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Graphical abstract

**Factors influencing the fate of Antibiotic Resistance Genes
during Thermochemical Pretreatment and Anaerobic Digestion
of Pharmaceutical Waste Sludge**

Juan Tong^{1,2} · Xueting Lu² · Junya Zhang^{1,2} · Irini Angelidaki³ · Yuansong Wei^{1,2*}

1. State Key Joint Laboratory of Environmental Simulation and Pollution Control,
Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences,
Beijing 100085, China

2. Department of Water Pollution Control, Research Center for Eco-Environmental
Sciences, Chinese Academy of Sciences, Beijing 100085, China

3. Department of Environmental Engineering, Technical University of Denmark,
Copenhagen Lyngby 2800, Denmark

Corresponding Author

Yuansong Wei*, Email: yswei@rcees.ac.cn

Juan Tong, Email: hittj@163.com

Abstract

The prevalence of antibiotic resistance genes (ARGs) in waste sludge, especially for the pharmaceutical waste sludge, presents great potential risks to human health. Although ARGs and factors affecting their spreading are of major importance for human health, the factors influencing the fate of ARGs during sludge treatment, especially for pharmaceutical sludge treatment are not yet well understood. In order to be able to minimize ARGs spreading, it is important to find what is influencing their spreading. Therefore, certain factors, such as the sludge characteristics, bacterial diversity and community composition, and mobile genetic elements (MGEs) during the advanced AD of pharmaceutical sludge with different pretreatments were studied, and their affinity with ARGs was elucidated by Spearman correlation analysis. Furthermore, multiple linear regression was introduced to evaluate the importance of the various factors. Results showed that 59.7% - 88.3% of the variations in individual ARGs and total ARGs can be explained by the corresponding factors. Bacterial diversity rather than specific bacterial community composition affected the fate of ARGs, whereas alkalinity was the most important factor on ARGs among all sludge characteristics investigated in this study. Besides, 66.4% of variation of total ARGs was driven by the changes of MGEs. Multiple linear regression models also reveal the collective effect of these factors on ARGs, and the contributions of each factor impact on ARGs. This study provides more comprehension about the factors impact on the fate of ARGs during pharmaceutical sludge treatment, and offers an approach to evaluate the importance of each factor, which method could be introduced for

evaluation of factors influencing ARGs during other types of sludge or wastewater treatment.

Keywords: antibiotic resistance genes (ARGs); mobile genetic elements (MGEs); bacterial community; sludge characteristics; multiple linear regression

1. Introduction

Wastewater treatment plants are important for controlling antibiotic resistance emergence and spreading, as these facilities are central collection points for antibiotic resistant bacteria (ARB), antibiotics, biocides, and heavy metals (Michael et al., 2013; Calero-Caceres and Muniesa, 2016; Manaia et al., 2018). Pharmaceutical wastewaters harbor more ARB and antibiotic resistance genes (ARGs) than municipal wastewater (Liu et al., 2012; Liu et al., 2014), and therefore require more attention. However, most countries are lack of specific legislation for antibiotic content and regulation disposal of these wastewaters. Therefore, attention is required due to the huge negative health impact could be caused by spreading of antibiotic resistance. Inadequate treatment of waste sludge from pharmaceutical wastewater treatment plants could impose a potential risk for resistance spreading. It is therefore imperative to elucidate the factors influencing the fate of ARGs during sludge treatment for developing effective technologies to control antibiotic resistance spreading. However, the factors that impact ARGs and influence their development are rather complex and remain a black box (Manaia et al., 2018).

Wastewater or sludge characteristics and environmental conditions are reported as important factors affecting the spreading of antibiotic resistance. For example, COD

could influence how antibiotics affect ARGs (Guo et al., 2017), and a higher COD loading rate could amplify the growth of all six types of ARB during wastewater treatment (Yuan et al., 2015). Temperature is another important factor influencing ARGs. However, conflicting results with respect to temperature for reducing ARGs are reported. For example, thermophilic AD of waste sludge was found to offer greater and more rapid reduction of ARGs than conventional mesophilic AD (Ghosh et al., 2009; Diehl and LaPara 2010; Burch et al., 2016), while others have found no difference on ARG reduction based on digestion temperature (Ma et al., 2011; Zhang et al., 2015). In addition, pH can influence ARGs. For example, the potential of tetracycline resistance genes transferred via genetic vectors was greatly promoted in acidic pH conditions (Huang et al., 2016). Alkaline fermentation (pH 10) of sludge led to a greater reduction in *sul* genes and *tet* genes than fermentation at neutral pH. The shift of ARG hosts range and restricted gene transfer potential at pH 10 might result in a critical effect on the attenuation of ARGs (Huang et al., 2017). It was found that the major reduction of the ARGs occurred at the acidogenic phase (with pH around 6) of a two-stage thermophilic digestion process, whereas an increase of the ARGs was observed at the subsequent methanogenic phase (Wu et al., 2016). These findings suggest that the influence of sludge characteristics and environmental conditions is complex and often lead to conflicting results.

Microbial community composition is important for the fate of ARGs (Miller et al., 2016; Zhang et al., 2016), which could affect the distribution of ARGs in the biosolids after AD of waste sludge (Ma et al., 2011). It was proposed that the variations

82 observed in ARGs during the AD of sewage sludge mixed with food waste were
83 mainly linked to bacterial community composition, rather than to the content of
84 mobile genetic elements (MGEs), and heavy metals (Zhang et al., 2016). Changes in
85 the structure of a microbial community may lead to changes in the biodegradation
86 capacity of antibiotics, which likely represents one of the factors underlying the
87 impact of the microbial community on ARGs (Akyol et al., 2016).

88 Wastewater and sludge contain various MGEs, including plasmids, transposons,
89 integrons and insertion sequences (Guo et al., 2017; An et al., 2018), which are
90 responsible for the horizontal gene transfer (HGT) of resistance genes (Hegstad et al.,
91 2010; Ma et al., 2014). Antibiotic resistance may be amplified from the progressive
92 enrichment of resistant bacteria, and/or transfer of resistance genes between microbial
93 members of a given community (Rizzo et al., 2013). In addition to the above, biocides,
94 heavy metals (Michael et al., 2013), and certain additives, such as zero valent iron
95 (Gao et al., 2016), may also affect the fate of ARGs. All the above suggest that the
96 interactions deciding the fate of ARGs during wastewater and sludge treatment is
97 volatile, complex and remain poorly understood.

98 The mechanisms determining the fate of ARGs are rather complex, and therefore it
99 still remains unclear which factors are the main drivers on the fate of ARGs.

100 Therefore, this study aims to provide a further understanding of the factors affecting
101 the fate of ARGs during pharmaceutical sludge treatment. In our previous study, the
102 occurrence of ARGs and MGEs in enterococci isolated from sludge, and in genomic
103 DNA during the pharmaceutical waste sludge treated by AD with different

pretreatments were investigated (Tong et al., 2017). Based on this research, sludge characteristics, bacterial community composition, bacterial diversity, MGEs, and their associations with the fate of ARGs were investigated. Additionally, stepwise multiple linear regression analyses were applied to evaluate the importance of sludge characteristics, bacterial community composition, bacterial diversity, and MGEs on the fate of individual ARGs and total ARGs during the pharmaceutical sludge treatment.

2. Materials and methods

2.1 Sludge resources and treatment processes

The sludge used in this study was collected from the sedimentation tank of a wastewater treatment system of a spiramycin production plant in Wuxi, China. The sludge was subjected in a number of treatments (microwave (MW), thermal hydrolysis (TH), and ozone oxidation) and subsequently was treated by anaerobic digestion as described in a previous study (Tong et al., 2017). Figure 1 shows the operation procedure of the sludge treatment. The sludge pretreatment and subsequent AD were repeated three times in independent experiments.

2.2 Analytical methods for sludge characteristics

The sludge samples were centrifuged at 4000 rpm for 10 min and filtered through a 0.45 μm cellulose membrane. The pH, alkalinity (ALK), soluble COD (sCOD) content, protein content, and polysaccharide content of the filtrate was analyzed. The total solid (TS), volatile solid (VS), ALK, and sCOD contents were measured according to the standard methods procedure (APHA, 2005). The pH was determined

using a pH meter (pHs-3C, Leici, China). The modified Lowry method was adopted to determine the protein content using bovine serum albumin as the protein standard (Frolund et al., 1995). Polysaccharides were measured by the Dubois method with D-glucose as the standard (Dubois et al., 1956).

2.3 DNA extraction and quantification

One milliliter of the pretreated sludge and 0.5 mL of raw sludge and digested sludge samples, which volume was optimized for DNA extraction, were centrifuged at 8000 rpm for 15 min, and the pellet from each centrifugation was subsampled for DNA extraction. DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, US) according to the manufacturer's instructions. Triplicate-extracted DNA of each sample was mixed to minimize the potential variations during the DNA extraction process. A NanoDrop 2000 (Thermo Scientific, USA) was used to determine the DNA concentration and purity.

The presence of different mechanisms of macrolide-lincosamide-streptogramin (MLS) resistance, i.e. the following genes were investigated: efflux pump genes *mefA/E*; esterase genes *ereA*; ribosomal methylase genes *ermB*, F and X; macrolide phosphotransferase genes *mphB*; and the ribosomal protection genes of tetracycline resistance, *tetM* and *tetO*, which were intimately connected with *ermB* (Roberts et al., 1999; Tremblay et al., 2011), MGEs (*Tn916/1545*, *ISCR1*, and *intI1*). The 16S rRNA genes were quantified using a Step One Plus real-time PCR system (ABI, USA). The primers and detail quantitative PCR procedure were described in our previous study (Tong et al., 2017).

2.4 High-throughput sequencing and bacterial community analysis

High-throughput sequencing was performed as previously described (Zhang et al., 2016). In brief, PCR primers 515F and 806R targeting the V4 region of the 16S rRNA gene for both bacteria and archaea. Sequencing was performed with Illumina high-throughput sequencing method (Caporaso et al., 2011). Barcodes unique to each sample were incorporated before the forward primers, which allowed for the identification of each sample in a mixture for an Illumina sequencing run. DNA was amplified in triplicate for each sample. The PCR amplicons were further purified with a DNA purification kit (Gel Purification kits, Sangon, China). The DNA concentrations were determined using a spectrometer (Qubit 2.0, Invitrogen, USA). Amplicons from different samples were then mixed to achieve equal mass concentrations in the final mixture, which were sent to Sangon Co., Ltd. in Shanghai for small fragment library construction and pair-end sequencing using the Illumina MiSeq sequencing system (Illumina, USA).

Sequencing reads were assigned to each sample according to the unique barcode of each sample. Pairs of reads from the original DNA fragments were first merged using FLASH, and then PRINSEQ was used for the quality control of these merged reads. The barcode and primers were then removed. All the reads were uploaded to MG-RAST (<http://metagenomics.anl.gov/linkin.cgi?project=mgp80384>). PCR chimeras were filtered out using UCHIME. After the above filtration, the average length of all clean reads was 250 bp and the average sequencing depth was 44,523 clean reads for the bacterial community analysis. The taxonomic classification of the

sequences was performed using the Ribosomal Database Project (RDP) Classifier at the bootstrap cutoff of 80% suggested by the RDP.

2.5 Data analysis

The Spearman correlation was performed using SPSS 20.0 (IBM, USA), and a p value < 0.05 was considered statistically significant. Multiple linear regression analyses (Neter et al., 1996) were performed by SPSS 20.0. Based on F-tests, the least significant variables were removed from the model via a stepwise variable reduction. To simplify the bacterial community composition in the multiple linear regression model, a dimension reduction for the dominant genera was first conducted based on a PCA using SPSS 20.0. Then, the top 5 master coordinates (Coord1, Coord2, Coord3, Coord4, and Coord5), with a cumulative variance proportion over 80% were adopted to express the bacterial community (Table S1, S2). A p value < 0.05 of each model was considered as statistically significant. The adjusted R^2 values of the linear regression models are indicators of how well the regression model explained the new observations. A principal component analysis (PCA) and Procrustes analysis were conducted using Canoco 5.0 (Microcomputer Power, USA). The Procrustes analysis was done by rotating the coordinate of changes of the bacterial community to match the profiles of ARGs based on a PCoA analysis (Lou et al., 2017). The values of explanatory variables accounted for the sludge treatment process showed how much evolution of ARGs could be explained the bacterial community succession during the sludge treatment. The heatmap illustrating the evolution of the quantified genes in each sample was built by Heml 1.0.

3 Results and discussion

3.1 Variations in sludge characteristics

The variations in sludge characteristics during sludge treatment are shown in Figure 2. The TS increased by 4.9% and 5.1% for the MW and ozone pretreatments because of evaporation, whereas the TS was reduced by 29.2% after the TH pretreatment because of dilution by the condensed process steam. The same trend was observed for the VS during the pretreatments. The TH pretreatment showed the highest release of soluble organics, with concentrations which were 15.0, 29.3, and 39.8 times higher compared to raw sludge for the sCOD, protein, and polysaccharide, respectively. The second best pretreatment for sludge was the MW (15.0, 29.8, and 32.3 times higher, respectively). The ozone pretreatment resulted in the lowest soluble organics release (6.5 times of sCOD). The AD with TH pretreatment presented the best VS reduction (39.3% reduction), which was followed by AD with the MW (26.4% VS reduction) and ozone pretreatments (25.0% VS reduction).

The initial pH of pharmaceutical sludge was 7.65, with 0.08~0.48 variations of pH after pretreatments. The pH of digested sludge ranged from 7.47~7.62, which was typical for normal AD. The ALK increased from 78.9% to 230.1% during AD, which variation trend was consistent with the results of the previous study (Zou et al., 2013). The increasing ALK after AD was mainly caused by the increasing ammonia produced by the degradation of protein and nitrogenous compounds during AD (Song et al., 2004).

3.2 Variations in bacterial diversity

Microbial biomass on basis of 16S rRNA, was reduced after the sludge pretreatment but increased during the subsequent AD (Figure 3). However, the bacterial diversity did not always show the same trends. The Chao 1 index, which represents the richness of microbial diversity; Pielou index, which expresses the evenness of microbial diversity; and Shannon index, which is considering both richness and evenness of microbial diversity were investigated in the present study. During the sludge pretreatment, the Shannon index was reduced from 5.00 for the raw sludge to 4.59, 2.63, and 4.69 for the MW-, TH-, and ozone-pretreated sludge, respectively; and 31.1%, 66.6% and 21.9% reduction for Chao 1 index. In addition, Pielou index decreased from 0.64 to 0.60, 0.39, and 0.61. The 16S rRNA decreased from 12.7 to 12.7, 11.7, and 12.5 log copies·g⁻¹, respectively. The radiation by MW treatment, heating and increased pressure of the TH treatment, and oxidation of the ozone treatment did not only reduce the microbial biomass, but also the microbial diversity (both in richness and evenness), indicating that the pretreatments were affecting the various taxa differently. The TH pretreatment showed the best reduction in microbial diversity, with 66.6%, 47.5%, and 39.4% reduction for Chao1, Shannon, and Pielou index, respectively. This result is in agreement with a previous study showing that TH pretreatment for waste sludge could lead to a reduction of microbial diversity (Ma et al., 2011).

The Chao1, Pielou, and Shannon index increased simultaneously both in the AD without pretreatment and AD with MW pretreatment, indicating the microbial diversity both in richness and evenness increased along with the biomass. However,

all these three diversity indexes decreased by 4.7%~10.0% after AD with TH pretreatment, showing that the microbial diversity reduced even when the biomass increased (with 2.3% increase) after AD, and that the TH was the harshest treatment permitting recovery of only few microorganisms during the AD process. Therefore, it can be concluded that sludge treated by TH has a lower microbial diversity which might affect the subsequent AD process.

3.3 Bacterial community succession

The bacterial community succession during the sludge treatments is shown in Figure 4. To simplify the results, the top 10 abundant phyla and top 10 abundant genera in each sample (23 abundant phyla and 61 abundant genera (the genera names shown in Table S3) were obtained from 36 samples) were selected for comparison. The top three dominant phyla were Proteobacteria (57.2%), Bacteroidetes (18.0%), and Planctomycetes (7.2%) for the spiramycin waste sludge, followed by Verrucomicrobia (3.7%) and Acidobacteria (3.3%) (Figure 4a). Likewise, Proteobacteria and Bacteroidetes were found dominant phyla in sewage sludge (Zhang et al., 2012; Zhang et al., 2016). However, Firmicutes and Actinobacteria were also found to be dominant phyla in sewage sludge which was not the case for the spiramycin waste sludge. These differences were also observed in the sludge after AD, with Proteobacteria, Bacteroidetes, Firmicutes, and Cloacimonetes the dominant phyla in this study, whereas Proteobacteria, Firmicutes, Bacteroidetes, and Chloroflexi were the dominant phyla in sewage sludge after mesophilic digestion (Bibby et al., 2010). It seems that the different sludge sources (sewage sludge vs.

pharmaceutical sludge) supported the development of different microbial communities.

For the TH-pretreated sludge, the abundance of Proteobacteria increased from 57.2% to 82.3%, indicating their robustness to TH conditions. On the contrary, the abundance of Bacteroidetes decreased from 18.0% to 7.4%, and Proteobacteria, Bacteroidetes, and Firmicutes became the dominant phyla. The abundance of Firmicutes increased after the pretreatments (from 2.1% to 6.2%, 6.9%, and 5.0%, respectively, for the control to MW, TH, and ozone pretreatments). Many Firmicutes produce endospores and therefore can survive extreme conditions, such as heat, desiccation, UV irradiation, and the presence of organic solvents and oxidizing agents (Galperin, 2013). Firmicutes are well-known to be acetogenic and syntrophic bacteria that can degrade VFA (Yi, et al, 2014). The increase of Firmicutes in the sludge could generate a favorable environment for fermentation (Ma et al., 2011), provides precursors for methanogenesis, and therefore plays a critical role in anaerobic digestion (Yi, et al, 2014). The augmentation of Firmicutes after pretreatments in this study might be an important reason for the higher methane production in the AD sludge with pretreatments.

At the genus level (Figure 4b), the abundance of genera belonging to Proteobacteria (i.e. *Pseudomonas*, *Acinetobacter*, and *Alcaligenes*, etc.) Firmicutes (such as *Clostridium*) increased or remained the same after the sludge pretreatments. *Acinetobacter* and *Pseudomonas* are opportunistic pathogenic genera in the Proteobacteria group. The abundance of *Pseudomonas* increased after all

pretreatments, especially the TH pretreatment (from 0.1% to 38.7%), whereas the abundance of *Acinetobacter* increased from 0% to 5.0% in TH-pretreated sludge but was 0-0.1% in other pretreated sludge. *Pseudomonas*, *Alcaligenes*, and *Clostridium* are acidogenic fermentation genera (Li et al., 2011). *Alcaligenes* might greatly contribute to the degradation of VFAs and stabilization of ammonium level in the anaerobic digester (Shoda and Ishikawa, 2014; Wu et al., 2016). *Clostridium* can produce cellulases that hydrolyze cellulosic biomass, and also contribute to acetate, propionate and butyrate fermentation (Li et al., 2011). Cooperation among these microorganisms playing their respective roles in one or more trophic levels including hydrolysis, fermentation, and acetogenesis is needed for mineralization of organic matter to biogas (Yi, et al, 2014). Therefore, the prevalence of the above fermentation genera could benefit for methane production. However, the abundance of *Zoogloea*, *Thiobacillus*, and *Lewinella* reduced after the sludge pretreatments. *Zoogloea* is an aerobic chemoorganotrophic bacterium responsible for forming zoogloes and is common in activated sludge (Shin et al., 1993). *Thiobacillus* includes obligate aerobes and facultative denitrifying types (Kelly and Wood 2000). *Lewinella* is also aerobic, and it cannot grow at temperatures above 35°C (Khan et al., 2007). The bacterial diversity in Chao 1, Shannon, and Pielou index of the digested sludge was 4.3%~181.3% higher than that of the pretreated sludge (Figure 3), resulted in complex variations in the bacterial community in the genus level during the AD stage.

Principal component analysis (PCA) showed that sludge pretreatments significantly changed the bacterial community composition of sludge (Figure 5). The

greatest dissimilarity in bacterial community composition was between TH pretreated and raw sludge. There were moderate changes of bacterial community composition after MW pretreatment, followed by ozone pretreatment. Likewise, AD significantly changed the microbial composition of sludge. The bacterial communities of the digested sludge from AD with MW and ozone pretreatments were similar to but different from those of the digested sludge from AD with TH pretreatment. These results indicate that compared with the other two pretreatments, TH pretreatment caused more radical changes in the bacterial community and this change could influence the bacterial community of the subsequent AD process.

3.4 Variations of ARGs during sludge treatment

The removal of ARGs and MGEs during sludge treatment are shown in Figure 6 (the distribution of ARGs and MGEs during sludge treatment seen in our previous study (Tong et al., 2017)). Most of the individual ARGs were reduced after pretreatments but increased after AD. The tendency of ARGs decreasing during pretreatment but enriching after the subsequent AD is consistent with previous studies dealing with pretreatment and AD of sewage sludge (Ma et al., 2011; Pei et al., 2016; Tong et al., 2016). It is interesting that the increases or reduction of *ereA*, *mefA/E*, *tetM*, *tetO*, *mphB*, and *Tn916/1545* in Figure 6 were more significant for the AD with pretreatment than that of the AD without pretreatment. For example, there was 1.21~2.02 log copies·g⁻¹ increase for *mefA/E* during AD with pretreatments, but 0.27 log copies·g⁻¹ increase for it during AD without pretreatment. These results indicate that the sludge pretreatments affect the final output of the individual ARGs after AD

324 process.

325 3.5 Correlations analysis between sludge characteristics and ARGs

326 It is known that the physical and chemical characteristics (Guo et al., 2017), MGEs
327 (Hegstad et al., 2010) and bacterial community composition (Miller et al., 2016)
328 might affect the fates of ARGs. Therefore, Spearman correlation analysis was applied
329 for evaluation the correlations between sludge characteristics, such as sludge
330 composition, bacterial diversity, abundance of dominated genera, MGEs and the
331 absolute abundance of ARGs (Table 1). The results identified a significant negative
332 correlation between *ereA/ermB/ermF/ermX*/total ARGs and the
333 sCOD/protein/polysaccharides contents ($p = 0.000\sim 0.022$). According to Breazeal et
334 al (Breazeal et al., 2013), the removal of ARGs was significantly correlated with the
335 corresponding protein and polysaccharide contents during the wastewater treatment.
336 In addition, *mefA/E* and *tetM* showed strong positive correlations with ALK (with p
337 value 0.002 and $p < 0.001$, respectively), whereas *mphB* presented a significant
338 negative correlation with ALK ($p = 0.036$). There was no significant correlation
339 between any individual ARGs/total ARGs and pH.

340 The increase of ALK during AD were mainly caused by the increasing ammonia
341 produced by the degradation of protein and nitrogenous compounds (Song et al.,
342 2004). Ammonia could inhibit the biodegradation of erythromycin during wastewater
343 treatment (Fan and He, 2011), which means reduction of selective pressure of
344 antibiotics to the ambient bacteria was inhibited. Furthermore, it was found that
345 abundant ammonia could promote horizontal gene transfer (HGT) during wastewater

treatment through conjugative transfer mechanisms (Guo et al., 2015). It can be argued that the host bacteria of *mefA/E* and *tetM* might be favorite to ALK or ammonia. Therefore, there might be more chances for some specific host bacteria of *mefA/E* and *tetM* to proliferate during sludge treatment, or more chances for the HGT of *mefA/E*, *tetM* under higher ALK condition. Further study is needed to provide more understanding about the mechanism behind these observations.

The relative abundances of MGEs showed significant correlations with ARGs in our previous study (Tong et al., 2017). In this study, the absolute abundances, i.e. the copy number of *ermB* and *ermX* all showed statistically significant correlations with *intI1*, *ISCR1*, and *Tn916/1545* ($p = 0.007\sim 0.033$). In addition, there was a positive correlation between *ereA* and *ISCR1* ($p = 0.002$), and between *tetO* and *Tn916/1545* ($p = 0.042$). The *tetM* gene is usually carried by the transposon *Tn916* or a related conjugative transposon. Moreover, a *tetO* gene integrated into a transposon carrying the macrolide resistance genes *mefA* and *msrD* was reported (Roberts, 2005). The transposon *Tn916/1545*-family containing *tetM* and *ermB* was also found in enterococci (Hegstad et al., 2010). The transposons that can carry specific resistance genes could contribute to multi-resistance of tetracyclines and macrolides. The affinity of MGEs and ARGs confirmed by the present study is supporting that MGEs is a key factor determining the fates of ARGs during sludge treatment.

Among all pretreated sludge, the TH pretreated sludge presented the lowest microbial diversity richness (Chao 1 index = 1925). Likewise, thermally hydrolyzed digested sludge showed the lowest microbial diversity richness (Chao 1 index = 5417)

368 compared with other digested sludge. A narrow host range for ARGs of TH pretreated
369 sludge, expressed as a relatively low diversity index, would contribute to increased
370 ARGs removal efficiency. This result is consistent with the previous study which
371 proposed that lower bacterial diversity could provide a narrower host range for ARGs
372 and result in fewer ARGs after the sludge treatment (Ma et al., 2011).

373 The correlation of variations of the abundance of 61 dominated genera and ARGs
374 were also analyzed (Table 1). The hosts of *mphB* were reported only as *Streptococcus*
375 and *Escherichia* according to Antibiotic Resistance Genes Database (ARDB).
376 However, the genera of *Brevundimonas*, *Longilinea*, *Smithella*, etc. might affect the
377 fate of *mphB*. Moreover, *Crocinitomix* and *Simplicispira* showed a positive correlation
378 with *ereA*. It is indicated that these genera might affect the distribution of the
379 corresponding ARGs and should be given more attention in future research.

380 To better explain the impact of succession of bacterial community on ARGs, a
381 Procrustes analysis was conducted (Figure 7). Procrustes analysis could provide the
382 overall correlations between ARGs and the microbial community composition in the
383 reactors (Zhang et al., 2016; Luo et al., 2017). The correlation coefficients between
384 the first two principal axes were 0.80 and 0.62, respectively. The explanatory
385 variables of bacterial community succession accounted for 43.8%, indicating 43.8%
386 of the evolution of ARGs in the sludge pretreatment and AD could be explained by
387 the changes of bacterial community. These results suggest that bacterial community
388 composition might play an important role on the fate of ARGs during the sludge
389 treatment, which was consistent with the results from previous reports (Luo et al.,

2017; Zhang et al., 2018).

3.6 Comprehensive evaluation of factors influencing the fate of ARGs

For evaluating the factors impacting the fate of ARGs, multiple linear regression analyses were applied, which can be used to explore the importance of various explanatory variables, i.e. various factors. (Czekalski et al., 2015; Herrig et al., 2015, Mao et al., 2015). The effects of bacterial community composition (in genera level), bacterial diversity (Chao 1, Pielou, and Shannon index), MGEs (*int1*, *ISCR1*, and *Tn916/1545*), and sludge characteristics (polysaccharide, protein, sCOD, pH, and ALK) on the absolute abundances of ARGs during the sludge treatments were analyzed.

The multiple linear regression models are shown in Table 2. The results showed that the models were highly significant for individual ARGs and total ARGs with *p* values range from 0.000 to 0.002, indicating linear relationships are not rejected. The adjusted R^2 values of the linear regression models ranged from 0.597-0.883, which suggests that 59.7%-88.3% of the variations in individual ARGs and total ARGs can be explained by the corresponding factors. According to the multiple linear regression models, 76.3%, 50.3%, and 59.7% variations of *ermB*, *ermX*, and *mphB* can be explained by the MGEs respectively, whereas the explanatory variables of bacterial diversity account for 78.8% and 80.0% of fates of *ereA* and *ermF*. Besides, the alkalinity explained 60.3%, 82.6%, and 27.5% of evolutions of *mefA/E*, *tetM*, and *tetO*. There were 66.4% of the variations in total ARGs can be explained by the changes of MGEs. These results indicate that bacterial diversity rather than bacterial

community composition affected the fate of ARGs, whereas alkalinity was the most important factor on ARGs among all sludge features investigated in this study. ALK in an AD reactor is mainly determined by the concentrations of VFA and ammonia, which are formed as a result of microbial activity in the reactor. In turn, bacteria could affect the fates of ARGs. Therefore, it seems that there is an indirect correlation between ALK and ARGs, which still need further investigation in the future. Moreover, the models also showed these factors affecting the fate of ARGs in a collaborative way. For example, 50.3% of the variation of *ermX* could be explained by the drive of MGEs (*intI1*), with 28.4% of it contributed by the change of bacterial diversity (Pielou index), and 7.1% for the change of bacterial community composition (coord4). The evolution of bacterial community composition led to the change of potential host bacteria range of ARGs and MGEs, resulting in different fate of ARGs. Moreover, the lower bacterial diversity might narrow the host range of ARGs and MGEs and then led to changes of ARGs (Ma et al., 2011).

Bacterial community shifts rather than MGEs have been reported as the major driver shaping antibiotic resistomes analyzed by partial Redundancy analysis (RDA) (Chen et al., 2016; Zhang et al., 2016; Zheng et al., 2017). The Partial RDA can assess the importance of 3 groups of factors, such as MGEs, bacterial community composition, and sludge characteristics, on the fate of ARGs. However, there are numerous factors contribute to the variations of ARGs, mostly in a complex interplay (Manaia et al., 2018). Based on multiple linear regression models, the MGEs might be the main driver on the fates of total ARGs. It can be argued that the following factors

might result in different main driver for the fate of ARGs: 1) the major influence factors for different ARGs were varied, 2) the composition of total ARGs and MGEs investigated in different studies were not the same, 3) the different level of bacterial community (phyla, genera, etc.) used during the analysis could lead to different results.

4 Conclusions

Pharmaceutical sludge harbors large amounts of ARGs with a potential risk for resistance spreading, which needs more information about the factors impact on ARGs during pharmaceutical sludge treatment. This study elucidates the factors having impact on the fate of ARGs during pharmaceutical sludge treatment. Each factor was evaluated for its impact on ARGs. Spearman correlation analysis showed the fate of ARGs presented significant correlations with the changes of sludge features, bacterial diversity, bacterial community composition, and MGEs during sludge treatment. Further evaluation with multiple linear regression models showed that 59.7%-88.3% of the variations in individual ARGs and total ARGs can be explained by the corresponding factors. And bacterial diversity rather than bacterial community composition affected the fate of ARGs, whereas alkalinity was the most important factor among the sludge characteristics on ARGs. Besides, 66.4% of the variation of total ARGs was driven by the change of MGEs. Multiple linear regression models also revealed the collective effect of these factors on ARGs, and how much contribution of each factor impact on ARGs. This study provides more comprehension about the factors impact on the fate of ARGs during pharmaceutical sludge treatment,

and the methodology applied in this study could be used for evaluation of other types of sludge or wastewater treatment.

Appendix A. Supplementary data

Supplementary data related to this article can be found at the online version.

Acknowledgments

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663 **Table 1** Correlation analysis between absolute abundance of ARGs, sludge
664 characteristics, MGEs, bacterial diversity and major bacterial genera (green: $p < 0.05$;
665 pink: $p < 0.01$).

666 **Table 2** Multiple linear regression models.

667

Table 1 Correlation analysis between absolute abundance of ARGs, sludge characteristics, MGEs, bacterial diversity and major bacterial genera (green: $p < 0.05$; pink: $p < 0.01$).

Correlation coefficient	<i>ereA</i>	<i>ermB</i>	<i>ermF</i>	<i>ermX</i>	<i>mefA/E</i>	<i>mphB</i>	<i>tetM</i>	<i>tetO</i>	Total ARGs
sCOD	-0.860	-0.727	-0.767	-0.666	-0.552	0.517	-0.580	-0.154	-0.832
Polysaccharides	-0.818	-0.755	-0.823	-0.746	-0.413	0.49	-0.462	-0.049	-0.79
Protein	-0.874	-0.699	-0.711	-0.651	-0.566	0.524	-0.566	-0.161	-0.818
ALK	0.469	0.098	0.392	0.07	0.804	-0.608	0.916	0.559	0.559
Shannon index	0.580	0.322	0.680	0.48	0.245	-0.65	0.343	-0.105	0.455
Pielou index	0.552	0.406	0.634	0.602	0.189	-0.545	0.252	-0.224	0.448
<i>intI1</i>	0.161	0.615	0.284	0.718	-0.084	0.399	-0.035	-0.399	0.315
<i>ISCR1</i>	0.795	0.697	0.561	0.725	0.462	-0.329	0.417	-0.018	0.729
<i>Tn916/1545</i>	-0.091	-0.629	-0.14	-0.732	0.021	-0.455	0.063	0.594	-0.252
<i>Brevundimonas</i>	-0.320	0.109	-0.364	0.082	-0.356	0.637	-0.449	-0.09	-0.191
<i>Comamonas</i>	-0.195	-0.487	-0.468	-0.58	0.004	0.103	0.06	0.54	-0.199
<i>Longilinea</i>	0.431	-0.141	0.375	-0.002	0.343	-0.76	0.396	0.117	0.198
<i>Smithella</i>	0.347	-0.214	0.244	-0.158	0.410	-0.581	0.515	0.221	0.193
<i>Paludibacter</i>	0.382	0.112	0.612	0.053	0.399	-0.427	0.641	0.263	0.403
<i>Geobacter</i>	0.246	0.593	0.216	0.429	0.200	0.088	0.074	-0.333	0.326
<i>Candidatus Cloacamonas</i>	0.316	-0.116	0.425	0.083	0.389	-0.596	0.575	0.221	0.267
<i>Crocinitomix</i>	0.580	0.021	0.292	-0.088	0.516	-0.636	0.548	0.375	0.399
<i>Methanolinea</i>	0.465	-0.163	0.357	-0.044	0.429	-0.777	0.501	0.305	0.265
<i>Sulfuricurvum</i>	0.369	-0.026	0.397	-0.113	0.467	-0.475	0.641	0.385	0.373
<i>Simplicispira</i>	0.591	0.591	0.369	0.474	0.263	-0.145	0.263	0.134	0.591
<i>Tissierella</i>	-0.112	0.407	-0.112	0.405	-0.056	0.614	-0.100	-0.383	0.080
<i>Sphingobacterium</i>	-0.624	-0.266	-0.763	-0.188	-0.349	0.643	-0.486	-0.055	-0.459

671 **Table 2** Multiple linear regression models.

Absolute abundance of ARG (log copies·g ⁻¹)	Multiple linear regression models	<i>p</i> Value ^a	Interpretation of models in variation of ARG (%)	Interpretation of factors in variation of ARG (%)	
				Factors	Interpretation (%)
<i>ereA</i>	<i>ereA</i> = 1.021+0.0005 Chao1+0.732 <i>ISCR1</i>	0.000	86.1	Chao1	78.8
				Chao1, <i>ISCR1</i>	86.1
<i>ermB</i>	<i>ermB</i> = 0.462+0.950 <i>intI1</i>	0.000	76.3	<i>intI1</i>	76.3
<i>ermF</i>	<i>ermF</i> = 4.339+0.637 Shannon	0.000	80.0	Shannon	80.0
<i>ermX</i>	<i>ermX</i> = -0.867+0.725 <i>intI1</i> +6.415 Pielou-0.265 coord4	0.000	85.8	<i>intI1</i>	50.3
				<i>intI1</i> , Pielou	78.7
				<i>intI1</i> , Pielou, coord4	85.8
<i>mefA/E</i>	<i>mefA/E</i> = 2.668+0.001 ALK+0.460 <i>intI1</i>	0.001	76.1	ALK	60.3
				ALK, <i>intI1</i>	76.1
<i>mphB</i>	<i>mphB</i> = 15.227-0.728 Tn916/1545	0.002	59.7	Tn916/1545	59.7
<i>tetM</i>	<i>tetM</i> = 8.189+0.0005 ALK+9.192×10 ⁻⁵ Chao1	0.000	88.1	ALK	82.6
				ALK, Chao1	88.1
<i>tetO</i>	<i>tetO</i> = 10.420+0.001 ALK-4.870 Pielou+0.355 Coord5	0.000	88.3	ALK	27.5
				ALK, Pielou	51.2
				ALK, Pielou, coord5	88.3
total ARGs	total ARGs= 32.391+4.892 <i>ISCR1</i>	0.001	66.4	<i>ISCR1</i>	66.4

672 Note: a—*p* value from the F-test. A *p* value<0.05 means that the multiple linear regression model is significant at 95% and linear relationships are not rejected.

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Figure 1 Scheme of the sludge treatment methods.

Figure 2 Variation of sludge characteristics during sludge treatment.

Figure 3 Variation in bacterial diversity during the sludge treatments.

Figure 4 Bacterial community succession during sludge treatment. (a) Relative abundance of bacterial at phylum level; and (b) Heat map of the top 10 genera in each sludge sample based on relative abundance (log 2 transformed).

Figure 5 Evolution of the microbial community during sludge treatment by principal component analysis (PCA) based on the top 10 abundant genera of each sample. Red solid circles: samples name; blue arrows: the top 10 abundant genera of each sample. Abbreviations are as follows: Raw, raw sludge; MW, microwave pretreated sludge; TH, thermal hydrolysis pretreatment sludge; O₃, ozone pretreated sludge; Raw ADin, inlet sludge of AD without pretreatment; Raw ADout, digested sludge of AD without pretreatment; MW ADin, inlet sludge of AD with microwave pretreatment; MW ADout, digested sludge of AD with microwave pretreatment; TH ADin, inlet sludge of AD with thermal hydrolysis pretreatment; TH ADout, digested sludge of AD with thermal hydrolysis pretreatment; O₃ ADin, inlet sludge of AD with ozone pretreatment; O₃ ADout, digested sludge of AD with ozone pretreatment; Number 1-61 expressed the 61 abundant genera (genera names shown in Table S3) of 36 samples.

Figure 6 Removal of ARGs and MGEs during sludge pretreatment and AD.

Figure 7 Procrustes analysis between ARG abundance (PCoA based on Bray-Curtis)

695 and bacterial composition (PCoA based on Bray-Curtis) for sludge pretreatment and

696 AD. Abbreviations are as defined in Figure 5.

Figure 1 Scheme of the sludge treatment methods.

Figure 2 Variation of sludge characteristics during sludge treatment.

Figure 3 Variation in bacterial diversity during the sludge treatments.

Figure 4 Bacterial community succession during sludge treatment. (a) Relative abundance of bacterial at phylum level; and (b) Heat map of the top 10 genera in each sludge sample based on relative abundance (log 2 transformed).

Figure 5 Evolution of the microbial community during sludge treatment by principal component analysis (PCA) based on the top 10 abundant genera of each sample. Red solid circles: samples name; blue arrows: the top 10 abundant genera of each sample. Abbreviations are as follows: Raw, raw sludge; MW, microwave pretreated sludge; TH, thermal hydrolysis pretreatment sludge; O₃, ozone pretreated sludge; Raw ADin, inlet sludge of AD without pretreatment; Raw ADout, digested sludge of AD without pretreatment; MW ADin, inlet sludge of AD with microwave pretreatment; MW ADout, digested sludge of AD with microwave pretreatment; TH ADin, inlet sludge of AD with thermal hydrolysis pretreatment; TH ADout, digested sludge of AD with thermal hydrolysis pretreatment; O₃ ADin, inlet sludge of AD with ozone pretreatment; O₃ ADout, digested sludge of AD with ozone pretreatment; Number 1-61 expressed the 61 abundant genera (genera names shown in Table S3) of 36

722 samples.

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724 **Figure 6** Removal of ARGs and MGEs during sludge pretreatment and AD.

Figure 7 Procrustes analysis between ARG abundance (PCoA based on Bray-Curtis) and bacterial composition (PCoA based on Bray-Curtis) for sludge pretreatment and AD. Abbreviations are as defined in Figure 5.

Highlights

- Factors impact on ARGs during pharmaceutical sludge treatment were studied.
- Multiple linear regression was used to assess the importance of factors on ARGs.
- Alkalinity showed more influence on ARGs than other sludge features.
- Bacterial diversity rather than community composition affected ARGs.
- Interaction effect and contributions of each factor on ARGs were elucidated.