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Characterization of microbial community and antibiotic resistance genes in activated sludge under tetracycline and sulfamethoxazole selection pressure

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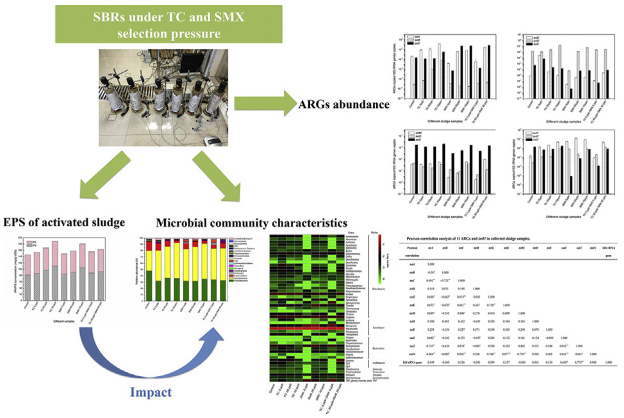
HIGHLIGHTS

- COD and NH_4^+—N removals significantly decrease under 10 ppm TC or SMX.
- Activated sludge EPS concentrations increase with increasing TC or SMX concentrations.
- TC and SMX affect the microbial community diversity of activated sludge.
- *Actinobacteria* abundances increase with increase of TC or SMX concentration.
- ARGs abundance increases with addition of TC or SMX.

GRAPHICAL ABSTRACT



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ABSTRACT

To investigate the microbial community characteristics, antibiotic resistance genes (ARGs), and bioreactor effluent quality change under tetracycline (TC) and sulfamethoxazole (SMX) selection pressure, sequencing batch reactors (SBRs) were used with environmentally relevant concentration and high-level of TC and SMX concentrations (0, 5 ppb, 50 ppb and 10 ppm). Chemical oxygen demand (COD) and ammonia nitrogen (NH_4^+—N) removals appeared unchanged ($p > 0.05$) with 5 and 50 ppb, but decreased significantly with 10 ppm ($p < 0.05$). Extracellular polymeric substances (EPS) concentrations increased significantly with increasing TC or SMX concentrations ($p < 0.05$). High-throughput 16S rRNA gene sequencing results suggested that *Proteobacteria*, *Actinobacteria* and *Bacteroidetes* were the three most abundant phyla in sludge samples. The *Actinobacteria* percentages increased with increasing TC or SMX concentration, while *Proteobacteria* and *Bacteroidetes* decreased. The microbial diversity achieved its maximum at 5 ppb and decreased with higher concentrations. The total ARGs abundances in sludge increased with addition of TC or SMX, and the higher relative abundances were in the order of *sul1* > *tetG* > *sul2* > *tetA* > *intI1* > *tetS* > *tetC*. Pearson correlation analysis showed most ARGs (*tetA*, *tetC*, *tetG*, *tetK*, *tetM*, *sul1*) were significantly correlated with *intI1* ($p < 0.01$).

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1. Introduction

Due to the intensive use of antibiotics for human, veterinary and agriculture purposes, various antibiotics have been frequently detected in hospital effluent, municipal wastewater, surface water, groundwater, and drinking water (Jiang et al., 2013; Kummerer, 2009; Yang et al., 2014). The antibiotics can reach wastewater treatment plants (WWTPs) from different routes and are usually detected at ppt to ppb levels (Shimizu et al., 2013; Zhang and Li, 2011; Zhou et al., 2013). Wide occurrence of antibiotics in the environment can lead to the emergence and spread of antibiotic resistance genes (ARGs) and bacteria (ARB), which reduce the therapeutic potential against human and animal pathogens, thus increasing risks to public health (Kemper, 2008; Rizzo et al., 2013; Zhang et al., 2009). Yang et al. (2014) identified 271 ARGs subtypes belonging to 18 ARGs types by the broad scanning of metagenomic analysis. Nowadays, WWTPs are considered to be the main 'hotspots' for the release of antibiotics and ARGs into the environment (Michael et al., 2013; Rizzo et al., 2013).

Previous studies have been conducted on the fate of antibiotics in the wastewater treatment process and the biological removal of antibiotics from wastewater (Kim et al., 2005; Le-Minh et al., 2010; Sahar et al., 2011; Watkinson et al., 2007). Some studies reported that different operational conditions could have a significant impact on microbial communities in activated sludge during the wastewater treatment process (Pala-Ozkok et al., 2014; Amorim et al., 2014). For example, Dorival-Garcia et al. (2013) suggested that nitrifying conditions could enhance the degradation of quinolones and affect the bacterial composition of sludge. It has been demonstrated that trace contaminants can be toxic to microorganisms or can potentially shape the bacterial community in WWTPs (Kohanski et al., 2010). Amorim et al. (2014) found that denitrifying activity was inhibited in a granular sludge sequencing batch reactor (SBR) and the denaturing gradient gel electrophoresis (DGGE) results revealed a dynamic bacterial community due to fluoroquinolones shock loading exposure at concentrations of 9 and 32 μM. Wunder et al. (2013) reported 3.33 ppb ciprofloxacin could affect both the structure and activity of biofilms growing in a continuous-flow rotating annular bioreactor. Since WWTPs rely on microorganisms, which might be affected by antibiotics (Li et al., 2011), further investigation in community shifts and activated sludge system responses to antibiotic selection pressure is crucial. However, little information is available on the relationship among microbial community, bioreactor effluent quality, extracellular polymeric substances (EPS) of activated sludge and induced ARG changes under antibiotic pressure.

In this study, tetracycline (TC) and sulfamethoxazole (SMX) were chosen as the antibiotic selection pressure because they are frequently found in wastewater and can only be partially removed in WWTPs (Batt et al., 2007; Gros et al., 2012; Miege et al., 2009). The environmental concentration of TC and SMX in wastewater is very low (ppt–ppb), but non-negligible concentrations are also found in pig slurry (up to 5 ppm) as well as drug manufacture effluents (Sengelov et al., 2003). According to pretest data, the main goal of this study is to evaluate the possible microbial community shift in an SBR system under different concentrations ranging from ppb to ppm, which simulate environmentally relevant concentration and high-level antibiotic concentration detected in wastewater, respectively. The abundance of ARGs exposed to TC and SMX was also measured. In addition, chemical oxygen demand (COD), ammonia nitrogen (NH_4^+—N), antibiotic removal performance and EPS changes of activated sludge were also taken into consideration. This study could provide the possible changes of bacterial community composition and wastewater treatment efficiency, as well as the ARGs in WWTPs under TC and SMX selection pressure.

2. Materials and methods

2.1. Chemicals

TC and SMX (>99% in purity) were purchased from Sigma-Aldrich Chemie (Steinheim, Germany). HPLC-grade water, HPLC-grade methanol, and HPLC-grade acetonitrile were supplied by Merck (Darmstadt, Germany). Other chemicals were analytically graded and obtained from Nanjing Chemical Reagent Factory, China. Milli-Q water, with a resistivity of at least 18.2 MΩ·cm, was produced from a Millipore purification system (Billerica, CA, USA).

2.2. SBR set up and operation

Six lab-scale SBRs with a working volume of 4 L were operated in this study. The column reactor had a total height of 60 cm and an internal diameter of 10 cm. The reactors were operated at room temperature (20 ± 5 °C) and at a hydraulic retention time (HRT) of 10 h. The solid retention time (SRT) was set to 20 d by applying a daily manual purge. The reactors were continuously operated following a 12 h cycle, repeated over time in the typical phase sequence: 30 min feeding, 600 min aeration, 60 min settling and 30 min withdraw. The reactors were protected from light in order to avoid possible antibiotics photolytic degradation. The dissolved oxygen (DO) concentration was controlled at around 3.8 ± 0.2 mg L^{-1}.

The SBRs were initially inoculated with seed sludge collected from the aeration tank of a WWTP in Nanjing, China. Before usage, the sludge was acclimatized for two weeks. The mixed liquor suspended solids (MLSS) concentrations in the six reactors were approximately 3000 mg L^{-1}. Synthetic wastewater simulated a municipal wastewater with 300 mg L^{-1} COD, 20 mg L^{-1} NH_4^+—N and 3 mg L^{-1} PO_4^{3-}—P as primary nutrients were selected in the feeding of SBRs. The artificial wastewater also contained $MgSO_4 \cdot 7H_2O$ 71 mg L^{-1}, $CaCl_2 \cdot 2H_2O$ 19.3 mg L^{-1}, $FeSO_4 \cdot 7H_2O$ 17.4 mg L^{-1}, $CuCl_2 \cdot 2H_2O$ 0.07 mg L^{-1}, $ZnSO_4 \cdot 7H_2O$ 0.13 mg L^{-1}, $Na_2MoO_4 \cdot 2H_2O$ 0.03 mg L^{-1}, $MnCl_2 \cdot 4H_2O$ 0.13 mg L^{-1}, KI 0.033 mg L^{-1} and H_3BO_3 0.15 mg L^{-1} as trace nutrients. The influent pH was maintained at 6.8–7.0 by using Na_2CO_3. To evaluate the potential effects of TC or SMX on the microbial community and ARGs in the SBR system, R_0 was the control experiment without antibiotic addition. R_1–R_5 were set in different concentrations of TC or SMX added into the synthetic wastewater (Table 1). After adaptation for thrice the SRT, the bioreactors can be recognized as stable. The sludge samples of each reactor were collected during the stable stage (60th and 120th day) for analysis.

2.3. Analytical methods

COD, NH_4^+—N and mixed liquor volatile suspended solids (MLVSS) of the sludge (MLVSS) were determined according to *Standard Methods for the Examination of Water and Wastewater* (APHA, 2005). DO concentration, pH and temperature values were measured using oxygen (SG6, METTLER TOLEDO Inc., USA) and pH meters (FE20, METTLER TOLEDO Inc., USA). The EPS of sludge samples during stable stage, which are mainly composed of proteins (PN) and polysaccharides (PS), were

Table 1
Antibiotic concentration conditions in SBRs.

Reactor code	Antibiotic concentrations	
	0–60 d	61–121 d
R_0	Control	Control
R_1	TC–50 ppb	TC–50 ppb
R_2	SMX–50 ppb	SMX–50 ppb
R_3	TC–5 ppb	TC–10 ppm
R_4	SMX–5 ppb	SMX–10 ppm
R_5	TC–5 ppb + SMX–5 ppb	TC–50 ppb + SMX–50 ppb

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