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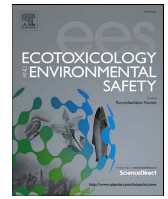
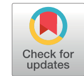


The effect of cadmium exposure on diversity of intestinal microbial community of *Rana chensinensis* tadpoles



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ABSTRACT

Cadmium is a natural and widely distributed toxicant, and can be commonly found in environment. Intestinal microbiota plays a very important role in maintaining its host's health. The effects of cadmium on the intestinal microbiota composition and stability of amphibians are little known. We exposed *Rana chensinensis* (*R. chensinensis*) embryos to different concentrations of cadmium (0, 112 and 448 μg Cd L^{-1}) until they reached Gosner stage 38, and analyzed their microbial communities using 16S rRNA amplicon sequencing. By measures of both alpha and beta diversity, intestinal microbial communities were significantly differentiated in 448 μg Cd L^{-1} exposure groups. Cadmium exposure significantly altered the intestinal microflora diversity and composition of *R. chensinensis*. At the phylum level, it is worth noting that Fusobacteria and Spirochaetae were not detected in 448 μg Cd L^{-1} exposure groups. Firmicutes rapidly decreased in 448 μg Cd L^{-1} exposure group. At the genus level, Succinispira (Firmicutes), Desulfovibrio (Proteobacteria) and Fusobacterium (Fusobacteria) vanished in 448 μg Cd L^{-1} exposure groups. Our results demonstrate that cadmium exposure changed the composition and decreased the community diversity of intestinal microbiota of *R. chensinensis* tadpoles. Our study may provide a new framework based on intestinal microbiota to evaluate the response of amphibians to environmental chemicals pollution.

1. Introduction

The decrease in the populations and diversity of amphibian species in their natural habitats has been happened for several years (Houlahan et al., 2000; Beebee and Griffiths, 2005). Chemical pollutants are main reasons that contribute to the decline of amphibian by altering aspects of physiology. Cadmium, a toxic metal, is one of the chemical contaminations. The major natural sources of cadmium are the volcanism, ocean spray, forest fires, and the release of metal-enriched particles from terrestrial vegetation (Burger, 2008). However, anthropogenic activities, such as the production of nickel cadmium batteries, stabilizers, synthetic pig-ment, and metals melting, contribute to an increase of cadmium in the environment (Bhattacharyya et al., 2000; Patar et al., 2016). The maximum allowable concentration of Cd in discharge water is 10 μg L^{-1} in China (Huang et al., 2015), but in a vast area of China levels of Cd can even reach to 100–900 μg L^{-1} in the polluted waters (Deng et al., 2014). Cadmium ion could be discharged into aquatic environment, resulting in high bioaccumulation tendency in living organisms (Martins et al., 2004; Wu et al., 2017). Cadmium has a long biological half-time of 10–30 years in human body (Järup and Åkesson, 2009). Thus, the adverse effects of cadmium on aquatic biota have aroused widely public concerns in recent years.

A large number of studies have shown that cadmium exposure produces many negative effects on amphibians. For example, cadmium affects growth, metamorphosis and gonadal sex differentiation of African clawed toad (*Xenopus laevis*) tadpoles, *Rana limnocharis tadpoles* and *Pleurodeles waltl* (urodele amphibian) larvae (Sharma and Patiño, 2009; Patar et al., 2016; Flament et al., 2003). Also, cadmium exposure results in the malformation of skin, eye, digestive system in the South African frog (Sunderman et al., 1991). In addition, cadmium induces histological lesions in liver and kidney damage of *Rana ridibunda* frog and *Triturus carnifex* (Amphibia, Urodela) (Loumbourdis, 2005; Capaldo et al., 2016), and causes liver damage of *Bufo gargarizans* (Wu et al., 2017).

The microbial community of gut plays an important role in general health and function of host. For instance, intestinal microbiota provides numerous nutritional functions to their hosts, such as synthesizing essential amino acids and fermenting fiber (Stevens and Hume, 2004). Intestinal microbiota even assists in the development of host tissue such as the brain, which in turn affects behavior of host (Heijtz et al., 2011). Additionally, loss of the microbial diversity may cause disturbances of the intestinal immune system and increased disease prevalence (Van

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et al., 1971; Mazmanian et al., 2008). According to previous reports, cadmium exposure has been linked to an increased incidence of diseases in animals and human (Satarug et al., 2011). Therefore, it is of particular interest to observe the intestinal microbial community changes associated with cadmium exposure. However, the effects of cadmium on the intestinal microbiota of amphibians remain unclear.

In the current study, we use *Rana chensinensis* tadpoles as a model system to explore the impact of cadmium exposure on the animal's intestinal microbiota. *R. chensinensis* belongs to *Ranidae Rana*, and is a wide distributed amphibian species in northern China. *R. chensinensis* is a species of frog endemic to East Asia. It is widely distributed from China to Mongolia, Russia and in Korea. Breeding usually occurred in February-March and the embryonic period is about 10 days. In addition, *R. chensinensis* has been used as an excellent indicator organism for evaluating adverse chemicals effects to amphibians in our laboratory (Chai et al., 2016).

In this study, we exposed larval *R. chensinensis* to cadmium in a controlled laboratory experiment, then applied high-throughput sequencing of V3-V4 segment of the bacterial 16S rRNA gene to profile intestinal microbial communities. The aim of the present study was to reveal the impact of anthropogenic environmental changes, toxic metal of cadmium contamination in particular, on the changes of intestinal microbial community diversity and structural of *R. chensinensis*. We hope that the results will help to elaborate the toxic effects of pollution on amphibians from a new standpoint.

2. Material and methods

2.1. Animals husbandry

Adult *R. chensinensis* were collected in February 2017 from Qinling Mountains, Shaanxi Province, China (109°06′52″E, 34°00′56″N). A pair of frogs were placed in 40 L glass aquaria with shallow naturally dechlorinated tap water (50 mm), which were induced to spawn naturally. The aquaria was kept at 18 ± 1 °C with a 12 h light:12 h dark photoperiod. The water dissolved oxygen was about 6.1–7.6 mg/L. The pH ranged from 6.9 to 7.3. Conductivity, total chlorine and TOC was 195–275 mS/cm, 0.1–0.4 mg/L and 2.49–4.59 mg/L, respectively. The embryos were not the progeny of multiple breeding group, rather were isogenic to reduce potential effects of host genotypic variation. After spawning, 90 embryos at Gosner stage 2 (Gosner, 1960) were randomly assigned to three plastic tanks (50 cm × 20 cm × 10 cm) containing 2 L treatment solution and each plastic tank has 30 embryos. The cultivated conditions of embryos were same as that of frogs. All larvae were offered boiled lettuces during the test period of cadmium exposure. The experimental procedures were approved by the Animal Use Ethics Committee of the Shaanxi Normal University and China Wildlife Conservation Association.

2.2. Cadmium exposure

Cadmium sulfate hexahydrate with a purity of approximately 99% was obtained from Sigma Corporation (Sigma-Aldrich, St. Louis, MO, USA). The stock solution were prepared by dissolving $Cd_2(so_4)_3{\cdot}6H_2O$ in dechlorinated tap water to a final concentration of 2240 μg Cd L^{-1}. Treatment solutions were prepared by diluting the stock solution with dechlorinated tap water to obtain the following target concentration: 112, 448 μg Cd L^{-1}. The control group was kept in dechlorinated water. During the process of raising tadpoles, the feces and food residue can contaminate the water quality in the plastic water tank. In order to maintain the water quality and appropriate concentrations of cadmium, test solutions were completely replaced every 72 h.

2.3. Sample collection

The embryos were divided into three experimental groups; a control group was kept in dechlorinated water, two groups were exposed to a nominal concentration of 112 and 448 μg Cd L^{-1}, respectively. Embryos were exposed in cadmium until they reached Gosner stage 38, and the sample animals were selected at random from three exposure group (0, 112 and 448 μg Cd L^{-1}). The development period of *R. chensinensis* from embryo to Gosner stage 38 is about 70 days. Each group performed three repetitions, and five intestines were pooled in each repetition to reduce inter-individual variation. A total of 15 tadpoles were selected for each concentration group. Manipulation instruments, glassware and 2 ml screw cap tube were autoclaved prior to use, and instruments and surfaces were cleaned with ethanol after each specimen. All work was performed in the super clean workbench. Each animal was euthanized in 1% buffered tricaine methanesulfonate, douched using sterile water three times, and intestinal contents were collected by removing the length of the intestinal (posterior of the esophagus to the vent) under a dissecting microscope. The excised intestines were douched three times using sterile water, then longitudinally opened and placed in a 2 ml screw cap tube. Samples were stored at − 80 °C until DNA extraction.

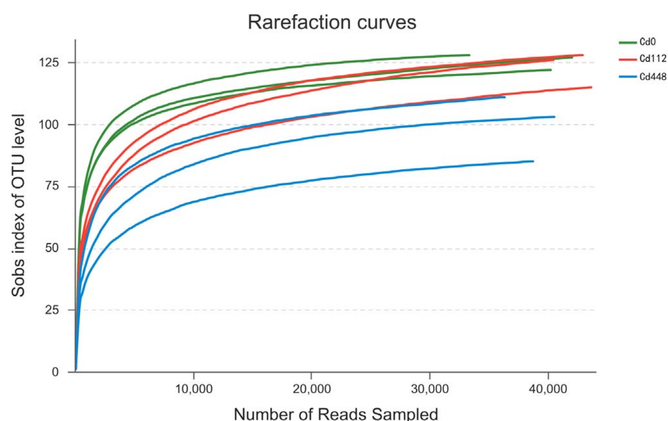

Fig. 1. Rarefaction curves of tadpoles intestinal microbial samples based on Illumina MiSeq sequencing. Horizontal axis: the amount of effective sequencing data; vertical axis: the observed number of operational taxonomic units. Total sample richness estimates were calculated by richness estimators Sobs.

2.4. DNA extraction and PCR amplification

The E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) was used to extract DNA from the intestinal contents samples with the manufacturer's protocol. Amplicon sequencing of 9 samples targeting the V3-V4 hypervariable regions of the bacterial 16S rRNA gene was performed following procedures. The primers 338F 5′-barcodeACTCC TACGGGAGGCAGCA-3′ and 806R 5′-GGACTACHVGGGTWTCTAAT-3′ were used, where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate using a 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA under the following conditions: 95 °C for 3 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 10 min.

2.5. Illumina Miseq and sequence processing

Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's protocols and quantified using QuantiFluorTM-ST (Promega, U.S.). The amplicons were pooled in equimolar ratios to generate amplicon libraries. Finally, samples were sequenced on the MiSeq Illumina Sequencing Platform (2 × 300) according to the standard protocols.

Raw 16S rRNA data were analyzed using the Quantitative Insights

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