bacteria for adhesin receptors in intestinal epithelial cells (Candela et al., 2008). Severe damage might lead to an invalid defensive strategy, causing an increase in specific microbial communities, such as infectious bacteria, which may lead to decreased biodiversity in those groups. Another study also suggested that NP exposure might induce an increase in certain groups of bacteria (increased abundance and decreased diversity of microbial community, Kang et al., 2021).

Furthermore, by comparing the Simpson and Shannon indices of the OTC and combined exposure groups, we found that in both the micro- and nano-plastic treatments, the combined exposure treatments tended to weaken the decrease in biodiversity caused by OTC exposure. This may be caused by the adsorption of microplastics to antibiotics (Han et al., 2021; Zhou et al., 2020a). Additionally, the biodiversity of the intestinal microbiome between the combined exposure and microplastic treatments was closer. Moreover, with the functional prediction of PICRUSt, we found that the main enriched second-level KEGG pathways in the plastic particles treatments and combined exposure were distinct from those in

Table 1
One-way ANOVA test of normalized abundance of ARGs and MGEs of different catalogs among all treatments ($P < 0.05$), and the table should be read horizontally.

	CR	OTC	MPs	NPs	MOTC	NOTC
Aminoglycoside	ab	c	a	bc	ab	abc
Beta lactam	b	b	b	ab	a	b
Chloramphenicol	Not significant					
Fluoroquinolone	ab	ab	c	bc	abc	a
MGEs	Not significant					
MLSB	Not significant					
Multidrug	c	c	b	a	c	ab
Sulfonamide	bc	c	a	b	a	c
Taxanomic	b	ab	b	ab	a	ab
Tetracycline	bc	c	a	abc	a	ab
Vancomycin	Not significant					
Others	Not significant					
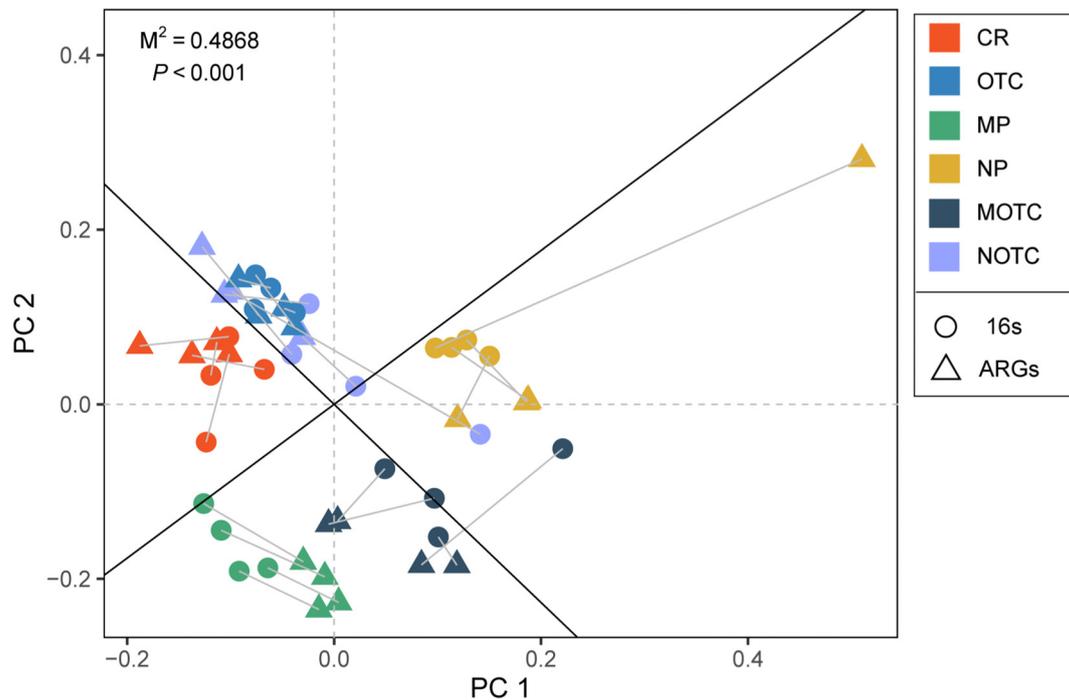
Fig. 8. Procrustes test showing the significant correlation between ARGs profile and zebrafish gut microbiota (OTU data) based on Bray − Curtis dissimilarity metrics (sum of squares $M^2 = 0.4868$, $P < 0.001$, 9999 permutations). The triangles indicate the ARGs in zebrafish intestine, circles indicate the OTU data of collembolan gut microbial composition, and squares indicate different treatments.

the OTC-treated group, which was also consistent with the mentioned alterations in microbial composition and diversity.

The results of HT-qPCR showed that significant differences in ARGs belonging to 11 types were observed among different treatments, indicating that polystyrene microplastics and OTC changed the profiles of ARGs in zebrafish intestine. We found that the abundance of ARGs in the MOTC treatment was significantly higher than that in the control and single exposure (MP and OTC), and a similar increase was observed in the NOTC treatment compared with the control and OTC treatments, suggesting that the co-exposure of microplastics and OTC will increase the incidence of ARGs.

However, the highest abundance of ARGs was detected in the NP treatment, which may be partly due to the highest abundance of MGEs detected in the NP treatment. The highest abundance of MGEs in NP treatment is due to the following reason: NPs have the ability to be internalized into cells due to their tiny size (Ramsperger et al., 2020), and they can adsorb and interact with biomolecules like free proteins and nucleic acid fragments with their large specific surface area and complex surface structure (Kelly et al., 2015; Lundqvist et al., 2008; Ramsperger et al., 2020), which means the possibility of NPs adsorbing MGEs like plasmid and insertional sequences, but more detailed and accurate explanations still require further investigation. MGEs represent an increased possibility of horizontal gene transfer (HGT), while HGT is an important pathway of ARG transmission (Drudge and Warren, 2012) and is relatively likely to occur in cell-to-cell contact bacteria. The large specific surface area and small size of microplastics bring higher nutritional availability and density of the microbiota (Aminov, 2011), making them hotspots of horizontal gene transfer (Arias-Andres et al., 2018). In addition, nano-plastics are more likely to internalize in the intestine of zebrafish (Sendra et al., 2021), rather than remaining in the intestinal lumen, prolonging the retention time of nano-plastics, which may increase the possibility of HGT. Another reason to explain the higher abundance of ARGs in the NP than in the MP treatment is that the severe intestinal damage observed in the NP treatment (as well as in the MOTC/NOTC treatments) might result in more microplastic retention in the gut, which may lead to an increased hotspot for ARGs and antibiotic resistance bacteria, thus exhibiting a higher ARGs abundance in those treatments (NP, MOTC, NOTC).

In the present study, we did not detect the highest tetracycline resistance genes in the OTC treated groups. Speculated reasons are: (i) a higher abundance of other types of antibiotic resistant genes was detected in the blank group (CR), indicating a higher background abundance of other types of antibiotic resistance genes. The relatively low exposure concentration of OTC may not trigger an increase in tetracycline resistance genes (studies suggesting that antibiotics exposure leads to an increase in corresponding types of antibiotic resistance genes are conducted at high concentrations, such as mg/L and mg/kg: Looft et al., 2012; Ma et al., 2019; Yin et al., 2015; Zhu et al., 2018a), which might explain the higher abundance of other types of antibiotic resistance genes than tetracycline resistance genes. (ii) There are co-occurrence patterns between ARGs, and different types of ARGs may be carried by the genome of the same microorganism (Botts et al., 2017; Peter et al., 2017; Poey and Laviña, 2018). The enrichment of specific types of antibiotic-resistant bacteria may be accompanied by other types of resistance genes (Martins et al., 2017). Various types of inherent resistance genes were detected in the zebrafish (ARGs in the control group), and a possible increase of tetracycline resistance genes may accompany other types of ARGs, which may also be the reason why tetracycline resistance genes are not higher than other types of resistance genes.

Another intriguing finding is the significant differences in ARGs profiles between the MP and NP treatments. As the highest Shannon index but lower abundance of ARGs were identified in the MP treatments, the lowest Shannon index and increased abundance of ARGs were detected under NP exposure, implying that NP exposure is inclined to enrich a certain type of ARG, while the distribution of different types of ARG in MP exposure was more proportional. However, co-exposure with OTC weakened this discrepancy, indicating that combined exposure with antibiotics will change the effects of microplastics exposure alone. The heatmap of enriched ARGs also presented an explicit distinction between single and combined exposure, suggesting that co-exposure altered the enrichment of ARGs compared with single exposure.

This variation might be attributed to the alteration of zebrafish gut microbiota, and previous studies have confirmed that the diversification of intestinal microbiota can influence the profile of ARGs in the intestine (Tian et al., 2012; Ding et al., 2019; Zhu et al., 2020). The gut microbiota

of zebrafish changed significantly after exposure to micro- and nano-plastics, which may be the dominant reason for the changes of ARGs abundance in zebrafish intestines (such as the increase of multidrug and the decrease of fluoroquinolone and sulfonamides resistance genes). Some bacteria in the environment are generally considered as possible harbors of ARGs and diverse ARGs host (Ma et al., 2020; Su et al., 2015; Zhu et al., 2017); thus changes in the bacterial community may lead to differences in ARGs. The correlation heatmap results suggested that the changes of aminoglycoside, tetracycline and sulfonamide resistance genes were positively correlated with the increase in *Pseudomonadales* abundance in the MP and MOTC treatments, as well as the correlation between *Burkholderia* abundance and β-lactam resistance genes in NP and MOTC treatments. These results were consistent with our results of the greatest abundance of aminoglycoside, tetracycline and sulfonamide resistance genes in the MP treatments (Table 1), which further suggested that the change of bacterial community would influence the abundance of resistance gene. However, it should be noted that this study only detected the abundance of ARGs based on HT-qPCR, further investigation using metagenome assembly and specific microbial culture are needed to verify the cooccurrence characteristics of the same or different types of genes, and whether specific bacteria have resistance genes or multiple antibiotic resistance.

The variation among treatments might be related to the difference of toxicological mechanisms between micro- and nano-plastics and their different adsorption capacities to OTC. A more accurate conclusion needs to comprehensively consider the adsorption and desorption of antibiotics by micro- and nano-plastics in zebrafish, as well as the metabolic coefficient and bioavailability of microplastics and antibiotics in zebrafish.

5. Conclusion

Plastics and antibiotics in the environment are of great concern considering their increasing amount and the ecological risk to ecosystems. This is the first study to compare the intestine histopathology, microbiota and ARGs profile in the zebrafish gut after exposure to OTC and micro- and nano-sized polystyrene particles. We found that the histological damage caused by plastic particles is size-dependent, and nano-sized plastics induced severe intestinal damage. The combined exposure of micro-plastics and OTC could significantly alleviate the intestinal damage of zebrafish induced by OTC, while no such moderation was observed in the nano-sized plastic treatments. The combined exposure of microplastics and OTC exhibited the opposite effects on the intestinal microbial diversity between micro- and nano-plastics compared with the control. Combined exposure increased the abundance of intestinal resistance genes. Nano-plastics mainly tend to enrich certain types of ARGs, while micro-plastics enrich different types of resistance genes, and the distribution of ARGs is more homogenous. In general, the combined exposure of microplastics and OTC significantly affected the intestinal histopathology, microbiota and abundance of ARGs in the zebrafish gut, and the effects between the micro- and nano-plastics-treated groups were distinguished. These results are essential to further understand the effects of antibiotics associated with micro- or nano-plastics on the ecotoxicology and transfer of ARGs in aquatic ecosystems. However, more comprehensive indicators are urgently needed to reflect the combined ecotoxicological effect between micro- and nano-plastics combined with antibiotics, and the underlying mechanism induced by the difference between micro- and nano-plastics still requires further investigation.

CRediT authorship contribution statement

Ziyue Yu: Methodology, Investigation, Writing - original draft. **Ling Zhang**: Software, Writing - review & editing. **Qiansheng Huang**: Writing - review & editing. **Sijun Dong**: Writing - review & editing, Funding acquisition. **Xinhong Wang**: Writing - review & editing. **Changzhou Yan**: Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

Declaration of competing interest

Acknowledgement

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.156917.

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