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Amoxicillin effects on functional microbial community and spread of antibiotic resistance genes in amoxicillin manufacture wastewater treatment system

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ABSTRACT

This study aimed to reveal how amoxicillin (AMX) affected the microbial community and the spread mechanism of antibiotic resistance genes (ARGs) in the AMX manufacture wastewater treatment system. For this purpose, a 1.47 L expanded granular sludge bed (EGSB) reactor was designed and run for 241 days treating artificial AMX manufacture wastewater. 454 pyrosequencing was applied to analyze functional microorganisms in the system. The antibiotic genes OXA-$_1$, OXA-$_2$, OXA-$_{10}$, TEM-$_1$, CTX-M-$_1$, class I integrons (intI1) and 16S rRNA genes were also examined in sludge samples. The results showed that the genera *Ignavibacterium*, *Phocoenobacter*, *Spirochaeta*, *Aminobacterium* and *Cloacibacillus* contributed to the degradation of different organic compounds (such as various sugars and amines). And the relative quantification of each β-lactam resistance gene in the study was changed with the increasing of AMX concentration. Furthermore the vertical gene transfer was the main driver for the spread of ARGs rather than horizontal transfer pathways in the system.

Introduction

The antibiotics in the aquatic environment have received considerable concerns in recent years. The main reason is that antibiotics emitted into receiving water systems have adverse effects on aquatic organisms (Batt et al., 2006; Jones et al., 2003), and easily lead to the development of multi-resistant strains of bacteria, even threat the health of human being (Li et al., 2010; Novo et al., 2013). Besides, antibiotics discharged into the wastewater treatment system restrain functional microbial community, and make troubles in the system (Amin et al., 2006a; Chelliapan et al., 2006). Therefore, the study on how antibiotics affect the microbial community structure in the system becomes necessary. Previous studies have proven this by investigating the impacts of some antibiotics on microbial communities, especially on bacterial and archaea communities (Aydin et al., 2015; Kor-Bicakci et al., 2016; Meng et al. 2015a). According to the previous studies, *Deltaproteobacteria* was the major bacterial groups in anaerobic reactors treating antibiotic-bearing (mainly streptomycin) wastewater (Deng et al. 2012b). The long-term adverse impact of tetracycline was quite variable for fermentative bacteria and methanogenic archaea (Rodriguez-Mozaz et al., 2015).

And most of previous research reveal the risks that antibiotic resistance genes (ARGs) threaten to human health have predominantly been found in the clinical setting (Prabhu et al., 2007; Tatavarthy et al., 2006). Therefore, the spread of ARGs in the wastewater system have also been recognized as a significant problem in the last few decades. Research shows that wastewater treatment plants contribute to the presence and spread of

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ARGs, because the high load of organic matter, and the bacterial density created an ideal condition for cell-to-cell contact and gene exchange (Sorensen et al., 2005). And some studies find that there is a positive correlation between ARGs and concentration of antibiotics (Rodriguez-Mozaz et al., 2015, Wang et al., 2015). Especially, the wastewaters coming from the manufacture of antibiotic products contain a higher level of organic matter and antibiotics which may create more favorable conditions for the spread of ARGs than other environmental water (Larsson et al., 2007; Saravanane et al., 2001).

Amoxicillin (AMX) is a broad-spectrum β-Lactam antibiotic that belongs to penicillin class organism (De Baere and De Backer 2007) and is the most common featured pollutant in antibiotic manufacture wastewater (Parmar et al., 2000). AMX manufacture wastewater mainly contains high concentration of chemical oxygen demand (COD) with reaching to several thousand mg/L. The ammonia–nitrogen and the high residual antibiotic reaching to several hundred mg/L also have been detected in the wastewater (Chen et al., 2011).

ARGs proliferate mainly through two processes: horizontal gene transfer (HGT) attributed to the transfer of ARGs between different bacterial cells *via* mobile elements, and vertical gene transfer attributed to the reproduction of bacterial hosts (Sørensen et al., 2005). For the class I integrons (intI1), the mobile bacterial genetic elements capable of acquiring and expressing genes embedded within gene cassettes (Stokes and Hall, 1989). And intI1 have also been commonly reported to contain antibiotic-resistance gene cassettes (Stalder et al. 2013b) and associated with other mobile elements such as plasmids and transposons (Stalder et al. 2012), which contribute to the spread of ARGs. As the most abundant integron in environmental bacteria, intI1 have been suggested as a proxy for antibiotic pollution (Stalder et al. 2013a). In addition, the bacterial reproduction which was attributed to the ARG proliferate has been suggested during sludge composting (Tian et al., 2016). Nevertheless, the understanding of specific development and spread of ARGs in anaerobic manufacture wastewater treatment process remains limited.

Therefore, in the study, 454 pyrosequencing of 16S rRNA genes was applied to explore the bacterial community structure. And β-lactam resistance genes were determined and quantified using PCR and quantitative PCR (Q-PCR) respectively. The intI1 which can be tracked as an indicator of horizontal gene transfer potential was also quantified using Q-PCR.

1. Material and methods

1.1. The operation of EGSB bioreactor

The laboratory-scale (1.47 L) expanded granular sludge bed (EGSB) bioreactor used in this study and its operation conditions were described in previous research (Meng et al., 2015b, 2015c). In short, AMX was introduced into the EGSB reactor with 19.7 (day 146) to 52.6 (day 166), 90.4 (day 190) and 214.7 mg/L (day 216) step by step under a hydraulic retention time of 20 hr and an average volumetric loading rate of 9.5 kg COD/(m^3·day) conditions. And the detail operation parameter is shown in Table S1.

1.2. Chemical analysis

Concentrations of COD and AMX were measured according the pervious methods by the Ultraviolet Spectrophotometry Method and High Performance Liquid Chromatography (HPLC) Method, respectively (Meng et al., 2015b).

1.3. Sampling and DNA extraction

Sludge samples were collected at days 145 (S1), 165 (S2), 189 (S3), 215 (S4), and 241 (S5). DNA was extracted from a 0.25-g sample of sludge with the PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA), according to the instructions of the manufacturer. Concentrations and quality of the extracted DNA were checked by spectrophotometric analysis on a NanoDrop ND-2000 (Thermo Fisher Scientific, USA) and electrophoresis on a 0.8% (*w/V*) agarose gel. Then extracted DNA was stored at −20°C until analysis.

1.4. Quantification of antibiotic resistance genes

Polymerase chain reaction (PCR) assays were used to determine the presence and absence of targeted ARGs. All PCRs were conducted in 25 μL of reaction mixture. The PCR was run using a S2000 thermal cycler (BioRad, Hercules, CA). Negative controls contained all components of the PCR mixture except the DNA template. The details of primer sequences and annealing temperatures of PCR and qPCR are described in Table S2 (in the supporting information). PCR products were analyzed by agarose gel electrophoresis (2.0%).

Q-PCR amplifications were conducted using the TransStart Top Green Q-PCR SuperMix (TransGen Biotech, China). Thermal cycling conditions consisted of 30 sec at 95°C followed by 40 amplification cycles of 10 sec at 95°C, 15 sec at an annealing temperature (Table S2), and 31 sec at 72°C. A melt curve profile was obtained by heating the mixture to 95°C, cooling to 65°C (15 sec), and slowly heating to 95 at 0.1°C/sec with continuous measurement of fluorescence. Plasmids integrated with targeted genes were cloned into *Escherichia coli* DH5α for the purpose of quantification. Standard curves were obtained by calibrating standard plasmids with amplification products at a 10-fold dilution from 10^9 to 10^3 copies/μL as shown in Table S3.

1.5. PCR amplification and 454 pyrosequencing

The DNA was amplified with a set of primers targeting the hypervariable V3–V4 region (about 375 bp) of the 16S rRNA gene. The forward and reverse primers were 5′- GTGYCAGCMGCC GCGGTA-3′, 5′-CCCCGYCAATTCMTTTRAGT-3′, respectively. Besides, the statistical analysis of 454 Pyrosequencing was showed as the previous study (Stadler and Love 2016).

1.6. Statistical analysis

The redundancy analysis (RDA) was conducted to investigate the relationships between bacterial community structure and environmental parameter including AMX concentrations and COD removal efficiency, using software package CANOCO version 4.5. To visualize the correlations between ARGs and bacterial taxa, 5 ARGs quantified by Q-PCR and 49 bacterial

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