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Multiple antibiotic resistance genes distribution in ten large-scale membrane bioreactors for municipal wastewater treatment

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HIGHLIGHTS

- Multiple antibiotic resistance genes were studied by GeoChip in 10 MBRs.
- Dominant antibiotic resistance gene groups were different in individual MBR.
- Antibiotic resistance genes were majorly from Proteobacteria and Actinobacteria.
- Influent, temperature and conductivity affected the resistance gene distribution.

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ABSTRACT

Wastewater treatment plants are thought to be potential reservoirs of antibiotic resistance genes. In this study, GeoChip was used for analyzing multiple antibiotic resistance genes, including four multidrug efflux system gene groups and three β-lactamase genes in ten large-scale membrane bioreactors (MBRs) for municipal wastewater treatment. Results revealed that the diversity of antibiotic genes varied a lot among MBRs, but about 40% common antibiotic resistance genes were existent. The average signal intensity of each antibiotic resistance group was similar among MBRs, nevertheless the total abundance of each group varied remarkably and the dominant resistance gene groups were different in individual MBR. The antibiotic resistance genes majorly derived from Proteobacteria and Actinobacteria. Further study indicated that TN, TP and COD of influent, temperature and conductivity of mixed liquor were significant ($P < 0.05$) correlated to the multiple antibiotic resistance genes distribution in MBRs.

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1. Introduction

Antibiotics have been widely used in medicine and agriculture settings since last century. However, only a small portion can be metabolized by humans and animals; the rest are released into the environment (Uyaguari et al., 2011; Wei et al., 2014). The accumulations of antibiotics have boomed the antibiotic resistance genes of bacteria (Zhu et al., 2013). Recently, more and more related genes have been detected in the settings, accompanied with the appearance of a few super recalcitrant antibiotic-resistant bacteria (Huang et al., 2012; Marti et al., 2014). The emergence of antibiotic resistance genes together with the mutation of microbes has become not only a public health problem, but also an immeasurable risk to the ecological stability. Wastewater treatment plants (WWTPs) are considered as a major sink which collects all the unmetabolized antibiotics from human activities (Aydin et al., 2016; Qiu et al., 2013). In addition to the incomplete degradation of antibiotics in WWTPs, the surplus sludge and effluent with the enriched antibiotic resistance genes are also a potential threat to environment (Chen et al., 2015; Gao et al., 2012; LaPara et al., 2011; Naquin et al., 2015; Rodriguez-Mozaz et al., 2015). It is critical to obtain a better understanding of the ecology of the antibiotic resistance genes in WWTPs and it will be consequently helpful to predict and counteract the negative impact that these genes might impose on ecology. The current studies on antibiotic resistance genes in WWTPs mostly confined to the analyses of several common antibiotics and corresponding antibiotic resistance genes such as tetracycline, erythromycin and sulfonamide resistance genes (Aydin et al., 2015; Munir et al., 2011). However, it is crucial to cover multiple antibiotics resistance genes because the microbial multiple antimicrobial resistance mechanisms involving the multidrug resistant efflux systems and

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β-lactamase have attracted global health concerns (Marquez, 2005).

The objective of this study was to illustrate the distribution and abundance of the major antibiotic resistance genes in large-scale WWTPs. A high-throughput array GeoChip was employed to analyze the antibiotic resistance genes of ten large-scale WWTPs in China. GeoChip covers four multidrug efflux system genes and three β-lactamase genes (Classes A–C), providing more insightful information than conventional microbiology approaches. All the selected ten plants are featured as a nutrient-removal process combined with a membrane bioreactor (MBR), which is commonly operated at longer sludge retention time (SRT) and higher sludge concentrations. These unique conditions may affect the development and proliferation of the antibiotic resistance genes and the corresponding microorganisms. This study focused on the overall distributions of antibiotic genes by a common wastewater treatment process, but from different geographic regions. The obtained information can provide helpful knowledge to estimate the environmental risk of antibiotic resistance genes.

2. Materials and methods

2.1. MBRs and physicochemical analysis

The ten selected large-scale WWTPs are located in four different cities from China: Beijing (B1, B2, B3 and B4), Wuxi (W1, W2, W3 and W4), Shiyan (S1) and Kunming (K1) (Fig. S1). These plants share a similar nutrient-removal anaerobic–anoxic–oxic process combined with a membrane bioreactor (A_1/A_2/O-MBR). The technical processes, operational parameters and performance of the ten plants were summarized in Supporting information (Tables S1, S2). Three replicate sludge samples were collected from the membrane tanks of each plant. The mixed liquor properties were measured immediately after sampling, including temperature, pH, dissolved oxygen (DO), conductivity, viscosity, the ratio of mixed liquor volatile suspended solids (MLVSS) and mixed liquor suspended solids (MLSS), sludge volume index (SVI) and supernatant substances (total organic carbon (TOC), polysaccharides, proteins and humic substances). The detailed information is shown in Table S3 and the related study has been published in the previous paper (Shen et al., 2012). Another set of the sludge samples was shipped to the lab on dry ice for DNA analysis.

2.2. GeoChip 4.0 assay for antibiotic resistance genes

Genomic DNA was extracted and quantified as previously described (Sun et al., 2014). The purified DNA (2 μg) was labeled with Cy3 using random primers and the Klenow fragment of DNA polymerase I (Wu et al., 2006), and then purified and dried in a Speed Vac at 45 °C for 45 min. The hybridization was performed at 42 °C with 40% formamide for 16 h on a MAUI hybridization station using GeoChip 4.0 (NimbleGen, WI, USA), which contains 2812-*mer*-oligo nucleotide probes in seven categories regarding different antibiotic resistance microbes. The GeoChips were scanned at a laser power of 100% after hybridization. The signal intensities were measured based on the scanned images. The spots with a signal to noise ratio (SNR < 2.0) were removed as poor-quality spots (He et al., 2010). Genes that were detected in only one sample out of the three replicates were considered ineffective and removed.

2.3. Data statistical analysis

All GeoChip data, mixed liquor properties, and operational conditions were normalized by mean for standardization. Microbial diversity and evenness were characterized by Shannon–Weiner index, Simpson's index and Pielou Evenness index. Detrended correspondence analysis (DCA) was used to determine the overall functional variations in the antibiotic resistance genes. The relationship among the mixed liquor properties, operational conditions and resistance genes was explained by Mantel test and canonical correspondence analysis (CCA). Linear regression was performed to test the influence of influent industrial wastewater on antibiotic resistance genes by using the lm function in the R Statistics Package version 2.1.3.

3. Results and discussion

3.1. Diversity of antibiotic resistance genes

The ten MBRs displayed variations of the detectable antibiotic resistance genes in terms of gene number and diversity indices. A total of 585–1305 antibiotic related genes were detected in these plants (Table 1), with the highest gene number was found in W2, followed by W1, B1 and B2; while W4 and B4 showed the fewest antibiotic gene number. According to the GeoChip 4.0 database, seven antibiotic resistance gene groups were all detected in the ten MBRs, including ATP-binding cassette (ABC), small multidrug resistance (SMR), major facilitator super family (MFS), Multidrug toxic compound extrusion (MATE) and β-lactamase (Classes A–C).

These MBRs showed a high gene overlap level. The proportion of the unique antibiotic genes in each MBR was considerably low (less than 3.2%, see Table 1). W1/W2 shared the most genes (86.9%); B1/B2, B1/W1, W3/S1, and S1/K1 also displayed a high overlap of approximate 80%, while W2 and W4 owned the fewest common genes (Table 1). These results indicated that considerable amounts of common resistance genes existed in WWTPs located at different geographic sites.

The diversity results showed that W2 had the highest diversity, followed by W1, B2 and B1 (Table 2). The antibiotic gene diversity in W4 and B4 were relative lower, which was consistent with the results of the detected gene number. The resistance gene evenness

Table 1
Antibiotic resistance genes uniqueness and overlap of ten MBRs.

	B1	B2	B3	B4	W1	W2	W3	W4	S1	K1
B1	11	1090	887	585	1137	1169	896	551	861	809
B2		2	860	572	1106	1113	875	545	840	792
B3			7	595	882	896	830	540	818	770
B4				6	580	587	583	467	585	568
W1					27	1191	893	549	851	803
W2						42	901	549	862	805
W3							2	541	841	798
W4								1	543	540
S1									6	784
K1										1
No. of genes	1215	1148	944	639	1256	1305	936	585	909	845

Table 2
Antibiotic resistance genes diversity indices and evenness of ten MBRs.

	H	J	1/D	Si
B1	7.03 ± 0.02 ef	0.9992	1129 ± 21 ef	0.9889
B2	6.98 ± 0.02 e	0.9994	1074 ± 22 e	0.9910
B3	6.78 ± 0.01 d	0.9994	876 ± 8 d	0.9911
B4	6.36 ± 0.01 b	0.9993	577 ± 3 b	0.9912
W1	7.08 ± 0.03 f	0.9993	1185 ± 20 gh	0.9893
W2	7.12 ± 0.02 f	0.9992	1228 ± 13 h	0.9888
W3	6.79 ± 0.02 d	0.9993	881 ± 12 d	0.9893
W4	6.21 ± 0.09 a	0.9994	501 ± 29 a	0.9928
S1	6.73 ± 0.02 cd	0.9993	834 ± 9 cd	0.9910
K1	6.67 ± 0.03 c	0.9993	783 ± 11 c	0.9902

H: Shannon index; J: Pielou evenness; 1/D: Simpson index; Si: Simpson evenness.
One way anova was performed to assess the significance among ten MBRs, with a–g indicating significant differences at the $p < 0.01$ level.

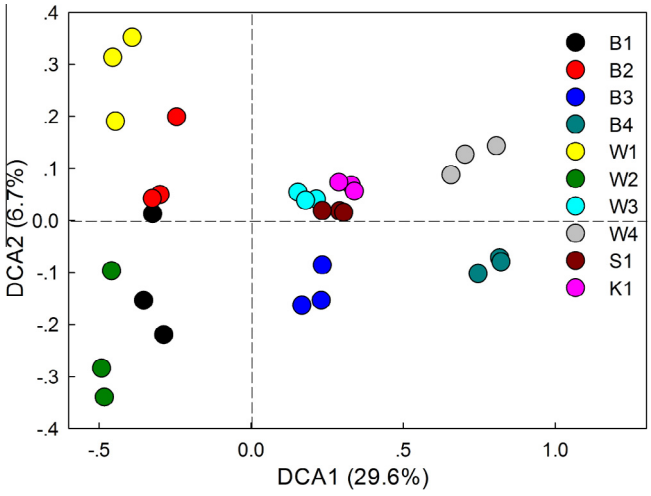

Fig. 1. Detrended correspondence analysis (DCA) of GeoChip data showing that the difference of antibiotic resistance genes among ten MBRs.

of MBRs was found to be at similar level among them. The influent industrial wastewater proportion didn't influence the antibiotic resistance gene diversity ($R^2 = 0.012$, p = 0.558).

The above-mentioned results indicated that geographic sites and the proportion of domestic and industrial wastewater had less effects on the antibiotic resistance gene number, diversity and evenness. Although the geographic regions showed a big variation, there were still many coexisted genes in the different WWTPs, an obvious contrast to the antibiotic resistance genes in soil or other environments that were significantly influenced by geographic position. It also illustrated the special ecological system of WWTPs which were largely different from natural environment. The influent components might play more roles in the antibiotic genes in WWTPs.

3.2. Dissimilarity and distribution of antibiotic resistance genes

The dissimilarity of antibiotic resistance genes was characterized by DCA, and two groups were observed. Group 1 contained W4, S1 and K1, indicating their similarity in the three MBRs (Fig. 1). B1 and B2 were clustered together, separated from the group 1. In addition, the others were independent from the above two groups, suggesting their respective specialties. The antibiotic resistance genes from different geographic zones were grouped together, showing the less influence of geographic factors on them again.

Phylogenetic analysis revealed that 95% antibiotic resistance genes mostly derived from bacteria. Gammaproteobacteria, Alphaproteobacteria and Betaproteobacteria of Proteobacteria, Actinobacteria of Actinobacteria, and Bacilli and Clostridia of Firmicutes were the main antibiotic resistance phyla. Further analysis on genus results suggested that resistance genes mainly originated from *Burkholderia*, *Pseudomonas*, *Streptomyces*, *Yersinia*, *Rhizobium* and *Mycobacterium*. Besides, the genes from *Vibrio*, *Bacillus* and *Bradyrhizobium* were also abundant in the ten MBRs. The antibiotic resistance bacteria were found to largely spread in Gram-negative and Gram-positive bacteria. Previous study also found that Alpha- and Beta-proteobacteria were popular in MBR, but Firmicutes and Bacteroidetes were dominant in upflow anaerobic sludge blanket (Qiu et al., 2013), and it might be determined by MBR operational characters and thus the ecological environment were more profitable to the growth of Proteobacteria. Other bacteria such as enterococci, *Klebsiella*, and *Pseudomonas* were also common antibiotic resistance bacteria (Bouki et al., 2013). Some studies reported discrepant microbial community composition in WWTPs, but they displayed similar function. The possible reason is that the complicated environment of WWTPs might cause the resistance gene transfer to non-resistant bacteria and develop some multiple antibiotic resistance bacteria (Bouki et al., 2013).

3.3. Abundance of antibiotic resistance genes

The abundance of resistances genes is related to antibiotic resistant bacteria and therefore it is an important index that reflects degree of antibiotics contamination in environment. To identify the major antibiotic resistance gene groups in MBRs, the average abundances of them were analyzed (Fig. 2). Results showed that the relative abundance of each resistance gene group was similar in the ten MBRs. The average signal intensity of MFS group was the highest among MBRs, and the relative abundance accounted for approximately 20% of the total. β-Lactanase A also had higher average signal intensity, accounting for about 18%. In contrast, β-lactanase B showed the lowest average signal intensity and occupied about 6% of the total (Fig. 2).

In order to further identify the absolute amount of the major resistance genes, the nominal signal intensity of them was analyzed. The absolute amount of resistance genes varied a lot among MBRs (Fig. 3). SMR was the highest in K1, which were also higher in W1, W3, B1 and B2, indicating that SMR were dominant antibiotic genes in MBRs. SMR protein family is known as the smallest drug efflux proteins (Marquez, 2005), and they demonstrated transport of lipophilic compounds, primarily quaternary ammonium compounds (QACs) and a variety of antibiotics such as aminoglycosides, chloramphenicol, erythromycin as well as tetracyclines (Bay et al., 2008). SMR were abundant in the five MBRs,

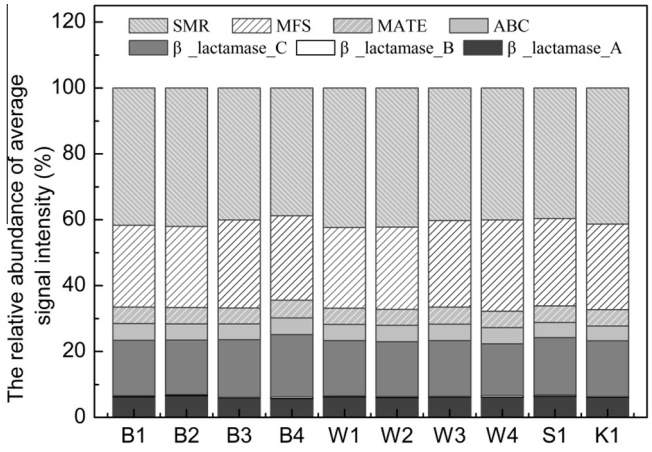

Fig. 2. The comparison of average signal intensity of each antibiotic resistance group among ten MBRs.

indicating that QACs and the above several antibiotics may also be abundant in these MBRs. QACs are used widely as detergents and disinfectants in domestic, industrial and clinical settings (Gaze et al., 2011), and the residues of QACs finally flow into wastewater treatment plant. The selection pressure of such compounds may lead to the accumulation of SMR in MBRs. Relevant study also demonstrated that the SMR family resistance genes (*qac* genes) were both detected in activated sludge and final effluents of WWTPs (Szczepanowski et al., 2009), suggesting that WWTPs was a major potential reservoir for resistance genes and the genes may enter into environment by disposal of sewage sludge.

The abundance of MATE was highest in B4, and MFS was abundant in W4, W3, B3 and S1 (Fig. 3). Compared with SMR gene family, MFS gene family displayed more broad-spectrum antibiotic resistance. Besides the above-mentioned antibiotics, MFS genes coding for antibiotic efflux pumps are relative to fluorochinolone, lincosamides, novobiocin and rifampin resistance (Mokracka et al., 2012). In Gram-positive bacteria, multi-drug resistance is mainly conferred by MFS efflux systems, such as the pumps Bmr and Blt in *Bacillus subtilis* and NorA of *Staphylococcus aureus* (Marquez, 2005). Tetracycline-specific efflux pumps, which are members of the MFS family and they are found in both pathogenic Gram-negative and Gram-positive bacteria. MFS tetracycline efflux pump genes such as *tetA*, *tetD*, *tetG*, *tetH*, *tetL* and *tetY* were all detected in WWTPs (Auerbach et al., 2007; Szczepanowski et al., 2009). An MFS efflux pump, Tap, conferring aminoglycosides and the tetracyclines resistance, has been detected in Mycobacteria, such as *Mycobacterium fortuitum* and *Mycobacterium tuberculosis*

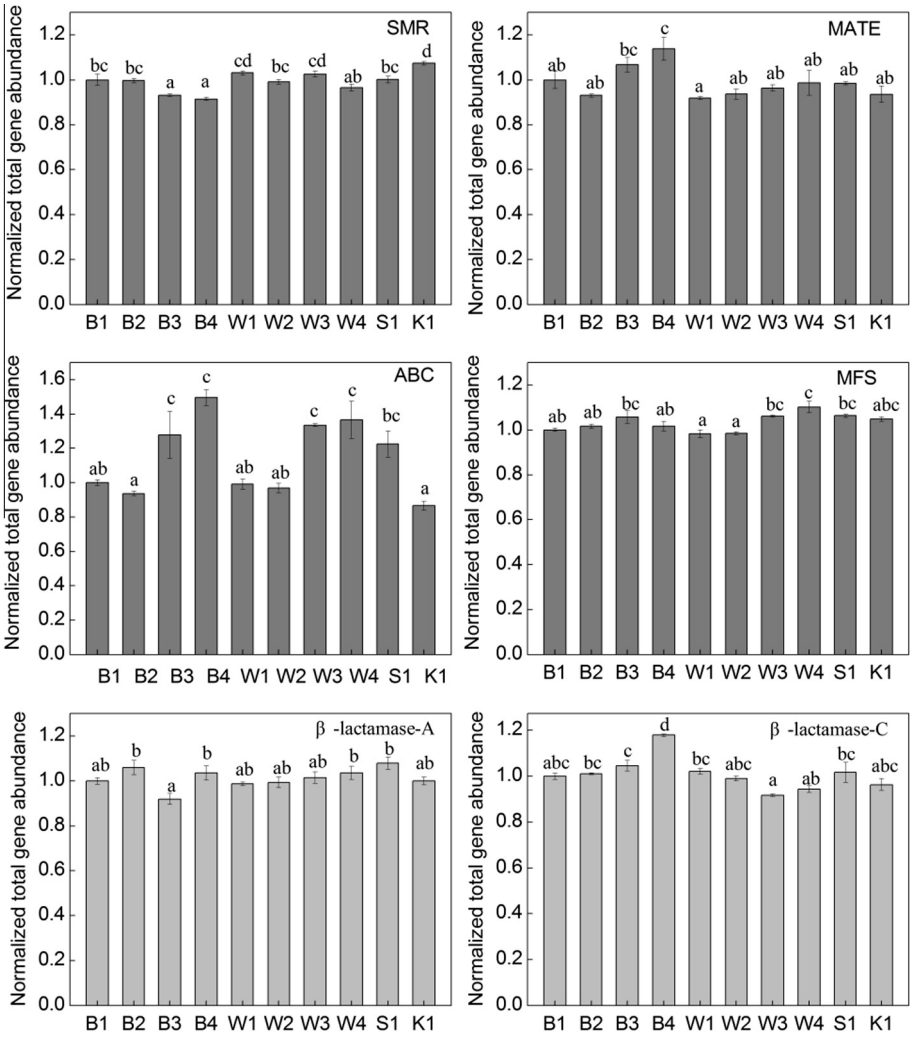

Fig. 3. The total abundance of antibiotic resistance genes (normalized to the abundance of B1). One way anova was performed to assess the significance among MBR plants, with a, b, c and d indicating significant differences at the $P < 0.05$ level.

Table 3
Mantel tests to examine the correlation between the antibiotic resistance genes and the environmental parameters.

Operational condition	Statistic (r)	Sig. (P)	Mixed liquid property	Statistic (r)	Sig. (P)
SRT	0.1016	0.109	Temperature	0.2938	**0.003***
HRT	0.0026	0.432	pH	0.0223	0.359
MLSS	−0.0377	0.705	DO	0.0593	0.195
F/M	−0.0006	0.705	Viscosity	−0.0794	0.822
Influent characters	Statistic (r)	Sig. (P)	Conductivity	0.1631	**0.041***
COD	0.2464	**0.003****	SVI	0.3389	**0.003****
NH_4^+-N	0.1383	**0.027***	MLVSS/MLSS	0.0810	0.132
TN	0.2364	**0.001****	TOC	−0.0373	0.638
TP	0.3868	**0.002****	Polysaccharide	0.0343	0.315
SO_4^{2-}	0.0791	**0.089***	Protein	0.1014	0.082
			Humics	0.0202	0.384

"*" indicated that the significant level was $p < 0.05$; "**" indicated that the significant level was $p < 0.005$.

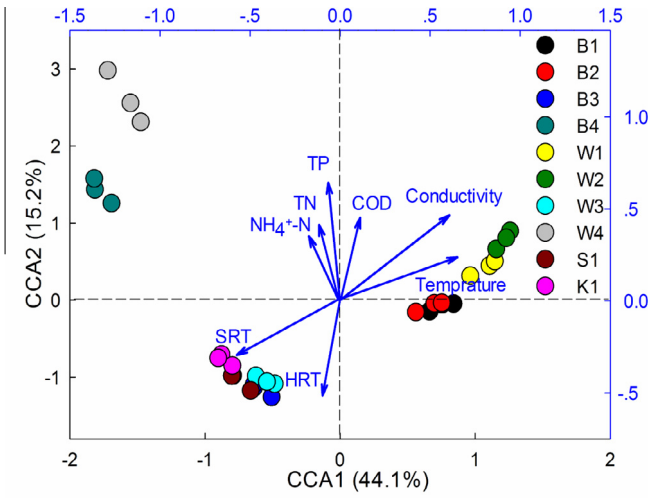

Fig. 4. Canonical correspondence analysis (CCA) of antibiotic resistance genes and operational conditions, influent characters and mixed liquor properties.

(Louw et al., 2009). In this study, both *Bacillus* and *Mycobacterium* were abundant antibiotic resistance bacteria, indicating their contribution to the accumulation of MFS genes.

The abundance of β-lactanase C was predominant in B4, and β-lactanase A in B2, B4, W4 and S1 was higher than other MBRs (Fig. 3). β-Lactam antibiotics was widely used, accounting for 60% of all used antibiotics in the last decades (Yang et al., 2011). The primary mechanism of bacterial resistance to this group of antibiotics was the production of β-lactamases (Uyaguari et al., 2011). In this study, β-lactam A and β-lactam C were both found in the ten MBRs, but they displayed different distribution among MBRs. Another study revealed that β-lactam C was higher than other β-lactam resistance genes. Noticeably, Mycobacteria was also reported to produce β-lactamases and intrinsically resistant to β-lactam antibiotics (Flores et al., 2005).This further explained the reason that abundant *Mycobacterium* was detected in MBRs. Overall, these results indicated that antibiotic resistance genes and bacteria were also existed in biosolids of MBR systems, and necessary measures for the treatment of biosolids were required to prevent them from integrating into ecological reservoirs. Previous study indicated that two-phase thermophilic digestion and microwave pretreatment could decrease the quantity of a few antibiotic resistance genes, but it did not play roles in many other antibiotic resistance genes in municipal wastewater sludge (Tong et al., 2016; Wu et al., 2016). Therefore, more efficient methods are needed to treat different kinds of biosolids and remove the antibiotic resistance genes from WWTPs.

The influence of influent industrial wastewater proportion on each antibiotic resistance gene abundance was investigated. Results suggested that the influent industrial wastewater proportion significantly influenced the β-lactamase C ($R^2 = 0.171$, $p < 0.05$) and MATE family ($R^2 = 0.150$, $p < 0.05$). The proportion of industrial wastewater displayed negative correlation with the abundance of β-lactamase C ($r = -0.414$) and MATE family ($r = -0.387$). The β-lactam are widely used to treat human and animal infectious disease, because they have high clinical efficacies and low toxicity (Yang et al., 2011). MATE family is responsible for efflux of antibiotics such as aminoglycosides, fluoroquinolones, chloramphenicol and so on. These antibiotics were also used in human and animals medicine and the unmetabolized portion were released into domestic wastewater. Thus it might be the reasons that low β-lactam C and MATE abundance was found in WWTPs with higher industrial wastewater portion. On the other side, it also illustrated that controlling the usage and disposal of antibiotics and decreasing the release to domestic wastewater would be very helpful to improve future environmental health.

3.4. Relationship between antibiotic genes and environmental parameters

To examine whether operational conditions, influent characters and mixed liquor properties affect the antibiotic resistance genes, Mantel test was performed with 20 individual variables. Results showed that the influent COD, TN, NH_4^+-N, TP, temperature, conductivity, and SVI of mixed liquor had significant correlations ($P < 0.05$) with antibiotic resistance genes (Table 3), suggesting their roles in shaping antibiotic resistance microbial community structure.

CCA was carried out to further discern the major environmental variables correlated with antibiotic resistance genes. A total of nearly 60% variation of the antibiotic resistance genes could be explained by the two axes (Fig. 4). W1, W2, B1 and B2 clustered together, conductivity and temperature seemed to be the major factors that affected the antibiotic resistance genes of the four MBRs. The conductivity was higher in W1 and W2 (Table S3), suggesting that metallic ions played important roles in shaping antibiotic resistance genes. The influent TN was highest in B4, and TP was highest in W4 (Table S3), the nutrient in wastewater might have effect on antibiotic microbes and then affect the antibiotic resistance genes of MBRs. In addition, operational conditions SRT and hydraulic retention time (HRT) showed important influence on B3, W3, S1 and K1.

The selective pressure of antibiotic residues was thought to be the major reason of antibiotic genes and microbes deposition in WWTPs. Thus, previous studies most focused on the relations between antibiotic concentration and antibiotic resistance genes

(Zhang et al., 2013). Some studies also suggested that the quantities of antibiotic resistance genes and microorganisms were not as positive correlations with corresponding antibiotics concentrations as expected (Gao et al., 2012; Novo et al., 2013; Xu et al., 2015). Therefore, their links were rather complex, and the other environmental parameters were probably involved in driving antibiotic resistance genes distribution.

The influent TN, TP and COD demonstrated significant effects on the distribution of antibiotic resistance bacteria and genes especially for B4 and W4. The difference of nutrients intensity and organic load could influence the growth and distribution of antibiotic resistance bacteria and genes, which was also mentioned in another study (Novo et al., 2013). Besides, temperature and conductivity of mixed liquor displayed strong correlations with antibiotic genes in MBRs. Temperature was a very important factor to growth of microorganisms, and it was also reasonable that it affected the antibiotic resistance bacteria and genes. The conductivity indicated the metal iron concentration in MBRs, and heavy metals were also found to be coexisted with antibiotics and caused the enrichment of some resistance genes (Ji et al., 2012; Mao et al., 2015). What's more, significant positive correlations were revealed between some antibiotic resistance genes and typical heavy metals such as Cu, Zn and Hg (Ji et al., 2012). Therefore, metals might accelerate the dissemination of antibiotic resistance genes in environment. Besides, the relation of antibiotic concentration and multiple antibiotic resistance genes needed to be addressed in future study.

4. Conclusion

The dominant multiple antibiotic resistance gene groups were different among MBR plants. Multiple antibiotic resistance genes were mostly from Proteobacteria and Actinobacteria. The TN, TP and COD of influent, temperature and conductivity of mixed liquor were the major factors that affected the distribution of multiple antibiotic resistance genes in MBRs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2016.09.117.

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