Fate of antibiotic resistance genes and its drivers during anaerobic co-digestion of food waste and sewage sludge based on microwave pretreatment

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HIGHLIGHTS

- AcoD reduced abundance of total ARGs compared with AD of mono-SS.
- AD effectively reduced both abundance and quantities of MRGs.
- AD of MW-SS was more effective than that of MW-FW for ARGs abundance control.
- Evolution of bacterial community was the main driver to the fate of ARGs.
- ARGs reduction may be associated with the decreased co-selection from heavy metals.

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ABSTRACT

In this study, anaerobic digestion of mono-SS, MW-SS:FW and SS:MW-FW was investigated to understand the fate of ARGs and its drivers. Anaerobic digestion was effective for the reduction of metal resistance genes (MRGs), and could reduce the abundance of *bla*_{OXA-1}, *sulI* and *tetG*, while *sulII* in co-digestion and *bla*_{TEM} and *ereA* only in MW-SS. ARGs reduction could be partly attributed to the reduction of co-selective pressure from heavy metals reflected by MRGs. However, the abundance of *mefA/E*, *ermB*, *ermF*, *tetM* and *tetX* increased significantly. Anaerobic co-digestion, especially for MW-SS, could reduce total ARGs abundance compared with mono-SS, and evolution of bacterial community was the main driver for the fate of ARGs.

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1. Introduction

Increasing acquired antibiotic resistance is among the greatest worldwide concerns for health care, and it is currently considered to be one of the most serious public health issues. It has been estimated that antibiotic resistance is responsible for more than 25,000, 23,000, and 38,000 deaths every year in the European Union, the United States, and Thailand, respectively (Berglund, 2015). This highlights the need for broad strategies to slow the rate at which resistance spreads, and proactive treatment of anthro-

pogenic waste containing ARGs may help mitigate the spread of ARGs (Pruden et al., 2013).

Municipal wastewater treatment plants (WWTPs) were considered as significant reservoirs of ARGs, and numerous studies have detected amounts of ARGs at every stage of the municipal wastewater treatment processes (Munir et al., 2011). The vast majority of these ARGs are discharged from excess sludge, which has higher contribution (ca. 1000 times) to the release of the ARGs into the environment compared with effluent (Munir et al., 2011). In addition, ARGs were found in various foods like pork, beef, raw fruits, fresh vegetables, etc. (Rolain, 2013; Ruimy et al., 2010; Costa et al., 2008), while food waste (FW) in our daily life is composed of those things. Thus, FW should also be a reservoir of ARGs.

Ca. 6.25 million tons (dry solids) of sewage sludge (SS) were produced in 2013 at an average annual growth of 13% from 2007 to 2013 in China (Yang et al., 2015), and ca. 6.0×10^7 tons of FW

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were produced according to China Statistical Yearbook 2011 with the annual increasing rate higher than 10% every year due to huge population and rising living standards (Zhang et al., 2016b). Anaerobic digestion (AD) due to the production of renewable energy was widely adopted to treat SS and FW, while low AD efficiency for SS caused by the slow hydrolysis process and C/N ratio and the accumulation of volatile fatty acids (VFAs) for FW due to the labile organic fraction made the anaerobic co-digestion (AcoD) of SS and FW become increasingly popular, with the advantages of adjusting the C/N ratio, increasing the methane yield, diluting harmful substances, and mediating the hydrolysis of FW and SS (Lee et al., 2009). Microwave pre-treatment (MW) has been proved to further enhance the AcoD (Zhang et al., 2016b). A few studies have investigated the fate of ARGs during AD of SS and suggested that AD could be used to reduce ARGs quantities (Ma et al., 2011; Diehl and Lapara, 2010; Ghosh et al., 2009). Generally, previous studies indicated that different ARGs responded differently under mesophilic or thermophilic conditions, with thermophilic digestion generally outperforming mesophilic digestion (Zhang et al., 2015). However, the fate of ARGs during the AcoD based on MW pretreatment has never been investigated. It may also contribute to the enhancement of ARGs reduction, and this deserves to be elucidated.

The co-occurrence of antibiotic and metal resistance in bacteria has been widely observed (Pal et al., 2015), which is caused by the cross- or co-resistance phenomena. Cross-resistance occurs when the same mechanism reduces the susceptibility to metals and antibiotics simultaneously, and co-resistance occurs when separate resistance genes are situated on the same genetic element (Pal et al., 2015). This fact may be of great importance in the case of SS, because it both contains significant amounts of heavy metals and antibiotics (Bondarczuk et al., 2016; Le-minh et al., 2010). Therefore, the presence of heavy metals in SS and its further treatment like land application may select for antibiotic-resistant bacteria, and it is imperative to figure out the evolution and effects of co-selection from heavy metals to ARGs during AD.

Thus, AD of mono-SS, MW-SS:FW (3:2, total solids, TS) and SS:MW-FW (3:2, TS) was carried out as previously suggested (Zhang et al., 2016b) to investigate the fate of eleven frequently detected ARGs in this study, as well as the evolution of class 1 integron (*int1*), as the representative of mobile genetic elements (MGEs) and multiple resistance, and three plasmid-borne heavy metal resistance genes (MRGs, *pcoA*, *copA* and *czcA*) representing the co-selection of heavy metals was followed. The aims of this study were to (1) find out whether AcoD of SS and FW could contribute to reduction of ARGs and MRGs in SS and FW; (2) determine the main driver influencing the fate of ARGs regarding to bacterial community, MGEs and co-selection of heavy metals in order to provide some basic advice on ARGs control in SS and FW during AD.

2. Methods

2.1. Experimental set-up

Food waste (FW) was collected from the dining hall of Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, China. After the removal of the hard matters and grease, FW was homogenized and crushed to particle size of ca. 2 mm and stored at 4 °C before use (TS, 136.83 ± 11.65 g/L). The feed sludge (TS, 125.87 ± 7.56 g/L) was the dewatered sewage sludge collected from Beijing Qinghe WWTP. Seeding sludge was collected from a mesophilic AD reactor treating SS in Beijing Xiaohongmen WWTP. The optimized ratio (3:2, total solids) of FW and SS for AcoD based on MW pretreatment was adopted. Briefly, SS

and FW were mixed thoroughly at the optimized ratio (3:2) according to total solid (TS). 1.8 L of the mixture of substances and inoculums at a ratio of 5:1 (TS) were transferred to each bottle, and the final TS was adjusted to about 7%. Then the bottles were incubated in a water bath to control temperature at 37 ± 0.5 °C. Details of the optimization, biogas production, chemical parameters and the evolution of bacterial community were presented in a previous report (Zhang et al., 2016b).

2.2. DNA extraction

Samples on days 1, 5, 12, 19 and 33 at the optimized ratio of FW and SS based on MW were adopted for DNA extraction. 4 mL of each sludge sample was centrifuged at 10,000 rpm for 10 min, and the pellet was used for DNA extraction using FASTDNA Spin Kit for Soil (MP Biomedicals, USA) in triplicate according to manufacturer's instructions, and the resulting extracts were composited to average out bias in sampling and extraction. Quality and concentration of the extracted DNA were determined through 1% agarose gel electrophoresis and NanoDrop ND-1000 (NanoDrop, USA), respectively.

2.3. Quantitative PCR (qPCR)

Eleven frequently detected ARGs including two β -lactam resistance genes (*bla*_{OXA-1} and *bla*_{TEM}), four macrolide resistance genes (*mefA/E*, *ereA*, *ermB* and *ermF*), two sulfonamides resistance genes (*sulI* and *sulII*) and three tetracycline resistance genes (*tetG*, *tetM* and *tetX*) were quantified by qPCR. These ARGs were selected based on their frequent detection in previous studies (Ma et al., 2011; Diehl and Lapara, 2010; Zhang et al., 2016a) and their representative resistance mechanisms in the target antibiotics (Table 1). Meanwhile, evolution of three heavy metal resistance genes (MRGs, *pcoA*, *copA* and *czcA*), one representative mobile genetic element (class I integron, *int1*) and 16S rRNA representing the biomass were determined. The primers used here and their corresponding target antibiotics and mechanisms were summarized in Table 1. The plasmids containing these specific genes, used as standards in a 10-fold dilution for making qPCR standard curve, were manufactured by Zhejiang Tianke Biotechnology Company (Zhejiang, China). The 25 μ L PCR reaction mixtures contained 12.5 μ L of SYBR Green qPCR Super-Mix-UDG with Rox (Invitrogen, USA), 0.5 μ L each of 10 μ M forward and reverse primers (final concentration, 0.2 μ M), 10.5 μ L of DNA-free water, and 10 ng of standard plasmid or DNA extract. The thermo-cycling steps for qPCR amplification were as follows: (1) 50 °C, 2 min; (2) 95 °C, 5 min; (3) 95 °C, 20 s; (4) annealing temperature, 30 s; (5) 72 °C, 31 s; (6) plate read, repeat steps (3) through (5) 39 more times; (7) melt-curve analysis: 60–95 °C, 0.2 °C read. The reaction was conducted using an ABI Real-time PCR system 7500 (ABI, USA). Primer specificity was confirmed by melting curves and gel electrophoresis. Each gene was quantified in triplicate for each sample using a standard curve and a negative control. The amplification efficiencies were between 90.3% and 100.1% and summarized in Table S1.

2.4. Data analysis

The generation of plots for the target genes was performed with OriginPro 9.0 (OriginLab, USA), and Excel 2013 (Microsoft, USA) was used to determine the averages and fold change values of ARGs. The gene copies indicated the absolute copy numbers present per unit of dry weight (DW), while the normalized copy number by 16S rRNA was regarded as the abundance. The Spearman correlation was performed using SPSS 21.0 (IBM, USA), and a *p* value <0.05 was considered statistically significant. Principal component analysis (PCA), redundancy analysis (RDA), partial RDA and

Table 1
Primers used in this study and their corresponding target and mechanisms.

Target	Target genes	Mechanism	Primer Sequences	Amplicon size(bp)	Annealing temp(°C)	References
β-Lactam	<i>bla</i> _{OXA-1}	Class D β-lactamase	OXA-F: 5'-ATATCTCTACTGTTGCATCTCC-3' OXA-R: 5'-AAACCTTCAAACCATCC-3'	619	48	Colom et al. (2003)
	<i>bla</i> _{TEM}	Class A β-lactamase	TEM-C: 5'-ATCAGCAATAAACAGC-3' TEM-H: 5'-CCCCGAAGAAGCTTTTC-3'	516	54	
Macrolide	<i>mefA/E</i>	Efflux pump	F: 5'-AGTATC ATTAATCACTAGTGC-3' R: 5'-TTCTTCTGGTACTAAAAGTGG-3'	348	50	Sutcliffe et al. (1996)
	<i>ereA</i>	Inactivation enzyme	F: 5'-AACACCTGAACCCAAGGGACG-3' R: 5'-CTTCACATCCGGATTCTCGA-3'	420	60	
Macrolide-lincosamide-streptogramin B	<i>ermB</i>	23s rRNA methyltransferase	91f: 5'-GATACCGTTTACGAAATTGG-3' 454r: 5'-GAATCGAGACTTGAGTGTGC-3'	364	58	Chen et al. (2007)
	<i>ermF</i>	23s rRNA methyltransferase	189f: 5'-CGACACAGCTTTGGTTGAAC-3' 497r: 5'-GGACCTACCTCATAGACAAG-3'	309	56	
Sulfonamide	<i>sull</i>	dihydropteroate synthase	sull-FW: 5'-CGCACCAGAAACATCGCTGCAC-3' sull-RV: 5'-TGAAGTTCCGCCGCAAGGCTCG-3'	163	65	Pei et al. (2006)
	<i>sulll</i>	dihydropteroate synthase	sull-FW: 5'-TCCGGTGGAGGCCGGTATCTGG-3' sull-RV: 5'-CGGGAATGCCATCTGCCTTGAG-3'	191	57.5	
Tetracycline	<i>tetG</i>	Efflux pump	TetG-FW: 5'-GCAGAGCAGGTCTGG-3' TetG-RV: 5'-CCYGCAGAGAAGCCAGAAG-3'	134	64.2	Aminov et al. (2002) Aminov et al. (2001) Ng et al. (2001)
	<i>tetM</i>	Ribosomal protection protein	TetM-FW: 5'-ACAGAAAGCTTATTATATAAC-3' TetM-RV: 5'-TGGCGTGCTATGATGTTTAC-3'	171	55	
	<i>tetX</i>	Inactivation enzyme	F: 5'-CAATAATTGGTGGTGACCC-3' R: 5'-TTCTTACCTTGGACATCCCG-3'	468	60	
Mobile genetic element	<i>int1</i>	Class I integron	HS463a: 5'-CTGGATTTCGATCAGGCACG-3' HS464: 5'-ACATGCGTGTAATCATCTCG-3'	473	55	Stokes et al. (2006)
Copper	<i>pcoA</i>	Multi-copper oxidase	F: 5'-GCTGCAGATGGCCAGTATGTAAA-3' R: 5'-CCCTCGAGCGTAACCGTCC-3'	147	60	Xiong et al. (2015) Roosa et al. (2014)
	<i>copA</i>	Multi-copper oxidase	F: 5'-ATGTGGAACASARATGCGKATGA-3' R: 5'-AGYTTACAGCCSGGAATACG-3'	193	63	
Co/Zn/Cd	<i>czcA</i>	Inner part of a Co/Zn/Cd efflux protein	F: 5'-TCGACGGBGCCGTGGTSMTCGAGAA-3' R: 5'-GTVAVSGGCAKCGVBGGAACA-3'	232	63	Suzuki et al. (2000)
	16s rRNA	Biomass	1369F: 5'-CGGTGAATACGTTTCYCGG-3' 1492R: 5'-GGWTACCTTGTACGACTT-3'	124	55	

Procrustes analysis were conducted using Canoco 5.0 (Microcomputer Power, USA). Bacterial community has been analyzed previously using high-throughput sequencing method targeting the 16S rRNA V4 region (Zhang et al., 2016b). To clarify the correlation between the evolution of ARGs and the bacterial community, a Mantel test was conducted using PALEontological STatistics software (PAST 3.07). The heatmap illustrating the evolution of the quantified genes in each sample was built by Heml 1.0. Network analysis based on the Spearman analysis between ARGs and the bacterial community (based on OTU), MRGs, *int1* and chemical parameters was determined using the Gephi platform (Gephi 0.8.2 beta).

3. Results and discussion

3.1. Evolution of ARGs gene copies

As shown in Fig. 1A and S1A, the ARGs in AcoD were significantly higher than mono-SS, which reflected that there were much more ARGs in FW than SS. The biomass reflected by 16S rRNA increased significantly after AD, and they all showed the same pattern that biomass increased on Day 5, and decreased on Day 12, further increased on Day 19 and then decreased. The absolute gene copies of total ARGs showed the similar pattern (Fig. S1), and total ARGs have significantly positive correlation ($p < 10^{-4}$) with biomass. This may be caused by the soluble organics released from the dewatered sludge after the hydrolysis during AD, and the biomass increased significantly at the presence of soluble organics which were reflected by the biogas production. However, not all ARGs gene copies had significantly positive correlation with biomass, e.g., *bla*_{OXA-1}, *ereA*, *sull* and *tetG*. They showed different

patterns responding to different treatments (Table 2). The gene copies of *ereA* and *tetG* showed some removal at the final for MW-SS, while increased in mono-SS and MW-FW, and *sull* decreased in MW-SS and MW-FW, while increased in mono-SS. Although *bla*_{OXA-1} increased at the beginning, it decreased significantly at the final for all treatments, and even was not detected in the end of AD of the mono-SS and MW-SS.

As for the mono-SS in this study, the gene copies of all ARGs increased significantly except *bla*_{OXA-1}, although it also had the minimum biomass reflected by 16S rRNA in the feed (Fig. 1). In comparison with MW-FW, MW-SS showed some advantages in ARGs gene copies removal as reflected by Table 2. The *sull* dominated in the feed, but decreased in AcoD along with increasing in mono-SS. The gene copies of *tetM*, *mefA/E*, *ermB* and *ermF* increased significantly after AD, and became dominated in the final. Also, the gene copies of *bla*_{TEM} increased after AD. The significant increase of these genes after AD has been widely confirmed (Table 2). Interestingly, *sulll* and *tetX* increased significantly after AD in this study, while the controversial studies existed for the profiles of them, and not entire consistence of the ARG removal efficiency reported by these previous studies generally existed, e.g., Ma et al. (2011) indicated that mesophilic anaerobic digestion at both 10 and 20 day of solids retention times (SRTs) significantly reduced *sull*, *sulll*, *tetG*, and *tetX* with longer SRT exhibiting a greater extent of removal, and Zhang et al. (2015) pointed out that *sull* increased significantly while *tetG* decreased significantly at mesophilic AD through metagenomics approach. However, Yang et al. (2014) found that the abundance of *tetG* changed little after AD. The reason for the inconsistency could be complicated, which might include the difference of digester operational parameters and feed sludge. Different from the primary and/or secondary sludge were

Fig. 1. Changes in the abundance of the ARGs (A), *intI1* and heavy metals resistance genes (B) in different treatments during AD. D referred to day.

used previously in AD, dewatered sludge was used as the feed in this study, and thus the initial abundance of ARGs may be quite different. For instance, the gene copies of *sulI* could reach to ca. 10^{10} DW⁻¹ in previous study (Ma et al., 2011), while it was ca. 10^9 DW⁻¹ in this study. Although the biomass reflected by 16S rRNA was ca. 2.0×10^{11} g⁻¹ DW, it was ca. 1.31×10^{10} g⁻¹ DW in this study, and the final biomass was almost the same (ca. 1.0×10^{11} g⁻¹ DW). Thus it was not comparable if the removal efficiency of ARGs was just considered as the comparison between the feed and the effluent.

3.2. Fate of ARGs abundance

MW-SS not only showed better biogas production than that of mono-SS and MW-FW as previously reported (Zhang et al., 2016b), but also were higher in the final reduction of the abundance of total ARGs (Fig. 1). The evolution of the absolute gene copies of ARGs indicated the profiles of the host bacteria during AD, and reflected the vertical gene transfer – normal gene replication due to the proliferation of the host bacteria. In order to determine the distribution of ARGs in response to the significant changes of the composition of bacterial community, each ARG was normalized to 16S rRNA. As shown in Fig. 2, the fate of each ARG was quite different in the same treatment and the fate of the same ARG was distinctive in different treatments. AcoD based on MW could reduce some ARGs abundance in comparison with mono-SS in which the abundance of ARGs increased significantly, and MW-SS showed better ARGs reduction than that of MW-FW (Fig. 1). This indicated that AcoD based on MW pre-treatment

contributed to the ARGs reduction. The abundance of *tetG* decreased in all treatments, and this was comparable to previous studies (Table 2), as well as the abundance of *bla*_{OXA-1} (Fig. 2). Although the abundance of *sulI* in all treatments decreased in this study the same as that by Ma et al. (2011), its increase was observed by Zhang et al. (2015) through metagenomics approach. The abundance of the three macrolide resistance genes (*mefA/E*, *ermB* and *ermF*) and *tetM* all increased (Fig. 2), and this was also comparable to previous studies (Table 2). The abundance of *tetX* increased in all treatments while decreased in previous studies. However, this may be not a bad thing, because the *tetX* gene encodes for an NADPH-requiring oxidoreductase, which inactivates tetracycline in the presence of oxygen and NADPH, that is, it cannot function during AD, but has only been found in a strict anaerobe, *Bacteroides*, where oxygen is excluded (Liu and Pop, 2009). The increase of the abundance of *tetX* after AD in this study could avail the degradation of tetracyclines for the further treatment at the presence of oxygen, and thus to reduce the selective pressure of tetracyclines. The abundance of *sulII* only decreased in AcoD but increased in mono-SS, while *ereA* and *bla*_{TEM} only decreased in MW-SS. AcoD, especially for MW-SS, could reduce total ARGs abundance in comparison with mono-SS. The difference could be due to the different evolution of bacterial community.

3.3. The role of *intI1* and potential mechanisms of resistance

An increase in gene abundance would suggest either that the organisms harboring genes encoding for resistance are multiplying or that the quantities of genes are increasing via horizontal gene

Table 2
The profiles of the target ARGs in previous and the present studies^a.

Target ARGs	Gene copies		Abundance					
	Munir et al. (2011)	Ma et al. (2011)	Ghosh et al. (2009)	Diehl and Lapara (2010)	mono-SS	MW-SS	MW-FW	MW-FW
<i>bla_{OXA-1}</i>					+	+	+	+
<i>bla_{TEM}</i>					+	+	+	+
<i>me[A/E]</i>					+	+	+	+
<i>ereA</i>					+	+	+	+
<i>ermB</i>		+			+	+	+	+
<i>ermF</i>		+			+	+	+	+
<i>sulI</i>	±				+	+	+	+
<i>sulII</i>					+	+	+	+
<i>tetG</i>					+	+	+	+
<i>tetM</i>					+	+	+	+
<i>terX</i>					+	+	+	+
<i>intI1</i>					+	+	+	+
<i>pcoA</i>					+	+	+	+
<i>copA</i>					+	+	+	+
<i>czcA</i>					+	+	+	+
Feed sludge	N.D.	Mixture	N.D.	Mixture	Dewatered	Dewatered	Dewatered	Dewatered
Operation condition	Mesophilic	Mesophilic thermophilic	Mesophilic thermophilic	Mesophilic thermophilic	Mesophilic	Mesophilic	Mesophilic	Mesophilic

^a +: the ARG increased; -: the ARG decreased; ±: the ARG changed little; the simultaneous emergence of these marks means controversial results appeared in the study. N.D.: not described; mixture: the mixture of primary and secondary solids.

transfer (HGT). Mobile integrons like Class I integrons (*intI1*), were often used to represent the HGT. Although they cannot mobilize and transfer themselves between microbes, they are often associated with genetic elements which can, such as conjugative plasmids, transposons and insertion sequences (Berglund, 2015). Thus, *intI1* was suggested as the proxy for anthropogenic pollution of ARGs (Gillings et al., 2014). Previous studies suggested that the abundance of *intI1* could be reduced through AD with some exceptions (Table 2). In this study, *intI1* could be reduced in AcoD, while increased in mono-SS, and *intI1* had significantly positive correlation ($p < 0.01$) with *ereA*, *sulI*, *sulII* and *tetG*. This association was often observed previously, and might indicate that *intI1* may be associated with their evolution.

The mechanisms of resistance to different antibiotics in AD were quite distinctive. The mechanism of tetracycline resistance during AD in this study may be the ribosomal protection protein reflected by the evolution of *tetM*, considering that *tetX* cannot function at the absence of oxygen and the decreasing abundance of *tetG*. As for macrolide resistance, it may be the efflux pump (*mefA/E*) and 23s rRNA methyltransferase (*ermB* and *ermF*) that dominated the resistance. Class A beta-lactamase, encoded by *bla_{TEM}*, exerted the resistance to beta-lactam. The TEM β-lactamase specified by plasmids in gram-negative bacteria are widely distributed in nature in terms of geographical distribution, association with plasmids of different incompatibility groups, and occurrence in different bacterial species, while the OXA-1 are specified by plasmids of three, four, and two different incompatibility groups, respectively (Foster, 1983). Undoubtedly, the widespread potential host availed its dominance in AD. Furthermore, it is demonstrated that factors that can indirectly affect the level of β-lactamase expression and hence the resistance level of the cell are plasmid copy number, e.g., the TEM β-lactamase, which may be higher at anaerobic growth (Foster, 1983). While the pressure from sulfonamides may be reduced due to the effective degradation of sulfonamides during AD demonstrated by Mohring et al. (2009).

3.4. Reduction of MRGs

It has been concluded that copper, silver, arsenic, antimony, cobalt, nickel, cadmium, iron, zinc and mercury are all potential co-selectors for strains resistant to, e.g. sulfonamides, beta-lactams, amphenicols, tetracyclines and aminoglycosides, and certain bacterial taxa comprising many pathogens were particularly prone to carrying both MRGs and ARGs, highlighting the potential clinical consequences of co-selection (Pal et al., 2015). However, the bio-availability should be taken into account concerning the co-selection of heavy metals, because it is the bioavailable fractions of heavy metals that impose selective pressure on microbes. Moreover, MRGs could show better illustration of the real response of bacteria to the selective pressures caused by heavy metals (Roosa et al., 2014).

Unlike ARGs, all the MRGs quantified in this study decreased along with AD, no matter considering the absolute gene copies (except *czcA* in mono-SS) or the abundance (Fig. 1B, 2 and S1B). The *copA* is encoding for a periplasmic multi-copper oxidase which specified in plasmids, and this system needs oxygen contrary to the other systems such P-type ATPases and cation diffusion facilitators (Roosa et al., 2014). This could explain the reduction of *copA* abundance and gene copies in AD of this study. The *pcoA* (also a plasmid-borne Cu resistance) encodes a periplasmic multi-copper oxidase, and the *pco* gene cluster comprises seven genes *pcoABCDRESE*. Besides, the *pco* system requires CopA activity to confer resistance. Presumably, proteins encoded in the *pco* gene cluster are responsible for the handling of periplasmic copper delivered by CopA from the cytoplasm, and also there is significant

Fig. 2. Heatmap of changes in the target genes during AD in different treatments. Values plotted are the natural log₂ transformed of the abundance of each ARG.

correlation between *pcoA* and *copA* in this study. The *czc* operon is driven by a H^+ ion gradient that allows *czcA* (plasmid-borne Cd/Zn/Co resistance) to pump heavy metals out of the cytoplasm, and it has been demonstrated that the measurement of *czcA* gene levels could be used to estimate Cd/Zn/Co bioavailability in sediment compartments (Roosa et al., 2014). This may indicate that the changes of the speciation of heavy metals happened, and bio-availability of heavy metals may be reduced during AD. Besides, there is no positively significant correlation ($p > 0.05$) between each MRG and 16s rRNA, and MRGs did not increased along with the increase of biomass. According to Spearman analysis, MRGs have some co-occurrence with specific ARGs. The ARGs decreased including *bla*_{OXA-1}, *ereA*, *sulI* and *tetG* all have positively significant correlation ($p < 0.05$) with each MRG including *pcoA*, *copA* and *czcA*. Besides, the evolution of *czcA* was significantly correlated with *sulII* and *intI1* which only decreased in AcoD. This indicated that the reduction of the selective pressure of heavy metals might favor the reduction of these ARGs.

3.5. ARGs profile responding to the changes of bacterial community

There was much difference of the evolution of bacterial community between mono-SS and MW-SS and MW-FW, and the details have been described as previously (Zhang et al., 2016b). The proliferation of ARGs still count on the activity of microbes, and the changes of bacterial community may have significant influence on the profiles of ARGs. In order to elucidate this, Procrustes analysis was conducted by rotating the ordination of changes in the bacterial community to match the profiles of ARGs based on PCA analysis, and the results indicated that there was positively significant correlation between the evolution of ARGs and bacterial community (Fig. 3). 77.2% of the variables of the evolution of ARGs could be explained by the changes of bacterial community through Procrustes analysis, and the correlation between the first two principal axes was significantly positive ($R = 0.9734$ and 0.7787 , respectively). A further Mantel test based on Bray–Curtis distance confirmed this ($R = 0.7833$, $p = 0.0003$, permutation $N = 9999$). The evolution of bacterial community had significant correlation with the fate of ARGs.

A previous study confirmed that network analysis could be used to provide new insights into ARGs and their possible hosts in complex environmental scenarios (Zhang et al., 2016a). As shown in Fig. 4, various ARGs quantified in this study were significantly ($p < 0.05$) correlated with various species. The significantly positive correlation between different ARGs and the same host bacteria indicated by network analysis demonstrated the co-occurrence of different ARGs. The network was clearly divided into two groups: the ARGs that increased, and the ARGs that decreased (Fig. 4).

Fig. 3. Procrustes analysis of the significant correlation between ARG profiles and the bacterial community (A) and Principal component analysis (PCA) of the changes of the bacterial community for AD in different treatments (B). Circles, stars and triangles represent samples from mono-SS, MW-SS and MW-FW, respectively.

According to the network analysis, the genus *Bacteroides* may be the host bacteria of *ermB*, *ermF*, *bla*_{TEM}, *tetM* and *tetX*, while it has been widely demonstrated that *ermB*, *ermF* and *tetX* often existed in *Bacteroides* (Liu and Pop, 2009). The significant correlation between *bla*_{TEM}, *tetM* and *Bacteroides* may be due to the functional correlation between their host bacteria and *Bacteroides*, or *Bacteroides* was their host bacteria through HGT, because *tetM* and *bla*_{TEM} are often associated with conjugative transposons which also have a very wide host range (Liu and Pop, 2009). Their wide host range can also be reflected by the significant correlation with bacterial diversity index (Simpson) in Fig. 4. As for the ARGs decreased, the genus *Aeromonas* was the potential host bacteria of *tetG*, *bla*_{OXA-1}, *ereA*, *sulI* and *sulII*, and it has been demonstrated that *Aeromonas* was the host bacteria of *ereA*, *sulI* and *sulII* (Liu and Pop, 2009). Network analysis further elucidated the primary effects of bacterial community on the evolution of ARGs by

Fig. 4. Network analysis showing the co-occurrence of ARGs, MRGs and environmental parameters together with their potential host bacteria. A connection represents a significant positive correlation ($p < 0.05$) according to spearman analysis.

determining their potential host bacteria. It has been demonstrated that environmental bacteria and pathogens harbor diverse and abundant ARGs (Forsberg et al., 2014). The potential host bacteria reflected by network analysis may be not antibiotic resistance bacteria (ARB) yet, and the significantly positive correlation with ARGs may be due to its functional connection with real ARBs. However, these potential host bacteria may have the biggest chance of becoming the ARBs through HGT due to their functional connection. This would make sense in case that the function connection was found between pathogens and non-pathogens.

3.6. The relationship among environmental factors, bacterial communities, MRGs and ARGs

RDA analysis was conducted to investigate the relationships between environmental factors, bacterial community, *intI1* and MRGs and ARGs (Fig. 5), and the results showed that the selected variables accounted for 98.2% of the total variation of the evolution of ARGs quantified in this study ($p = 0.002$). The contributors for the ARGs profiles in different stages are quite different. *Proteobacteria*, *Actinobacteria* and *Planctomycetes* accounted for the patterns at the beginning, and could explain the evolution of the ARGs decreased including *bla*_{OXA-1}, *sulI*, *sulII*, *tetG* and *ereA*. *Firmicutes* mainly accounted for the evolution of the ARGs increased including *tetX*, *bla*_{TEM}, *ermB*, *ermF* and *tetM*, while *Bacteroides* may account for the pattern of ARGs at D5 in MW-SS and MW-FW, and *Firmicutes* was also previously demonstrated to contribute to the elevated abundance and enrichment of ARGs (Zhang et al., 2016a). Thus, ARGs reduction by reducing the abundance of *Firmicutes* in AD may be feasible, but the dominance of *Firmicutes* often occurred in various AD (Zhang et al., 2016b).

Fig. 5. Redundancy analysis (RDA) results of the relationship between environmental factors, bacterial community, MRGs and *intI1* and ARGs profiles in AD of mono-SS, MW-SS and MW-FW, respectively. The blue lines and red lines represent the species variables and environmental variables, respectively, during the RDA analysis through Canoco 5.0 in this study. Black circle, star and triangle represented samples from mono-SS, MW-SS and MW-FW, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

As far as the environmental factors were concerned in this study, there was significant correlation between pH and *mefA/E* (Fig. 4), and proteins correlated significantly to the patterns of

ARGs at D2 for mono-SS, while polysaccharide was significantly correlated with the ARGs decreased. This indicated that changes of the environmental factor influenced the evolution of ARGs. For instance, the easier degradation of polysaccharide than proteins may lead to the dominance of fast growing host bacteria, and higher pH may resulted in the SOS response for some microbes, while the SOS could avail the increase of specific ARGs (Miller et al., 2014). To determine the key contributor to the explanatory variation as a whole and separate the influences of bacterial community, environmental factors concerned, *intI1* and MRGs, partial RDA was conducted by designating the explanatory variables and covariates (Table S2). It was concluded that the bacterial community contributed the most to the ARG changes, followed by environmental factors, *intI1* and MRGs. Indeed, the evolution of the bacterial community was the main driver for the changes in ARGs rather than HGT induced by MGEs or co-selection/co-occurrence determined by MRGs. The dominant contribution of the bacterial community to ARG profiles has been elucidated in various environments (Zhang et al., 2016a), including soils, rivers, WWTPs, sludge composting etc., and this was the first report that elucidated the dominant contribution of bacterial community in AD. Nevertheless, the less frequent HGT incidents should not be overlooked, as even one HGT event into a human pathogen has the potential for great harm (Zhang et al., 2016a).

Besides, the sludge retention time (SRT) was considered as one of the most important operation parameters for AD. It seemed the same importance for the ARGs reduction. The extension of SRT could favor the reduction of ARGs during AD, and the detail evolution of the abundance of each ARG was shown in Fig. 2 and Table S3. The abundance of ARGs increased to the maximum on D12 for both mono-SS and MW-FW, while it increased to the maximum on D19. But the best ARGs reduction occurred on D33. This may indicate that longer time was needed to exhibit a great extent of reduction ARGs during AD, which was also demonstrated previously (Ma et al., 2011). From the point view of the abundance reduction of ARGs in this study, it seemed that the mono-SS showed the highest risks on Day12, while it was on Day19 for AcoD. The evolution of absolute gene copies of ARGs showed the same pattern (Fig. S1).

4. Conclusions

Anaerobic co-digestion (AcoD) showed some advantages over mono-SS digestion, and MW-SS was better than that of MW-FW considering both the methane production and the reduction of ARGs abundance. AcoD could reduce total ARGs abundance and should be adopted more widely concerning the ARGs control in SS and FW. The decrease of selective pressure from heavy metals due to AD may partially contribute to the reduction of ARGs. Evolution of bacterial community composition is the main driver for the fate of ARGs, not HGT.

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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.2016.02.140>.

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