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Co-selection of antibiotic resistance via copper shock loading on bacteria from a drinking water bio-filter[☆]

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ABSTRACT

Heavy metal contamination of source water frequently occurred in developing countries as a result of accidents. To address the problems, most of the previous studies have focused on engineering countermeasures. In this study, we investigated the effects of heavy metals, particularly copper, on the development of antibiotic resistance by establishing a copper shock loading test. Results revealed that co-selection occurred rapidly within 6 h. Copper, at the levels of 10 and 100 mg/L, significantly increased bacterial resistance to the antibiotics tested, including rifampin, erythromycin, kanamycin, and a few others. A total of 117 antimicrobial-resistance genes were detected from 12 types of genes, and the relative abundance of most genes (particularly mobile genetic elements *int*land transposons) was markedly enriched by at least one fold. Furthermore, the copper shock loading altered the bacterial community. Numerous heavy metal and antibiotic resistant strains were screened out and enriched. These strains are expected to enhance the overall level of resistance. More noticeably, the majority of the co-selected antibiotic resistance could sustain for at least 20 h in the absence of copper and antimicrobial drugs. Resistance to vancomycin, erythromycin and lincomycin even could remain for 7 days. The prominent selection pressure by the copper shock loading implies that a real accident most likely poses similar impacts on the water environment. An accidental release of heavy metals would not only cause harm to the ecological environment, but also contribute to the development of bacterial antibiotic resistance. Broader concerns should be raised about the biological risks caused by sudden releases of pollutants by accidents.

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1. Introduction

Water pollution is a serious problem worldwide. The frequent occurrence of accidental source water pollution undoubtedly intensified the severity of current situation (Hou and Zhang, 2009). According to the data of Ministry of Environmental Protection and China News, nearly 6700 water pollution accidents occurred from 2000 to 2008, annually more than 1700 accidents happened in the last decade (Yao et al., 2016; Zhang et al., 2011b). Among the four major types of accidental water pollution reported (i.e., toxic organic, heavy metal, oil-spill, and biological contamination), heavy metal pollution ranked the highest on the list (Bruyninckx et al.,

2007; Qu et al., 2016; Yao et al., 2016). For example, the Dawu River in Jiangxi was contaminated by waste water containing copper in 2003. In the contaminated region, the cupric concentration ranged from 15 to 30 mg/L (Huang et al., 2004). The leakage of copper contaminated effluent (50–500 mg/L) into the Ting River, Fujian resulted in enormous economic loss and serious negative implications on the environmental ecology (Miao et al., 2015). Although acute toxicity and corresponding countermeasures for the accidental source water pollution have been reported (Wang et al., 2013; Xue et al., 2012; Zhang et al., 2011b), biohazards, especially potential contributions of pollutants to the co-selection of bacterial antibiotic resistance, have rarely been addressed.

Antibiotic resistance (ABR), posing large health risks to humans, is mainly caused by the overuse of antibiotics in humans and animals (Pruden et al., 2006). However, many contaminants, like heavy metals, organic compounds, and biocides could promote ABR through co-selection. The term “co-selection” refers to “the pressure from an adverse environmental condition confers resistance to

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one of the antibiotics or metals and then is enough for maintain (select for) all the antibiotic resistance mechanisms" (Icgen and Yilmaz, 2014). It involved in three mechanisms: co-resistance, cross-resistance and co-regulation (Baker-Austin et al., 2006). Heavy-metal pollutants have been reported as notable contributors to the development of ABR and its determinants (Alonso et al., 2001; Baker-Austin et al., 2006). Berg's study demonstrated that Cu-resistant bacteria which isolate from the soil contaminated with copper for eighty years, showed prominent resistance to tetracycline, olaquinox, nalidixic acid, chloramphenicol, and ampicillin (Berg et al., 2010). While, in the study of Zhu et al., the abundance of 63 antibiotic resistance genes (ARGs) in the animal farms where heavy metals were used as feed supplements has been enriched 192-folds up to 28,000 folds, suggesting heavy metals to be a factor that causing the release of antibiotic resistant bacteria (ARB) and ARGs to the environment (Zhu et al., 2013).

The present study was designed to explore the co-selection effects of bacterial antibiotic resistance, resulting from the source water pollution caused by an accidental release of heavy metals. We chose copper, a common type of pollutant during the accidental source water pollution, and microbes from a bio-filter in a drinking water system to simulate a copper shock loading test. In a drinking water system, bio-filter governs the bacterial community structure (Pinto et al., 2012). The microbiome in a drinking water bio-filter is a part of the raw water and more likely to access to the human dominated areas via forming bacterial community in the distribution system. Investigation of bacterial resistome changes in it helps better predict the human health risks and threats to public. This is an original study that simulated the accidental source water pollution in relation to the development of ABR among bacteria, particularly focused on co-selection resulting from a heavy-metal shock loading. In this work, we confirmed the co-selective effects and the endurable capacity of the co-selected antibiotic resistances under the absence of copper and antibiotics.

2. Materials and methods

2.1. Bacterial sample preparation

Biofilm-covered sand was collected from the surface of a biologically active sand filter (bio-filter) in the Tianma Water Supply Plant located in Xiamen, Fujian Province, southeastern China. After being uniformly mixed in a sterile plastic bag, the sand was stored at 4 °C and transported to the laboratory. Approximate 400 g of sand was distributed in several 50 mL sterile centrifuge tubes and sonicated for 10 min at 38.5–40.5 KHz with 30–45 mL 0.9% NaCl solution to harvest the microbes (Teitzel and Parsek, 2003). Then, the liquid was pooled and centrifuged at 460 × g for 2–4 min to remove as much sand as possible. The suspensions were transferred to clean tubes and centrifuged (3500 × g for 10 min). The combined pellets were washed and re-suspended in 5 mL 0.9% NaCl solution.

One mL of the aforementioned re-suspended solution was added to 100 mL R2A medium in a 250 mL sterile shaker flask for enrichment, because of the inadequate quantity of the microbes obtained from the surface of the sands (5.2×10^4 colony-forming units per gram [cfu/g] wet weight). The flasks were aerobically incubated at 28 °C in a shaker at 180 rpm for 48 h, and the enriched microbes were used in the follow-up process.

2.2. Copper-shock loading test

The tested copper concentrations were selected according to the incidents that happened in recent years. These incidents included the Dawu River copper pollution and the Ting River pollution in

China in 2003 and 2010, respectively; the cyanide pollution happened in Szamos-Tisza in Romania, 2000 and the mine tailing spill accident in Aznalcollar, SW Spain, 1998 (Grimalt et al., 1999; Ju et al., 2012; Kraft et al., 2006). The cupric concentration in these cases ranged from 8 to 500 mg/L. For instance, the cupric concentration in Dawu River was from 12 to 30 mg/L. The considerably high concentrations of copper (80–320 mg/L) were detected in Lapos watercourse and the contamination lasted for at least 6 h. In this study, 10 mg/L copper was chosen as a possible real case scenario and 100 mg/L to simulate an extreme condition to investigate the full potential of co-selection by copper challenge.

The enriched microbes were centrifuged at 3500 × g for 10 min, washed, and re-suspended in 0.9% NaCl solution to adjust the initial bacterial concentration to around 10^9 cfu/mL. Then, 2 mL of the bacterial suspension was mixed with 18 mL 3-morpholino-2-hydroxypropanesulfonic acid (MOPSO)-buffered saline medium (Teitzel and Parsek, 2003) containing 10 or 100 mg/L Cu^{2+} ($\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$) or without copper (control) in 50 mL sterile flasks. These flasks were aerobically incubated for 6, 12, and 24 h at 28 °C and 180 rpm. The MOPSO-buffered saline medium used in this experiment was amended by R2A medium to achieve a final organic carbon content <5 mg/L (detected by total organic carbon analyser, TOC-Vwp, Shimadzu, Japan). The low TOC was maintained to simulate the relatively low carbon source condition.

2.3. Determination of antibiotic resistance

Samples exposed to copper for different durations were serially diluted in 0.9% NaCl solution, and 100 μL aliquots were placed on R2A agar containing different antibiotics (Table S1). The antibiotic concentrations were chosen according to the clinically relevant minimal inhibitory concentration (MIC) of *Escherichia coli* (as a representative Gram-negative bacterium) and *Staphylococcus aureus* (as a representative Gram-positive bacterium) which reported by the National Committee for Clinical Laboratory Standards (Jorgensen, 1993; Pitkälä et al., 2004). For a certain antibiotic, the higher one of the MICs of the two bacteria was chosen to enumerate the ARB (Lin et al., 2016a; Mohanta and Goel, 2014; Nwakaeze, 2013).

The co-resistant bacteria (resistant to both copper and antibiotics) in the indigenous bacterial community could exaggerate the role of the copper and bring out the false positive results. Control groups were set to exclude the impact of co-resistant bacteria on estimating copper co-selection. After the same dilutions, a 100 μL aliquot taken from the control group at different times was placed in R2A agar amended with 10 or 100 mg/L Cu (II) and different concentrations of antibiotics as shown in Table S1. The total number of viable bacteria in each sample was determined by heterotrophic plate counts of antibiotic-free and copper-free R2A agar plates. All culture plates were incubated at 28 °C for 48 h before counting. All copper shock loading experiments were conducted in triplicate.

2.4. Quantification principle of antibiotic resistance

To quantify the change in antibiotic resistance induced by copper, we used the parameter ΔN , which is defined as

$$\Delta N = \frac{\log_{10}(r_i/r_{i0}) - \log_{10}(r_j/r_{j0})}{|\log_{10}(r_j/r_{j0})|} \quad (1)$$

Where r_{i0} and r_i are the numbers of ARB, respectively, before and after the exposure to copper; and r_{j0} and r_j are the numbers of indigenous bacteria, respectively, before and after the exposure to both antibiotic and copper. The principle of this formula is

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