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Environmental concentrations of antibiotics impair zebrafish gut health☆

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ABSTRACT

Antibiotics have been widely used in human and veterinary medicine to both treat and prevent disease. Due to their high water solubility and low bioavailability, many antibiotic residues have been found in aquatic environments. Fish are an indispensable link between the environmental pollution and human health. However, the chronic effects of environmental concentrations of antibiotics in fish have not been thoroughly investigated. Sulfamethoxazole (SMX) and oxytetracycline (OTC) are frequently detected in aquatic environments. In this study, zebrafish were exposed to SMX (260 ng/L) and OTC (420 ng/L) for a six-week period. Results indicated that exposure to antibiotics did not influence weight gain of fish but increased the metabolic rate and caused higher mortality when treated fish were challenged with *Aeromonas hydrophila*. Furthermore, exposure to antibiotics in water resulted in a significant decrease in intestinal goblet cell numbers, alkaline phosphatase (AKP), acid phosphatase (ACP) activities, and the anti-oxidant response while there was a significant increase in expression of inflammatory factors. Antibiotic exposure also disturbed the intestinal microbiota in the OTC-exposed group. Our results indicated that environmental antibiotic concentrations can impair the gut health of zebrafish. The potential health risk of antibiotic residues in water should be evaluated in the future.

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1. Introduction

Antibiotics are natural or synthetic drugs with the capacity to kill or inhibit the growth of bacteria. In addition to treating infectious diseases, some antibiotics are used as feed supplements to promote growth of livestock (Carvalho and Santos, 2016). In the United States of America and Europe, the annual usage of antibiotics has been estimated to be in the range of tens of thousands of tons (Food and Drug Administration, 2009; European Medicines Agency, 2011). In China, approximately 162,000 tons of antibiotics were used in 2013 (Zhang et al., 2015). Most antibiotics are poorly absorbed by humans or animals after intake, with approximately 25–75% of antibiotics discharged into aquatic environment as unchanged or modified as metabolites via feces or urine (Karthikeyan and Meyer, 2006). Due to their low bioavailability, high water solubility, and widespread misuse, various antibiotic residues have been detected in many aquatic environments (Chang et al., 2010; Chen et al., 2015a; Godeaux, 2015; Leung et al., 2012; Ma et al., 2015; Wei et al., 2011).

Antibiotic residuals in water have been reported to cause adverse effects on aquatic organisms. However, information on ecological toxicity is generally limited to acute lethal effects, which occurs at concentrations in the mg/L range (Ji et al., 2012). Generally, the environmental concentrations of antibiotic residues in water range from ng/L to μg/L, several orders of magnitude lower than concentrations resulting in acute effects. More recently, studies have been conducted to evaluate the influence of antibiotics using concentrations relevant to those in the environment *in situ* but they are mainly limited to short-term tests (Carlson et al., 2017;

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Elizalde-Velazquez et al., 2017; Oliveira et al., 2013). As many aquatic species are continuously exposed to water over long periods of time or perhaps over their entire life cycle, it is more apposite to measure the chronic influence of antibiotics using environmental concentrations on aquatic animals (Fent et al., 2006). Fish are particularly important targets for evaluating environmental risks, as they provide an indispensable link between antibiotics released into aqueous environments and human health (Zhao et al., 2015). Furthermore, they are good models for chronic toxicity analyses (Nannou et al., 2015).

A functional and stable gut microbiota is important to host health. Antibiotics can deplete commensal microbiota or directly affect host tissues, causing adverse effects on animal physiology (Morgun et al., 2015). Intact intestinal structures and normal intestinal function help animal hosts prevent many diseases. For example, it has been shown that an intact intestine affords a barrier to exogenous toxins through oxidative and reductive processes which are the key components of metabolic pathways of xenobiotics, including drugs (Rodrigues et al., 2017). Although the intestine is the main target of antibiotic absorption, previous studies have mainly focused on the functions of liver, kidney or gills when the risks of antibiotics were evaluated (Madureira et al., 2012; Rodrigues et al., 2017; Yonar et al., 2011). Recent studies have suggested involvement of the gut microbiota in the development of a wide range of disorders, including metabolic dysfunctions and inflammatory disease (Fei and Zhao, 2013; Xiao et al., 2014). Furthermore, strong evidence also showed the critical role of intestinal microbiota in drug metabolism and toxicity (Choi et al., 2013).

Sulfamethoxazole (SMX) and oxytetracycline (OTC) are two commonly used antibiotics in human and veterinary medicine (Rigos and Troisi, 2005; Trovó et al., 2009). Consequently, SMX and OTC have been frequently detected in surface water, groundwater and seawater (Nie et al., 2013). The concentrations of these antibiotics vary in different places or seasons. It has been observed that the concentrations of SMX range from 2 to 11600 ng/L in natural or waste water (Gust et al., 2012; Terzic et al., 2008). In Shanghai for example, a mean concentration of 259.6 ng/L SMX was found in Huangpu River (range of 2.2 ng/L to 764.9 ng/L) (Chen and Zhou, 2014). The geometric mean concentration of OTC in Tha Chin River was 220 ng/L in the dry season and 500 ng/L in the wet season (Rico et al., 2014). The mean concentration of OTC in water from the Hailing Bay Region was found to be 417.76 ng/L with a maximum concentration of 15.16 μg/L (Chen et al., 2015b) (Table S1).

Similar to other antibiotics, the risk assessment for these two antibiotics and their effect on aquatic animals is mainly obtained from acute toxicological research studies. For example, a combination of SMX and trimethoprim at a concentration of 40 ppm increased plasma cortisol and glucose and resulted in a decreased *C*-reactive protein concentration in gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*), after one hour of treatment (Yildiz and Altunay, 2011). Acute exposure (96 h) of rainbow trout (*Oncorhynchus mykiss*) to OTC at a concentration ranging from 5 μg/L to 50 mg/L did not change catalase (CAT) activity in gill tissues of rainbow trout. However, chronic exposure (28 days) to OTC at a concentration ranging from 0.3125 μg/L to 5 μg/L decreased CAT activity in gills (Rodrigues et al., 2017), indicating that tissues function differently when exposed to chronic and acute antibiotic exposure. However, there is very limited information relating to environmental concentrations of these two widely distributed antibiotics especially at ng/L level, and how they can adversely influence fish physiology.

Considering that the gut is the main area of antibiotic absorption and that intestinal health is very important to fish, the aim of this study was to identify the chronic influence of environmental concentrations of antibiotics on the intestinal health of fish. Zebrafish were exposed to SMX and OTC at ng/L levels for a six-week period. Gut histological morphology, oxidative stress status, inflammatory reactions, and microbiota composition were investigated to explore the influence of antibiotic residues in water at ng/L concentrations on gut health of zebrafish.

2. Materials and methods

2.1. Ethical considerations

All experiments were conducted under the Guidance of the Care and Use of Laboratory Animals in China. This research was approved by the Committee on the Ethics of Animal Experiments of East China Normal University.

2.2. Experimental animals and antibiotic exposure

Six hundred zebrafish (0.1 g each) were purchased from Yuyi tropical fish farm (Shanghai, China). Before the experiment, fish were allowed to acclimatize for two weeks in 100 cm × 45 cm × 45 cm tanks with aerated dechlorinated tap water and a natural photoperiod of 12 h light and 12 h dark. The water temperature and pH were kept at 26 ± 2 °C and pH 6.8–7.5, respectively. During acclimatization, fish were hand-fed a commercial diet (Shengsuo Co., Shandong, China) twice daily.

Sulfamethoxazole (SMX, CAS:723-46-6) and oxytetracycline (OTC, CAS: 615 3-64-6) were purchased from Yuanmu Biotechnology Co., Ltd, Shanghai, China. SMX is dissolved in water with a concentration at 260 mg/L as a stock solution while OTC is dissolved in water with a concentration at 210 mg/L as a stock solution. Based on the available literature on the environmental concentrations of the two antibiotics, the concentrations of SMX and OTC used in the present study were set at 260 ng/L and 420 ng/L, respectively (Chen and Zhou, 2014; Chen et al., 2015b). After two weeks of acclimatization, 600 zebrafish were randomly divided into three triplicate groups: control, SMX-bath and OTC-bath groups. During the experiment, fish were hand-fed a basic diet at a ration of 2% of body weight, twice daily (Supplemental Table S2). The weight of the fish in each tank was recorded during the experiment, and the quantity of feed was adjusted correspondingly. All treatments were performed in clean tanks, and half of the water from each tank was replaced every two days. On each occasion when the water was changed, the water in the antibiotic-treated tanks was replaced by an equal amount of water containing the initial concentration of antibiotics in order to maintain the antibiotic concentrations within the test range throughout the experiment. Water in the control tank was equally replaced with clean and fresh dechlorinated tap water. The duration of the experiment lasted for six weeks. At the end of the experiment, fish were captured and their final weight was determined.

2.3. Determination of oxygen consumption rate

The metabolic rate was evaluated by examining the oxygen consumption rate (OCR). The OCR of zebrafish was measured at the end of the experiment using a Strathkelvin Instrument 782 Oxygen Meter (Strathkelvin Instruments Limited, North Lanarkshire, Scotland, UK). Zebrafish were fasted for 24 h to exclude interference with postprandial metabolism. Before the measurement, ten fish were placed in one chamber with 650 ml air-saturated water at 24 °C until the baseline on the instrument shows a smooth curve. To minimize the effect of low oxygen concentration on the metabolic rate and oxygen consumption, the time allowed for detecting the oxygen consumption was limited for 5 min and the oxygen

concentration of the water was never allowed to fall below approximately 80 percent saturation before recording (Xuan et al., 2013). The experiments were repeated three times with independent samples. The chamber without fish was used as the control. OCR was calculated using the amount of O_2 in the measured water sample according to the following equation: OCR = [ΔO_2 concentration (mg/L) × water volume (L)]/[fish mass (g) × time (h)].

2.4. Bacterial challenge

At the end of the experiment, *Aeromonas hydrophila* was used to challenge the zebrafish in this study (Fig. S1). The bacterial challenge was conducted in the three treatment groups (control, SMX–bath and OTC-bath) and each treatment had 25 fish in the three replicate groups. A single colony of *A. hydrophila* was grown in Luria-Bertani medium (LB) at 37 °C with shaking at 200 rpm for 14 h. The overnight culture was then inoculated into 100 mL of fresh LB medium (1:100). Zebrafish were immersed in water containing the bacteria at a final concentration of 5×10^8 CFU/ml for six days. The water and bacteria were changed every two days and mortality was recorded every day.

2.5. Biochemical assays

In order to minimize bias, each treatment contained 30 fish for biochemical analysis. The whole gut contents of each fish were weighed and homogenized with 9 vol (v/w) of 0.8% physiological saline. Then, the homogenate was centrifuged at 2500 × g at 4 °C for 10 min and the supernatant was collected for biochemical assays according to the manufacturer's instructions. Malondialdehyde (MDA) was measured using an enzyme-linked immune sorbent assay (ELISA) kit (Hengyuan, Shanghai). Superoxide dismutase (SOD), peroxidase (POD), reduced glutathione (GSH), acid phosphatase (ACP), and alkaline phosphatase (AKP) were measured using related commercial assay kits (Nanjing Jiancheng Institute, China). Results were recorded on a microplate reader (Epoch, Bio-Tek, USA).

2.6. Gut morphology

Three fish per tank were collected and approximate 0.5 cm-length segments of the foregut were fixed in 4% paraformaldehyde for 24 h. Then, the paraformaldehyde-fixed samples were transferred to different concentrations of ethanol (75%, 85%, 95%, and 100%) for dehydration, followed by transfer to xylene for transparency, processing, and embedding in paraffin wax. The foreguts were sliced transversely into 5 μm sections and stained with hematoxylin-eosin (H & E). Stained slides were observed under microscopy (Nikon, Eclipse, TS100).

2.7. Quantitative Real-time PCR

Total RNA was extracted from zebrafish gut using Tri Pure Reagent (Aidlab, China). A NaNo DROP 2000 Spectrophotometer (Thermo Scientific, MA, USA) was used to measure the amount of RNA. Visualization of the 28S/18S ribosomal RNA ratio on a 1% agarose gel was used to assess the RNA quality for each sample. Total RNA (1 μg) was used to synthesize the complementary DNA by a PrimeScript™ RT Reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. Real-time PCR was conducted in the CFX Connect Real-Time System (Bio-Rad). Genes related to inflammation (TNF-α, IL-1, and TGF-β) were chosen in this study. EF-1α was chosen as the internal control to normalize the data. The primers for EF1α and target genes for quantitative PCR (qPCR) are listed in Supplemental Table S3. The mixture for qPCR contained 10 μL of 2 × SYBR qPCR Mixture (Aidlab, China), 2 μL cDNA, 1.6 μL of qPCR primers (4 μM), and 6.4 μL nuclease-free water. The program for the qPCR reaction was 94 °C for 3 min, 40 cycles at 94 °C for 6 s and 60 °C for 30 s. The melting curves for the amplified products were generated to ensure the specificity of assays at the end of each PCR. Gene expression quantification was calculated using the comparative ΔΔCT method, and the gene expression level in the control group was used as the comparator. All assays were performed in triplicate.

2.8. Bacterial genomic DNA extraction

The intestines from three fish in each group were aseptically dissected and the intestinal contents were used for bacterial composition analysis. Total bacterial DNA was isolated using the E.Z.N.A.® Soil DNA Kit (OMEGA, USA) according to the manufacturer's instructions. DNA yield was measured in a NaNo DROP 2000 Spectrophotometer (Thermo, USA).

2.9. Illumina high-throughput sequencing of barcoded 16S rRNA genes

The V3-V4 region of the bacteria 16S ribosomal RNA gene was amplified by PCR using primers 338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3'. Unique eight-base barcodes were added to each primer to distinguish the different PCR products. PCR reactions were performed in a 20 μL mixture containing 4 μL of 5 × Fast Pfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase (TransGen, China), and 10 ng of template DNA. The PCR conditions were as follows: 95 °C for 3 min to allow DNA denaturation, with the amplification lasting 29 cycles (95 °C for 30 s, 55 °C for 30 s and 72 °C for 45 s), and the final extension period lasted for 10 min at 72 °C. Purified PCR products were subjected to Illumina based high-throughput sequencing (Majorbio Bio-Pharm Technology, Co., Ltd., Shanghai, China). The sequences obtained in this paper are available in SRA with the accession number SRP111618.

2.10. Bioinformatics analysis

The raw pair-end readings were then subjected to a quality-control procedure using QIIME (version 1.17). The qualified reads were clustered to generate operational taxonomic units (OTUs) at the 97% similarity level using UPARSE (version 7.1). Chimeric sequences were identified and removed using UCHIME (version 4.1). The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed using RDP Classifier against the silva 16S rRNA database using a confidence threshold of 70%. Taxonomic richness and diversity estimators were determined in Mothur. ACE and Chao were used to reflect community richness, and diversity was assessed by using Shannon and Simpson indices. All these indices were estimated based on OTU abundance matrices using Mothur. Principal component analysis and heat-map analysis were performed under a MATLAB R2016a environment. Forty-three OTUs were selected for heatmap analysis based on, 1) the abundances of these OTUs were higher than 0.01% in each sample and 2) the abundance of these OTUs were significantly different for SMX-bath or OTC-bath treatment compared with the control group using the Student's t-test with subsequent Bonferroni correction.

2.11. Statistical analysis

All results are presented as mean ± standard error (SE). The Student's *t*-test was used to independently compare each group treated with antibiotics with the control group. All statistical

analyses were conducted using SPSS software (version 18.0, Chicago, IL, USA). A value of $P < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1. Effects of antibiotic exposure on growth, metabolic rate, and mortality in response to bacterial challenge of zebrafish

At the end of the experiment, there was no significant difference in final body weight between the control and antibiotic treated groups ($P > 0.05$) (Fig. 1A). The whole fish oxygen consumption rate in the SMX-bath and OTC-bath groups was significantly higher than that observed in the control group ($P < 0.05$) (Fig. 1B). When fish were challenged with *A. hydrophila,* the accumulated mortality of fish in the antibiotic treated groups was higher than that observed in the control group (Fig. 1C). The SMX-bath treated group showed significantly higher mortality than the control group on days 1, 2, and 3 ($P < 0.05$), while the OTC-bath group had a higher mortality rate than the control group on day 4 and day 6 ($P < 0.05$). These results indicated that antibiotic residues in water increased the metabolic rate of zebrafish while decreasing their resistance to *A. hydrophila.*

3.2. Influence of antibiotic exposure on the intestinal structure

The number of goblet cells is an important parameter for evaluating innate immunity in fish. In order to detect the influence of antibiotic residues on the intestinal structure, H&E staining was used to show the intestinal structure. Results indicated that the number of goblet cells decreased significantly ($P < 0.05$) in the foregut of SMX-bath and OTC-bath treated groups compared with the control group (Fig. 2). This suggests that antibiotic exposure could impair zebrafish innate immunity through reduction of gut goblet cells.

3.3. Effects of antibiotic exposure on non-specific immune reactions in the gut

AKP (alkaline phosphatase) and ACP (acid phosphatase) activities were detected in each group. Results showed that AKP activity in the SMX-bath and OTC-bath groups decreased significantly ($P < 0.05$) compared with the control group (Fig. 3A). However, the ACP activity decreased significantly ($P < 0.05$) in the SMX-bath group only, whereas a decreasing trend was detected in the OTC-bath group compared with the control but was not significantly different (Fig. 3B).

3.4. Effects of antibiotic exposure on expression of inflammation-related genes in the gut

The relative expressions of immune-related pro-inflammatory cytokines (TNF-α, IL-1) and the anti-inflammatory cytokine (TGF-β) in the gut were tested to evaluate the influence of antibiotic exposure on inflammatory status. The relative expression of TNF-α was higher in the SMX-bath and OTC-bath groups compared with the control group ($P < 0.05$; Fig. 4A). In the SMX-bath group, the relative expression of IL-1 was significantly higher than in the control group ($P < 0.05$; Fig. 4B), whereas an increasing trend was observed in the OTC-bath treatment group compared with the control group, although this was not significantly different from the control group ($P > 0.05$; Fig. 4B). There was no significant difference in the expression of TGF-β between the experimental groups ($P > 0.05$; Fig. 4C).

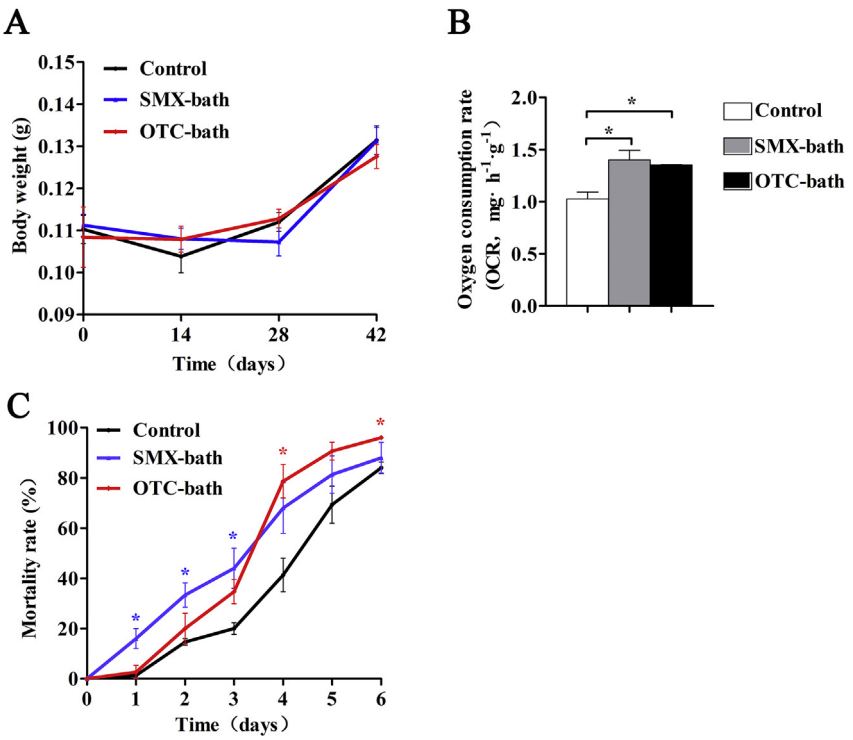


Fig. 1. Influence of SMX and OTC exposure on the body weight, oxygen consumption rate, and mortality rate of zebrafish. (A) Growth curve of zebrafish during the six-week period. (B) Oxygen consumption rate of fish. (C) The mortality of zebrafish infected with *A. hydrophila.* Student's *t*-test was used to detect the significant differences between the control and antibiotic exposed groups, *$P < 0.05$.

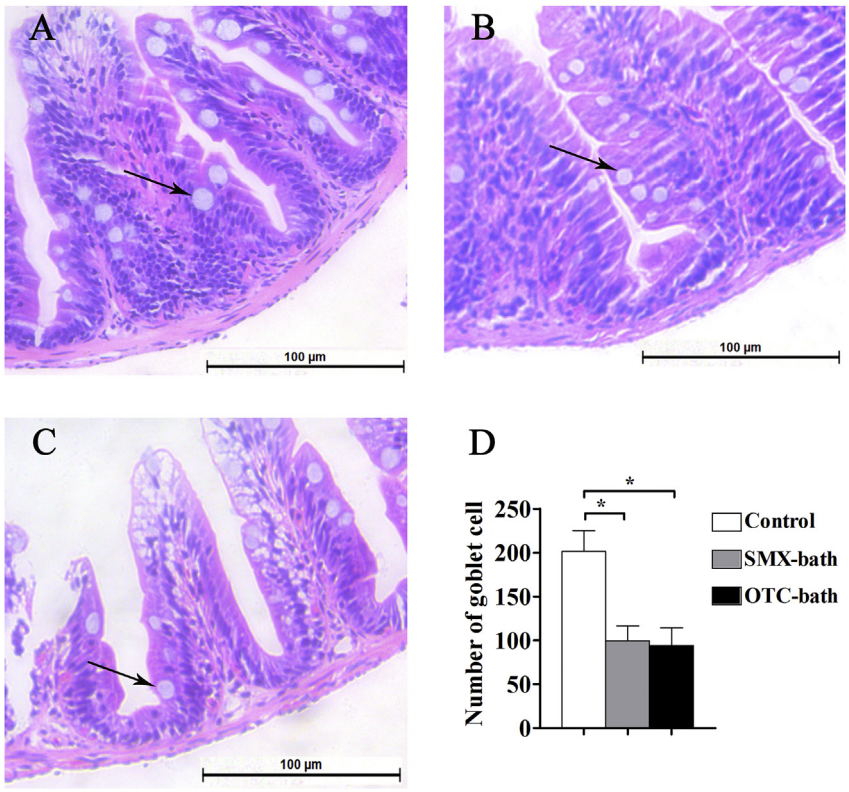


Fig. 2. Hematoxylin-eosin staining of zebrafish gut in control and antibiotic exposed groups, 200 × , scale bars 100 μm. (A) Control group; (B) SMX-bath group; (C) OTC-bath group; (D) The number of goblet cells (as indicated by the black arrows) in zebrafish intestinal tracts was statistically analyzed based on three separate samples and Student's *t*-test was used to detect the significant difference between the control and antibiotic exposed groups, *P < 0.05.

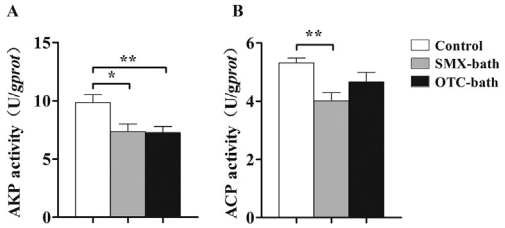


Fig. 3. Effect of antibiotic exposures on (A) the alkaline phosphatase activity in zebrafish intestine and (B) acid phosphatase activity in zebrafish intestine. Student's *t*-test was used to detect the significant difference between the control and antibiotic exposed groups, *P < 0.05.

3.5. Oxidative stress response to antibiotic exposure in the gut

Compared to the control group, MDA content increased significantly (P < 0.05) in the SMX-bath group only (Fig. 5A). Zebrafish in the SMX-bath and OTC-bath treatment groups showed lower activities (P < 0.05) of SOD and POD compared to the group control (Fig. 5B and C). Moreover, GSH content in the SMX-bath and OTC-bath groups decreased significantly (P < 0.05) compared with the control group (Fig. 5D). We also detected the activities of MDA, SOD and POD in muscle and liver. With exception of SOD in the OTC group, which decreased in muscle, the other measured parameters remained broadly similar for the treatments (Fig. S2, Fig. S3).

3.6. Gut microbial communities in zebrafish

Bacterial quantity was detected by conventional plate count method and the results showed no significant difference between

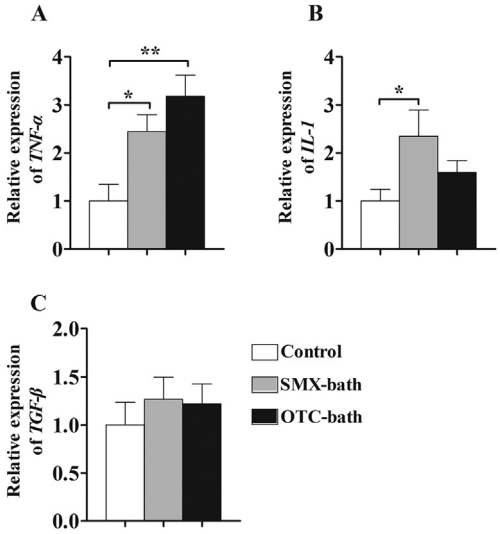


Fig. 4. Effect of antibiotic exposures on the expression of (A), tumor necrosis factor alpha (TNF-α), (B) interleukin-1 (IL-1) and (C) Transforming growth factor-beta (TGF-β) in zebrafish intestines. Student's *t*-test was used to detect for a significant difference between the control and antibiotic exposed groups, *P < 0.05.

the control and antibiotic exposure groups (Fig. S4). A total of 360,875 high-quality sequences were produced in this study, with an average of 40,097 sequences per sample. The coverage for each group exceeded 99%, indicating that the 16S rRNA gene sequences identified in the groups represent the majority of bacteria present in the samples of this study. The number of OTUs, the estimators of

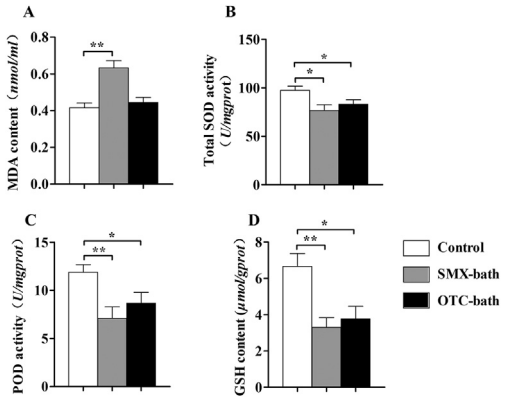


Fig. 5. Effect of antibiotic exposures on (A) Malondialdehyde content (MDA), (B) total superoxidase dismutase (SOD) activity, (C) peroxidase (POD) activity and (D) reduced glutathione (GSH) content in zebrafish intestine. Student's *t*-test was used to detect for significant differences between the control and antibiotic exposed groups, *P < 0.05.

community richness (Ace and Chao) and diversity (Shannon and Simpson) are shown in Table 1. Results indicated a statistically significant reduction in OTU Ace and Chao indices in the SMX-bath and OTC-bath treatment groups compared to the control group ($P < 0.05$). This suggests that SMX-bath and OTC-bath treatment groups had lower bacterial richness compared with the control group. The microbial diversity from the SMX-bath and OTC-bath treatment groups showed a decreasing trend compared with the control group but this was not statistically significant ($P > 0.05$).

The relative abundance (%) of gut microbiota in each sample at the phylum level is shown in Fig. 6A. The dominant phyla in the three groups were Proteobacteria, Firmicutes, Fusobacteria, Bacteroidetes, and CKC4 (Table 2). In the control group, the relative abundances of these five phyla were 51.3%, 25.7%, 6.7%, 10.9%, and 2.8%, respectively. In the SMX-bath treatment group, the relative abundances of the five phyla were 50.2%, 24.7%, 10.0%, 8.8%, and 3.8%, respectively. In the OTC-bath treatment group, the relative abundances of those five phyla were 30.4%, 20.2%, 22.2%, 8.6%, and 16.0%, respectively. The relative abundances of Proteobacteria and Planctomycetes were significantly decreased in the OTC-bath treatment group ($P < 0.05$), while Fusobacteria and CKC4 were significantly increased in the OTC-bath group ($P < 0.05$) compared with the control group (Fig. S5).

Principal component analysis (PCA) was used to characterize the distribution of variations in community composition among the three treatments, with most of the variation explained by the first two coordinates (62.5% and 20.1%, respectively; Fig. 6B). Samples from the control and the SMX-bath treatment group clustered together, with the exception of one sample in each group. The OTC-bath treatment group was distinctly separated from the control group. Significant difference has been detected in PC1 between the control and OTC-bath group based on ANOVA analysis ($P < 0.05$) but no significant difference has been found between the control and SMX-bath group ($P = 0.429$). These results indicate that OTC exposure had a greater influence on gut bacterial communities than SMX exposure.

Forty-three OTUs were selected for heatmap analyses and among them 23 OTUs showed significant difference between the control and antibiotic exposed groups (Fig. 7). Eighteen OTUs decreased significantly in antibiotic exposed groups and these OTUs were distributed in the Proteobacteria, Bacteroidetes and Planctomycetes. Five OTUs increased significantly in the antibiotic exposed groups and these OTUs were distributed in the Proteobacteria, Fusobacteria and CKC4. OTU 453 (Cetobacterium) and OTU413 (CKC4) increased in the antibiotic exposed groups, especially in those treated with OTC.

4. Discussion

In this study, the influence of antibiotic residues in water at ng/L concentrations was detected on fish growth conditions and their metabolic rate. Long-term exposure to OTC or SMX at environmentally relevant concentrations did not influence the weight gain of zebrafish. These data are consistent with a previous study that examined exposure to SMX at concentrations ranging between 2 and 200 μg/L for 150 days and no apparent effects on the growth conditions of zebrafish was noted (Yan et al., 2016). Indeed, for higher concentrations of antibiotics (200 μg/L), the weight gain of zebrafish was actually found to be suppressed (Yan et al., 2016). Melvin et al. (2014) also found that exposure to SMX (10 μg/L), alone or combined with other pharmaceuticals, did not affect the growth of the Australian striped marsh frog tadpole (*Limnodynastes peronii*) (Melvin et al., 2014). However, in the present study, although fish growth was not affected by the antibiotic residues, we found that the fish exposed to antibiotics consumed more aqueous oxygen, suggesting that those fish exposed to low concentrations of antibiotics required extra energy to tolerate the toxicological stress instead of using this for growth (Meade et al., 2002).

Fish mainly depend on innate immune responses to environmental insults, and these appear to be more potent than in higher vertebrates (Yildiz and Altunay, 2011). In the present study, environmental concentration of antibiotics decreased the number of goblet cells, which play important roles in host defense against physical and chemical injury caused by endogenous and exogenous irritants, or microbial attachment and invasion (Kim and Ho, 2010). Exposure to environmental antibiotic residues decreased the activities of ACP and AKP in zebrafish gut, which are important components of the non-specific immune system. With exception to the innate immune system, an early pivotal event in the inflammatory process is the increase in the transcription of pro-inflammatory cytokines, which enable organisms to respond to different types of challenge (Chen and Nunez, 2010). In our present study, antibiotic exposure increased the expression levels of TNF-α and IL-1 in zebrafish, indicating that concentration of antibiotics in water disturbed the intestinal inflammation process (Barros-Becker et al., 2012). This is consistent with previous studies that 48 h of exposure to OTC (750 ppm) triggered a widespread inflammatory response that persisted until 96 h of exposure in zebrafish (Barros-Becker et al., 2012). It has been shown that long-term exposure to SMX at environmentally relevant concentrations (2 μg/L) may not

Table 1
Alpha diversity of intestinal microbiota in zebrafish *(Danio rerio)*.

	Reads	OTUs	Ace	Chao1	Shannon	Simpson	Coverage
Control	36506 ± 4625	349 ± 33	366 ± 34	373 ± 35	3.40 ± 0.26	0.086 ± 0.026	99.89% ± 0.03%
SMX-bath	34823 ± 3887	203 ± 78*	213 ± 77*	219 ± 78*	3.08 ± 0.36	0.099 ± 0.025	99.95% ± 0.02%*
OTC-bath	37354 ± 6344	259 ± 34**	276 ± 38*	275 ± 34**	3.15 ± 0.10	0.099 ± 0.016	99.92% ± 0.04%

Note: * represents significant difference ($P < 0.05$) compared with Control group.
** represents significant difference ($P < 0.01$) compared with Control group.

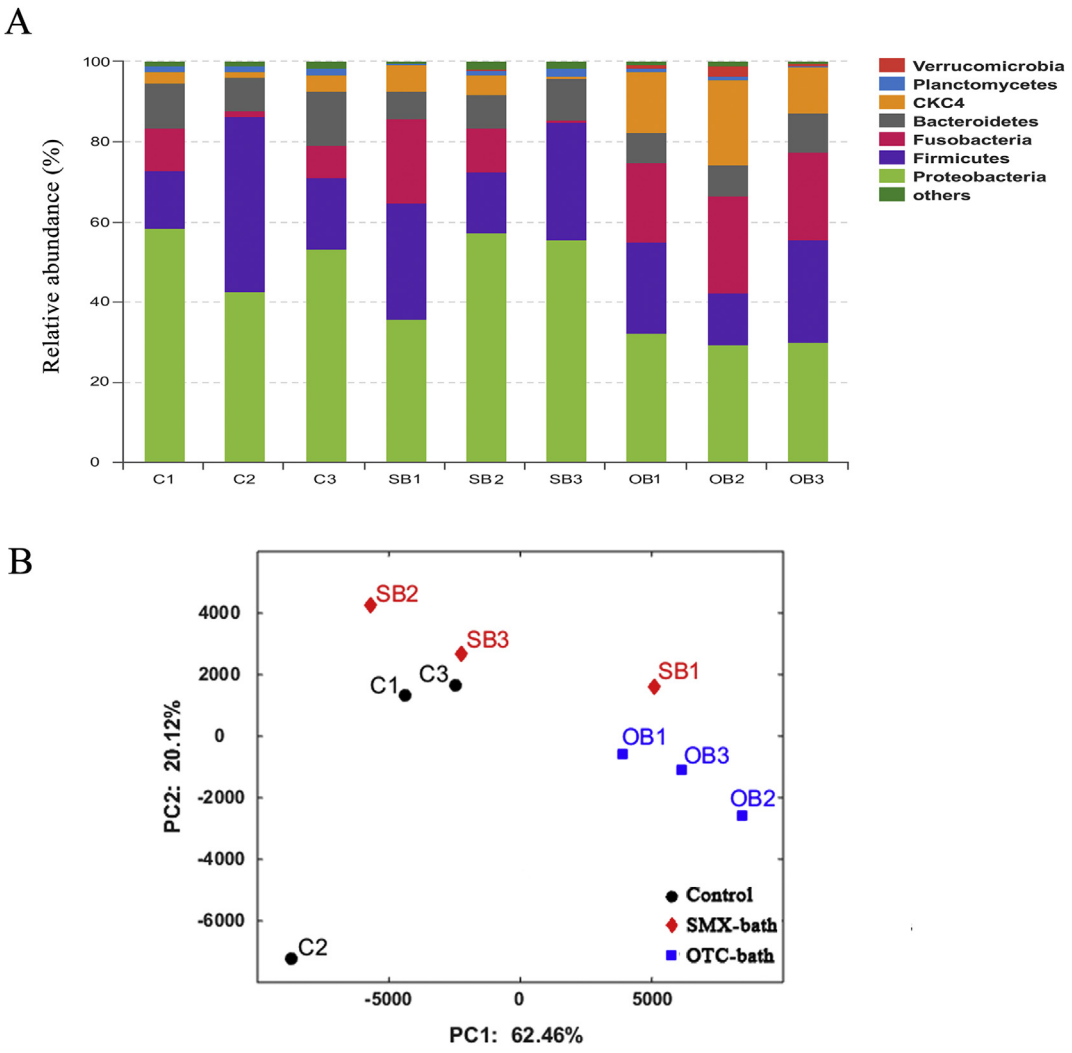


Fig. 6. Effect of antibiotic exposures on the intestinal microbial composition. (A) Gut microbiota composition at the phylum level. (B) Principal component analysis of intestinal bacteria in the three groups. SB denotes SMX-bath group, OB denotes OTC-bath group and C denotes Control group.

Table 2
The percentages of major phyla in zebrafish.

	Control (%)	SMX-bath (%)	OTC-bath (%)
Proteobacteria	51.3 ± 4.7	50.2 ± 7.0	30.4 ± 0.8
Firmicutes	25.7 ± 9.2	24.7 ± 4.6	20.2 ± 3.9
Fusobacteria	6.7 ± 2.8	10.0 ± 5.9	22.2 ± 1.2
Bacteroidetes	10.9 ± 1.4	8.8 ± 1.1	8.6 ± 0.7
CKC4	2.8 ± 0.7	3.8 ± 1.7	16.0 ± 2.9

pose a significant threat to the growth, development, and reproduction of zebrafish (Yan et al., 2016). However, in the present study we found that bacterial challenge of zebrafish significantly increased the mortality of fish exposed to antibiotics. Impairment of intestinal structure and function may be a possible reason for this observed increase in mortality, suggesting that although the environmental concentrations of antibiotics was very low, there was still an augmented risk to fish health through impaired immune function and increased inflammation in the gut.

Increased oxidative stress is a common toxicological effect caused by many environmental pollutants (Liu et al., 2014; Yan et al., 2016). Dysfunction of the anti-oxidative system has been reported in some fish species. For example, oral administration of OTC (100 mg/kg body weight) to rainbow trout for a period of 14 days increased the concentration of MDA, while decreasing the concentration of SOD, GSH and CAT in plasma, liver and kidney, respectively (Yonar et al., 2011). The decrease in antioxidant enzyme (SOD and CAT) activity has also been demonstrated in the liver of rainbow trout exposed to sub-lethal eprinomectin at concentrations ranging from 0.001 μg/L to 0.05 μg/L for 24–96 h (Alak et al., 2017). Furthermore, enhanced SOD activity has been found in the livers of goldfish exposed to SMX with concentrations ranging from 3.2 μg/L to 0.4 mg/L over a period of seven days (Liu et al., 2014). In the present study, decreased SOD, POD, and GSH, and increased MDA content was observed in the guts of zebrafish treated with antibiotics. However, no significant oxidative damage was observed in muscle or liver, except for SOD which decreased in muscle of fish exposed to OTC (Fig. S2, Fig. S3). Compared with other organs such as muscle and liver, this indicates that the gut is a more sensitive organ to antibiotic residues in water at ng/L concentrations. This suggests that the gut is likely to be the primary organ affected by environmental antibiotic residues. In addition, it also indicates that the target organs and the corresponding antibiotic concentrations should be carefully evaluated in future studies.

The intestinal microbiota are essential for the preservation of

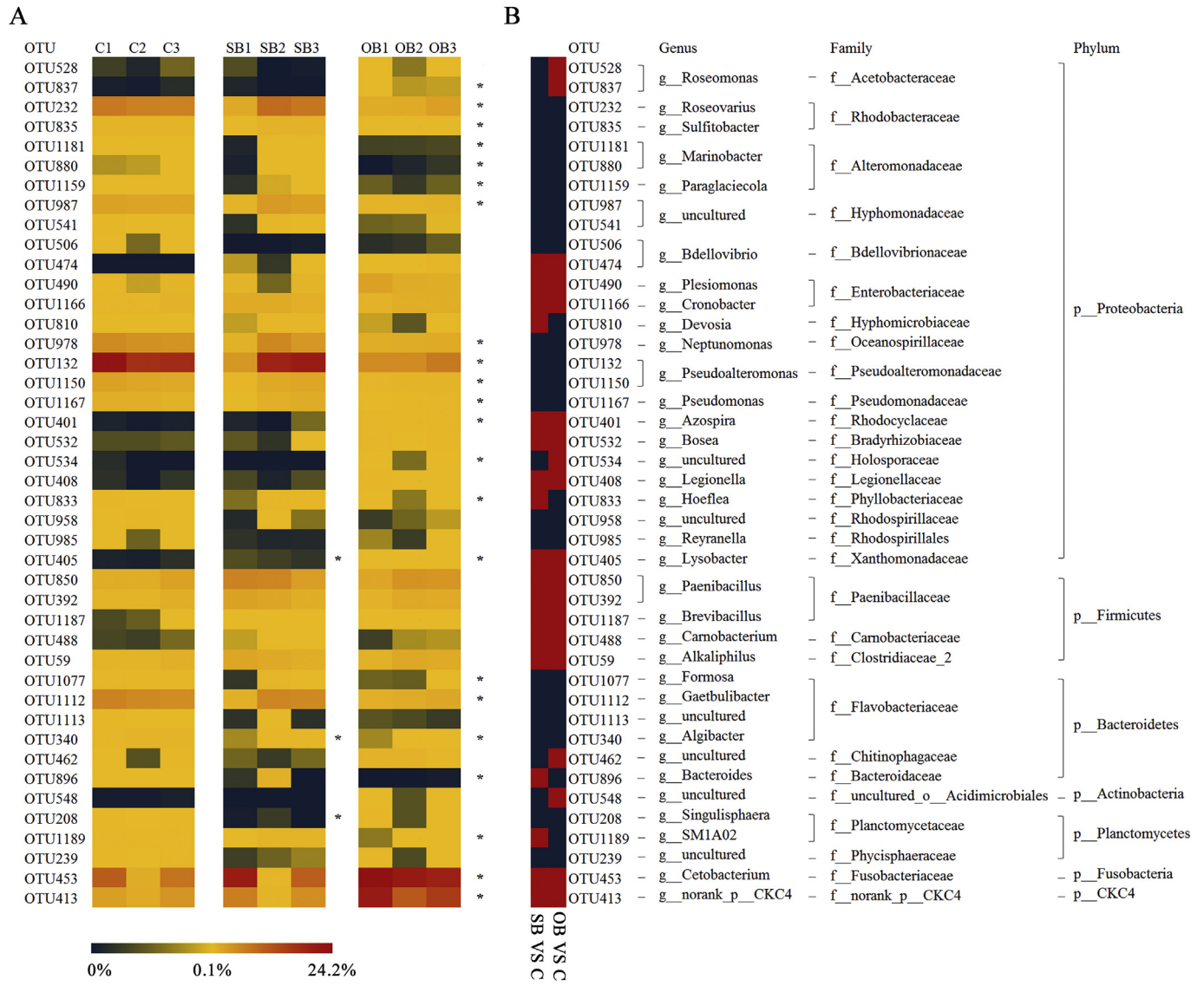


Fig. 7. Heatmap analysis of 43 OTUs. (A) The color bar of each OTU in three samples in each group. (B) Red bar suggests that the abundance of OTUs is higher compared with the control group, and blue bar means the abundance of OTUs is lower compared with the control group. The taxonomy of the OTUs (genus, family and phylum) is depicted on the right. Asterisk (*) represents a significant difference compared with the control group based on Student's *t*-test with subsequent Bonferroni correction. SB denotes SMX-bath group, OB denotes OTC-bath group and C denotes Control group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

the integrity of the mucosal barrier function and should be considered when intestinal health is evaluated (Sommer and Backhed, 2013). Previous studies have shown that exposure to OTC at concentrations exceeding 250 mg/L caused significant mortality in intestinal bacteria of grass shrimp. However, lower OTC concentrations (1, 16, and 32 mg/L) did not show significant bacterial mortality (Uyaguari et al., 2009). Oral OTC treatment (75 mg/kg per day) did not influence the bacterial quantity but did change the bacterial composition (Navarrete et al., 2008). In our previous research, we found that the intestinal microbiota of aquatic animals have not been affected significantly by living water (Zhang et al., 2016), so only intestinal contents of zebrafish from each tank were collected for the bacterial composition analysis. The results indicated that no significant difference was found in bacterial quantity between the control and antibiotic exposed groups (Fig. S4) but the bacterial composition changed dramatically in the OTC-exposed group. Together, these results indicate that compared with bacterial quantity, bacterial diversity or composition is more susceptible to influence by low concentrations of some antibiotics. It is possible that the number of antibiotic-sensitive bacteria decreased while antibiotic-resistant bacteria increased in response to antibiotic treatment. Proteobacteria are dominant members in zebrafish (Roeselers et al., 2011). In our study, the abundance of Proteobacteria decreased in the OTC-exposed group, while OTUs related to Cetobacterium or CKC4 increased significantly in the antibiotic treated groups. It has previously been demonstrated that the abundance of Cetobacterium increased in the gut of *Gambusia affinis* exposed to the antibiotic rifampicin (Carlson et al., 2017). These results show that antibiotic residues in water can eradicate susceptible microorganisms and promote opportunists that may occupy previously unavailable ecological niches (Navarrete et al., 2008).

5. Conclusion

The environmental concentrations of antibiotics at ng/L levels

enhanced the metabolic rate of zebrafish. Furthermore, exposure to such low levels of antibiotics increased the mortality rate of zebrafish, when they were challenged with *A. hydrophila*, while they also impaired the intestinal structure, function, and symbiosis of intestinal microbiota. To our knowledge, this is the first study to assess the long-term biological effects of SMX and OTC at nanogram per liter concentrations and the results reveal the potential ecological risk of both antibiotics in aquatic environments. Our study indicates that there is an urgent need to reduce antibiotic pollution of the aquatic environment.

Conflicts of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2017.12.073.

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