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.

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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 (Cetecioğlu et al., 2013).

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 ARGs, not only in the receiving water systems, but also in the environment. Therefore, understanding the potential for antibiotic resistance genes and resistance to be transferred into the environment is of important.
ARGs, because the high load of organic matter, and the bacterial density created an ideal condition for cell-to-cell contact and gene exchange (Sørensen 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 has 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 has also 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). 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/(m³ 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 10⁸ to 10⁵ 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′- GTG YCA CCG MGC C GCC GGT A-3′, 5′-CCCCGYCAATT CMT TTRAGT-3′, respectively. Besides, the statistical analysis of 454 Pyrosequencing was showed as the previous study (Stalder 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
genera identified by pyrosequencing were combined, and a co-occurrence network was constructed by the random matrix theory-based network (Deng et al., 2012a). A correlation matrix was constructed by calculating all possible pairwise Pearson correlations and then converted into the similarity matrix by taking absolute values (0.43). Network analyses were performed using an online analysis pipeline at http://ieg2.ou.edu/MENA. To construct networks, negative edges were excluded. Network visualization was conducted on the interactive platform of Cytoscape (version 3.2.0).

2. Results

2.1. Reactor performance

The results of the mean COD and AMX removal efficiency with different concentrations of AMX are demonstrated in Fig. 1. It can be seen that around 85% COD and 80% AMX could be removed when the concentration of AMX increased to 214.7 mg/L. The operation condition and the performance of the reactor were described in detail in our previous paper (Meng et al., 2015c).

2.2. Diversity and abundance of bacteria at the gene level

Investigations into the stability and dynamics of the microbial community are crucial for better understandings of the microbial mechanism response to the effect of AMX on EGSB running, so 454 pyrosequencing was used to analyze shifts of microbial community structure in the study.

At genus level, we could detect the core of taxa common to all the investigated samples, such as Ignitibacterium, Phocenobacter, Spirochaeta, Aminobacterium and Cloacibacillus belonging to the Gram-negative. And they can utilize various sugars and amines to produce organic acids (e.g., acetate, propionate and butyrate) (Baena et al., 1998; Foster et al., 2000; Ganesan et al., 2008; Iino et al., 2010), which contribute to the high removal efficiency of those organic pollutants in the system. Besides, RDA analysis was conducted to investigate the relationships between environmental factors (COD removal efficiency and AMX concentration) and bacterial community (based on the relative abundance of genus), and the two axes accounted for 74.22% of the observed variation, with the first and second axes explained 64.20% and 10.02%, respectively. As shown in Fig. 3, RDA results showed that the above mentioned genera (except Spirochaeta) were positive correlations with the AMX concentration, and seemed to be adaptive to high concentration AMX pressure.

RDA results also showed that COD removal rate was inversely associated with various AMX concentrations and Bellilinea and Longilinea which are capable of degrading carbohydrates and amino acids (Yan et al., 2015), and Chelonobacter which produce acid fermentatively from glucose (Gregersen et al., 2009) exhibited a positive relationship with COD removal efficiency, while a strong inverse correlation with AMX concentrations. Especially, the relative abundance of Chelonobacter sharply decreased from 3.32% in S1 sample to 0.15% in S5 sample as shown in Fig. 2.

2.3. Abundance and correlation analysis of ARGs and intI1 in antibiotic wastewater treatment system

Five ARGs belonging to β-lactam resistance genes and intI1 were quantified by Q-PCR in five sludge samples. As shown in Fig. 4, the different concentrations of AMX affected the spread of β-lactam resistance genes. For OXA-1 genes, the peak relative quantification was showed in the S4 (AMX 100 mg/L) compared with the others samples. Besides, as shown in STab.4 (in the supporting information), the gene copies of OXA-1 from 1.92 × 10^2 to 9.54 × 10^4 copies/ng DNA were generally similar to sewage treatment plants (Yang et al., 2012). The relative quantification of OXA-2 increased in the S2 (AMX 20 mg/L), whereas it decreased with AMX concentration increased in the S3, S4 and S5. And the gene copies of OXA-3 were higher than sewage treatment plants (1-4 logs) as shown in Table S4 (Yang et al., 2014). However, it seemed that AMX around 100 mg/L is most optional to OXA-10. The CTX-M-1 was not widely detectable in WWPTs, indicating that they might not be the dominant β-lactam resistance genes in the WWPTs. However, in the study, similar to OXA-10, the relative quantification of CTX-M-1 reached to highest level with AMX around 100 mg/L and decreased with the high AMX concentration around 200 mg/L, and the gene copies of CTX-M-1 ranged from 6.95 × 10^3 to 6.53 × 10^3 copies/ng DNA. The TEM-1 gene was predominant and its relative quantification (0.15–0.35) was higher than other ARGs in the system. Besides, it was enriched with the increasing concentrations of AMX, especially under the high AMX concentration condition.

intI1 which integrates and transfers ARGs via gene cassettes, is the most prevalent mobile genetic elements in various environments (Mazel 2006). Therefore, quantitative change of intI1 gene was tracked as an indicator of the HGT potential. In the study, the relative quantification of intI1 reached the peak with the AMX concentration increasing to 50 mg/L, whereas it decreased when the AMX concentration continuously increased. Besides, there was no significantly positive correlation (p < 0.001) between intI1 and ARGs, indicating that HGT didn’t occur frequently in the system as shown in Table S5 (in the supporting information).
2.4. The role of the bacterial community in the spread of ARGs

The previous study confirmed that network analysis could be used to provide new insights into ARGs and their possible hosts in complex environmental scenarios (Deng et al., 2012a; Tian et al., 2016). As shown in Fig. 5, various ARGs quantified in this study were significantly \((p < 0.05)\) and positively correlated with various species. And the detailed co-occurrence between ARGs and bacterial taxa was summarized in Table 1. The four ARGs, including OXA-1, OXA-2, TEM-1 and CTX-M-1, had the most diversity in terms of host bacteria, with 6, 9, 10 and 6 potential hosts, respectively. However, there were only three potential hosts found for OXA-10. *Ignavibacterium* might be the hosts of resistance gene OXA-1, TEM-1 and CTX-M-1, and *Aminobacterium* and *Phocoenobacter* might contain resistance gene TEM-1 and OXA-10, respectively. *Bellilinea*, *Longilinea* and *Chelonobacter* were inferred to be the hosts of OXA-2.

### 3. Discussion

The performance of EGSB and the bacterial community dynamics analysis under different AMX concentrations condition had been described in previous researches (Meng et al., 2015b). However, in this study, 454 pyrosequencing methods can generate large amounts of DNA reads with a length of up to 370 bps, and then is better able to reveal the diversity and abundance of bacterial community.

The genera of *Ignavibacterium*, *Phocoenobacter*, *Spirochaeta*, *Aminobacterium* and *Cloacibacillus* belonging to Gram-negative kept the higher abundance than other genera during the operation stage and had the function of degradation of different organic compounds. These genera contributed to the EGSB consistently achieving typical COD reduction of 85%. What should be noted is that AMX is grouped into \(\beta\)-lactam antibiotics and mainly active against Gram-positive bacteria by acting on the bacterial wall, while Gram-negative bacteria are resistance to \(\beta\)-lactam antibiotics. Meanwhile, Gram-negative bacteria have been isolated with multiple plasmids that can carry resistance genes or different \(\beta\)-lactamas in the same organism (Lauretti et al., 1999; Sykes and Matthew, 1976).

Different class antibiotics had different inhibition functions on the special microbial growth. For example, erythromycin representing macrolide, a protein synthesis inhibitor, has been found significantly inhibiting ammonification and nitrification when concentration was higher than 20 mg/L (Alighardashi et al., 2009) and Tugce Katipoglu-Yazan et al., (2013) had found that both steps of nitrification were totally blocked by erythromycin. Besides, the conversion of butyric acid was inhibited when erythromycin was present in anaerobic treatment system (Amin et al., 2006b). However, tetracycline inhibits protein synthesis by disrupting amino acid chain elongation at the 30S subunit of ribosome (Backhaus and Grimme, 1999). And it is active \textit{in vitro} against gram-positive and gram-negative bacteria, and reducing nitrification rates and growth of bacteria (Halling-Sørensen, 2001), but has little effect on fungi (Anderson and Domsch, 1973). In the study, AMX concentrations was

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**Fig. 2** – Taxonomic classification of 454 pyrosequences from bacterial communities at the genus levels.

**Fig. 3** – RDA analysis of bacterial community structure and environmental parameter including AMX concentrations and COD removal efficiency. RDA: redundancy analysis; COD: chemical oxygen demand; AMX: amoxicillin.
inversely associated with the growth of some genera such as *Bellilinea*, *Longilinea* and *Chelonobacter* degrading carbohydrates, amino acids and glucose, and then COD removal efficiency was slightly affected.

The purpose of this study was also to explore the mechanisms behind the spread of ARGs with the increasing concentration of AMX. As mentioned above, the change in relative quantification of each ARG was quite different under the effect of increasing AMX concentration. The spread of ARGs in different environmental conditions has been speculated to be associated with horizontal transfer pathways, especially when ARGs were positively correlated with intI1 (Di Cesare et al., 2016; Li et al., Tian et al., 2016). However, 5 ARGs detected in this study did not display any positive correlation with intI1 ($p < 0.05$) during the operation period. So the horizontal transfer pathways might not be the main driver for the spread of ARGs in the system.

![Fig. 4](image1.png)

**Fig. 4** – Changes in relative quantification of the ARGs and IntI 1 with the increasing of AMX concentration. ARGs: antibiotic resistance genes; AMX: amoxicillin.

![Fig. 5](image2.png)

**Fig. 5** – Network analysis shows the co-occurrence of ARGs and their potential host bacteria. ARGs: antibiotic resistance genes.
Therefore, it was inferred that vertical gene transfer mainly contributed to the ARG profiles and the similar result had also been suggested in various environments (Aydin et al., 2016; Czekalski et al., 2014; Forsberg et al., 2014; Yang et al., 2014; Zhang et al. 2016).

With AMX concentration increasing, both the abundance and diversity of the bacterial population were changed. The possible host information for individual ARGs, the co-occurrence patterns between the 5 ARGs and bacterial taxa during the operation period were investigated using network analysis. In this study, the relative quantification of OXA-1 increased with the increasing of AMX concentration. And the relative abundance of OXA-1 gene potential hosts, such as Kosmotoga, Aminomonas and Proteiniphilum, also increased with the increasing of AMX concentration. And the relative abundance of OXA-1 gene potential hosts, such as Kosmotoga, Aminomonas and Proteiniphilum, also increased with the increasing of AMX concentration. And the relative abundance of OXA-1 gene potential hosts, such as Kosmotoga, Aminomonas and Proteiniphilum, also increased with the increasing of AMX concentration. And the relative abundance of OXA-1 gene potential hosts, such as Kosmotoga, Aminomonas and Proteiniphilum, also increased with the increasing of AMX concentration. And the relative abundance of OXA-1 gene potential hosts, such as Kosmotoga, Aminomonas and Proteiniphilum, also increased with the increasing of AMX concentration.

With the TEM-1 gene, it’s interesting that the TEM-1 gene has more potential hosts than other ARGs in the study. According to the risk ranking in the antibiotic resistome published previously (Martínez et al., 2015), genes encoding β-lactamases, including TEM-1, belong to the highest risk category. However, in the study, the gene was enriched, especially under high AMX concentration. And the TEM-1 gene was also frequently detected in different environmental conditions (Henriques et al., 2006, Li et al., 2009). Brevundimonas diminuta, Brachymonas denitrificans, Psychrobacter spp and Bacillus endophyticus have been described containing TEM-1 in the penicillin production wastewater treatment plant and the receiving river (Li et al., 2009). However, in the study, the potential hosts of TEM-1 resistance gene were different with the previous study. The reason of inconsistency might be that hosts of ARGs had mainly been found under different environmental conditions. Therefore, for further make network analysis valid, isolate bacterial hosts of ARGs from various environments should be concerned.

### 4. Conclusions

The Gram-negative bacteria were the main genera to keep the relative high COD removal efficiency during the operation period. In this study, the relative quantification of each β-lactam resistance gene in the study changes quite differently with the increasing of AMX concentration. Especially noted that the TEM-1 gene can be enriched with high AMX concentration (exceed 200 mg/L). And the vertical gene transfer was concluded to be the main driver for the spread in ARGs rather than horizontal transfer pathways.

<table>
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<th>Genus</th>
<th>ARGs subtype</th>
<th>Bacterial relative abundance</th>
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Table 1 – ARGs host information revealed by co-occurrence between ARGs subtype and bacterial taxa.

*a:* The reference listed in this column verified the same host information for the related ARGs as that revealed by co-occurrence results.
Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jes.2017.09.020.

REFERENCES


