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Monitoring and evaluation of antibiotic resistance genes in four municipal wastewater treatment plants in Harbin, Northeast China☆

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ABSTRACT

The development and proliferation of antibiotic resistance in pathogenic and environmental microorganisms is of great concern for public health. In this study, the distribution and removal efficiency of *intI1* and eight subtypes of antibiotic resistance genes (ARGs) for tetracycline, sulfonamides, beta-lactams resistance in four municipal wastewater treatment plants (WWTPs) in Harbin, which locates in Songhua River basin in cold areas of China, were monitored by real-time fluorescent quantitative PCR. The results showed that *intI1* and 6 ARGs except for bla_{TEM} and bla_{SHV} were detected in wastewater and sludge samples and 0.3–2.7 orders of magnitude of ARGs removal efficiency in the four WWTPs were observed. The investigation on the removal of ARGs of different treatment units in one WWTP showed that the biological treatment unit played the most important role in ARGs removal (1.2–1.8 orders of magnitude), followed by UV disinfection, while primary physical treatment units can hardly remove any ARGs. Although all the WWTPs can remove ARGs effectively, ARGs concentrations are still relatively high in the effluent, their further attenuation should be investigated.

1. Introduction

Antibiotics play a major role in both humans and animal husbandry, they are extensively used for therapeutic treatment and are also used as growth promoters to improve the growth rate in aquaculture and farming (Sapkota et al., 2008; Xu et al., 2015). Long-term overuse of antibiotics has posed a potential threat to human health and the environment (Pruden et al., 2006; Kümmerer, 2009), because residual antibiotics in the environment would cause the selection pressure on the presence of antibiotic-resistant bacteria (ARB) and propagation of antibiotic resistance genes (ARGs) through horizontal gene transfer (HGT) (Zhang et al., 2009a, 2009b; 2009c; Dodd, 2012), which associated with the acquisition of clinical drug resistance (Prabhu et al., 2007; Abriouel et al., 2008). It is well-known that ARB and ARGs are ubiquitous in nature (Alonso et al., 2001), and ARGs have been detected in various water environment all over the world (Sapkota et al., 2007; Deekshit et al., 2012), including drinking water and recycling water (Pruden et al., 2006). Therefore, removal and reduction of antibiotic resistance determinants are urgently required.

In recent years, at least 39 different tetracycline genes have been detected in different kinds of environmental samples, including genes encoding efflux proteins (*tetA*, *tetB*, *tetC*, *tetD*, *tetE*, *tetG*, *tetH*, *tetK*, *tetL*), genes encoding ribosomal protection proteins (*tetM*, *tetO*, *tetQ*, *tetS*, *tetT*, *tetW*), genes encoding modification enzyme (*tetX*) and other unknown mechanism of resistance (Zhang et al., 2009a, 2009b; 2009c; Zhang and Zhang, 2011). Chen et al. (2007) detected six resistance genes encoding macrolide (*ermA*, *ermB*, *ermC*, *ermF*, *ermT*, *ermX*) in livestock wastewater. Other resistance genes encoding sulfonamides (*sulI*, *sulII*, *sulIII*, *sulA*) and beta-lactam resistance genes (*OXA-1*, *OXA-2*, *OXA-10*, *ampC*, *TEM-1*) have also been detected in the environment (Pei et al., 2006; Yang et al., 2012).

WWTPs have played important roles in recombination, exchange, and propagation of ARGs in the environment, they are considered to be hotspots for the development of ARGs and ARB (Rizzo et al., 2013). Some research indicated that the conventional biological treatments in WWTPs can not eliminate ARGs completely, because high microbial density and diversity of activated sludge in the biological treatment stage mixing with nutrients and antimicrobial agents may create a favorable environment to contribute to horizontal gene transfer and occurrence of

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multidrug-resistance bacteria (Lindberg et al., 2004). However, although WWTPs could serve a vital role to remove nutrients and pathogenic micro-organisms and limit the dissemination of ARGs, there are still relatively high levels of ARGs and ARB detected in the effluent (Auerbach et al., 2007; Gao et al., 2012; Novo et al., 2013). It has been reported that about 10^9–10^{12} Colony Forming Units (CFU) per day, per inhabitant equivalent in the final effluent was discharged into the river, and about 10^7–10^{10} among these have acquired antibiotic resistance (Novo and Manaia, 2010; Rizzo et al., 2013), even multiple resistance (Bell et al., 1983). The subsequence sludge from WWTPs has generally been made use in the application in agricultural fields as fertilizer, which will increase the loads of ARGs in the environment (Gaze et al., 2011; Amos et al., 2014).

With the development and promotion of sewage disposal and sanitation in China, more and more attention is paid to revealing the role of WWTPs in propagation of environmental ARGs. Several studies have characterized the distribution of ARGs and mobile genetic elements (MGEs) in WWTPs in different regions in China, like Luo et al. (2013) and Mao et al. (2015) reported the ARGs distribution in Haihe River basin. Zhang et al. (2015) investigated the ARGs distribution in Tai Lake basin, and Li et al. (2015) investigated both ARGs and metal resistance genes in the Pearl River basin. However, the information of environmental ARGs distribution in Songhua River Basin, which is the fourth longest river in China, is absent. Besides, Songhua River is a representative river locates in cold area in China, investigation on ARGs distribution considering the effect of low temperature in winter is rare. Harbin locates in Songhua River Basin, the average lowest temperature in January is −24 °C. The investigation on the distribution and removal of ARGs in WWTPs in Harbin is essential to supplement the fundamental data of ARGs information in China. As tetracycline and sulfonamide antibiotics were used in large quantities in livestock husbandry (Chopra and Roberts, 2001; Boxall et al., 2003) and beta-lactam antibiotics were widely used in clinical applications (Zhang and Zhang, 2011), eight ARGs including *tetA*, *tetO*, *tetW* (resistance to tetracycline), *sulI*, *sulII* (resistance to sulfonamides), bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M}$ (resistance to beta-lactams) and the class 1 integrase gene intI1 were selected and monitored in this study to investigate and analyze the contamination and removal of ARGs in four WWTPs in Harbin, China. The research also attempted to assess the propagation of ARGs and analyze the removal efficiency of ARGs in full-scale WWTPs with different treatment processes. Such information would be helpful in choosing an appropriate treatment process considering ARGs removal in cold areas.

2. Materials and methods

2.1. WWTPs and sampling

Four full-scale WWTPs (plant A, B, C, D) with different biological treatment processes in Harbin, China, were selected for the investigation in the present study. The daily average flow rate of A and B are 100,000 m^3/d and 325,000 m^3/d, respectively, treating domestic sewage using anaerobic-anoxic-oxic (A^2/O) process and anaerobic-oxic (A/O) process, respectively. The daily average flow rate of C is 150,000 m^3/d, which use cyclic activated sludge system (CASS) to treat domestic sewage. And plant D, with a daily average flow rate of 150,000 m^3/d, used CASS to treat mainly domestic sewage. Two sampling events were conducted from plant A, B and C in November 2013 and April 2014, and plant D was sampled once a month from November 2013 to April 2014. The flow diagrams for the four WWTPs and the sampling locations are shown in Fig. 1.

Water samples were taken from the raw influent, influent and effluent of the biological treatment unit and the final effluent every 6 h one day. One liter of 24 h mixed aqueous sample at each sampling site was collected in pre-sterilized polypropylene flasks individually. Sludge samples were collected from excess sludge. While in plant D the samples from (a) coarse screen effluent, (b) fine screen effluent, (c) grit chamber effluent, (d) sedimentation tank effluent, (e) CASS effluent and (f) UV disinfection effluent were taken. Then all samples were refrigerated and transported immediately back to the lab for subsequent processing.

2.2. DNA preparation

All water samples were filtered through a 0.22 μm-pore-size member filter (Millipore, USA) with a vacuum filtration device. The filters were cut into pieces in sterile centrifugal tubes, then separately stored at −20 °C and were analyzed within three days. Excess sludge samples were kept in −20 °C to freeze completely, then frozen dried by a vacuum dryer and 0.1 g lyophilized samples were acquired for subsequent DNA extraction.

Substance on the filter membranes and sludge samples were cleaned by PBS, then harvested by centrifugation at 9000 rpm for 3 min. The medium of the harvested samples were removed completely, 180 μL TE buffer and 30 μL lysozyme were added, and then incubated at 30 °C overnight for complete digestion. Digested products were performed at 9000 rpm for 5 min. Then DNA was extracted using Omega E.Z.N.A. Bacterial DNA Kit (Omega, USA) according to the manufacture's protocol with some modifications. The quality and concentration of extracted DNA were determined by 0.8% agarose gel electrophoresis and spectrophotometer analysis (NanoDrop ND-2000C, Thermofisher, USA).

2.3. Detection of ARGs

The presence of ARGs and *intI1* was checked by qualitative PCR in all the wastewater and sludge samples. The PCR primers and conditions for the amplifications of the ARGs and *intI1* are listed in Table S1. The PCR assays were carried out in 50 μL reaction systems including: 0.25 μL of Takara ExTaq (5 U/μL) (Japan), 1.0 μL of each primer, 4.0 μL of dNTP Mixture, 5.0 μL of 10 × ExTaq Buffer (Mg^{2+} plus) and 4 μL of template DNA. The thermal cycle procedure was: initial denaturation at 95 °C for 5 min, followed by a 35 cycles of 95 °C for 45 s, annealing (varied according to the information listed in Table S1) for 45 s, 72 °C for 60 s, with a final extension of 72 °C for 5 min. Duplicate PCR assays were performed to ensure the reproducibility, and each assay contained negative controls.

2.4. Real-time qPCR for ARGs

The purified PCR amplicons were ligated into the pGEM-T Easy vectors (Promega, Beijing, China) and then cloned into *Escherichia coli* DH5α (Transgen Biotech). Plasmids carrying target genes were extracted using TaKaRa MiniBEST Plasmid Purification Kit Ver.4.0 (Takara, Japan) and quantified by spectrophotometer. The log copy number of genes per μL DNA template solution was calculated according to Pei et al.'s (2006): $\text{logcopy of genes}/\mu\text{LDNA} = \log\frac{b\times c}{L\times a\times 10^{12}}$, where a represents the weight of kb DNA per pmol (1 kb DNA = 0.66 μg/pmol), b represents the Avogadro's constant (6.022×10^{23}/mol), L represents the length of template containing the target gene and c represents the concentration of template in μg/μL. Standard curves for qPCR were acquired by 10-fold serial dilutions of purified plasmid extracts, ranging from 10^8 to 10^2 gene copies μL^{-1}. Negative controls were conducted using double distilled water as a replacement of template DNA added into the qPCR assays. The standard curves were obtained with high R^2 values and high efficiency illustrating the linearity and sensitivity for each qPCR assay (see Table S2). Then the