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# 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.



## 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 anthropogenic 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. $6.0 \times 10^7$ 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 (*intI1*), 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, *intI1*) 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 μL PCR reaction mixtures contained 12.5 μL of SYBR Green qPCR Super-Mix-UDG with Rox (Invitrogen, USA), 0.5 μL each of 10 μM forward and reverse primers (final concentration, 0.2 μM), 10.5 μL 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