Journal of Microbiology (2020) Vol. 58
DOI 10.1007/s12275-020-9309-y
eISSN 1976-3794
pISSN 1225-8873

Exploring the antibiotic resistome in activated sludge and anaerobic digestion sludge in an urban wastewater treatment plant via metagenomic analysis[§]

Keunje Yoo[1], Hyunji Yoo[2], Jangho Lee[2], Eun Joo Choi[3], and Joonhong Park[2*]

[1]*Department of Environmental Engineering, Korea Maritime and Ocean University, Busan 49112, Republic of Korea*
[2]*Department of Civil and Environmental Engineering, Yonsei University, Seoul 03722, Republic of Korea*
[3]*Department of Systems Biology, Yonsei University, Seoul 03722, Republic of Korea*

(Recevied Jun 20, 2019 / Revised Oct 8, 2019 / Accepted Nov 3, 2019)

Antibiotic resistance genes (ARGs) are emerging contaminants that pose a potential threat to human health worldwide. Urban wastewater treatment plants (WWTPs) are a main source of both antibiotic-resistant bacteria and ARGs released into the environment. Nevertheless, the propagation of ARGs and their underlying mechanisms and the dynamics of mobile genetic elements (MGEs) in WWTPs have rarely been investigated in South Korea. In this study, shotgun metagenomic analysis was used to identify comprehensive ARGs and their mechanisms, bacterial communities, and MGEs from 4 configurations with 2 activated sludge (AS) and 2 anaerobic digestion sludge (ADS) samples. A total of 181 ARG subtypes belonging to 22 ARG types were broadly detected, and the ARG abundances in the AS samples were 1.3–2.0 orders of magnitude higher than in the ADS samples. Multidrug and bacitracin resistance genes were the predominant ARG types in AS samples, followed by ARGs against sulfonamide, tetracycline, and β-lactam. However, the composition of ARG types in ADS samples was significantly changed. The abundance of multidrug and β-lactam resistance genes was drastically reduced in the ADS samples. The resistance genes of MLS were the predominant, followed by ARGs against sulfonamide and tetracycline in the ADS samples. In addition, plasmids were the dominant MGEs in the AS samples, while integrons (*intI1*) were the dominant MGEs in the ADS samples. These results provide valuable information regarding the prevalence of ARG types and MGEs and the difference patterns between the AS and ADS systems.

Keywords: antibiotic resistance genes (ARGs), Mobile genetic elements, urban wastewater treatment plant, metagenomics

*For correspondence. E-mail: parkj@yonsei.ac.kr; Tel.: +82-2-2123-5798; Fax: +82-2-312-5798
[§]Supplemental material for this article may be found at http://www.springerlink.com/content/120956.

Introduction

According to the UN environment report (https://www.unenvironment.org/resources/frontiers-2017-emerging-issues-environmental-concern), growing antibiotic resistance is one of the major emerging global public health concerns. The presence of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) is a serious problem because of the prevalence of horizontal gene transfer (HGT), the process by which bacteria acquire genes from the environment. Wastewater treatment plants (WWTPs) are one of the main reservoirs regarded as hotspots for the release of both ARGs and ARB into the environment because sewage from households and hospitals contains massive amounts of antibiotics and bacteria of human origin, potentially providing selective pressure for ARB and ARGs (Rizzo *et al*., 2013; Manaia *et al*., 2018). In addition, it has been suggested that certain conditions within the WWTPs might increase the number of ARB and ARGs during the treatment process (Yang *et al*., 2014; Li *et al*., 2015; Ju *et al*., 2016; Gupta *et al*., 2018).

From the perspectives of energy savings and resource recovery in WWTPs, activated sludge (AS) processes are less effective than anaerobic digestion processes (McCarty *et al*., 2011). However, from the perspective of managing ARGs as a public health issue, it remains unclear which WWTP process exhibits a higher microbial hazard and/or risk (Kim and Aga, 2007; Guo *et al*., 2017). Currently, there are few comparative studies on the overall occurrence and diversity of various types of ARGs involved in AS and anaerobic digestion sludge (ADS) processes from urban WWTPs (Bouki *et al*., 2013; Mao *et al*., 2015; Zhang *et al*., 2015; Guo *et al*., 2017). Although some studies have been carried out to determine the differences in occurrence, abundance, and diversity of ARGs and mobile genetic elements (MGEs) in AS and ADS processes, studies regarding which contaminant antibiotics are a result of human activities in urban WWTPs are still limited (Guo *et al*., 2017; Manaia *et al*., 2018). Indeed, it remains unclear which ARGs are critically acquired and transferred among bacterial communities via MGEs during the AS and ADS processes. Metagenomic approaches based on high-throughput sequencing are now regarded as the most reliable and cost-effective methods for gaining deep insights into the diversity and composition of the microbial community of environmental samples (Li *et al*., 2015; Yoo *et al*., 2018). Therefore, it is useful to apply metagenomics to understand variations within the profiles of ARGs and MGEs in wastewater treatment systems (Li *et al*., 2015; Guo *et al*.,

2017).

The increase in ARGs and ARB is a major public health concern in South Korea (Park *et al.*, 2017). In South Korea, the level of antibiotic usage is much higher than the average level of antibiotic usage in other Organisation for Economic Cooperation and Development (OECD) countries (Park *et al.*, 2017). Nevertheless, relatively few studies have investigated the dynamics of ARG contamination in urban WWTPs in South Korea. In particular, wastewater treatment processes significantly affect the fate of ARGs, but ARGs have not been fully explored in AS and ADS processes in South Korea. Characterization of the prevalence of ARGs in urban WWTPs in Republic of Korea is essential to establish a strategy for preventing their proliferation because approximately 20% of South Korea's total population lives in Seoul, South Korea, which as the capital of South Korea is an overcrowded city that may be suffering from critical issues related to water management and public health. Therefore, the aim of this study was to comparatively investigate the diversity and occurrences of ARGs, resistance mechanisms, and MGEs in AS and ADS in the metropolitan city of Seoul using shotgun metagenomics.

Materials and Methods

Study area and sampling

As shown in Supplementary data Fig. S1, 500 ml of mixed liquor suspended solids (MLSS) from the aerobic zone of the Modified Ludzack Ettinger (MLE) and 500 ml of anaerobic digested sludge from the anaerobic digester were collected at the Jung-Rang wastewater treatment plant (37°33′28.2″N, 127°03′54.9″E), Seoul, South Korea in January (AS1 and ADS1 samples) and November (AS2 and ADS2 samples) 2015, respectively. The treatment capacity of the WWTP was approximately 1.6 million tons of wastewater per day. The sludge samples were collected with a sludge sampler and immediately transferred to sterilized bottles and stored in a refrigerator (-80°C) prior to molecular analysis.

DNA extraction and high-throughput sequencing

The FastDNA Spin Kit for Soil (MP Biomedicals) was used to extract genomic DNA from each sample, following the manufacturer's instructions. The concentration and quality of extracted DNA was assessed by electrophoresis and spectrophotometry (260/280 nm ratio, NanoDrop, Thermo Fisher Scientific).

The extracted DNA passed the criteria of the library size check (470 bp) and library quantity check (DNA concentration > 2 nM). This process was performed using the TruSeq DNA PCR-Free Kit (Invitrogen, Inc.) followed by the library protocol by Macrogen. Each sample was also barcoded, and library construction was then performed. After the library was constructed, Illumina HiSeq was performed using a 2 × 101 bp paired-end protocol with a HiSeq 2000 platform by Macrogen.

Bioinformatic analyses

Raw sequences from the Illumina HiSeq data were trimmed to remove adapters and low-quality nucleotide stretches using TrimGalore! version 0.5.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) with the default settings. Trimmed and filtered sequences were then used as input for all further analyses. The clean metagenomic data were processed against the Structured ARG (SARG) database (containing 23 ARG classes and 1277 ARG subtypes) through the ARGs-OAP online pipeline (http://smile.hku.hk/SARGs) following the published procedure (Yin *et al.*, 2018) to identify ARG types and subtypes. The UBLAST algorithm, using a Perl script supplied by the platform, was run to prescreen ARG-like and 16S rRNA gene sequences (Eq. 1).

$$\text{Abundance} = \sum_{1}^{n} \frac{N_{\text{ARG-like sequence}} \times L_{\text{reads}} / L_{\text{ARG reference sequence}}}{N_{\text{16S equence}} \times L_{\text{reads}} / L_{\text{16S sequence}}} \quad (1)$$

The candidate ARG sequences were matched against the SARG databases using BLASTX. The sequences that met the BLASTX criteria (alignment length 25 aa, similarity 80%, and e-value $1e^{-5}$ [Yang *et al.*, 2016]) were classified according to the SARG hierarchy. ARG abundances (copies of ARGs per copy of 16S rRNA) in the metagenomic data were calculated and used.

Local BLASTn was employed to align our sequencing data against the databases of integrons and plasmids in order to characterize MGEs in the AS and ADS samples. An integron database was constructed according to the nucleotide sequences of all integrases available in the INTEGRALL database (http://integrall.bio.ua.pt/), and a plasmid database was developed on the basis of the plasmid sequences of the NCBI RefSeq database (http://www.ncbi.nlm.nih.gov/refseq). A read was annotated as an integron sequence if the nucleotide sequence identity of the best BLASTn hit was higher than 90% with an alignment length of at least 50 bp, and the plasmid-like tags in all data sets were determined by the alignments with a nucleotide sequence identity above 95% over a length of at least 90 bp (Kristiansson *et al.*, 2011; Fang *et al.*, 2019). The abundances of MGEs were presented using the unit of copies/16S rRNA gene. The calculation of MGE abundance was adapted from ARG abundance calculation, which is described by Yang *et al.* (2016).

To identify the microbial communities in AS and ADS samples, MetaPhlAn2 was used (Truong *et al.*, 2015). The MetaPhlAn2 software and the database of the markers can be downloaded from http://huttenhower.sph.harvard.edu/metaphlan/. All the parameters of MetaPhlAn2 utilized default settings. The metagenomes generated in this study are publicly available via MG-RAST under sample IDs (mgm4785060.3 and mgm4785061.3).

Results

Abundance and diversity of ARG types and subtypes

In total, 181 ARG subtypes belonging to 22 ARG types were identified in AS and ADS samples (Fig. 1, Supplementary data Table S1). The overall ARG abundances of four different samples were determined in the range 3.14×10^{-4} – 1.57×10^{-1} copies per 16S rRNA gene with an average value of 3.65×10^{-2} copies per 16S rRNA gene for AS and the range 5.80 ×

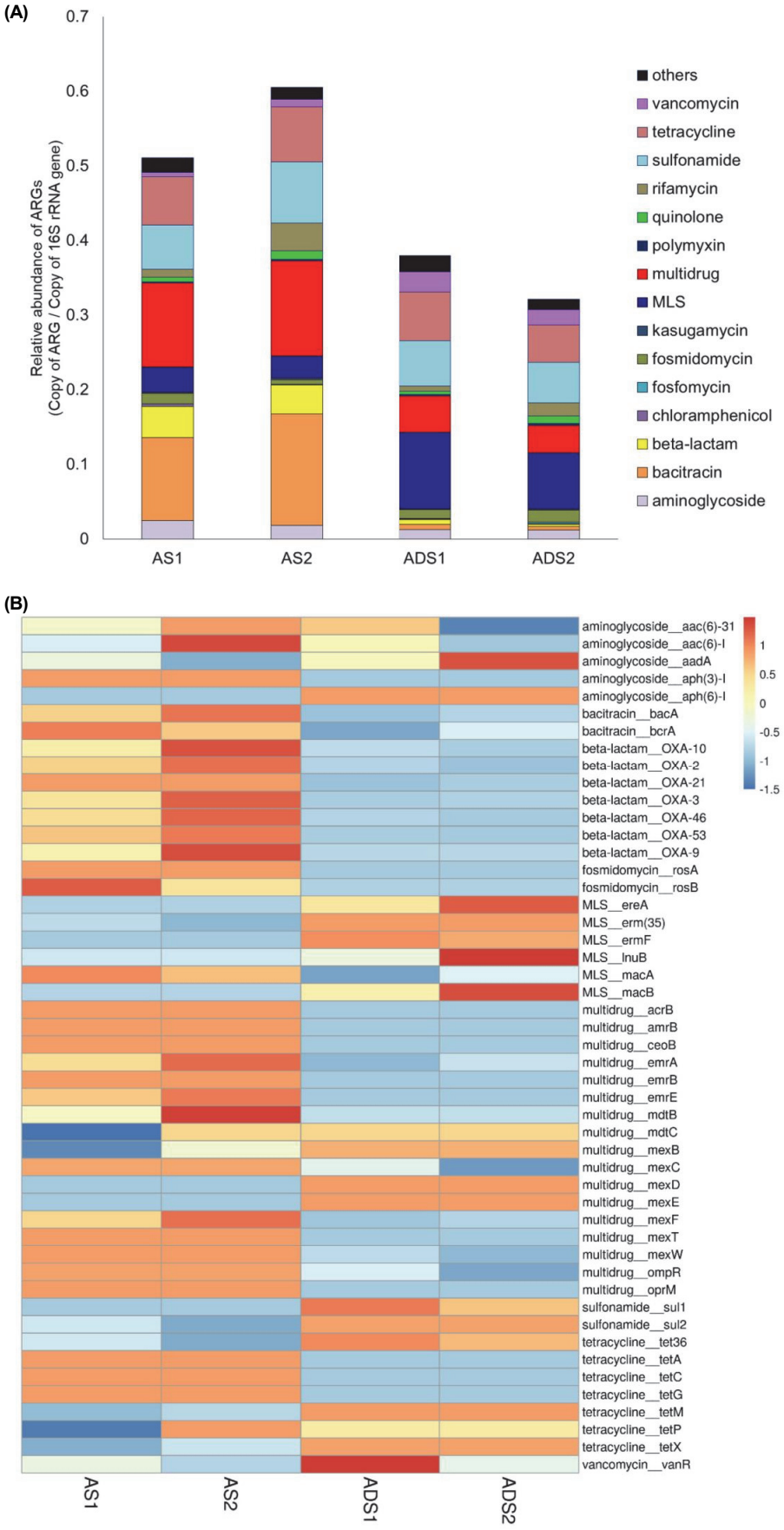

Fig. 1. The diversity and abundance of ARG types (A) and subtypes (B) in the AS and ADS samples. The abundances of the top 50 ARG subtypes (ARG per copy of 16S rRNA gene copies) are illustrated by a heatmap using normalized z-scores in all samples. AS1 and ADS1 indicate the January sample in 2015; AS2 and ADS2 indicate the November sample in 2015.

10^{-5} – 1.10×10^{-1} copies per 16S rRNA gene with an average value of 2.19×10^{-2} copies per 16S rRNA gene for ADS. The ARG abundances were 1.3–2.0 orders of magnitude higher in the AS samples than in the ADS samples. The most prevalent ARG types found in the total samples were genes conferring multidrug, macrolide-lincosamide-streptogramin (MLS), bacitracin, sulfonamides, tetracycline, and β-lactam resistance (Fig. 1A). Multidrug and bacitracin resistance genes were the predominant ARG types in the AS samples (1.45×10^{-1} copies/16S rRNA gene, 1.21×10^{-1} copies/16S rRNA gene, respectively), followed by ARGs against sulfonamide (7.17×10^{-2} copies/16S rRNA gene), tetracycline (6.93×10^{-2} copies/16S rRNA gene), and β-lactam (4.03×10^{-2} copies/16S rRNA gene). However, the composition of ARG types in ADS samples was significantly different. The abundance of multidrug and β-lactam resistance genes was drastically reduced in ADS samples. Genes for resistance to MLS (8.93×10^{-2} copies/16S rRNA gene), sulfonamide (5.74×10^{-2} copies/16S rRNA gene), and tetracycline (5.71×10^{-2} copies/16S rRNA gene) were the major ARG types found in ADS samples.

From the occurrences and diversities of ARG subtype results, we observed that different subtypes of ARGs had various degrees of enrichment from each sample. Not only the abundance of ARGs but also the ARG diversity was greatly reduced in the ADS samples compared to the AS samples (Fig. 1B). In the AS samples, 174 ARG subtypes were identified, whereas 112 ARG subtypes were identified in the ADS, which was comparable to the AS results, although some of the ARG abundance in ADS was higher than that in AS. In the AS samples, activation of the *bac*A gene (13.45% relative abundance with respect to 16S rRNA genes), bla_{oxa} genes (9.32% relative abundance with respect to 16S rRNA genes), *tet* genes (*tet*A, *tat*C, and *tat*G) (6.84% relative abundance with respect to 16S rRNA genes), *mdt*B and *emr*B genes (5.86% relative abundance with respect to 16S rRNA genes), and *mac*A gene (5.48% relative abundance with respect to 16S rRNA genes) was predominant over other resistance (Fig. 1B). However, the profiles of ARG subtypes in ADS samples were significantly different. The *ermF* and *mac*B genes (18.22% relative abundance with respect to 16S rRNA genes), *sul* genes (*sulI*, *sulII*) (13.34% relative abundance with respect to 16S rRNA genes), and *tet* genes (*tetM*, *tetP*, and *tetX*) (5.06% relative abundance with respect to 16S rRNA genes) were enriched in the ADS samples.

The ARGs detected in all samples encompassed the two major resistance mechanisms – extrusion by efflux pumps and target modification. There was a significant difference in the relative abundance of resistance mechanisms between the AS and ADS samples (Supplementary data Fig. S2). Efflux pumps were the main resistance mechanisms (45.34–45.94%) in the AS samples, followed by target modification (25.37–28.42%), antibiotic inactivation (17.44–19.12%), and target bypass (8.21–10.17%). However, target modification was the predominant resistance mechanism (47.66–48.13%) in the ADS samples, followed by efflux pumps (28.95–30.58%), target bypass (13.51–14.14%), and antibiotic inactivation (8.25–8.78%).

Detection of the occurrence and abundance of MGEs

Shotgun metagenomics sequences were aligned against the NCBI RefSeq and INTEGRALL databases of plasmid and integrons to understand their occurrence and abundance in AS and ADS samples. Figure 2 shows the detection frequency for MGEs of plasmids, integrons, and others among the samples. A large variety of integrons and plasmids were found in both the AS and ADS samples. Generally, abundant MGEs were detected in the AS and ADS samples, and the total quantity of plasmid-like sequences was substantially greater than that of integron-like sequences in all samples. In the AS samples, approximately 3.85×10^{-2} copies/16S rRNA gene and 6.12×10^{-3} copies/16S rRNA gene were plasmid and integron genes, compared with the corresponding values of 1.52×10^{-2} copies/16S rRNA gene and 1.14×10^{-2} copies/16S rRNA gene for plasmid and integron genes in the ADS samples, respectively. The abundance of major plasmids showed remarkably distinct patterns between the AS and ADS samples (Supplementary data Table S2). In contrast to the plasmid results, approximately 83% of integrons were shared by all samples. Although *intI*1, *intI*3, and unknown integrase genes were widely detected in this study, the *intI1* gene, like various recombinase-encoded genes, was predominant in both the AS and ADS samples (Supplementary data Table S3). The relative abundance of *intI*1 was determined by 94.2% and 97.8% of alignment hits of the AS and the ADS samples, respectively.

Microbial taxonomic composition among the AS and ADS samples

In this study, most metagenomic reads (96.8–98.4%) were taxonomically assigned to bacteria. During the different wastewater treatment processes between AS and ADS samples, changes in the physical and chemical conditions affect the composition of the microbial community. To better under-

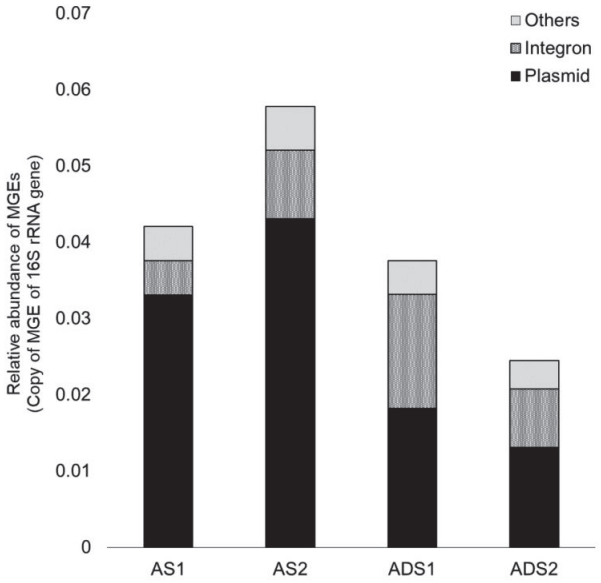

Fig. 2. Relative abundances of MGEs (MGE per copy of 16S rRNA gene copies) in the AS and ADS samples. The category 'others' indicates MGEs that were not identified as associated with plasmids or integrons in this study. AS1 and ADS1 indicate the January sample in 2015; AS2 and ADS2 indicate the November sample in 2015.

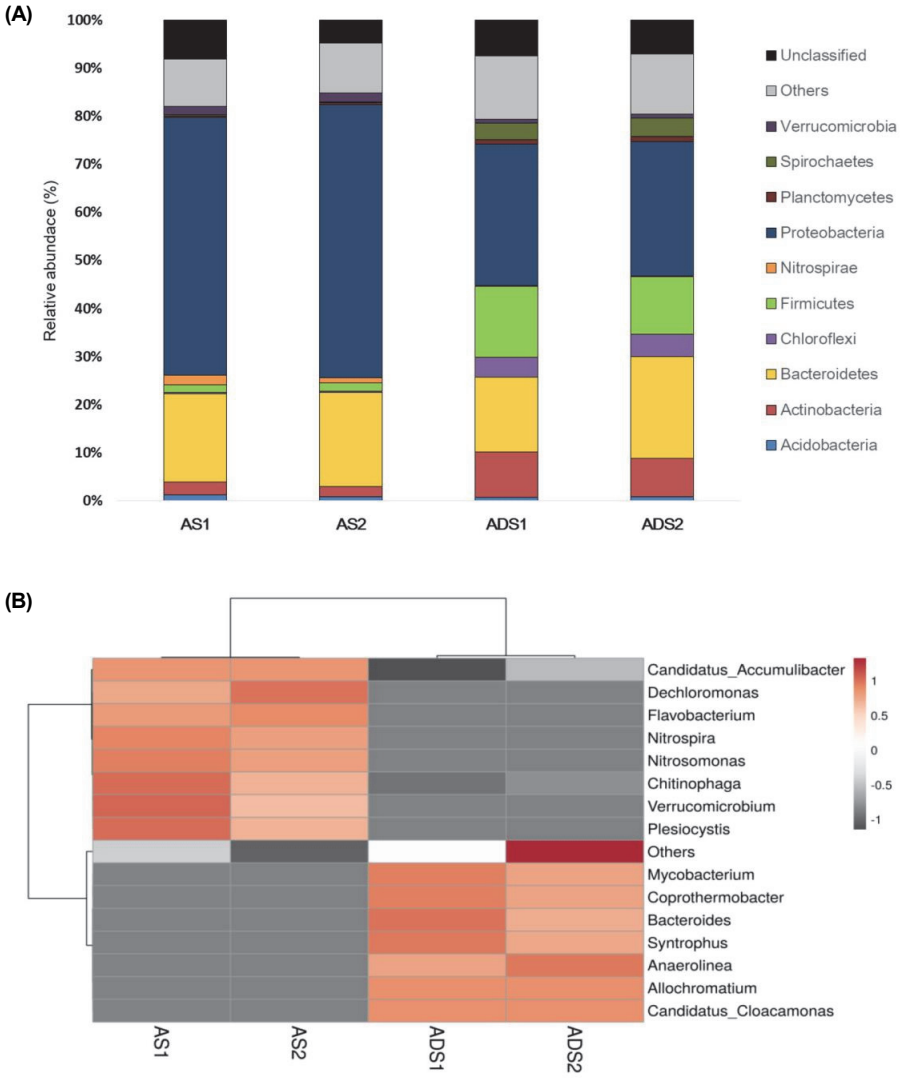

Fig. 3. Microbial community compositions in AS and ADS samples from an urban WWTP in Korea. Relative abundance at the phylum level (A) and genus level (B) in the AS and ADS samples. 'Others' indicates the sum of the relative percentages of phyla and genera with a maximum abundance lower than 1% in any sample. AS1 and ADS1 indicate the January sample in 2015; AS2 and ADS2 indicate the November sample in 2015.

stand the microbial communities in the AS and ADS samples, the taxonomic affiliation at the phylum and genus level was investigated. The microbial taxonomic compositions showed marked differences between AS and ADS samples. As shown in Fig. 3A, the dominant phylum in the AS samples was Proteobacteria (57.82–59.58%), followed by Bacteroidetes (20.32–22.24%), Actinobacteria (6.72–7.60%), and Nitrospirae (3.18–5.32%). However, the Proteobacteria (23.18–28.34%), Bacteroidetes (16.78–18.32%), and Firmicutes (13.22–14.42%) phyla were predominant in the ADS samples. The different wastewater treatment processes and stresses result in different physiochemical conditions between the AS and ADS samples and consequently in distinctive genus compositions. At the genus level, the AS samples were dominated by nitrifiers (*Nitrosomonas*, 11%, and *Nitrospira*, 10%), denitrifiers (*Dechloromonas*, 22%), and *Chitinophaga* (15%), whereas *Dechloromonas* (23%), *Anaerolinea* (18%), *Candidatus cloacamonas* (16%), *Bacteroides* (13%), and *Mycobacterium* (9%) were prevalent in the ADS samples (Fig. 3B).

Discussion

WWTPs are regarded as the hotspot for ARGs and ARB (Rizzo *et al*., 2013; Guo *et al*., 2017). To deeply investigate ARG contamination in urban WWTPs, shotgun metagenomics analysis was applied. Differences in the abundance and occurrence of ARGs, MGEs, and microbial communities were found between AS and ADS samples. The profiles of major ARGs that confer bacitracin, β-lactam, multidrug, macrolide, sulfonamide, and tetracycline resistance were abundant in Korea urban WWTPs. These antibiotics are among the most commonly prescribed drugs in Korea (Park *et al*., 2017) and are among the major pharmaceutical products found in the influents of WWTPs (Zhang *et al*., 2015; Park *et al*., 2017). In addition, genes conferring resistance to sulfonamides and MLS are frequently detected in WWTPs worldwide, along with those conferring resistance to tetracyclines and beta-lactams (Gao *et al*., 2012; Li *et al*., 2015; Guo *et al*., 2017). Because of the strong sorption of the tetracycline and MLS antibiotics, their mobility in the environment may be facilitated by transport with wastewater (Kolz

et al., 2005; LaPara *et al*., 2011). Relatively high multidrug resistance in aerobic processes has also been reported (Yang *et al*., 2013; Zhang *et al*., 2015) and potentially explained by the presence of many microstressors in wastewater, which select for bacteria with multiple defense mechanisms and dissemination of their resistance through HGT (Christgen *et al*., 2015). In addition, change from anoxic and aerobic conditions during the treatment process in the bioreactor might influence the HGT mechanism, potentially affecting the selection of multidrug resistance genotypes (Pál *et al*., 2005). This explanation is plausible because bacterial stress responses indicate HGT (Chen *et al*., 2016), and a change in redox conditions due to changes in anoxic and aerobic conditions would increase bacterial stress. Sulfonamides with high solubility and chemical stability can persist in the environment for a long period of time (Wang *et al*., 2013), resulting in the high abundance of *sul*I and *sul*II in the WWTPs. Therefore, sulfonamide resistances are frequently detected in effluent (Bouki *et al*., 2013; Rizzo *et al*., 2013; Xu *et al*., 2015), dewatered sludges (Munir *et al.,* 2011), and surface water (Li *et al*., 2015; Fang *et al*., 2019), indicating a general resilience to the wastewater treatment process.

Generally, AS processes involve relatively high bacterial diversity and density, biofilm formation, and metabolic activity during the treatment process, which can create an environment potentially suitable for the development and spread of resistance (Munir *et al*., 2011; Devarajan *et al*., 2015; Lu *et al*., 2015). Therefore, we anticipated that the anaerobic digestion process would achieve substantially better reductions in ARGs because it is well established that high-temperature and high-pressure treatment processes are effective at inactivating ARGs and pathogenic bacteria (Pruden *et al*., 2013; Ju *et al*., 2016). However, our results showed that certain resistance genes, such as MLS, sulfonamide, and tetracycline, were enriched in the ADS samples compared to the AS samples (Fig. 1). This finding implies that specific ARGs may have enhanced resistance under favorable operating conditions and selective pressure in the thermophilic digesters of the ADS system (Baquero *et al*., 2008; Mao *et al*., 2015). As a previous study showed, some of the ARGs were significantly enriched in the sludge remaining after the anaerobic digestion process (Zhang *et al*., 2015; Guo *et al*., 2017).

In particular, sequences related to subtypes of MLS resistance genes (*erm*F) were enriched by more than 10 times in ADS compared with AS (Fig. 1B). A previous study reported that *Erm* resistance genes, such as *erm*B and *erm*F, were prevalent in the ADS process (Zhang *et al*., 2015). The enrichment of certain genes might be a result of host bacterial cells harboring such genes that are subject to amplification via cell growth or HGT or attenuation via differential survival with respect to the digester operating conditions (Wu *et al*., 2016; Zhang *et al*., 2016b). These results implied that anaerobic bacteria might carry more MLS resistance genes than aerobic bacteria, although further analysis is needed to verify the cause of the higher abundance of ARGs in ADS than AS. Moreover, some previous studies (Ghosh *et al*., 2009; Zhang *et al*., 2015; Ju *et al*., 2016) suggested that anaerobic digestion is an inefficient process for the removal of ARGs, such as tetracycline, MLS, class 1 integrons, and others, due to stronger adaptation of the bacterial community to operational conditions and selective pressure (Baquero *et al*., 2008; Mao *et al*., 2015). Therefore, more studies are absolutely required to better understand the emergence of ARGs in the ADS process.

HGT via MGEs easily occurs in niches with high biomass. Antibiotics, nutrient substances and high biomass in biological nutrient removal processes can obviously accelerate ARG replication on MGEs in microbial populations and facilitate their horizontal transfer among bacterial cells by plasmids or integrons (Gaze *et al*., 2011; Hsu *et al*., 2014). *Tet* and *sul* genes are strongly associated with MGEs and thus move readily from one species to another. Tet genes with high abundance are usually carried by MGEs and can be transferred among the bacteria in the environment (Allen *et al*., 2010; Chen *et al*., 2016; Manaia *et al*., 2018). Considering the transfer mechanisms of sulfamethoxazole, *sul*I was carried on *int*1, but *sul*II was detected on a broad range of host plasmids; this could lead to the widespread detection of *sul*I in aquatic environments (Ma *et al*., 2011; Chen *et al*., 2013). Furthermore, this observation is consistent with previous studies in the aquatic environment (Pruden *et al*., 2013). The BacA gene, which encodes bacitracin resistance, was found to be abundant in drinking water and wastewater (Jia *et al*., 2015). Because the *bac*A gene product is essential for the biosynthesis of peptidoglycan and other cell wall components, the *bac*A gene and bacteria harboring *bac*A can survive under external stress (Christgen *et al*., 2015; Jia *et al*., 2015; Chen *et al*., 2016).

Different MGE patterns between the AS and ADS samples might be affected by different types of stressors, such as the temperature, nutrient load, HRT, SRT, and pH of the biological process, which will exert a selective effect on the types of ARB and ARGs in the bioreactor (Zhang *et al*., 2015; Guo *et al*., 2017). In particular, the ADS samples overall showed enhanced abundance of class 1 integrons and enhanced diversity of gene cassettes containing various ARGs, which may accelerate the mobility of some ARGs in the ADS samples (Ghosh *et al*., 2009; Gillings *et al*., 2014; Wu *et al*., 2016). The variations in the abundances of MGEs were not consistent in the aerobic treatment systems (Su *et al*., 2015; Zhang *et al*., 2016a). However, most previous studies demonstrated that integrons were a major indicator of MGEs during anaerobic fermentation and treatment (Chen *et al*., 2013; Christgen *et al*., 2015; Guo *et al*., 2017).

The possibility of extensive lateral gene transfer within the municipal wastewater treatment process is an emerging concern because it suggests that anaerobic digestors could be a source of new ARB (Guo *et al*., 2017). This would be a substantial paradigm shift from our original viewpoint, which was that municipal wastewater was an important reservoir of resistant bacteria and that municipal wastewater treatment processes could be used to decrease the size of this reservoir of resistance.

The taxonomic profiles at the phylum and genus levels were investigated to interpret the differences in the bacterial community structure between AS and ADS samples (Fig. 3). Proteobacteria are believed to be involved in the removal of organic pollutants, such as nitrogen, phosphorus, and aromatic compounds (Wagner and Loy, 2002). Bacteroidetes are a group of fermentative bacteria involved in the acidogenic phase of the digestion process (Traversi *et al*., 2012).

Firmicutes are well-known fermenters and syntrophic bacteria that can degrade various substrates (Garcia-Peña *et al.*, 2011). In addition, Firmicutes are a butyrate-utilizing microbial community (Ariesyady *et al.*, 2007). These results indicated that the abundance of Proteobacteria in the ADS samples possibly resulted from the feed sludge since Proteobacteria was enriched in AS (Zhang *et al.*, 2011).

The high inconsistency in the presence or absence of species in the results between the AS samples and ADS samples indicated that changes resulting from anoxic/aerobic/anaerobic conditions during the treatment process in the bioreactor may have influenced the bacterial community. Although the reason for the high abundance is unclear, differences in process operation (pH, temperature, time, etc.) or in feed sludge (salinity, sulfate, etc.) may increase the persistence of conditions and/or resources that positively affect bacterial communities between AS and ADS (Zhang *et al.*, 2015; Ju *et al.*, 2016). Thus, such distinct differences show the substantial influence of unnatural and rapid changes in physicochemical state on the bacterial community of a sample.

According to previous studies, bacterial communities often benefit from MGE-carrying genes under harsh conditions (Ju *et al.*, 2016; Guo *et al.*, 2017). Thus, the distribution of MGE-carrying genes among bacterial hosts could be important to the composition and structure of the bacterial community. However, another approach is that the major driver of antibiotic resistome alteration is a bacterial community shift rather than MGE reproduction or transfer in a drinking water system (Jia *et al.*, 2015) and composting sewage sludge (Su *et al.*, 2015). These observations further support the conclusion that bacterial community composition plays an important role in driving similar responses of ARGs to treatments. Therefore, it is absolutely necessary to investigate ARG profiles as well as microbial communities during the long-term monitoring of WWTPs to further delineate the relationship between ARGs and bacterial communities and to provide more information on the control of ARGs.

Although the major hosts of ARGs in microbial communities and their roles in driving the dissemination of ARGs remain unclear, this study provides information on the differences in the antibiotic resistome and MGEs between AS and ADS, which may illuminate the mechanisms by which antibiotic resistance is promoted by wastewater treatment management in South Korea. However, additional studies should be carried out in the future to better understand these relationships to control the emergence of ARB and ARGs in WWTPs and facilitate their removal. The results from this work and others call for extended studies on several urban WWTPs with differential processes to better understand ARG dynamics and the process efficiency of ARG removal.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2018R1-A6A1A08025348).

References

Allen, H.K., Donato, J., Wang, H.H., Cloud-Hansen, K.A., Davies, J., and Handelsman, J. 2010. Call of the wild: antibiotic resistance genes in natural environments. *Nat. Rev. Microbiol.* **8**, 251.

Ariesyady, H.D., Ito, T., and Okabe, S. 2007. Functional bacterial and archaeal community structures of major trophic groups in a full-scale anaerobic sludge digester. *Water Res.* **41**, 1554–1568.

Baquero, F., Martínez, J.L., and Cantón, R. 2008. Antibiotics and antibiotic resistance in water environments. *Curr. Opin. Biotechnol.* **19**, 260–265.

Bouki, C., Venieri, D., and Diamadopoulos, E. 2013. Detection and fate of antibiotic resistant bacteria in wastewater treatment plants: A review. *Ecotoxicol. Environ. Saf.* **91**, 1–9.

Chen, Q., An, X., Li, H., Su, J., Ma, Y., and Zhu, Y.G. 2016. Long-term field application of sewage sludge increases the abundance of antibiotic resistance genes in soil. *Environ. Int.* **92–93**, 1–10.

Chen, B., Yang, Y., Liang, X., Yu, K., Zhang, T., and Li, X. 2013. Metagenomic profiles of antibiotic resistance genes (ARGs) between human impacted estuary and deep ocean sediments. *Environ. Sci. Technol.* **47**, 12753–12760.

Christgen, B., Yang, Y., Ahammad, S.Z., Li, B., Rodriquez, D.C., Zhang, T., and Graham, D.W. 2015. Metagenomics shows that low-energy anaerobic-aerobic treatment reactors reduce antibiotic resistance gene levels from domestic wastewater. *Environ. Sci. Technol.* **49**, 2577–2584.

Devarajan, N., Laffite, A., Graham, N.D., Meijer, M., Prabakar, K., Mubedi, J.I., Elongo, V., Mpiana, P.T., Ibelings, B.W., Wildi, W., *et al.* 2015. Accumulation of clinically relevant antibiotic-resistance genes, bacterial load, and metals in freshwater lake sediments in central Europe. *Environ. Sci. Technol.* **49**, 6528–6537.

Fang, H., Huang, K., Yu, J., Ding, C., Wang, Z., Zhao, C., Yuan, H., Wang, Z., Wang, S., Hu, J., *et al.* 2019. Metagenomic analysis of bacterial communities and antibiotic resistance genes in the *Eriocheir sinensis* freshwater aquaculture environment. *Chemosphere* **224**, 202–211.

Gao, P., Munir, M., and Xagoraraki, I. 2012. Correlation of tetracycline and sulfonamide antibiotics with corresponding resistance genes and resistant bacteria in a conventional municipal wastewater treatment plant. *Sci. Total Environ.* **421–422**, 173–183.

Garcia-Peña, E.I., Parameswaran, P., Kang, D.W., Canul-Chan, M., and Krajmalnik-Brown, R. 2011. Anaerobic digestion and co-digestion processes of vegetable and fruit residues: Process and microbial ecology. *Bioresour. Technol.* **102**, 9447–9455.

Gaze, W.H., Zhang, L., Abdouslam, N.A., Hawkey, P.M., Calvo-Bado, L., Royle, J., Brown, H., Davis, S., Kay, P., Boxall, A.B.A., *et al.* 2011. Impacts of anthropogenic activity on the ecology of class 1 integrons and integron-associated genes in the environment. *ISME J.* **5**, 1253.

Ghosh, S., Ramsden, S.J., and LaPara, T.M. 2009. The role of anaerobic digestion in controlling the release of tetracycline resistance genes and class 1 integrons from municipal wastewater treatment plants. *Appl. Microbiol. Biotechnol.* **84**, 791–796.

Gillings, M.R., Gaze, W.H., Pruden, A., Smalla, K., Tiedje, J.M., and Zhu, Y.G. 2014. Using the class 1 integron-integrase gene as a proxy for anthropogenic pollution. *ISME J.* **9**, 1269.

Guo, J., Li, J., Chen, H., Bond, P.L., and Yuan, Z. 2017. Metagenomic analysis reveals wastewater treatment plants as hotspots of antibiotic resistance genes and mobile genetic elements. *Water Res.* **123**, 468–478.

Gupta, S.K., Shin, H., Han, D., Hur, H.G., and Unno, T. 2018. Metagenomic analysis reveals the prevalence and persistence of antibiotic-and heavy metal-resistance genes in wastewater treatment plant. *J. Microbiol.* **56**, 408–415.

Hsu, J.T., Chen, C.Y., Young, C.W., Chao, W.L., Li, M.H., Liu, Y.H., Lin, C.M., and Ying, C. 2014. Prevalence of sulfonamide-resis-

tant bacteria, resistance genes and integron-associated horizontal gene transfer in natural water bodies and soils adjacent to a swine feedlot in northern Taiwan. *J. Hazard. Mater.* **277**, 34–43.

Jia, S., Shi, P., Hu, Q., Li, B., Zhang, T., and Zhang, X.X. 2015. Bacterial community shift drives antibiotic resistance promotion during drinking water chlorination. *Environ. Sci. Technol.* **49**, 12271–12279.

Ju, F., Li, B., Ma, L., Wang, Y., Huang, D., and Zhang, T. 2016. Antibiotic resistance genes and human bacterial pathogens: Co-occurrence, removal, and enrichment in municipal sewage sludge digesters. *Water Res.* **91**, 1–10.

Kim, S. and Aga, D.S. 2007. Potential ecological and human health impacts of antibiotics and antibiotic-resistant bacteria from wastewater treatment plants. *J. Toxicol. Environ. Heal. Part B* **10**, 559–573.

Kolz, A.C., Ong, S.K., and Moorman, T.B. 2005. Sorption of tylosin onto swine manure. *Chemosphere* **60**, 284–289.

Kristiansson, E., Fick, J., Janzon, A., Grabic, R., Rutgersson, C., Weijdegård, B., Söderström, H., and Larsson, D.G.J. 2011. Pyrosequencing of antibiotic-contaminated river sediments reveals high levels of resistance and gene transfer elements. *PLoS One* **6**, e17038.

LaPara, T.M., Burch, T.R., McNamara, P.J., Tan, D.T., Yan, M., and Eichmiller, J.J. 2011. Tertiary-treated municipal wastewater is a significant point source of antibiotic resistance genes into Duluth-Superior Harbor. *Environ. Sci. Technol.* **45**, 9543–9549.

Li, B., Yang, Y., Ma, L., Ju, F., Guo, F., Tiedje, J.M., and Zhang, T. 2015. Metagenomic and network analysis reveal wide distribution and co-occurrence of environmental antibiotic resistance genes. *ISME J.* **9**, 2490.

Lu, X., Zhang, X.X., Wang, Z., Huang, K., Wang, Y., Liang, W., Tan, Y., Liu, B., and Tang, J. 2015. Bacterial pathogens and community composition in advanced sewage treatment systems revealed by metagenomics analysis based on high-throughput sequencing. *PLoS One* **10**, e0125549.

Ma, Y., Wilson, C.A., Novak, J.T., Riffat, R., Aynur, S., Murthy, S., and Pruden, A. 2011. Effect of various sludge digestion conditions on sulfonamide, macrolide, and tetracycline resistance genes and class I integrons. *Environ. Sci. Technol.* **45**, 7855–7861.

Manaia, C.M., Rocha, J., Scaccia, N., Marano, R., Radu, E., Biancullo, F., Cerqueira, F., Fortunato, G., Iakovides, I.C., Zammit, I., *et al.* 2018. Antibiotic resistance in wastewater treatment plants: Tackling the black box. *Environ. Int.* **115**, 312–324.

Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q., and Alvarez, P.J.J. 2015. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. *Water Res.* **85**, 458–466.

McCarty, P.L., Bae, J., and Kim, J. 2011. Domestic wastewater treatment as a net energy producer–can this be achieved? *Environ. Sci. Technol.* **45**, 7100–7106.

Munir, M., Wong, K., and Xagoraraki, I. 2011. Release of antibiotic resistant bacteria and genes in the effluent and biosolids of five wastewater utilities in Michigan. *Water Res.* **45**, 681–693.

Pál, C., Papp, B., and Lercher, M. J. 2005. Adaptive evolution of bacterial metabolic networks by horizontal gene transfer. *Nat. Genet.* **37**, 1372–1375.

Park, J., Han, E., Lee, S.O., and Kim, D.S. 2017. Antibiotic use in South Korea from 2007 to 2014: A health insurance database-generated time series analysis. *PLoS One* **12**, e0177435.

Pruden, A., Larsson, D.J., Amézquita, A., Collignon, P., Brandt, K.K., Graham, D.W., Lazorchak, J.M., Suzuki, S., Silley, P., Snape, J.R., *et al.* 2013. Management options for reducing the release of antibiotics and antibiotic resistance genes to the environment. *Environ. Heal. Persp.* **121**, 878–885.

Rizzo, L., Manaia, C., Merlin, C., Schwartz, T., Dagot, C., Ploy, M.C., Michael, I., and Fatta-Kassinos, D. 2013. Urban wastewater treatment plants as hotspots for antibiotic resistant bacteria and genes spread into the environment: A review. *Sci. Total Environ.* **447**, 345–360.

Su, J.Q., Wei, B., Ou-Yang, W.Y., Huang, F.Y., Zhao, Y., Xu, H.J., and Zhu, Y.G. 2015. Antibiotic resistome and its association with bacterial communities during sewage sludge composting. *Environ. Sci. Technol.* **49**, 7356–7363.

Traversi, D., Villa, S., Lorenzi, E., Degan, R., and Gilli, G. 2012. Application of a real-time qPCR method to measure the methanogen concentration during anaerobic digestion as an indicator of biogas production capacity. *J. Environ. Manage.* **111**, 173–177.

Truong, D.T., Franzosa, E.A., Tickle, T.L., Scholz, M., Weingart, G., Pasolli, E., Tett, A., Huttenhower, C., and Segata, N. 2015. MetaPhlAn2 for enhanced metagenomic taxonomic profiling. *Nat. Methods* **12**, 902–903.

Wagner, M. and Loy, A. 2002. Bacterial community composition and function in sewage treatment systems. *Curr. Opin. Biotechnol.* **13**, 218–227.

Wang, Z., Zhang, X.X., Huang, K., Miao, Y., Shi, P., Liu, B., Long, C., and Li, A. 2013. Metagenomic profiling of antibiotic resistance genes and mobile genetic elements in a tannery wastewater treatment plant. *PLoS One* **8**, e76079.

Wu, Y., Cui, E., Zuo, Y., Cheng, W., Rensing, C., and Chen, H. 2016. Influence of two-phase anaerobic digestion on fate of selected antibiotic resistance genes and class I integrons in municipal wastewater sludge. *Bioresour. Technol.* **211**, 414–421.

Xu, J., Xu, Y., Wang, H., Guo, C., Qiu, H., He, Y., Zhang, Y., Li, X., and Meng, W. 2015. Occurrence of antibiotics and antibiotic resistance genes in a sewage treatment plant and its effluent-receiving river. *Chemosphere* **119**, 1379–1385.

Yang, Y., Jiang, X., Chai, B., Ma, L., Li, B., Zhang, A., Cole, J.R., Tiedje, J.M., and Zhang, T. 2016. ARGs-OAP: online analysis pipeline for antibiotic resistance genes detection from metagenomic data using an integrated structured ARG-database. *Bioinformatics* **32**, 2346–2351.

Yang, Y., Li, B., Ju, F., and Zhang, T. 2013. Exploring variation of antibiotic resistance genes in activated sludge over a four-year period through a metagenomic approach. *Environ. Sci. Technol.* **47**, 10197–10205.

Yang, Y., Li, B., Zou, S., Fang, H.H.P., and Zhang, T. 2014. Fate of antibiotic resistance genes in sewage treatment plant revealed by metagenomic approach. *Water Res.* **62**, 97–106.

Yin, X.L., Jiang, X.T., Chai, B.L., Li, L.G., Yang, Y., Cole, J.R., Tiedje, J.M., and Zhang, T. 2018. ARGs-OAP v2.0 with an expanded SARG database and Hidden Markov Models for enhancement characterization and quantification of antibiotic resistance genes in environmental metagenomes. *Bioinformatics* **34**, 2263–2270.

Yoo, K., Yoo, H., Lee, J.M., Shukla, S.K. and Park, J. 2018. Classification and regression tree approach for prediction of potential hazards of urban airborne bacteria during Asian dust events. *Sci. Rep.* **8**, 11823.

Zhang, J., Chen, M., Sui, Q., Tong, J., Jiang, C., Lu, X., Zhang, Y., and Wei, Y. 2016a. Impacts of addition of natural zeolite or a nitrification inhibitor on antibiotic resistance genes during sludge composting. *Water Res.* **91**, 339–349.

Zhang, J., Chen, M., Sui, Q., Wang, R., Tong, J., and Wei, Y. 2016b. Fate of antibiotic resistance genes and its drivers during anaerobic co-digestion of food waste and sewage sludge based on microwave pretreatment. *Bioresour. Technol.* **217**, 28–36.

Zhang, T., Yang, Y., and Pruden, A. 2015. Effect of temperature on removal of antibiotic resistance genes by anaerobic digestion of activated sludge revealed by metagenomic approach. *Appl. Microbiol. Biotechnol.* **99**, 7771–7779.

Zhang, T., Zhang, X.X., and Ye, L. 2011. Plasmid metagenome reveals high levels of antibiotic resistance genes and mobile genetic elements in activated sludge. *PLoS One* **6**, e26041.