RESEARCH ARTICLE

Effects of thallium exposure on intestinal microbial community and organ functions in zebrafish (Danio rerio)

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Thallium (Tl) is a highly toxic trace metal widely distributed in water environments, which may threaten the water quality and aquatic organisms at excessive levels due to increased anthropogenic activities. This study investigated the changes in microbial communities of intestines and organs of zebrafish. The toxic response assessments include intestinal microbiota composition and the histopathology of zebrafish’s gill and liver tissues under exposure of Tl at environmental-relevant levels. The results support that the intestinal microbial community of zebrafish greatly changed under a relatively high Tl concentration (1000 ng/L). A significant increase of pathogenic intestinal bacteria such as Mycobacterium in the intestine of zebrafish exposed at Tl levels over 500 ng/L was found. Additionally, the gill and liver tissues displayed different degrees of damage under Tl exposure, which possibly leads to mating behavior changes and death of zebrafish. The results indicate that low doses of Tl in the aquatic environment induce high toxicity on zebrafish and may pose pathological threats to the gill and liver of zebrafish. In addition, Tl exposure gives rise to increasing abundance of pathogenic intestinal bacteria and changes the community structure of intestinal microorganisms.

Keywords: Zebrafish, Thallium, Chronic effect, Intestinal microorganism

1. Introduction

Thallium is a highly toxic element dispersed in the atmosphere, pedosphere, and hydrosphere (Liu et al., 2019; Lopez-Arce et al., 2019; Wang et al., 2020, 2021; Zhou et al., 2020). Its appearance in water is mainly originated from illegal or unintentional drainage of Tl-bearing materials mining, which greatly threatens water quality and the aquatic organisms (Cheam et al., 2000; Twiss et al., 2003; Liu et al., 2018). Due to its similar ionic ratio to K+, Tl+ can interfere with the potassium-uptake efficiency and therefore cause physiological toxicity via a similar way to be taken up (Xiong, 2009; Hou et al., 2017; Osorio-Rico et al., 2017). Acute Tl poisoning in humans covers pathological changes in organs such as stomach, liver, brain, and intestine, along with chronic effects like mental disorders or polyneuritis (Voegelin et al., 2015; Vaněk et al., 2018; Jiang et al., 2018; Liu et al., 2021).

It is reported that chronic toxic effects may occur in zebrafish when it is exposed to Tl concentration levels from 0.10 to 100 μg/L (Campanella et al., 2017; Liu et al., 2019). In 2014, severe Tl contamination in drinking water (15 μg/L in the spring water) was discovered in northern Tuscany, Italy, resulting in a severe Tl accumulation in urine and hair of local residents (Campanella et al., 2016; Biagioni et al., 2017). China has abundant Tl-bearing mineral resources, and the mining and smelting activities have released large amounts of Tl into aquatic environments (Liu et al., 2010; Xu et al., 2019; Liu et al., 2020). Several rivers in South China such as the North River in Guangdong province, the Hejiang River in Guangxi province, and Xiannv Lake in Jiangxi province were contaminated by the discharge of wastewater containing high Tl. In order to prevent potential adverse health effects caused by Tl contamination, the maximum contaminant level in drinking water was set at 0.1 μg/L in China (State Environmental Protection Administration, 2006) and 2.0 μg/L in the United States (U.S. Environmental Protection Agency, 2018).
Thallium in water can pose health risks to aquatic organisms, which eventually causes detrimental effects on human health through food chains (Twiss et al., 2003; Jardine et al., 2019). Some investigations have been carried out to study the toxic effects of Tl on typical aquatic organisms such as zebrafish (Danio rerio), trout-perch (Percaopsis omiscomaycus), and brown trout (Salmo trutta; Hou et al., 2017; Li et al., 2020). For example, the average content of Tl in the oolith of trout-perch from the Athabasca River near an open-pit mining in northern Alberta was found up to 170 ± 4.5 μg/kg (William et al., 2018). Hou et al. (2017) discovered that exposure to low-level Tl may damage the organs and enzyme activities of zebrafish. However, the changes in intestinal microbial community associated with organ damage of Tl-exposed zebrafish remain largely unknown.

This study aims to (1) reveal survival situation of zebrafish under different environmental-relevant Tl levels, (2) evaluate organs (gill and liver) effects caused by long-term Tl exposure and accumulation, and (3) demonstrate the changes of the intestinal microbial composition of zebrafish induced by Tl. This work will provide a comprehensive evaluation of the biological responses of zebrafish toward Tl toxicity, especially on the changes of intestinal microorganism.

2. Materials and methods

2.1. Zebrafish exposure experiments

Thallium contaminated water under different exposure levels was prepared by dissolving Tl(NO₃)₂ in oxygenated and charcoal dechlorinated tap water (pH 7.0 ± 0.1). The treatments for the Tl exposure displayed as controlled (Group A), 100 ng/L (Group B), 500 ng/L (Group C), and 1000 ng/L (Group D), respectively, in tanks (20 L; Hou et al., 2017; Yang et al., 2017). Each individual experiment was performed in triplicate.

Four-month-old zebrafish (weight: 0.90 ± 0.02 g and length: 3.00 ± 0.02 cm) were prepared and then cultivated in the above four tanks under different Tl exposure levels. Thirty zebrafish at a sex ratio of 1:1 (male: female) were placed into each tank. The temperature was kept at 25 °C, with a 14 h light/10 h dark cycle (Hou et al., 2017; Yang et al., 2017). The zebrafish were fed twice a day with clean commercial food (Zhang et al., 2019). In this study, all the experiments, animal care procedures, and protocols were conducted in accordance with the national and institutional ethical guidelines for the protection and use of laboratory animals (Zhang et al., 2019). Thallium concentration in the solutions collected from all treatment groups after exposure was determined using inductively coupled plasma mass spectrometry (Perkin Elmer ElanDRCe, PerkinElmer, Waltham, MA, USA) under standard analytical condition (Zhang and Shi, 2017).

2.2. Death record and behavior observations

Paired zebrafish (one male and one female) were selected from each group and put into a fish tank observation system for observation of mating behaviors. A digital camcorder (BASLER acA1300-30gm, Germany) was used to record the behaviors of the paired zebrafish every 10 min (Frankel et al., 2016; Hou et al., 2018). The approach index of male and female zebrafish was analyzed through video analysis software. The dead zebrafish under high concentrations of Tl exposure were collected immediately from the tanks and recorded daily.

2.3. Organs histological examination

Ten zebrafish from each group of different Tl levels were selected for gill and liver histological examination. The liver and gills were excised and fixed for 24 h in Bouin solution. Tissues were then dehydrated using an ethyl alcohol series, hyaline in xylene baths, and embedded in paraffin (Montalbano et al., 2020). After solidification of the paraffin block, thin Sections (5 μm) were cut using a rotary microtome (Cut 4055; Olympus American, Melville, NY, USA). Sections were flatten, stained with hematoxylin and eosin, mounted on slides with neutral resins, and examined via light microscopy.

2.4. Intestinal microorganism detection via 16S rDNA analysis

The DNA from a mixture of zebrafish intestines was extracted by Soil DNA Kit. The concentration of DNA samples was measured by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). The MetaVx™ library construction kit (South Plainfield, NJ, USA) was used to construct the sequencing library. The hypervariable V3-V4 16S rRNA gene region was amplified by the polymerase chain reaction (PCR) primers (5’-CTACGGGRBGCASCAGKVRVGAAT-3’) and (5’-GGACTACNVGGGTWTCTAATCC-3’). The PCR reaction was set up according to the following procedures: initial denaturation at 94 °C for 3 min, followed by 24 cycles of denaturing at 94 °C for 5 s, annealing at 57 °C for 30 s and extension at 72 °C for 10 s, and single extension at 72 °C for 5 min. The PCR products were analyzed by 2% agarose gel stained with ethidium bromide (0.5 μg/mL). Library quality was measured by Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) and library concentration was measured by Qubit2.0 Fluorometer (Invitrogen, Carlsbad, CA). After the DNA library was mixed, PE250/300 double-end sequencing was performed by Illumina MiSeq (Illumina, San Diego, CA, USA) instrument instructions. Finally, the sequence information was read by MiSeq Control Software.

In order to obtain the bacteria distribution information of each sample, the taxonomic analysis of the representative sequences of operational taxonomic unit (OTU) was performed. Based on the results of OTU analysis, various alpha diversity indexes are then analyzed for each sample to obtain bacteria richness and diversity for each sample. Statistical analysis of the community structure based on taxonomic information was performed using unweighted pair group method with arithmetic mean (UPGMA) and principal component analysis (PCA). Linear discriminant analysis effect size (LEfSe) analysis was then applied to identify the microbial species with obvious differences in the different groups.
3. Results and discussion

3.1. Vital and behavioral changes of zebrafish

Death numbers of zebrafish in different groups were recorded daily and shown in Figure 1. The results indicate the death rate increases as Tl concentration in water elevates. The average death number of zebrafish in Group D is over 10, while that of Groups A, B, and C is much less (<5). Exposure with high Tl concentration (1000 ng/L) results in large amounts of death in the first 15 days. This could be explained mainly by Tl toxicity toward specific fish organs or the nervous system since fierce trembling behaviors of the zebrafish have been observed before death.

The results of behavior index analyzed by the video analysis software suggest that the increase of Tl concentration affects the contact intention between male and female zebrafish (Figure 1). Tl exposure could induce considerable damage to gonad tissues and consequently affect the normal courtship behaviors, which were consistent with the report by Hou et al. (2017).

3.2. Gill and liver tissues toxicity

As displayed in Figure 2, the structure of gills is arranged in a double row, protruding laterally with a battery of alternately arranged secondary lamellae (respiratory lamellae), which consists of a monolayer of cells. The ends of gill lamella in C and D groups show obvious hyperplasia under Tl exposure of 500 and 1000 ng/L, respectively (Figure 2). Additionally, hypertrophic fusion of the end of secondary lamellae, anomalous inter-lamellar spaces, and circulatory obstruction of secondary lamellae are observed in the gill tissues of zebrafish in both group C and D.

Gills are the main organs of fish breathing and filtering and hence are considered a first reacted organ to aquatic pollutants. They offer an important way for the absorption and excretion of poisons due to their large surface area, short distance between internal and external media, and direct contact with the ambient environment (Hou et al., 2017; Liu et al., 2019; Paravani et al., 2019). For example, Tu et al. (1994) demonstrated that bioaccumulation of the rare earth elements such as lanthanum, gadolinium, and yttrium in carp was largest in visceral, followed by gill, bone, and muscle. Zitko et al. (1975) also confirmed that Tl accumulation followed the order: gills > liver > gonads > muscle. Considering the fact that the gill is a highly efficient bioaccumulation organ of fish, its pathological analysis is essential for studying Tl effects on organisms.

As shown in Figure 3, in the liver tissue of control fish (Tl = 0 ng/L), hepatocytes with healthy development are arranged closely, with cordlike structures or hepatic cell cords (Hou et al., 2017; Fathi et al., 2019; Teng et al., 2019). While in high concentration Tl groups (Group B, C, and D), hyperplasia hepatocytes without roundish, polygonal cell body, and hepatic vacuolization are found. Kupffer cells and apoptosis also have a conspicuous increase in liver tissues of zebrafish exposed to 1000 ng/L Tl. The liver is the primary target organ for all toxic substances and is responsible for detoxification and foreign body metabolism (Mukhopadhyay and Chattopadhyay, 2014). Liver is also one of the most sensitive organs to changes in tissue structures, biochemistry, and physiology, followed by exposure to various types of environmental contaminants (Kotsanis and Ilipoulou-Georgudaki, 1999). The microsomes, mitochondria, and cytoplasm of the liver contain multifarious metabolic enzymes that also rely on the cytochrome P450 monooxygenase system. Drugs and poisons primarily lead to toxic substance-induced liver injury or hepatotoxicity, including oxidative stress and apoptosis (Bunchornvatakul and Reddy, 2013).

The liver is the largest organ in vertebrates and its cells play significant roles in the metabolisms of carbohydrates, fats, proteins, vitamins, and hormones in the regulation of detoxification (Gebhardt and Matz-Soja, 2014; Adeva-Andany et al., 2016). The results showed that zebrafish exposed to high concentrations of Tl in water suffered from pathological changes in both gill and liver, which might be one of the most important reasons for its severe toxicity.
3.3 Differences between microbial community

The indices of community richness (Ace) and community diversity (Shannon) show an obvious difference in all samples (Table 1). The lowest richness is found in D2 (41.000), whereas the highest in D1 (82.698). The lowest value of Shannon is observed in B2 (0.922), whereas the
The mortality of zebrafish at high TI concentrations may be related to changes in the intestinal microbial community.

### 3.4. Changes in intestinal microbiota composition (phylum level)

The top 13 bacteria at phylum level are displayed in **Figure 6**. Group A is dominated by *Proteobacteria* and *Fusobacteria*, followed by *Firmicutes* and *Synergistetes*. The dominant phylum in Group B is *Proteobacteria*, whose relative abundance increases by about 30% compared with Group A. Its relative abundance eventually reaches at 80%, while the relative abundance of *Fusobacterium* and *Firmicutes* significantly decreases. In Group C, the relative abundance of *Proteobacteria* is nearly 95%, and the relative abundance of *Actinobacteria* is similar to that of *Firmicutes*. The main bacteria in Group D are still *Proteobacteria*, but the types of other phyla greatly vary. *Actinomycota* sharply increases to a relative abundance of nearly 30% and *Cyanophyta* also elevates. However, it is obvious that all the microorganisms except *Proteobacteria* decreases or even becomes extinct with the increase of TI concentration in water, which play important roles in the physiological function of zebrafish. For example, *Firmicutes* can facilitate the absorption of fat in the intestinal epithelium and liver tissues and facilitate the formation of lipid droplets (Semova et al., 2012). The reduction or extinction of *Firmicutes* may be the dominant reason of liver problems. The results show that microorganisms at phylum level in Group A, B, and C are normally dominated as *Proteobacteria* *Firmicutes* and *Fusobacteria*, which is consistent with previous results. However, the dominant bacteria are significantly

| Sample | Ace     | Shannon |
|--------|---------|---------|
| A1     | 59.261  | 2.785   |
| A2     | 44.498  | 1.365   |
| A3     | 70.749  | 2.612   |
| B1     | 58.000  | 1.209   |
| B2     | 63.864  | 0.922   |
| B3     | 71.467  | 1.033   |
| C1     | 60.427  | 1.312   |
| C2     | 55.767  | 1.655   |
| C3     | 44.539  | 2.111   |
| D1     | 82.698  | 3.323   |
| D2     | 41.000  | 2.323   |
| D3     | 81.474  | 3.799   |

**Figure 4.** Principal component analysis of the microbial communities from different groups of zebrafish exposed to thallium concentrations of 0 ng/L (control, Group A), 50 ng/L (Group B), 500 ng/L (Group C), and 1000 ng/L (Group D). DOI: https://doi.org/10.1525/elementa.2021.00092.f4

**Figure 5.** The unweighted pair group method with arithmetic mean tree presenting clusters of microbial communities based on unweight UniFrac with 100% support at all nodes. DOI: https://doi.org/10.1525/elementa.2021.00092.f5

The mortality of zebrafish at high TI concentrations may be related to the changes of the intestinal microbial community.
changed in Group D, with an increase of Actinomycota followed by Proteobacteria. This indicates that the microbial changes may be responsible for the mass death of zebrafish in Group D.

3.5. Changes in intestinal microbiota composition (genus level)

The relative abundance of microbes at genus level in four groups are Aeromonas, Vibrio, Plesiomonas, Cetobacterium, Mycobacterium, and Chitinbacic. Vibrio is present in all groups, while Aeromonas is the main microorganism only in Groups C and D. Mycobacterium dominates in Group D, followed by Aeromonas and Vibrio. The major bacteria genera in Group D were significantly different from those in Groups A, B, and C, indicating that exposure to high concentrations Tl has a significant effect on the zebrafish intestinal microbial composition. With increasing Tl concentration, Mycobacterium, Vibrio and Aeromonas, and Acinetobacter Bacillus are prevalent in the zebrafish intestine. In addition, it was found that Cetobacterium appeared in Groups A and B but not in Groups C and D. All these results show that microbes in the zebrafish intestine exposed to high concentrations Tl has a significant effect on the zebrafish intestinal microbial composition. With increasing Tl concentration, Mycobacterium, Vibrio and Aeromonas, and Acinetobacter Bacillus are prevalent in the zebrafish intestine.

Vibrio is one of the most common bacterial groups in the natural environment. It is widely distributed in the coastal and estuary seawater, the surface of marine life and the intestinal tract of some animals. In addition, it was reported that most species of Vibrio have benign symbiotic relationships with various aquatic animals (Rubio-Portillo et al., 2014). Cetobacterium, a Gram-negative bacterium commonly found in the intestines of freshwater fish (Charbonneau et al., 2016), is observed in Groups A and B but not in Groups C and D. Tsuchiya et al. (2008) showed that Cetobacterium in fish can produce vitamin B12 and acetic acid, which can promote the metabolism of proteins, carbohydrates, and fats for use by the host and play important roles in its growth and development. Aeromonas dominated in Groups C and D are widespread in nature and can cause a variety of diseases in farmed animals, such as Saprophytic syndrome, grass carp intestinal hemorrhage, allogynogenetic silver carp hemolytic ascites disease, carp red spot disease, and vertical Squamous disease, eel redfin disease, and perforation of tadpoles (Nriagu, 1998). Aeromonas is an important pathogen in aquatic ecosystems, which can cause diseases in a variety of aquaculture animals. At present, it has become a significant and important pathogen for human and animal coinfection and has attracted the attention of the aquatic, veterinary, and medical communities. In addition, Mycobacterium in Group D is pathogenic and can cause chronic long-term persistent infections, accompanied by granulomas (Cosma et al., 2003; Meijer et al., 2005; Hu et al., 2019). This study reveals that the dominated microorganisms are Vibrio and Cetobacterium in Groups A and B, which are normal and beneficial ones in the intestinal environment. By contrast, under high Tl exposure conditions (Groups C and D), the dominated microorganisms, that is, Mycobacterium and Aeromonas, are pathogenic bacteria for zebrafish, which might represent the main inducing bacteria under Tl exposure.
3.6. Dominant species under Tl exposure (LEfSE analysis)

As shown in Figure 8, the dominant genus of Group A, Cetobacterium, was a Gram-negative bacterium commonly found in the intestine of freshwater fish (Charbonneau et al., 2016). The dominant genus of Group D, Mycobacterium, is a kind of slender and slightly curved, sometimes with branches or filaments. At present, Mycobacterium has been classified into actinomycetes in taxonomy. This species belongs to nontuberculous Mycobacteria. The dominant bacteria analysis showed notably different intestinal microbial responses toward environmental-relevant Tl exposure. The dominant bacteria at genus level in Group A, Cetobacterium, was beneficial for zebrafish, while Mycobacterium in Group D might pose threats and cause specific diseases. The results indicated that high Tl concentrations in aquatic environment can lead to negative effects on intestinal microbiota communities of zebrafish.

4. Summary and conclusion

This study investigated the changes in gill, liver, and intestinal microorganism of zebrafish under different

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**Figure 8.** Linear discriminant analysis effect size identified the most differentially abundant taxon between different groups of zebrafish exposed to thallium concentrations of 0 ng/L (control, Group A, red legend), 50 ng/L (Group B, green legend), 500 ng/L (Group C, blue legend), and 1000 ng/L (Group D, purple legend). Small circles and shading with different colors in the diagram represent the abundance of those taxa in the respective treatment group. Yellow circles represent nonsignificant differences in abundance between different groups. The brightness of each dot is proportional to its effect size. DOI: https://doi.org/10.1525/elementa.2021.00092.f8
levels of Tl exposure (0, 50, 500, and 1000 ng/L) in freshwater. The results reveal that a relatively high concentration of Tl (>500 ng/L) leads to a high death rate (about 50%) of zebrafish and greatly affects the courtship behavior between male and female zebrafish. The results show that zebrafish exposed to high Tl concentration have suffered from severe deformation in gill tissues and an obvious increase in Kupffer cells and apoptosis in liver tissues. The 16S rDNA analysis indicates that the microbes in the zebrafish intestines exposed to high concentrations of Tl (500 and 1000 ng/L) were significantly different from those in the normal or low Tl concentration (0 and 50 ng/L) influenced zebrafish intestine. The increase of Tl concentration can lead to a decrease of some beneficial intestinal bacteria (e.g., Cetobacterium and Vibrio) while an elevation of deleterious bacteria (e.g., Mycobacterium). All these results can provide a comprehensive understanding of Tl toxicity to aquatic organisms.

Data accessibility statement
The raw sequence reads were deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database and the accession number was PRJNA668743. Raw data concerning Figure 1 are available in Supplemental file which is deposited on Mendeley. Other additional data (if any) would be available on the request of the corresponding authors.

Supplemental files
The supplemental file for this article can be found as follows: https://data.mendeley.com/datasets/pd4fdd6gyd/1.

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Competing interests
The authors have no competing interests to declare.

Author contributions
Yuxuan Wang and Yuting Zhou contributed equally to this work.
Contributed to the data analysis and interpretation: YJ, QZ, JC, YL, LW, JS.
Contributed to acquisition of data: XW, YC, SZ, WL.
Contributed to the project administration and funding acquisition: JW, JL.
Drafted and /or revised the article: JW, JL, JB, YZ, YW.
Approved the submitted version for publication: all coauthors.

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