Title
Fate of Antibiotic Resistance Genes in Activated Sludge Processes and Anaerobic Digestion at Six Wastewater Treatment Plants in California

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Publication Date
2021

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Fate of Antibiotic Resistance Genes in Activated Sludge Processes and Anaerobic Digestion at Six Wastewater Treatment Plants in California

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Civil Engineering

by

Renjie Li

2021

ABSTRACT OF THE DISSERTATION

Fate of Antibiotic Resistance Genes in Activated Sludge Processes and Anaerobic Digestion at Six Wastewater Treatment Plants in California

by

Renjie Li

Doctor of Philosophy in Civil Engineering

University of California, Los Angeles, 2021

Professor Michael K. Stenstrom, Co-Chair

Professor Jennifer A. Jay, Co-Chair

Antibiotic resistance has been a topic of increasing concern for several decades. Wastewater treatment plants are one of the potential sources of antibiotic resistance genes (ARGs) and antibiotic resistant bacteria. Activated sludge processes (ASP) are the most widely used biological secondary treatment processes for wastewater while anaerobic digestion (AD) processes are used for treating biosolids, both of which play an import role in degrading and removing most of the organic wastes. In our study, we investigated six wastewater treatment plants located near Los Angeles operated at long and short solids retention times (SRTs) for the activated sludge processes and under thermophilic and mesophilic conditions for the anaerobic digestion processes. The prevalence and dynamics of five selected ARGs (*sul*1, *sul*2, *tet*A, *tet*W, and *erm*B), class 1 integron

(*intI*1), and the total 16S rRNA were selected and evaluated by means of quantitative polymerase chain reaction (qPCR) in influent and effluent samples in six activated sludge processes and in digester feed and digested sludge in four anaerobic digestion processes.

Among the six full-scale activated sludge processes, the average removal efficiency of all target ARGs was 99% (1.98 log removals) at the three long SRT ASPs, which was higher than the 95% (1.29 log removals) observed at three short SRT ASPs. The relative gene abundance per gram volatile suspended solids (VSS) was introduced to study the relationship between organic materials and ARG levels and reinforced the hypothesis that both long and short SRT ASPs can remove ARGs and long SRT ASP also showed higher removal efficiencies. In addition, significant ($p < 0.05$), strong, and positive ($r > 0.7$) correlations were found between VSS concentrations and the total 16S rRNA. Furthermore, potential horizontal gene transfer (HGT) was indicated at all the six plants' ASPs in terms of the correlations between *intI*1 and target ARGs. These correlations were found more frequently at short SRT plants than at long SRT plants. In general, our results concluded that ASPs could remove the absolute gene abundance of selected ARGs but might in some cases increase the relative gene abundance. Also, ASP operating at long SRT achieved higher and less variable removal efficiencies than at short SRT.

Among the four full-scale anaerobic digestion processes, most of the ARGs' absolute abundance were significantly ($p < 0.05$) removed up to 1.58 logs. The lowest absolute gene abundance of all target ARGs and 16S rRNA were found at AD3, the only thermophilic anaerobic digestion process. And the relative gene abundance per VSS for wastewater samples were similar to the abundance gene abundance per g ds for sludge samples. There was evidence for HGT at all the four AD

processes, especially *sul*1, *sul*2, and *erm*B, which were all found to be significantly and positively correlated with *intI*1. Therefore, anaerobic digestion processes can remove the target ARGs but potential HGT may have occurred.

Ten heavy metal contents (barium, copper, iron, manganese, lead, rubidium, strontium, titanium, zinc, and zirconium) during three anaerobic digestion processes were also studied and the relationships between these heavy metal contents as well as phosphorus and five ARGs as well as *intI*1 were evaluated. In the results: Fe was observed with the highest heavy metal concentration while Rb was the lowest heavy metal concentration at all the three AD processes; 14 out of 30 observations were decrease observations with the highest removal efficiency of 61% in AD4 for Mn but 16 out of 30 observations were increase observations with the highest increase percentage of 124% in AD6 for Sr; redundancy analysis showed that Zr and Ba significantly ($p < 0.05$) explained the changes in ARGs, which accounted for 54.9% of the total variation in the environmental factors.

The dissertation of Renjie Li is approved.

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2021

Dedicated to my parents Yuxiang Ren and Shuqing Li

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Acknowledgements

This dissertation would have not been accomplished without the support of my professors, my family, and my friends.

I would like to express my deepest gratitude to my advisor Dr. Michael. K. Stenstrom for the continuous support of my Ph.D study and related research, for his inspiration, patience, motivation, and immense knowledge. I appreciate him as my academic advisor as well as my mentor in my life in the last six years. His patience, motivation, and immense knowledge resolving challenge inspires me and will always be assets in my life.

Besides my advisor, I would like to thank the rest of my committee: Dr. Sanjay Mohanty, Dr. Irwin Suffet, and Dr. Jennifer A. Jay, for their insightful comments, suggestions, and encouragements. I would like to specifically extend my sincere gratefulness to Dr. Jennifer A. Jay for allowing me to use her lab during the whole six years with guidance and unparalleled support.

I would like to thank the following lab mates for the stimulating discussions and selfless helps: Wayne Hung, Megyn Rugh, Vanessa Thulsiraj, and Victoria Whitener. I would also like to thank all my friends for their support and encouragements. I would specially like to thank Yuge Bian for happy distractions to rest my mind outside of my research during the last two of the toughest years.

I gratefully acknowledge the funding received towards my Ph.D from the Water Research Foundation 5028 Project. I would also like to thank all the members of the wastewater treatment plants for their assistance and collaborations.

Finally, I would like to give the deepest gratefulness to my parents for their unconditional love, financial and emotional support, and encouragements.

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Echeverria-Palencia CM, Thulsiraj V, Tran N, Ericksen CA, Melendez I, Sanchez MG, Walpert D, Yuan T, Ficara E, Senthilkumar N, Sun F, Li R, Hernandez-Cira M, Gamboa D, Haro H, Paulson SE, Zhu Y, Jay JA. Disparate Antibiotic Resistance Gene Quantities Revealed across 4 Major Cities in California: A Survey in Drinking Water, Air, and Soil at 24 Public Parks. ACS Omega. 2017 May 31;2(5):2255-2263. doi: 10.1021/acsomega.7b00118. Epub 2017 May 24. PMID: 30023659; PMCID: PMC6044758.

Chapter 1: Fate of Antibiotic Resistance Genes and Antibiotic-Resistant Bacteria in Water Resource Recovery Facilities

1.1. Introduction

Antibiotic failure due to increasing antibiotic resistance is a worldwide threat to public health. According to the Center for Disease Control (CDC), in 2019 in the United States alone, about 2,900,000 people were infected with antibiotic-resistant bacteria, and 32,000 died as a result (CDC, 2013). Globally, current antibiotic resistance accounts for at least 700,000 lives lost per year, and it is projected that an unabated rise in antimicrobial resistance may lead to 10 million deaths per year by 2050 (Liu et al., 2016; May, 2016; O'Neill December, 2014). Antibiotic overuse is one of the most important causes for the loss of antibiotic effectiveness. Even in developed countries, where antibiotic usage in medicine is strictly supervised, they are still widely overused in human daily life and on farms. In 2014, for example, outpatient healthcare providers in the United States wrote over 266 million antibiotic prescriptions, amounting to 835 antibiotic prescriptions for every 1,000 people. However, it is believed that at least 30 percent of oral antibiotics prescribed may be unnecessary (CDC, 2017).

In food animal production, approximately 9.7 million kilograms, or 21.4 million pounds, of antibiotics considered important for human use were sold for use in animal agriculture in 2015, which is 26 percent increased since 2009 (CDC, 2017). According to the report of Statista, for European Union's countries, Cyprus had the greatest antibiotics usage in 2015, which was almost 400 mg of antibiotics for every kilogram of meat produced, followed by Italy with 341 mg/kg and Hungary with 246 mg/kg in Europe. The average consumption for the European Union counties

was 152 mg/kg in livestock production. Table 1-1 shows the total antibiotic consumption and the consumption per kg of meat (beef, pork, chicken, and lamb) in some developed countries and indicates the antibiotics used in the meat production. The United States had an annual antibiotic consumption of 266 mg/kg from meat consumption, which was 100 mg/ kg more than the average of the European Union countries. Meat production is not the only source of antibiotics, and large amounts of antibiotics are utilized in a clinical setting. In 2015 in the United States, healthcare providers wrote 269.4 million antibiotic prescriptions, which is equivalent to 838 anti- biotic prescriptions per 1,000 persons (CDC, 2015). Assuming one adult weighs 70 kg and a typical amoxicillin dosage of 500 mg (because amoxicillin was the most commonly used antibiotic in the United States in 2015, according to Outpatient Antibiotic Prescriptions—United States, 2015), the consumption is 5.98 mg/Kg per 500 mg dosage. Assuming a typical pre- scription instruction every 8 hours for 1 week, the consumption is 125.6 mg/kg per prescription (MAYO CLINIC, 2017). The average number of prescriptions was one prescription for one person in the United States in 2015. Therefore, an adult consumed on average 8,792 mg of antibiotics in 2015. However, the annual possible antibiotic intake from meat production was approximately 30.5 g/person in 2015, which is three times more than consumption from antibiotic prescriptions.

Table 1-1. Antibiotic consumptions as a function of meat production in different countries

Country	Antibiotic Consumption (mg)	Ratio (mg/kg)
U.S.	9.7×10^{12}	266.2
E.U.	6.7×10^{12}	152.0
Cyprus	2.9×10^{10}	396.5
Italy	1.2×10^{12}	341.0
Hungary	2.0×10^{11}	245.5
Spain	6.5×10^{11}	242.0
Germany	1.1×10^{12}	204.8
Belgium	2.9×10^{11}	161.1

UK	2.2×10^{11}	62.0

Note: 1. The meat consumption data are cited from OECD data (https://data.oecd.org/agroutput/meat-consumption.htm) and statistical data (https://www.statista.com/statistics/679528/per-capita-meat-consumption-european-union-eu/).

In the EU, the level of usage exists in spite of their 20-year history of bans or partial bans on the use of antimicrobials for growth promotion. The reduced use of antibiotics may be the reason for the decreasing bacterial resistance to antibiotics in some European countries. For example, *Enterococcus faecium* from broiler chickens in Denmark are showing decreased resistance to avilamycin and macrolide antibiotics (tylosin is one type of macrolide antibiotic; Maron, Smith, & Nachman, 2013). In the United Kingdom, studies following the ban on antibiotic growth promoters in pigs showed that resistance to erythromycin in *Campylobacter coli* and *Enterococcus faecium* decreased from 85% in 1999–2000 to 36% in 2007 (Carol, Herman, & Christina, 2011). These results are very encouraging and may be a good example for the United States in anticipation of future regulations. Apart from chemical pollution caused by antibiotics themselves, widespread usage accelerates the development of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB), which can influence the health of both humans and animals. The World Health Organization in 2017 published its first ever list of antibiotic-resistant "priority pathogens," which is a catalogue of 12 families of bacteria that pose the greatest threat to human health (Table 1-2), which reinforces the urgency and need for the study of the emergence and fate of antibiotic resistance genes and bacteria and their threats to the environment and human health (Tacconelli et al., 2018). This list is guided by both small and large pharmaceutical companies applying a multicriteria decision analysis, a method that incorporates both expert opinion and evidence-based data in a transparent, explicit, and deliberative fashion (Tacconelli & Magrini, 2017). The following ten criteria were selected to perform pairwise comparison: all-cause mortality,

healthcare and community burden, prevalence of resistance, 10-year trend of resistance, transmissibility, preventability in hospital and com- munity settings, treatability, and current pipeline. ARGs and ARB are clearly starting to be a serious concern.

Table 1-2. WHO priority pathogens list for Research & Development (R&D)

Priority[1]	Bacteria	Antibiotic-resistant
Critical	*Acinetobacter baumannii*	carbapenem-resistant
	Pseudomonas aeruginosa	carbapenem-resistant
	Enterobacteriaceae	carbapenem-resistant, ESBL-producing
High	*Enterococcus faecium*	vancomycin-resistant
	Staphylococcus aureus	methicillin-resistant, vancomycin-intermediate and resistant
	Helicobacter pylori	clarithromycin-resistant
	Campylobacter spp.	fluoroquinolone-resistant
	Salmonellae	fluoroquinolone-resistant
	Neisseria gonorrhoeae	cephalosporin-resistant, fluoroquinolone-resistant
Medium	*Streptococcus pneumoniae*	penicillin-non-susceptible
	Haemophilus influenzae	ampicillin-resistant
	Shigella spp.	fluoroquinolone-resistant

Note: 1. The priority level is according to the urgency of need for new antibiotics.

Antibiotic-resistant bacteria spread via physical forces, such as wind, water environment, and soil, or biological forces, including human activities, animals, insects, and birds (Allen et al., 2010). These bacteria may also be transported from the environment to humans via direct or indirect contact (Allen et al. 2010, Davies & Davies, 2010; Zhang, Zhang, & Fang, 2009). Therefore, knowing the origins of ARGs to the environment plays an important role in understanding transport mechanisms. Hospitals and farms are two major sources (Baquero, Martinez, & Canton,

2008; Timraz, Xiong, Al Qarni, & Hong, 2017) introducing ARGs and ARB to the environment. The discharge of antimicrobial agents, detergents, disinfectants, and residues from industrial pollution such as heavy metals also contributes to the evolution and spread of such resistant organisms in the water environment (Baquero et al., 2008). Water resource recovery facilities are major receptors of ARGs and are potentially major sources of ARGs to the environment. Treated effluents are usually discharged to lakes, rivers, and oceans, or reused for human activities such as agricultural or landscape irrigation and reclamation with potential human contact. The City of Los Angeles proposed an indirect potable reclamation program that will eventually result in deliberate introduction of highly treated domestic wastewater into drinking water in order to reduce dependence on imported water. Thus, water resource recovery facilities play an important role in the water field. Water resource recovery facilities are rarely equipped with the processes specifically for removing antibiotics, ARGs, or ARB. For this reason, many current studies focus on the efficiency of the existing processes to remove ARGs or ARB (Gerrity, McLain, Rock, Dickenson, & Batista, 2018), which is also a major objective of this literature review.

This paper summarizes what has been reported about the performance of different types of water resource recovery facilities in removing ARGs and ARB, with particular emphasis on fate of ARGs and ARB in different processes. In addition, the factors potentially affecting the removal efficiency of water resource recovery facilities are discussed.

1.2. Types of environmental antibiotic resistance genes

Long-term applications of antibiotics in the protection of humans and animals and in agricultural growth promotion have exerted a major impact on bacterial communities. These applications have

resulted in bacteria possessing various resistances to antibiotics that are generally conferred by ARGs. The following mechanisms create increasing resistance in a bacterial community: (a) target bypass, which allows some bacteria to become refractory to specific antibiotics by bypassing the inactivation of a given enzyme; (b) efflux pump, which is the mechanism that prevents the antibiotic from penetrating the outer and/or cytoplasmic membrane by decreasing the uptake of the antimicrobial molecule (Munita & Arias, 2016); (c) antibiotic inactivation, which inactivates the active antibiotic molecule directly; and (d) target modification, which modifies action sites of antibiotics (Džidić, Šušković, & Kos, 2008; Munita & Arias, 2016; Zhang et al., 2009). Furthermore, the resistance of certain antibiotics might be associated with more than one mechanism.

Generally, several types of antibiotic resistance genes (ARGs) have been found in the environment, including genes resistant to tetracycline, sulfonamide, aminoglycoside, macrolide–lincosamide–streptogramin (MLS), chloramphenicol, vancomycin, and β-lactam antibiotics. Each type has its own characteristics and is discussed in detail.

ARGs related to tetracycline

Tetracycline-resistant bacteria emerge in environments with the introduction of tetracycline, and more than 22 tetracycline (*tet*) or oxytetracycline (*otr*) resistance genes have been found in bacterial isolates from water environments. There have been at least 38 different *tet* genes and *otr* resistance genes characterized to date. Of these genes, 23 tetracycline resistance genes code for efflux pump mechanism, 11 genes contain ribosomal protection proteins (target modification mechanism), and three genes inactivate enzymes (Zhang et al., 2009). Most environmental *tet*

genes code for transport proteins, which pump the antibiotics out of the bacteria cell and keep the intercellular concentrations low to make ribosomes function normally (Roberts, 2002). Studies showed that tetracycline resistance genes were detected above background levels in the sediments near a fish farm, even after antibiotic usage had stopped 6 years before sampling, suggesting the high persistence of tetracycline resistance genes in aquatic environments (Gao, Munir, & Xagoraraki, 2012). They determined that 7% and 8% of culturable bacteria are found to be tetracycline-resistant in the pre- and post-chlorinated samples of a water resource recovery facility, respectively, and are discharged into a nearby river water where similar percentages of culturable bacteria were found to be resistant to tetracycline (Munir, Wong, & Xagoraraki, 2011). Recently, the tetracycline resistance genes including *tet*A, *tet*B, *tet*BP, *tet*G, *tet*M, *tet*O, *tet*Q, *tet*S, *tet*T, *tet*W, *tet*X, and *tet*Z have been detected in anthropogenic or pristine environments; of these, *tet*M, *tet*O, tetS, *tet*Q, and *tet*W contain coding for ribosomal protection proteins and have been detected in microbial communities of water resource recovery facility, hospital or animal protection wastewaters, and even in natural water environments (Munir et al., 2011). Additionally, Pruden, Pei, Storteboom, and Carlson (2006) reported that *tet*B, *tet*P, *tet*O, *tet*S, *tet*T, and *tet*W were detected in the presence of ditch water and dairy lagoon water in Northern Colorado in the United States. Tetracycline-resistant bacteria have been frequently detected in many water sources.

ARGs related to sulfonamide

Sulfonamides are the first antibiotics developed for large-scale clinical use, which target dihydropteroate synthase (DHPS), a catalytic enzyme in the folic acid biosynthesis pathway (Alekshun & Levy, 2007). They found that sulfonamides compete with the structural analog *p*-aminobenzoic acid for binding to DHPS, thus preventing bacterial growth by inhibiting the

formation of dihydrofolic acid. Resistance to sulfonamides in *Escherichia coli* can result from mutations in the chromosomal DHPS gene (*folP*) or more frequently from the acquisition of an alternative DHPS gene (*sul*), whose product has a lower affinity for sulfonamides (Perreten & Boerlin, 2003). Different mechanisms have been found to confer sulfonamide resistance, mostly based on changes in the *sul* genes and mediation by mobile elements (Zhang et al., 2009). Four *sul* genes (*sul*I, *sul*II, *sul*III, and *sul*A) have been found in bacteria of environmental origin. *Sul*I and *sul*II have been detected in bacteria isolates from fecal slurry of dairy farms, water or sediments of aquaculture areas, and even from the river or seawater without evidence pollution. In China, *sul*I and *sul*III were detected in both rural wastewater treatment and municipal water resource recovery facilities in Zhejiang province (Chen & Zhang, 2013). This suggests that sulfonamide genes are worthy of concern.

ARGs related to macrolide

Antibiotics of macrolide are often investigated simultaneously for microbial resistance, because certain macrolide resistance genes (*erm*) encode resistance to at least two macrolide, lincosamide, and streptogramin antibiotics, although they are structurally unrelated to macrolide antibiotics (Roberts et al., 1999). Until 2009, more than 60 different genes, which confer total resistance to one or more of the macrolide–lincosamide–streptogramin (MLS) antibiotics, have been identified, including the genes associated with ribosomal RNA (rRNA) methylation, efflux, and inactivation. MLS resistance is mostly mediated by rRNA methylases (encoded by *erm* genes), which methylate the adenine residues to prevent the three antimicrobials from binding to ribosomal protein (Zhang et al., 2009). The *erm* genes can easily be transferred from one host to another, since they are usually acquired and associated with mobile elements, such as plasmids and transposons. Chen

and Zhang (2013) detected several *erm* genes in *Enterococcus* spp. isolated from poultry wastewater and environmental DNA extracted from livestock manures. Six classes of *erm*A, B, C, F, T, and X genes have been detected and quantified in the samples from animal production manures, lagoons, and a biofilter system treating hog house wastewater. Among the macrolide resistance determinants, *erm*B is considered the most prevalent gene in environmental microorganisms, especially in the strains of *Enterococcus* and *Streptococcus spp* (Zhang et al., 2009).

ARGs related to other types

Other types of ARGs, including those conferring resistance to aminoglycoside, vancomycin, and β-lactam antibiotics exist in the environment as well, which are potential pollutants to human and animals. More than 50 modification enzymes have been found so far (Chen & Zhang, 2013). These enzymes are divided into three groups based upon their biochemical actions on the aminoglycoside substrates. These include acetyltransferases, phosphotransferases, and nucleotidyltransferases (adenylyltransferases), which are encoded by three types of genes, namely, *aac*, *aph*, and ant (*aad*), respectively. Vancomycin resistance emerged first in enterococci. Recently, the resistance has also been detected in *Staphylococcus aureus*. So far, six types of vancomycin resistance genes (*van*) are known, and *van*A and *van*B are the most prevalent ones in water environments. Thirty-five ARGs are listed in Table 1-3 and discussed in the following sections, although there are more ARGs that have been found in the water resource recovery facility and natural environment. Major biological sources for individual ARGs are summarized as suggestions for future studies of antibiotic-resistant bacteria detection. However, other bacteria that are not listed in the table may contain these ARGs as well.

Table 1-3. Antibiotic resistance genes and their biological sources

ARG	Category	Biological source	ARG	Category	Biological source
*tet*A	Tetracycline resistance genes	*Aeromonas, Alcaligenes, Arthrobacter, Comamonas, Escherichia, Listeria, Pseudomonas, Salmonella, and Vibrio; Plasmids pB10, pTB11 and pRSB101*	bla_{TEM}	β-Lactam resistance genes	*Escherichia*
*tet*B		*Afipia, Alcaligenes, Arthrobacter, Burkholderia, Escherichia, Pseudomonas, Serratia, Staphylococcus, and Vibrio*	bla_{VIM}		*Pseudomonas aeruginosa*
*tet*C		*Aeromonas, Alcaligenes, Arthrobacter, Brevibacterium, and Pseudomonas*	bla_{SHV}		*Klebsiella pneumoniae*
*tet*E		*Aeromonas, Pseudoalteromonas, and Vibrio*	bla_{CTX-M}		*Klebsiella pneumoniae, Escherichia coli, Pseudomonas aeruginosa*
*tet*G		*Pseudomonas; microbial community*	*amp*(A)		*N/A*
*tet*M		*Aeromonas, Bacillus, Escherichia, Lactococcus, Pseudoalteromonas, and Vibrio; microbial community*	*mec*A		*Staphylococcus*
*tet*O		*Paenibacillus, Pseudoalteromonas, Shewanella, Sporosarcina, and Vibrio; microbial community*	*dfrA1*	Dihydrofolate reductase encoding genes	*Aeromonas, Escherichia, and Salmonella*
*tet*Q		*Microbial community*	*dfrA12*		*Aeromonas, Escherichia, and Salmonella*

*tet*S		*Lactococcus and Vibrio; microbial community*	*erm*B	Macrolide resistance genes	*Bacillus and Enterococcus*
tetT		*Microbial community*	*erm*F		*Microbial community*
*tet*W		*Microbial community*	*mef*A		*Streptococcus pyogenes, Enterococcus faecalis, Bacteroides ovatus, Neisseria gonorrhoeae, Exiguobacterium sp.*
*tet*X		*clostridium tetani, bacteriodes fragilis, clostridium sp. RKD, riemerella anatipestifer (Moraxella anatipestifer), actinoplanes sp., corallococcus coralloides Actinomycetales, Afipia, Brevibacterium, Burkholderia, Dietzia, Leucobacter, and Microbacterium*	*ere*A	Erythromycin encoding genes	*Escherichia coli, Achromobacter denitrifican, Zobellia galactanivorans, Proteus mirabillis, Salmonella choleraesuis, Aeromonas hydrophila, Vogesella fluminis*
*tet*Z			*mar*A	Multiple antibiotic resistance genes	*Salmonella enteritidis*
*sul*I	Sulfonamide resistance genes	*Aeromonas, Escherichia, and Listeria; Plasmids pB2, pB3, pB8, and pB10; Microbial community*	*qnr*S	fluoroquinolone resistance genes	*Escherichia coli, Providencia rettgeri, Morganella morganili, pseudomonas guangdongensis*
			*van*A	Vancomycin resistance genes	*Enterococcus and Staphylococci*
*sul*II		*cinetobacter, Escherichia, Salmonella, and Vibrio; Microbial community*	*str*A	Aminoglycoside resistance genes	*Listeria, Salmonella and Vibrio; Plasmids pB4 and pB10*
*sul*III		*Escherichia; Microbial community*	*str*B		*Salmonella and Vibrio; plasmids pB4 and pB10*
*sul*A		*Microbial community*	*aphA2*		*Escherichia*

Note: Biological sources are cited from http://www.uniprot.org/.

Techniques for the detection of ARGs

Selecting methods to detect ARGs and determine their treatment efficiency is a critical research decision to positively obtain results of the ARGs from water resource recovery facilities. Wastewater samples may be collected before and after treatment processes and stored at appropriate temperatures for transportation to laboratories. Wastewater sources and types of water resource recovery facilities are important factors to understand the results. Samples are usually concentrated with vacuum filtration apparatus onto different filters with various pore sizes or materials depending on water quality and study objectives. In addition, DNA extraction is usually performed, and there are several alterative DNA extraction instruments used in previous research with their own specific protocols (Guo, Li, Yang, Yang, & Yin, 2014; Munir et al. 2011). There are a number of methods for detecting and characterizing ARGs and ARB, but the four most commonly used methods are DNA hybridization, PCR (simple and multiplex PCR), quantitative PCR, and DNA microarray. A less commonly used method is Illumina HiSeq 2000 (Aydin, Ince, & Ince, 2015). Molecular hybridization has been used to detect the presence or absence of specific ARGs for three decades and is still often applied to distinguish different ARGs within one group or to identify the presence of specific genes in certain environments (Zhang et al., 2009). Fluorescence in situ hybridization (FISH), an important non-radiolabeled method, has been established and applied to detect clinical microbial resistances and for the rapid identification of macrolide resistances caused by ribosomal mutations (Russmann et al., 2001). PCR, including simple and multiplex PCR, has been widely used in both pure cultures and mixed environmental samples to detect different kinds of ARGs, such as resistance encoded by tetracycline and β-lactam (Jacobs & Chenia, 2007; Taviani et al., 2008). The multiplex PCR method has been often used to simultaneously detect more than one ARG, which can reduce time and effort compared to simple PCR. DNA microarray is a hybridization application that analyzes gene expression or screens

samples for single nucleotide polymorphisms (SNPs). This allows DNA and/or RNA hybridization analysis to be carried out in micro- miniaturized highly parallel formats (Heller, 2002). One advantage of DNA microarray is that microarray allows detection of antibiotic resistance determinants within a few hours, saving time and being used as a convenient tool supporting conventional resistance detection methods (Antwerpen, Schellhase, Ehrentreich-Foerster, Witte, & Nuebel, 2007). The core and one of the most widely used methods is quantitative polymerase chain reaction (qPCR) utilizing fluorescent DNA-intercalating dyes (e.g., SYBR Green) and/or fluorophore-tagged hybridization dyes (e.g., TaqMan probes) to determine the presence and absence of targeted ARGs after extraction and normalization (Dodd, 2012). It is an enabling technology of molecular diagnostics with a capacity to detect and measure minute amounts of nucleic acids in a wide range of samples from numerous sources (Bustin et al., 2009). Primers may be selected to detect targeted genes with corresponding melting temperature applied during the thermal cycles determined based on genes for running qPCR. Other methods may also be applicable, but qPCR has been the major application in previous studies (Li, Li, & Zhang, 2015). Most of the selected papers in this review used qPCR to amplify targeted DNA molecules. Significant statistical analysis of the experimental results is required, and techniques have been demonstrated (Liu et al., 2014; Narciso-da-Rocha, Varela, Schwartz, Nunes, & Manaia, 2014; Pruden et al., 2006). Measurement units are generally in gene copies/16S. Correlations between several factors are assessed using redundant analysis.

1.3. Antibiotic resistance genes in water resource recovery facilities

Effluents from water resource recovery facilities are an important, direct receptor of ARGs and ARB. Wastewater influents come from various genetic reactors, such as the human and animal

microbiota, hospitals, long-term healthcare facilities, farms, or other places where susceptible individuals are in close proximity and exposed to bacteria exchange. Farms and hospital/household wastewater are two major sources of influents. Municipal treatment plants are one of the treatment systems where ARGs are most frequently detected, where wastewaters mostly originate from household wastes and toilets. Guo et al. (2014) showed that incomplete metabolism in humans and improper disposal of antibiotics have been a main source of antibiotics released into the environment through municipal water resource recovery facilities. There are some other types of water resource recovery facility that have been optimized for different treatment purposes, such as industrial water resource recovery facilities and agricultural water resource recovery facilities. However, there are currently no water resource recovery facilities specifically designed to remove ARGs and ARB, due to many factors such as unknown mechanisms for removing ARGs and ARB and potentially high costs of facility improvements. Fortunately, many previous studies have reported that some current treatment plant designs may remove ARGs and ARB, at least partially, and different processes have their own characteristics for different types of ARGs. For example, studies have found that solids retention time may be an important factor to the treatment removal efficiency of ARGs in activated sludge processes (De Sotto et al., 2016; Neyestani, Dickenson, McLain, Obergh, et al., 2017; Neyestani, Dickenson, McLain, Robleto, et al., 2017). Characteristics of different treatment processes are discussed below in detail.

The presence of ARGs in treatment plants is often documented by comparing the detections in both influents and effluents. Naquin, Shrestha, Sherpa, Nathaniel, and Boopathy (2015) detected ARGs in a treatment plant in Thibodaux, Louisiana. ARGs were detected in the raw wastewater influent and the treated wastewater after being treated with UV disinfection from February 2014

until November 2014 (Naquin et al., 2015). ARGs, including *erm*B, *sul*I, *tet*A, *tet*X, and *mec*A, (conferring resistance to erythromycin, sulfonamide, tetracycline, and methicillin, respectively) were all detected in both the raw and treated wastewater. Their results showed that the levels of these ARGs from raw wastewater were higher than those from effluents of the water resource recovery facilities, and they hypothesized that the UV disinfection might play an important role in reducing the ARGs.

Munir et al. (2011) also studied five water resource recovery facilities and found that the concentrations of two tetracycline resistance genes (*tet*W and *tet*O) and one sulfonamide resistance gene (*sul*I) declined significantly in the treated effluent compared to the raw influents. They also concluded that water resource recovery facilities are a potential source of ARGs as well as a potential way to remove ARGs and ARB (Munir et al., 2011).

The other main potential source of ARGs flowing into water resource recovery facilities, especially in urban areas, is hospital wastewater where antibiotics are frequently prescribed and toilets containing excreta with antibiotics are flushed into wastewater systems. Thus, detection of ARGs in municipal water resource recovery facilities is frequent. Narciso-da-Rocha et al. (2014) detected three ARGs in a hospital-urban water resource recovery facility system: bla_{TEM} that is associated with Enterobacteriaceae; *van*A that is frequently used in clinical isolates, and *mar*A that is multiple antibiotic-resistant. They observed that the relative abundance of bla_{TEM} (ratio of bla_{TEM} to 16S rRNA) decreased, while the ratio of *van*A to 16S rRNA did not vary and the ratio of *mar*A to 16S rRNA increased (Narciso-da-Rocha et al., 2014). Although the overall results are not conclusive,

the impacts of water resource recovery facilities in municipal areas are a concern for ARGs release to the environment.

Although many studies have found that water resource recovery facilities may remove a fraction of ARGs and ARB, the study of individual processes is more useful and practical to determine mechanisms of removal and potentially to optimize removal. Processes found and reported are described in the following sections.

1.4. Antibiotic resistance genes in physicochemical treatment processes

ARGs in coagulation, sedimentation, and clarification

Physicochemical treatment processes at water resource recovery facilities are nonbiological processes and include coagulation, sedimentation, disinfection, and clarification. According to Guo et al. (2014) in the Yangtze River Delta, China, these processes only slightly reduced the relative abundance of ARGs, indicating poor ARG removal, although another study (Li, Sheng, Lu, Zeng, & Yu, 2017) in a full-scale water resource recovery facility found that coagulation with $FeCl_3$ or polyferric chloride removed certain amount of ARGs. Generally, these nonbiological treatment processes appear to be less able to remove antibiotic resistance genes and resistant bacteria than other processes.

ARGs in filtration process

Filtration is a widely used process in water resource recovery facilities and is capable of removing a variety of contaminants. Nevertheless, research is needed to understand its removal efficiency

for ARGs. One study (Tawfik El-Zanfaly, Reasoner, & Geldreich, 1998) found that both sand filters and granular activated carbon columns reduced certain amounts of ARGs in a pilot water resource recovery facility. Other workers (Breazeal, Novak, Vikesland, & Pruden, 2013) showed that membranes of 100 kDa or smaller achieved significant removal of ARGs. They found that membrane filtration of deionized water spiked with ARGs removed them, and interestingly, the addition of wastewater containing protein, polysaccharide, and total organic carbon improved removal efficiency. They also found that 0.1-um alumina membranes removed ARGs from spiked deionized water more effectively than the more common 0.1-um polyvinylidene fluoride membranes. The addition of wastewater materials enhanced removal efficiency of polyvinylidene membranes more than alumina membranes. Interestingly, however, this improvement was only observed in the presence of wastewater materials. Filtration can be regarded as a potential ARGs and ARB removal processes.

ARGs in chlorination process

Chlorination, one of the most widely used processes in water resource recovery facilities, disinfects the effluent from upstream processes, by oxidation of nucleic acids and cell membranes of microorganisms (Zhang, Zhuang, et al., 2015). Owing to its ability to destroy microorganisms, it can remove ARGs as well.

Guo et al. (2014) in seven municipal water resource recovery facilities in China found that chlorine was effective in attenuating total absolute quantities for most resistance genes, especially for *sul*I, *sul*II, *tet*G, and *tet*A, with the removal per- centage of more than 98%. Similarly, Zhang, Han, et al. (2015) and Zhang, Zhuang, et al. (2015) had the same results for the reduction of ARGs after

chlorination with free chlorine under laboratory conditions. Furthermore, they investigated the significant ARG removal at a concentration-contact time (CT) value of 450 mg (Cl_2) min/L, (30 mg/L free chlorine added from NaClO with 15 min contact time), especially for tetracycline and sulfonamide genes. They found that chlorination selectively removed certain ARGs but did not remove others including *sul*I, *sul*II, *tet*G, and *tet*A. Low-level genes (*tet*O, *tet*M, and *tet*W) were difficult to eliminate, and interestingly, these three genes, which were present in the influent wastewater, encoded ribosomal protection proteins, became much more prevalent after chlorination compared to their nonchlorinated abundance. They hypothesized that chlorination was effective for the majority of the bacterial community but might function as a selector for some chlorine-tolerant bacteria. Zhang, Han, et al. (2015) and Zhang, Zhuang, et al. (2015) hypothesized chlorination may affect bacterial populations differently based upon water properties and original ARG concentrations. Furthermore, they investigated the effect of NH_3-N on chlorination disinfection in removing ARGs and found that higher NH_3-N concentration (15mg/L) resulted in lower ARGs removal, which may be attributed to the form of the chlorine, which is more likely to be combined chlorine at higher NH_3- N. They also stated that in order to obtain ARG reduction in the presence of ammonia the application of Cl_2: NH_3-N mass ratio should be greater than 7.6:1, or beyond the break point. Chlorination may be an effective process for the removal of ARGs.

Similarly, Guo et al. (2014) also found that chlorine dose significantly impacts the removal efficiency of ARB. They found that low chlorine CT (up to 40 free chlorine mg-min/L) highly promoted the frequency of conjugative transfer (one of the three major mechanisms for horizontal gene transfer) by twofold to fivefold for *E. coli*. The chlorine dose induced more pilus on the surface of conjugative cells, which acted as pathways for ARGs transfer. Also, the generated

chloramine stimulated the bacteria and improved the cell permeability (Guo et al. 2014). High-chlorine contact time (>80 free chlorine mg-min/L) greatly suppressed the frequency of ARG transfers. Chlorination enhanced the transfer of ARGs at low CT and decreased the transfer at high CT.

ARGs in ozonation process

Ozonation is a treatment process that can improve the removal of organic compounds as well as disinfecting bacteria and viruses. Molecular ozone is a strong oxidant and can form hydroxyl radials, another strong oxidant, as the ozone decomposes. Different researchers have observed widely varying results, often with contradictory conclusions. Ozonation effectively removed *erm*B, but it increased the amount of bla_{VIM} and *van*A at the same time (Alexander, Knopp, Dotsch, Wieland, & Schwartz, 2016). Another research group found that treatment with ozone decreased the total number of bacteria but increased the percentage of culturable antibiotic-resistant bacteria, especially such as bacteria *E. coli* isolates and *staphylococci* isolates (Luddeke et al., 2015). However, they found that ozonation followed by filtration led to an additional reduction of total ARB of these species by 0.8 and 1.1 log- units, as compared with the currently established removals in tertiary wastewater treatment (flocculation and filtration). Some researchers (Czekalski et al., 2016) also found that ozonation reduced selective ARGs with different doses suggesting that some ARGs may be more resistant to ozonation than others. Therefore, dosage may be one of the important considerations for future application of ozonation for target genes. Additionally, a research group (Sousa et al., 2017) compared the removal efficiency of ozonation under different contact times (15, 30, and 60 min) and found that the largest reduction occurred in the first 30 min and no quantifiable reduction of ARGs was observed after 60 min. Thus, they

hypothesized that a contact time of 30 min might be adequate but further studies should confirm this hypothesis.

The reason that ozonation inefficiently removes or even increases antibiotic resistance genes may be due to the environment of ozonation process. One research group (Alexander et al., 2016) found that a sublethal concentration of bacterial antibiotics in batch experiments in the presence of ozone stimulated the formation of intracellular, highly reactive hydroxyl radicals that contributed to the killing efficiency of bacterial antibiotics. Nevertheless, they also found that the induction of oxidative stress by bactericidal antibiotics may induce sublethal stress response mechanisms in bacteria that not only deal with the adaptation to the original drug target (antibiotic resistance development), but also activate antioxidative mechanisms and oxidative damage-associated responses, resulting a considerable advantage in surviving oxidative wastewater treatment. Experimental verification in full-scale treatment plants is needed to further validate their results.

ARGs in UV disinfection process.

UV radiation is a disinfection process growing in popularity because it reduces or eliminates the need to transport and store chlorine gas. It has been reported to remove ARGs or inactivate ARB in wastewaters. UV light can penetrate UV-transparent structures in cells and be sorbed by the nucleobases comprising DNA and RNA, removing ARGs. It has the additional advantage of producing few disinfection by-products (DBPs; Zhang, Zhuang, et al., 2015). Exposure to UV produced no or very small change in an *E. coli* strain's resistance to 384 amoxicillin (AMX; minimum inhibiting concentration (MIC) > 256 mg/L) and sul- famethoxazole (SMZ; MIC > 1,024 mg/L; Rizzo et al., 2013). This result is consistent with other ARB as well. Another research group

(Lee et al., 2017) found that several antibiotic-resistant bacteria were reduced by 34%–74% in two water resource recovery facilities after UV disinfection (27 mJ/cm^2), but found the amount of ARGs was not significantly reduced. McKinney and Pruden (2012) found that a UV dose of 200–400 mg/cm^2 was required to reduce ARGs in the magnitude of 3–4 orders in a filtered-wastewater matrix. In order to improve removal of ARGs at smaller dosage, it is possible to add certain chemicals in the wastewater to promote removal efficiency. For instance, H_2O_2 improved UV disinfection efficiency. The researchers found that UV/H_2O_2 at the dose of 50–130 mJ/cm^2 could achieve 4-log reduction of ARG concentration at pH 7 following first-order kinetics (Yoon et al., 2017). Because of OH (from H_2O_2), the ARGs' structural integrity of extracellular plasmid changed more rapidly in UV/H_2O_2 than without H_2O_2, which enhanced ARG destruction during UV/H_2O_2. Zhang, Han, et al. (2015) and Zhang, Zhuang, et al. (2015) also studied the sequential combination of chlorination and UV disinfection, resulting in the higher removal efficiency of ARGs than chlorination or UV disinfection alone. The improvement might be due to the decreased bioactivity affected by UV irradiation, causing the chlorine to react with the cells more readily. Furthermore, the sequential UV/ chlorination disinfection process has the advantages of reducing the demand dosages of chlorine and the possibility of reducing DBPs formation at the same level of removal efficiency (Zhang, Zhuang, et al., 2015). Therefore, combined UV with other oxidants may be better than UV alone for the future applications.

1.5. ARGs in biological treatment

Biological treatment processes use organisms to break down organic substances in wastewater and are often used as secondary treatment processes. They are the major processes of either enriching or removing antibiotic-resistant bacteria. Aerobic treatment processes and anaerobic treatment

processes are two major categories for biological treatment. For example, researchers studied the potential proliferation of ARGs in a sequencing batch reactor operated in a successive arrangement of aerobic and anoxic cycles and membrane bioreactor (MBR) in the full-scale treatment plants in China (Wang, Mao, Mu, & Luo, 2015). Other biological treatment processes, such as anaerobic digesters, have also been found to influence the amount of some kinds of antibiotic resistance genes under the positive influence of the influent sludge microbial composition (Miller, Novak, Knocke, & Pruden, 2016). Activated sludge process, anaerobic digestion, and constructed wetland are dis- cussed in detail.

ARGs in activated sludge process

The activated sludge process (ASP) is the most widely used process in water resource recovery facilities, especially for large treatment plants. The activated sludge process reduces organic matter in wastewater by using a complex biological community in the presence of oxygen to convert the organic matter to new cell mass, carbon dioxide, and energy. It also produces solids capable of bio- flocculating and settling out in a clarifier to produce an effluent low in biological oxygen demand (BOD) and total suspended solids (TSS). Removal of microcontaminants and their fates is an increasing concern, and researchers are investigating the removal of ARGs and ARB in activated sludge process (Korzeniewaska & Harnisz, 2018). Previous work has shown that certain ASP processes, those with longer solids retention time (SRT), are much better in removing trace contaminants than short SRT processes (Leu, Chan, & Stenstrom, 2012; Soliman et al., 2007). They identified more than 100 contaminants in literature and their experiments that are better or completely removed at longer SRT. Contradictory results from different researchers, as well as the

impacts of other factors such as food-to-mass ratio (F/M), are discussed in the following sections and show the need for more research to better understand the removals of ARGs and ARB.

In order to observe the ability of activated sludge processes to remove ARGs and ARB, some researchers simulated mechanisms in laboratory-scale reactors. Huang, Tang, Zhang, Xu, and Ren (2014) sampled activated sludge from a water resource recovery facility in Nanjing, China, and detected the abundance of tetracycline and sulfonamide resistance genes. They found that 11 *tet* genes were present in the sludge, including *tet*A, *tet*B, *tet*C, *tet*G, *tet*K, and *tet*P(A) encoding tetracycline efflux proteins; *tet*M, *tet*O, *tet*S, and *tet*W encoding ribosomal protection proteins; and *tet*X encoding enzymatic modification protein. The amount of all of the tet genes increased, while sulII was decreased (Huang et al., 2014). Similarly, in a bench-scale activated sludge process with 500 μg/L tetracycline influent, an increase in the proportion of *tet*A and *tet*B as well as both tetracycline-intermediate-resistant and tetracycline- resistant heterotrophic bacteria occurred (Yu, Liu, & Li, 2015). Yu et al. (2015) hypothesized that the tetracycline-resistant genes created by efflux pump spread earlier and more quickly to encode resistance to tetracycline in activated sludge, because efflux pump mechanisms generally show an easier response to resistance of tetracycline. Interestingly, when tetracycline resistance genes exist in activated sludge together with other ARGs such as *sul*II, the occurrence and diversity of nontetracycline ARGs decreased (Huang et al., 2014). They found that the considerable decrease in the abundance of *sul*II (from 83.49% to 14.76%) might be attributed to the domination of tetracycline in the sludge, owing to the higher abundance in the sludge without tetracycline stress than with tetracycline stress. They hypothesized that the tetracycline treatment reduced the abundance of *sul*II. *Sul*II is located on the genome of Salmonella enterica plasmid pCVM19633_110 and the genome *Pasteurella multocida*

plasmid pCCK381; tetracycline reduces the predominance of these two genomes in the biomass. In full-scale water resource recovery facilities, the observed removal ability of ARGs in the activated sludge process also varies. For example, full- scale wastewater treatment plants with activated sludge processes showed partial removal of 13 tetracycline-, sulfonamide-, streptomycin-, and β-lactam resistance genes in three water resource recovery facilities in Jiangsu, China (Zhang, Han, et al., 2015). They observed that the presence of 13 ARGs (*sul*I, *sul*II, *sul*III, *sul*A, *tet*A, *tet*B, *tet*E, *tet*A, *str*A, *str*B, bla_{TEM}, bla_{SHV}, and $bla_{\text{CTX-M}}$) was detected at a higher frequency in ARBs from the influent than the effluent samples, except for *sul*A and $bla_{\text{CTX-M}}$. In another wastewater treatment plant using an anaerobic–anoxic–oxic configuration activated sludge process (sometimes called A2O), eight tet genes (*tet*A, *tet*B, *tet*C, *tet*E, *tet*M, *tet*O, *tet*S, and *tet*X) were removed after the treatment process, especially for *tet*C, *tet*M, *tet*O, and *tet*X with removal efficiencies of 94.9%, 88.5%, 77.7%, and 74.9%, respectively (Huang, Zhang, Liu, & Hu, 2015). The activated sludge plants in Jiangsu, China, consists of anoxic, anaerobic, and aerobic compartments (Zhang, Han, et al., 2015). They found that the abundance of the ARGs increased in aerobic sludge as compared to anaerobic sludge. They also observed that the level of *tet*A was higher in the aerobic tank than the anoxic tank, while *tet*B showed a decreased abundance. *Tet* gene concentrations were similar among anaerobic, anoxic, and aerobic tanks for the improved anaerobic–aerobic–oxic treatment plant (Huang et al., 2015). They also observed that there was no significant difference between aqueous and biosolids samples from these tanks. The results from different activated sludge processes removing ARGs vary more widely and not just among the different compartments of process modifications. Those results are summarized in Table 1-4. For example, different removal efficiencies were observed for tet and sul genes in the same treatment plants in different seasons (Zhang, Han, et al., 2015), indicating possible effects of temperature on

ARGs removal. Furthermore, other parameters play an important role in ASP removal efficiency for ARGs and ARBs, such as pH, F/M, hydraulic retention time (HRT), and sol- ids retention time (SRT).

Table 1-4. Summary of ARGs Treatment in Different Treatment Processes

Process	Scale	Total ARG Detected		Location	Comments	References
		Decreased/Total	Increased/Total			
AAA[1]	F[8]	10/12	1/12	China	no significant change for CTX-M.	Zhang et al. (2015)
AAO[2]	F	8/8	0/8	China	-	Huang et al. (2015)
CASP[3]	F	3/3	0/3	China	-	Gao et al. (2012)
ASP[4]	F	1/1	0/1	USA	-	Munir et al. (2011)
ASP	F	2/2	0/2	Sweden	non-nitrifying ASP	Börjesson et al. (2010)
ASP	F	1/2	1/2	Saudi Arabia	on-site Hospital WWTP	Timraz et al. (2017)
SBR[5]	F	8/8	0/8	China	A sequencing batch reactor operated in a successively arrangement of aerobic and anaerobic stages	Wang et al. (2015)
PFR[6]	F	8/8	0/8	China	-	Wang et al. (2015)
CASP	F	8/8	0/8	China	-	Wang et al. (2015)
AAO	F	6/8	2/8	China	-	Wang et al. (2015)
SBR	L[9]	0/2	2/2	China	Factory wastewater	Yu et al. (2015)
ASP	L	0/3	3/3	China	activated sludge was sampled from full-scale WWTP	Huang et al. (2014)
AD[7]	F	11/11	0/11	China	T and long SRT improved efficiency	Sui et al. (2016)
AD	L	8/14	5/14	China	*sul*II showed no significant change	Wu et al. (2016)
AD	L	4/4	0/4	UK	Mesophilic AD was more susceptible to ARG intrusion than thermophilic AD.	Miler et al. (2016)
AD	L	3/3	0/3	Brazil	AD may be influenced by the ambient temperature.	Resende et al. (2014)
AD	L	4/4	0/4	USA	T higher than 55°C didn't offer better removal.	Burch et al. (2016)

Note: 1. AAA = anoxic, anaerobic, and aerobics;
2. AAO = anaerobic, anoxic, and oxic;
3. CASP = conventional activated sludge process;
4. ASP = activated sludge process;
5. SBR = sequencing batch reactor;
6. PFR = Plug-flow Reactor;
7. AD = anaerobic digestion;
8. F = full scale; 9. L = lab scale

The F/M is considered to be one of the most important factors that influence organic degradation and bacterial growth in the activated sludge process, and is inversely related to the SRT. Yuan, Guo, and Yang (2015) evaluated the effect of the F/M on the fate of six antibiotic-resistant bacteria (tetracycline-, sulfadiazine-, cephalexin-, vancomycin-, erythromycin-, and gentamicin-resistant heterotrophic bacteria) in a bench-scale activated sludge treatment process. They observed that the growth rates of most ARB were increased with increasing F/M from 0.24 to 0.42 kg TCOD/(kg MLSS day) but did not increase beyond 0.42. A similar result was also observed in the batch-scale sequencing batch reactors (SBRs) for tetracycline- resistant bacteria (Kim, Jensen, Aga, & Weber, 2007).

Two possible reasons might account for this ARB amplification with an increased F/M or decreasing SRT (Yuan et al., 2015). First, Yuan et al. (2015) hypothesized that most antibiotic-resistant bacteria in activated sludge at high F/M were surrounded by an environment with higher antibiotic concentrations, which required them to become resistant for survival. Also, nutrients are essential for horizontal gene transfer (HGT), resulting in more frequent transfer of ARGs among bacteria in the presence of higher nutrient concentrations that exist at higher F/M (Yuan et al., 2015). Nevertheless, other possibilities may explain this amplification and the influence of F/M on ARGs and ARB is needed further study. In brief, the F/M is an important factor for controlling and removing the amount of ARGs and ARB in the activated sludge process.

Another important factor used to operate and control activated sludge processes is solids retention time (SRT) which is inversely related to the F/M and is usually expressed in days. The SRT has

been observed to be significant to the removal of most of the organic chemicals (Leu et al., 2012). Leu et al. (2012) found that removals of trace organics such as ibuprofen (almost 100%) and several other antioxidants (the variety between 43% and 99%) increased at higher SRTs. In laboratory-scale research, the proliferation of antibiotic resistance genes and antibiotic-resistant bacteria had decreased and sometimes increased at higher SRT (Neyestani, Dickenson, McLain, Obergh, et al., 2017). Neyestani et al. found that the prevalence of ARB in the activated sludge process increased at higher SRTs (2,7, and 20 days) and was more pronounced at higher temperatures. De Sotto et al. (2016) found similar results in laboratory-scale SBRs for the treatment of carcass leachate on the fate of tetracycline resistance. Neyestani, Dickenson, McLain, Robleto, et al. (2017) suggested that longer SRTs might select for resistant bacteria and/or result in false positives for antibiotic resistance. They also hypothesized that the increase in ARB might also be due to high antibiotic concentrations that facilitate selective pressure. However, there are few researchers focusing on the influences of SRTs for the removal of ARGs and ARB in full-scale water resource recovery facilities. Therefore, the detection and fate of ARGs in full-scale water resource recovery facilities and the influence of operating parameters such as SRT are import objectives of future research.

ARGs in anaerobic digestion

Anaerobic digestion (AD) is a series of biological processes in which microorganisms break down biodegradable mate- rial in the absence of oxygen. It degrades and stabilizes organic materials under anaerobic conditions by microbial organisms and forms biogas (a mixture of carbon dioxide and methane, a renewable energy source) and microbial bio- mass (Kelleher et al., 2002). Anaerobic digestion has been widely used for the treatment of municipal sludge and limited

application in the treatment of other organic industrial wastes (Parkin & Miller, 1983). Anaerobic digestion processes are critical for reducing residual sludge from other processes, reducing viral and bacterial pathogens, as well as dewatering post-treatment sludge. Digested biosolids can be further treated by composting or utilized for dairy bedding, applied to cropland, or converted into other products. Because of the potential widespread to use of anaerobically digested sludge in agricultural applications, it is possible that antibiotic resistance genes and antibiotic-resistant bacteria may be released to the environment (Munir et al., 2011). Therefore, the performance of anaerobic digestion processes in reducing ARGs and ARB is an important concern.

The performance of AD for removal of antibiotic resistance genes and antibiotic-resistant bacteria varies, according to the types of ARGs and ARB. Sui et al. (2016) studied the removal efficiency of full-scale mesophilic anaerobic digesters (35°C) in two water resource recovery facilities treating wastewater from swine farms, where antibiotic resistance genes and antibiotic-resistant bacteria are frequently found. ARGs of tetracycline (*tet*G, *tet*M, and *tet*X), sulfonamide (*sul*I and *sul*II), and macrolide (*erm*B, *erm*F, *ere*A, and *mef*A); class 1 integron gene (*intI*1); and bacterial 16S rRNA gene were detected in the biosolids and partially decreased after anaerobic digestion (Sui et al., 2016). Resende et al. (2014) obtained similar results in the laboratory-scale mesophilic digester showing that *erm*B, *aph*A2, and $bla_{\text{TEM-1}}$ decreased. The authors documented their methods to quantify removal or increase of ARGs and also noted the effects of process conditions. Ambient temperature may influence the performance of anaerobic digestion processes when treating ARGs and ARB, and anaerobic digestion was found to be more effective in reducing ARGs during the summer as compared to winter (Resende et al., 2014).

Thermophilic anaerobic digestion is an alternative to mesophilic digestion and usually operates at approximately 55°C (Iranpour et al., 2002). In the United States, thermophilic digestion is increasing in popularity due to its ability to produce Class A biosolids and has additional benefits such as a higher degree of waste stabilization and more thorough destruction of pathogens and biosolids. There are previous studies focused on the ARG removal performance of thermophilic digesters. Burch, Sadowsky, and LaPara (2010) studied the performances of laboratory-scale thermophilic anaerobic digesters on treating three antibiotic resistance genes (*tet*W, *tet*X, and *qnr*A) in untreated municipal wastewater solids. They found removal efficiency of approximate 99% at 56°C for these three antibiotic resistance genes Wu et al. (2016) also found a two-stage batch-scale thermophilic digestion process (55°C) reduced the presence of *tet*A, *tet*G, *tet*X, *sul*1, *erm*B, *dfr*A1, *dfr*A12, and *intI*1 by 0.1-0.72 log unit removal (approximately 20.57 to 80.95%). In contrast, they found that genes copies of *tet*O, *tet*W, *sul*II, *erm*F, and bla_{TEM} increased in comparison with number in the feed (untreated samples before anaerobic digestion) and s*ul*II showed no significant change (Wu et al., 2016).

Interestingly, Burch et al. (2010) also studied the influence of temperature operations on the thermophilic anaerobic digestion system, and they found that the removals of these three ARGs were increased with increasing temperature up to approximately 55°C. Over 55–63°C, the removal rate was not consistently better than that at 55°C (Burch et al., 2010). Unfortunately, there were no further studies explaining the reasons of these results Additionally, longer hydraulic residence times (HRTs) may also improve the reduction efficiency of ARGs in the full-scale anaerobic digestion process (Sui et al., 2016). However, the optimal HRTs for removing ARGs are still

unknown. Therefore, appropriate operating temperatures and HRTs for the anaerobic digestion process to remove ARGs is a topic for future study.

ARGs in constructed wetlands

Constructed wetlands consist of an artificial wetland created for the purpose of treating anthropogenic discharges using the natural functions of vegetation, soil, and organisms to treat different water streams. Although constructed wetlands are not normally used for treating large volumes of wastewater due to land requirements, they can be an effective process for removing antibiotic resistance genes.

In Zhejiang, China, Chen and Zhang (2013) identified previous research documenting removal of ARGs in constructed wetlands and in their own work found that constructed wetlands played a major role in the removal of ARG genes, especially *sul* genes, and also provided 2 log unit removals of fecal coliforms. Similarly, in Xiamen, China, Liu et al. (2013) found similar results that constructed wetlands significantly reduced the wastewater antibiotics content with the following sequence of elimination rates—oxytetracycline HCl>ciprofloxacin HCl>sulfamethazine (Liu et al., 2013). Additionally, they detected a higher concentration of antibiotics accumulated in the soil than in the media and vegetation, indicating that soil might be the dominant sink for antibiotics removal from waste- water in vertical flow constructed wetlands. The media and vegetation exhibited lower removal efficiency of 50% and one order of magnitude, respectively. This finding is consistent with Chen and Zhang's mechanism, indicating that plant roots in constructed wetlands encourage microbial attachment, which may be regarded as a mini aerobic/anoxic biological treatment system (Chen & Zhang, 2013). Removals in constructed wet-

lands vary with season, and temperature is significant factor affecting the removal efficacy of sulfonamide and tetracycline resistance genes, which range from 2.98×10^{-5} to 1.27×10^{-1} gene copies/16S rRNA for *sul* genes and 4.68×10^{-6} to 1.54×10^{-1} gene copies/16S rRNA for *tet* genes after treatment (Liu et al., 2014). Constructed wetlands are a potentially viable process for ARG removal, with possible advantages of inexpensive construction cost and easy operation, but further study is recommended.

1.6. Summary

Antibiotic resistance genes are contaminants of increasing concern, and their fate in water resource recovery facilities is not fully understood. Many studies, both laboratory scale and full scale, have found that treatment processes may in many but not all cases decrease the amount of some ARGs. A few other studies show an increase in ARGs and ARB. Table 1-5 summarizes the previous research found in this review in both laboratory- and full-scale studies. Thirty-five ARGs were detected and quantified in 25 independent studies with 215 observed results. Among the 215 results, 152 showed a decrease, while 26 showed no change, and 37 showed an increase. All of the results were reported in relative counts by per 16S. Table 1-6 summarizes nine different treatment processes that were categorized into three general groups, including secondary treatment processes (activated sludge, membrane bioreactor, and constructed wetlands), postsecondary treatment processes (filtration and coagulation), and disinfection treatment processes (UV disinfection, anaerobic digester, ozonation, and chlorination). Note that genes co-located on a plasmid and transferred together were both counted in this analysis. Thus, transfer of plasmids carrying multiple genes resulted in a higher score in this analysis. The activated sludge process was the most often studied (64/215 observations) and showed the most removal observations, with 54 observations of

decreases in 35 ARGs. Anaerobic digestion showed 21 removal results, followed by 13 observations for ozonation. Only one coagulation study was found, and it showed a decrease in ARGs. The results are confusing because some researchers documented increases in ARGs in the same processes. Nine of the 63 activated sludge observations showed increases in ARGs. There were other observations of increasing ARGs in treatment processes. The decreases, increases, and unchanged observations are shown in Figure 1-1.

Table 1-5. Treatment processes in 25 studies and their ARGs treatment performances

Treatment Process	Scale	References	Country	*tetA*	*tetB*	*tetC*	*tetE*	*tetG*	*tetM*	*tetO*	*tetQ*	*tetS*	*tetT*	*tetW*	*tetX*	*tetZ*	*sulI*	*sulII*	*sulIII*	*sulA*	bla_{TEM}	bla_{OXA}	bla_{SHV}	bla_{CTX-M}	*ermB*	*ermF*	*ereA*	*marA*	*mecA*	*mefA*	*qnrS*	*vanA*	*strA*	*strB*	*ampt(A)*	*dfrA1*	*dfrA12*	*aphA2*
Secondary Treatmet Processes																																					
ASP[1]	F[15]	Zhang et al. (2015)	China	↓	↓		↓										↓	↓	↓	→	↓		↓	↑								↓	↓					
ASP	F	Huang et al. (2015)	China	↓	↓	↓	↓		↓	↓		↓		↓		↓																						
ASP	F	Gao et al. (2012)	USA							↓							↓																					
ASP	F	Timraz et al. (2017)	Saudi Arabia													↓	↑																					
ASP	F	Wang et al. (2015)	China						↓	↓	↓		↓	↓			↓	↓							↓													
ASP	F	Wang et al. (2015)	China						↓	↓	↓		↓	↓			↓	↓							↓													
ASP	F	Wang et al. (2015)	China						↓	↓	↓		↓	↓			↓	↓							↓													
ASP	F	Wang et al. (2015)	China						↓	↑	↑		↓	↓			↓	↓							↓													
ASP	F	Timraz et al. (2017)	Saudi Arabia													↑	↓	↑																			
ASP	F	Munir et al. (2011)	USA														↓																				
ASP	L[14]	Huang et al. (2014)	China	↑		↑		↑																													
MBR[2]	F	Munir et al. (2011)	USA							↓				↓																							
MBR	F	Wang et al. (2015)	China						↓	↑	↑		↓	↓			↓	↓							↓													
CW[3]	L	Liu et al. (2014)	China						→	→				→			↑	↑																			
CW	L	Liu et al. (2013)	China							↓																											
CW	L	Liu et al. (2013)	China						↓	↓				↓																							
Post-Secondary Treatment Presesses																																					
SF[4]	F	[illegible]	Switzerland														↓																				
GSF[5]	F	Wang et al. (2015)	China						↑	↓	↑		↓	↑			↑	↑							↓													
Coagulation	F	Li et al. (2016)	China							↓	↓			↓			↓	↓																			
Disinfection Treatment Processes																																					
UV	L	McKinney and Pruden (2012)	USA	↓																								↓			↓			↓			
UV	L	Huang et al. (2016)	China																																		
UV	L	Zhang et al. (2015)	China					↓							↓		↓																					
UV	L	Sousa et al. (2017)	Portugal														↓				↓										↓	↓						
UV/H_2O_2	L	Ferro et al. (2016)	Italy											↓							↓										↓							
AD[6]	L	Resende et al. (2014)	Brazil														↓				↓				↓												↓
AD	L	Wu et al. (2016)	China	↓				↓		↑				↑	↓		↓		↑		↑				↓	↑									↓	↓	
AD	L	Miller et al. (2016)	USA					↑																													
AD	F	Sui et al. (2016)	China					↓	↓						↓		↓	↓							↓	↓	↓		↓								
AD	F	Munir et al. (2011)	USA							↓				↓																							
O_3	L	[illegible]	Switzerland														↓																				
O_3	L	Sousa et al. (2017)	Portugal														↓				↓											↓					
O_3	L	Sousa et al. (2017)	Portugal														↓				↓										↓	↓					
O_3/GAC^7	L	Alexander et al. (2016)	Germany																			↑			↓							↑					
O_3	F	[illegible]	Switzerland														↓																				
O_3	F	Guo et al. (2014)	China		↑			↓	↑	↑				↑			↓	↓																				
Cl_2^8	L	Zhang et al. (2015)	China					↓							↓		↓																					
Cl_2	F	Gao et al. (2012)	USA							↓				↓			↓																					
Cl_2	F	Guo et al. (2014)	China	↓	↑	→		↓	↑	→	↑			↑			↓	↑	→	↑	→																	
Others																																					
STP[9]	F	Naquin et al. (2015)	USA	→										→	→		→								→			→									
Pchem.[10]	F	Guo et al. (2014)	China	→	→	→		→	→	→				→	→		→	→																			
DWTP[11]	F	Guo et al. (2014)	China	↓	↓	↓		↓	↓	↓				↓	↓		↓	↓																				
N/S[12]	F	Chen et al. (2013)	China						↓	↓	↓			↓			↓	↓																				
N/S	F	Narciso-da-Rocha et al. (2014)	Portugal																		↓							↓			↓						

Note: 1. ASP = activated sludge process; 2. MBR = membrane bioreactor; 3. CW = constructed wetland; 4. SF = sand filter; 5. GSF = granular sand filter; 6. AD = anaerobic digester; 7. GAC = granular activated carbon; 8. Cl_2 = chlorination; 9. STP = sewage treatment plant (no specific treatment processes studied; 10. Pchem. = physicochemical treatment process; 11. DWTP = drinking water treatment plant (similar processes were used with wastewater treatment plant); 12. N/S = not specified (Either wastewater treatment plant or treatment processes were not introduced in the reference); 13. F = full-scale; 14. L = lab-scale; 15. Arrows indicate the fate of ARGs in the treatment processes ("↓" = decreased; "↑" = increased; "→" = unchanged)

Table 1-6. Summary of ARG fates in wastewater treatment processes

ARGs	*tetA*	*tetB*	*tetC*	*tetE*	*tetG*	*tetM*	*tetO*	*tetQ*	*tetS*	*tetT*	*tetW*	*tetX*	*tetZ*	*sul1*	*sul11*	*sul111*	*sul4*	bla_{TE}	bla_{VIM}	bla_{SHV}	bla_{CTX-M}	*ermB*	*ermF*	*ereA*	*marA*	*mecA*	*mefA*	*qnrS*	*vanA*	*strA*	*strB*	*amp(A)*	*dfrA1*	*dfrA12*	*aphA2*	Total
Secondary Treatment Processes																																				
ASP[1]	2	2	1	2	0	5	5	3	1	4	5	1	1	8	5	1	0	1	0	1	0	4	0	0	0	0	0	0	0	1	1	0	0	0	0	54
	1	0	1	0	1	0	1	1	0	0	0	0	1	1	1	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	9
MBR[2]	0	0	0	0	0	1	1	0	0	1	2	0	0	1	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	8
	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
CW[3]	0	0	0	0	0	1	2	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	4
	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	2
Total	2	2	1	2	0	7	8	3	1	5	8	1	1	9	6	1	0	1	0	1	0	5	0	0	0	0	0	0	0	1	1	0	0	0	0	66
	1	0	1	0	1	0	2	2	0	0	0	0	1	2	2	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	13
	3	2	2	2	1	7	10	5	1	5	8	1	2	11	8	1	0	1	0	1	1	5	0	0	0	0	0	0	0	1	1	0	0	0	0	79
Post Secondary Treatment Processes																																				
Filtration	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	4
	0	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
Coagualtion	0	0	0	0	0	0	1	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Total	0	0	0	0	0	0	2	1	0	1	1	0	0	2	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	9
	0	0	0	0	0	1	0	1	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	5
	0	0	0	0	0	1	2	2	0	1	2	0	0	3	2	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	14
Disinfection Treatment Processes																																				
UV	1	0	0	0	1	0	0	0	0	0	1	1	0	2	0	0	0	1	0	0	0	0	0	0	0	1	0	1	2	0	0	1	0	0	0	12
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
AD[4]	1	0	0	0	2	1	1	0	0	0	1	2	0	2	1	0	0	1	0	0	0	3	1	1	0	0	1	0	0	0	0	1	1	1	21
	0	0	0	0	1	0	1	0	0	0	1	0	0	0	0	1	0	1	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	6
O_3	0	0	0	0	1	0	0	0	0	0	0	0	0	5	1	0	0	2	0	0	0	1	0	0	0	0	0	1	2	0	0	0	0	0	0	13
	0	1	0	0	0	1	1	0	0	0	1	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	6
Chlorination	1	0	0	0	2	1	2	0	0	0	1	1	0	3	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12
	1	0	0	0	1	1	1	0	0	0	1	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7
Total	3	0	0	0	6	2	3	0	0	0	3	4	0	12	3	0	0	4	0	0	0	4	1	1	0	1	1	2	4	0	0	1	1	1	1	58
	1	1	0	0	2	2	3	0	0	0	3	0	0	1	1	1	0	1	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	19
	4	1	0	0	8	4	6	0	0	0	6	4	0	13	4	1	0	5	1	0	0	4	2	1	0	1	1	2	5	0	0	1	1	1	1	77

Note: 1. ASP = activated sludge process; 2. MBR = membrane bioreactor; 3. CW = constructed wetland; 4. AD = anaerobic digester; 5. Green zone indicates numbers of decreased observations; red zone indicates numbers of increased observations.

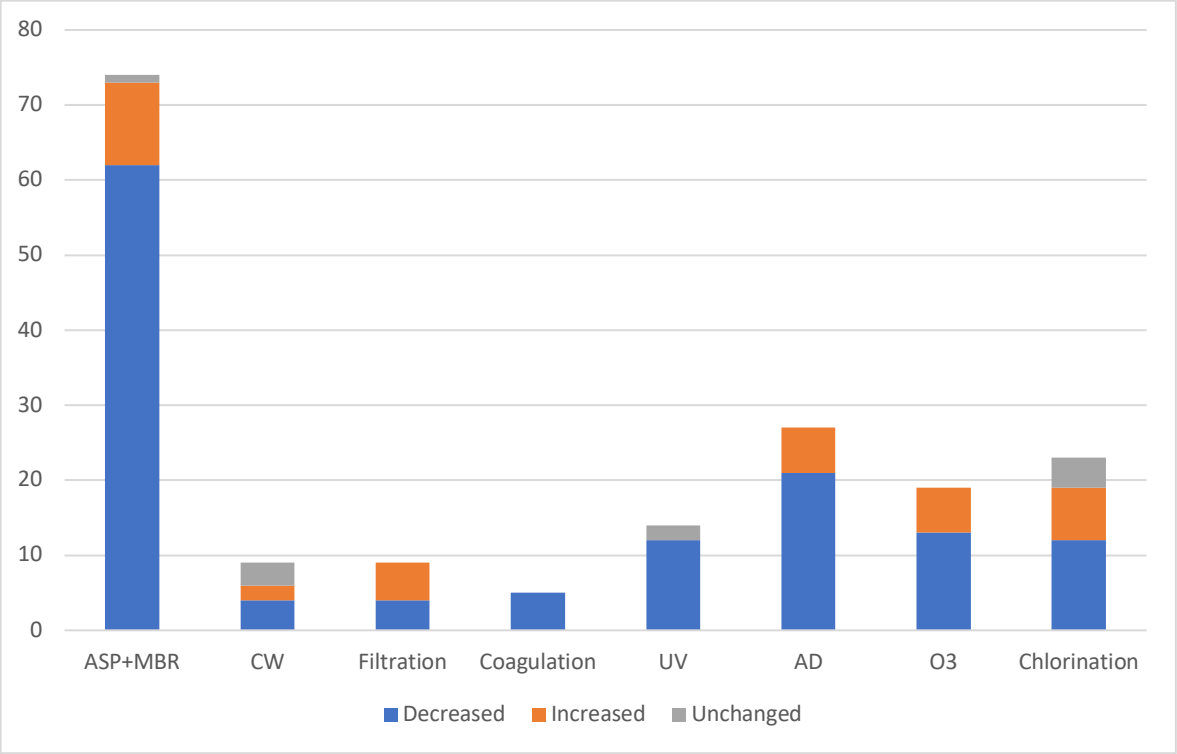

Figure 1-1. Comparison of ARGs decreased, increased, and unchanged observations in treatment processes, including activated sludge process (ASP) and membrane bioreactor (MBR), constructed wetland (CW), filtration, UV disinfection, anaerobic digester (AD), ozonation (O3), and chlorination.

For all processes, there were more decreases than increases in ARGs levels relative to the 16S gene levels, which indicates that wastewater treatment processes tend to reduce ARG release to the environment. The results are still confusing, and more research is needed. For example, the activated sludge process decreased ARGs in 54 of 64 observations, but increased in 9 of 64 observations. However, the factors that cause the proliferation of ARGs are still unknown, because of the design factors of treatment processes as discussed previously, horizontal gene transfer, or other natural influence. One potentially important factor is SRT. SRT is correlated with the growth rate of microorganisms; therefore, higher SRT allows enriching the process with slowly growing bacteria and establishing a more diverse microbial community with broader physiological

capabilities for better removal or biodegradation in activated sludge processes (Kreuzinger, Clara, Strenn, & Kroiss, 2004).

As discussed above, longer SRT showed greater removal efficiency for more than 100 contaminants, such as ibuprofen, oxybenzone, and benzophenone (Leu et al., 2012). We hypothesize that longer SRT may have a similar influence on the abundance of ARGs and ARB. Unfortunately, few studies have reported and verified this hypothesis. All of these hypotheses need further confirmation and study, and they will be further studied in our future research. Different treatment processes may also have specific influences on the amount and types of antibiotic resistance genes. Figure 1-2 shows the comparison of tetracycline (tet), sulfonamide (sul), β-lactam resistance genes, and the rest of the 12 ARGs that are decreased or increased in different treatment processes. It shows that proliferation of ARGs specifically in the activated sludge processes was studied and reported most often. The previous researchers found both increases and decreases for all three types of ARGs, with tetracycline resistance genes exhibiting the most increased results, 23 of 37 observations. Microbiological mechanisms in the biological treatment processes are complex, and any result showing an increase in ARG level indicates the proliferation of an antibiotic resistance gene. Other ARGs may also be similarly affected. Therefore, the optimal removal for any water resource recovery facilities should be no proliferation of ARGs.

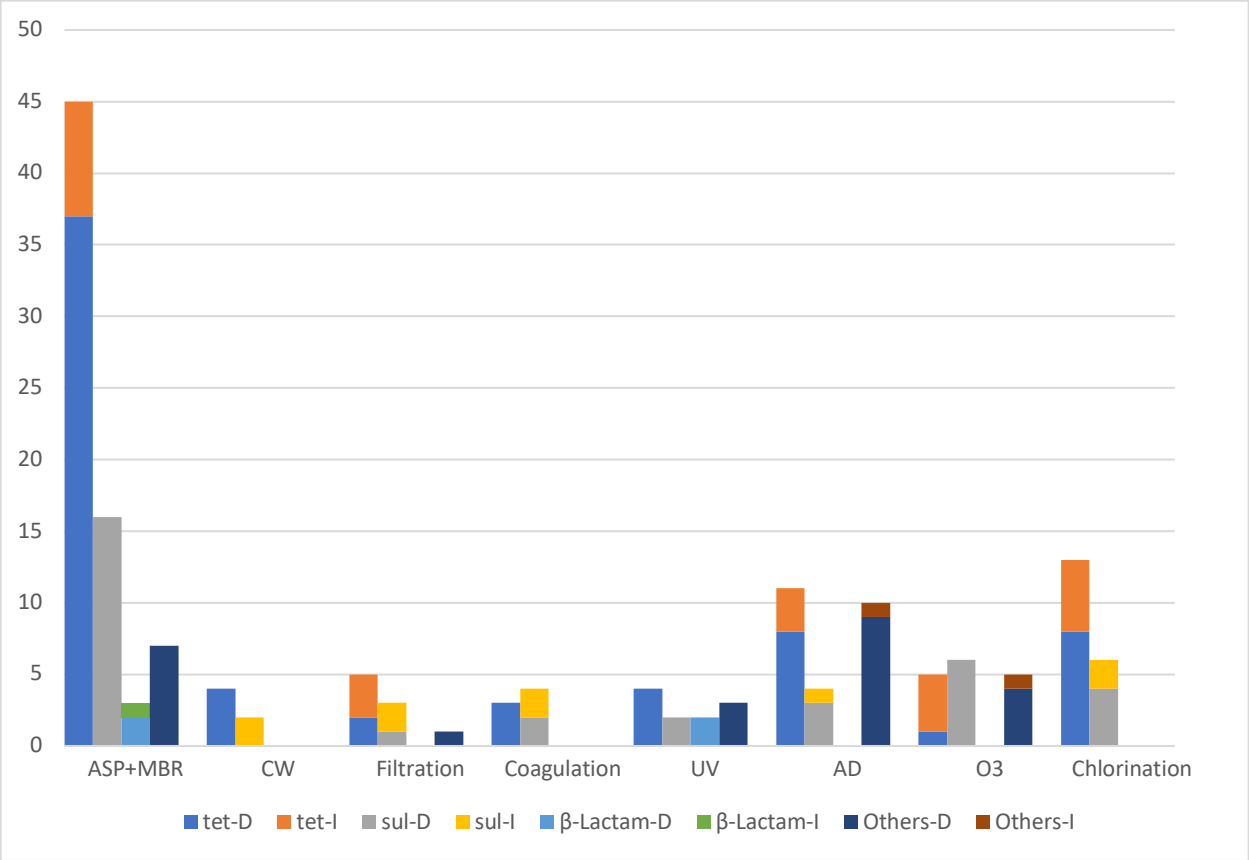

Figure 1-2. Comparison of tetracycline (*tet*), sulfonamide (*sul*), β-Lactam and other antibiotic resistance genes that are decreased and increased in treatment processes, including activated sludge process (ASP) and membrane bio-reactor (MBR), constructed wetland (CW), filtration, UV disinfection, anaerobic digester (AD), ozonation (O3), and chlorination. (Note: D indicates decrease and I indicates increase. For example, tet-D indicates numbers of decreased observations for tetracycline resistance genes and tet-I indicates numbers of increased observations for tetracycline resistance genes.)

UV disinfection was the only process studied that decreased ARGs for all observations (Figure 1-2). UV disinfection typically efficiently eliminates enteric bacteria, viruses, bacterial spores, and parasite cysts with the advantages of reduced disinfection by- product formation and not requiring transportation or storage of a hazardous chemical, for example, chlorine gas (Koivunen & Heinonen-Tanski, 2005). Organisms grown in the presence of anti- biotics can develop resistance mechanisms, but they have no expo- sure to UV light during their growth processes and therefore would not have a selective pressure to develop resistance to UV. Therefore, UV disinfection may

have an additional advantage over other dis- infection methods and may be valuable for reducing ARGs.

Anaerobic digestion showed increased results of different ARGs in only 5 of 24 observations. Anaerobic digestion is an important process for reducing exposure to ARGs because waste activated sludge or trickling filter sludge would otherwise be an important vector for spreading ARGs. Previous studies detected high concentrations of ARGs in biosolids (Munir et al., 2011). The efficiency of anaerobic digestion in reducing ARGs is important because it may reduce the ARGs not eliminated or reduced in the activated sludge process or other biological treatment processes. In addition, anaerobic digesters can be fed with purpose-grown energy crops as a source of renewable energy, which may increase the possibility of spreading ARGs. Therefore, anaerobic digestion is important and merits more study.

1.7. Conclusion

Antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) are of great concern because of the growing loss of effectiveness of important antibiotics. The World Health Organization has published a list of 12 pathogens that need urgent new anti- biotics due to the loss of current antibiotic effectiveness. Water resource recovery facilities have recently been implicated as vec- tors for increasing the occurrence of ARGs and ARB. To under- stand the potential contribution of treatment plants to the spread of ARGs and ARB, a survey was made of current literature with the specific objective of understanding their fates in treatment processes. References were carefully screened to understand the processes involved, which was difficult because many of the investigators reported only changes throughout an entire treatment plant.

Thirty-five ARGs were detected and quantified in 25 independent studies with 215 observed results. Among the 215 results, 152 showed a decrease, while 26 showed no change, and 37 showed an increase. All of the results were reported in relative counts by per 16S. The group of thirteen tetracycline resistance genes was studied the most, with 118 observations. Sulfonamide resistance genes were also frequently studied with 53 total observations of four kinds of sul genes. Other genes such as β-lactam and multidrug-resistant genes were studied less frequently with only 44 observations in total.

Nine different treatment processes were categorized into three general groups, including secondary treatment processes (activated sludge process, membrane bioreactor, and constructed wetlands), postsecondary treatment processes (filtration and coagulation), and disinfection treatment processes (UV disinfection, anaerobic digester, ozonation, and chlorination). Their treatment efficiency for reducing ARGs and ARB varied depending on the processes and its operating conditions.

The *sul*I gene, associated with resistance to sulfonamide anti- biotics, was the gene most studied with 32 observations including 25 decreased observations, four increased observations, and three unchanged observations in all treatment processes. Thirteen of the 32 observations were in disinfection treatment processes, and 12 of the 13 showed reductions. The activated sludge process with its variants such as MBRs was effective in reducing the *sul*I gene in 9 of 10 observations. There were only four observations for *sul*I gene in postsecondary treatment processes and constructed wetlands with two increasing and two decreasing.

There were 118 studies of 13 tetracycline-resistant genes among all treatment processes. In the activated sludge process and its variants such as MBRs, resistant genes decreased in 37 of 45 observations and increased in 8 of 45 observations. In disinfection processes, resistant genes decreased in 21 of 33 observations. Observations in plant-wide studies, provided without specific process information, showed decreases in 12 of 23 observations. Among all processes, tetracycline and sulfonamide resistance genes declined in approximately 70% of the observations and increased in 20% of the observations. ARGs and ARB for all genes decreased in approximately 70% of the observations and increased in 18% of observations, and 12% were unchanged.

The conclusion of this review is that treatment plants decreased the prevalence of ARGs and ARB in approximately 70% of the previous studies. Activated sludge and disinfection are the most effective. There is limited understanding as to what aspects of the activated sludge process are the most effective. Activated sludge plants with long SRT are more effective at removing trace organics and emerging contaminants, but there is no evidence for improved or decreased ARGs and ARB removal by long SRT plants. An important topic for future research is the fate of ARGs and ARB in long SRT processes, since the need for nutrient and improved trace organics removals is increasing. It is important for investigators examining the fate of ARGs and ARB in treatment plants to report as much as possible on the type of treatment process and the parameters of operation, since it is possible that treatment variations may have major impacts.

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Chapter 2: Fate of Antibiotic Resistance Genes in Activated Sludge Processes at Three Long SRT and Three Short SRT Wastewater Treatment Plants

2.1. Introduction

Antibiotic resistance has been increasingly impairing our ability to treat infectious diseases over the last few decades. While the first antibiotic discovered, arsphenamine, effectively treated syphilis beginning 1909, it was not until 1928 that antibiotics were widely used in clinics, after penicillin was discovered to be effective against *Staphylococcus*. Over the decades, numerous antibiotics have been approved for use, and the appearance of resistant clinical strains has followed. Until 2017, the World Health Organization published its first-ever list of antibiotic-resistant "priority pathogens", including 12 families of bacteria that pose the greatest threat to human health due to antibiotic inactivation (Li et al., 2019).

Overuse of antibiotics in human activities such as clinical and agricultural areas is one of the major causes resulting in antibiotic resistance. "Superbugs" have emerged as bacteria have developed resistance to many different types of antibiotics, and ARGs are now considered the emerging contaminants in the environment. ARGs have been found in different sources, including hospitals (Rodriguez-Mozaz et al., 2015; Wang et al., 2018), agricultural areas (Storteboom et al., 2010; Sun et al., 2020), and wastewater facilities (Li et al., 2019). Our previous research reviewed observations of ARGs in wastewater-impacted environments and wastewater facility systems worldwide (Li et al., 2019). Another previous study of ours showed dramatic geographical differences between cities in ARG levels in drinking water and soil (Echeverria-Palencia et al., 2017), although impacts on human health are not certain.

In our previous literature review, we summarized 25 independent studies worldwide and found 215 different observations for 35 ARGs at either lab or full scale (Li et al., 2019). We found that nearly 70% of observations showed decreased gene abundance relative to 16S rRNA after treatment while 18% of observations showed increases. Tetracycline and sulfonamide types of resistance genes were the most studied. Among all the nine treatment processes, activated sludge processes (ASP) were mostly studied. Activated sludge processes are the most widely used secondary treatment processes because of their high treatment efficiencies for removing the majority of organic and inorganic wastes. Solids retention time (SRT) is one of the key process control parameters to meet quality standards and additional environmental goals. Generally, the activated sludge processes at long SRT achieve better removal efficiency than at short SRT. Improved removal occurs at longer SRT because bacteria, growing slowly on complex and recalcitrant compounds, can be retained. This is supported by our previous review, which identified more than 100 contaminants that are better or completely removed at longer SRT nearly 100% (Leu et al., 2012). Therefore, ARG removal will likely be also influenced by SRT. However, few previous researchers focused on or even documented SRT. We believe that this will be of significant importance for the wastewater treatment industry and ARG environment.

To evaluate the hypothesis that SRT influences ARG removal, six California activated sludge wastewater treatment plants (WWTP) were selected, including three long SRT plants and three short SRT plants. Short SRT is defined as 1 to 3 days, which is low enough to completely avoid nitrification. Long SRT is defined as 8 days and longer which insures complete nitrification. Operation at medium SRT where the process provides only partial nitrification or drifts in an out

of nitrification is never advisable due to operational difficulty. Five ARGs (*sul*1, *sul*2, *tet*A, *tet*W, *erm*B), the mobile genetic element (MGE) *intI*1, and the 16S rRNA gene were monitored to assess the removal of ARGs in the six plants. Removal efficiencies of seven genes were evaluated and compared. This study provides further observations on the relation between the fate of ARGs and the SRT of activated sludge processes. Additionally, the correlation between 16S rRNA and volatile suspended solids (VSS) is introduced, showing how VSS can be used to help understand the fate of ARGs.

2.2. Materials and methods

Sample Collection

Six WWTPs, located near Los Angeles were selected for the collection of wastewater samples. Three WWTPs are long SRT plants (Plant 1,2, and 5) with SRTs ranging from 10 to 12 days and three are short SRT plants (Plant 3, 4, and 6) with SRTs ranging from 1 to 2 days. Wastewater temperatures ranged from 18 to 28^{o} C. The flow rates ranged from 57 to 1703 m^3/day. All six plants are municipal wastewater treatment plants but have some contribution of pretreated industrial wastewaters. The schematic layout of the six WWTPs is shown in Figure 1. Samples were collected over four or eight weeks, and details are shown in Table 2-1. 2-L water samples were collected from secondary influent (primary effluent) and secondary effluent in sterile containers at each WWTP (Figure 2-1). Four 500 mL samples were collected at each plant over a five minute period to obtain a 2-L extended grab sample (Ma et al., 2009). Samples were kept on ice during transport and stored at 4°C until the time of processing. Samples were then processed using vacuum-filtration with 47 mm diameter, 0.4 μm pore-size mixed cellulose esters (MCE)

filters (EMD Millipore, Billerica, MA). Wastewater samples were filtered until clogging and multiple filters were used in some cases to obtain sufficient sample sizes. After filtration, filters were carefully folded and placed into 1.7 mL sterile snap cap tubes and subsequently stored at -20°C, awaiting DNA extraction. All processing was performed in triplicate and all samples were processed within 24 hours of collection.

Table 2-1. Plant Information

Plant	Type	SRT4 (Days)	Average Primary Eff. TSS5 (mg/L)	Average Secondary Eff. TSS (mg/L)	Flow Rate (m3/day)	Sampling Time	Max. amount of filtration until clogging	
							Influent (mL/Filter)	Effluent (mL/Filter)
1	Long SRT MLE1	10-12	72	5	3.03×10^8	11/2018 & 11/2019	20/40	240/300
2			135	3	0.56×10^8	07/2019	40	500
5	ASP2		83	4	0.76×10^8	11/2020	20	300
3		1-1.5	182	17	17.0×10^8	07/2021	20	60/80
4	Short SRT	1.5-2	179	13	15.1×10^8	07/2019 & 11/2019	40	120
6	HPO3 ASP	1-2	85	4	3.79×10^8	02/2021 & 03/2021	20	250

Note: 1. MLE = Modified Ludzack-Ettinger model; 2. ASP = Activated sludge process; 3. HPO = High purisity Oxygen; 4. SRT = Solids retention time; 5. TSS = Total suspended solids

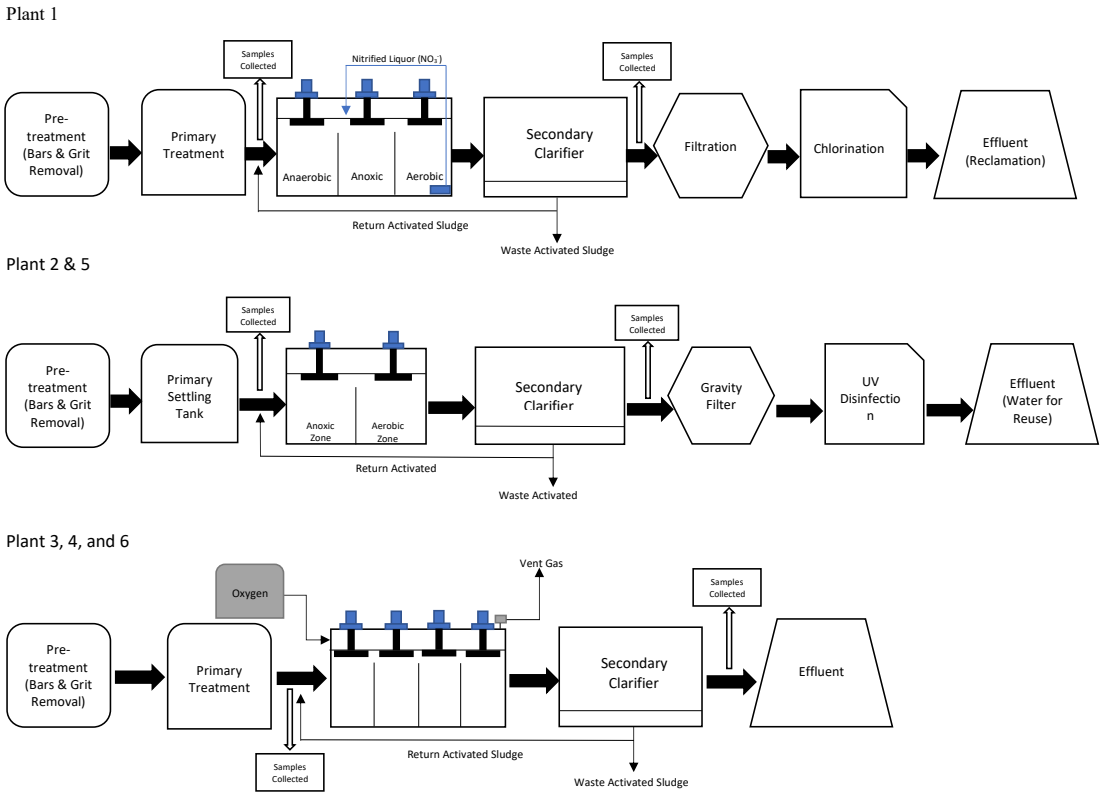


Figure 2-1. Schematic layout of the six wastewater treatment plants with activated sludge processes.

DNA Extraction

DNA was extracted from wastewater samples using the MP Biomedicals FastDNA SPIN Kit (MP Biomedicals, LLC, Irvine, CA). Extractions proceeded as per the manufacturer's guidelines except for sample homogenization, which was performed with a Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK) running for two, one-minute intervals. In order to meet the requirement of the purity of the DNA, samples were purified by adding prepared SEWS-M solutions to purify DNA three times instead of one time suggested by the protocols. Eluted DNA was determined via a Nanodrop 2000C (Thermal Scientific, Waltham, MA) at 260/280 nm absorbance ratios above 1.80.

Gene recoveries were calculated for each sample via a matrix spike and found to be within 80-120% (Armstrong et al., 2007).

Quantitative Polymerase Chain Reaction (qPCR)

Five ARGs, including two *tet* genes (*tet*A and *tet*W), two *sul* genes (*sul*1 and *sul*2), and one erythromycin resistance gene (*erm*B), class I integron gene (*intI*1), and the 16S rRNA gene were selected for quantitative detection using SYBR Green Master Mix qPCR. These ARGs were chosen because our literature review found that they were frequently studied and represented a variety of different antibiotic classes and resistance mechanisms. The bacterial 16S rRNA gene was determined by qPCR in order to enumerate the total bacterial community and to calculate the relative abundance of ARG relative to the 16S rRNA copy numbers.

All assays used a 20-µL reaction volume consisting of 10-µL of SYBR™ Select Master Mix (Thermo Fisher Scientific, Waltham, MA), 1-µL of forward primer working concentrations, 1-µL of reverse primer working concentrations, 4-µL of molecular grade RNAse-free molecular biological grade water (Fisher Scientific, Pittsburgh, PA), and 4-µL of diluted DNA template. DNA template was diluted before qPCR to exclude inhibition effects, as confirmed and clarified previously. All assays were performed in 96-well reaction plates using an Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA). Reaction conditions, including cycling conditions, primer sequences, and concentrations are shown in Table 2-2. Each plate included a 5-point standard curve positive control, all applicable extraction samples, and a negative control of molecular grade RNAse-free molecular biological grade water in triplicate. Target-containing DNA fragments served as positive controls and were analyzed using Geneious coupled with NCBI

Database information and ordered through IDT Technologies (Coralville, IA). Melt curves were used to further verify correct target gene quantification. Efficiencies ranged from 90 to 110 % and R^2 values were > 0.99 for all standard curves. Similar techniques have been used previously by our group (Echeverria-Palencia et al., 2017).

Table 2-2. Primer sequences used for qPCR

Target ARG	Primer	Concentration (nM)	Sequence (5'-3')	Annealing Temperature (°C)	Amp. Efficiency (%)	Reference
*sul*1	*sul*1-F	200	CGCACCGGAAACATCGCTGCAC	65 °C/30s	96	(Pei et al., 2006)
	*sul*1-R		TGAAGTTCCGCCGCAAGGCTCG			
*sul*2	*sul*2-F	200	CTCCGATGGAGGCCGGTAT	60 °C/30s	97	(Luo et al., 2010)
	*sul*2-R		GGGAATGCCATCTGCCTTGA			
*tet*A	*tet*A-F	200	GCTACATCCTGCTTGCCTTC	55 °C/30s	97	(Ng et al., 2001)
	*tet*A-R		CATAGATCGCCGTGAAGAGG			
*tet*W	*tet*W-F	200	GAGAGCCTGCTATATGCCAGC	60 °C/30s	98	(Aminov et al., 2001)
	*tet*W-R		GGGCGTATCCACAATGTTAAC			
*erm*B	*erm*B-F	500	AAAACTTACCCGCCATACCA	60 °C/30s	99	(Knapp et al., 2010)
	*erm*B-R		TTTGGCGTGTTTCATTGCTT			
*intI*1	*intI*1-F	200	CCTCCCGCACGATGATC	55 °C/30s	96	(Goldstein et al., 2001)
	*intI*1-R		TCCACGCATCGTCAGGC			
16S rRNA	16S rRNA-F	250	CCTACGGGAGGCAGCAG	56 °C/30s	92	(Ji et al., 2012)
	16S rRNA-R		ATTACCGCGGCTGCTGG			

Statistical Analysis

The change in relative gene abundance (ΔRGA) was calculated by normalizing to the bacterial 16S rRNA gene as shown in Equation 1 by subtracting relative gene abundance of secondary effluents (RGA_{eff}) with relative gene abundance of secondary influents (RGA_{inf}).

$$\Delta\text{RGA} = RGA_{eff} - RGA_{inf} \quad (1)$$

The relative gene abundance per mg volatile suspended solids (VSS) (RGA_{VSS}) was found by absolute gene abundance (AGA) divided by mg/mL VSS. VSS data for secondary influent and effluent were provided by wastewater treatment plants or estimated as 85% of the total suspended solids (TSS) (Karia and Christian, 2013). Equation 2 is shown below.

$$RGA_{VSS} = \frac{AGA\ (gene\ copies/mL)}{VSS\ (mg\ /mL)} \quad (2)$$

The change in relative gene abundance per mg VSS was calculated by normalizing to per mg VSS (Equation 2) by subtracting relative gene abundance of secondary influents ($RGA_{VSS|inf}$) to relative gene abundance of secondary effluents ($RGA_{VSS|eff}$) as shown in Equation 3.

$$\Delta RGA_{VSS} = RGA_{VSS|eff} - RGA_{VSS|in} \quad (3)$$

Log gene abundance and log removal values were calculated using a base-10 logarithm for the evaluation of removal efficiencies of the individual treatment plants. Relative *intI*1 abundance was correlated to all other ARG relative abundances. Correlations were calculated using Pearson's bivariate correlation coefficient (Galvin et al., 2010; Narciso-Da-Rocha et al., 2014).

Statistical analyses including two-tailed t-test were performed with GraphPad Prism version 8 (San Diego, CA) and Rstudio (Boston, MA). The differences at $p < 0.05$ level among samples were considered statistically significant. Correlation analysis using Pearson' correlation between the

removal of ARGs were studied and the strength of correlations were defined as strong ($r > 0.7$), moderate ($0.5 < r \leq 0.7$), weak ($0.3 < r \leq 0.5$), none or very weak ($r \leq 0.3$) (Moore and Kirkland, 2007).

2.3. Results and Discussion

Absolute abundance of ARGs at the six activated sludge plants

Figure 2-2 shows the absolute gene abundances in average for all six treatment plants. All target ARGs were consistently detected in the six wastewater treatment plants before and after secondary treatment processes, including ARGs belonging to the *tet* and *sul* genes. The absolute gene abundance was described as gene copies/mL in base-10 logarithm. The total 16S rRNA gene was detected in the highest levels at the influent of Plant 6 (8.91 log) but observed levels were much lower at the effluent of Plant 2 (6.83 log). At Plant 1, the highest absolute abundance before treatment was observed for *erm*B (6.93 log), which belongs to the macrolide resistance determinants and is considered the most prevalent gene in environmental microorganisms (Zhang et al., 2009); *sul*1 was the most abundant after the treatment (5.68 log). *Sul*1 was also the most abundant at Plants 5 and 6 before and after ASP with the range of 5.47 and 7.11 log. A previous study (He et al., 2020) also found that tetracycline and sulfonamide resistance genes are generally the most abundant ARGs of all classes. At Plants 2, 3, and 4, *intI*1 had the highest abundance before and after treatment (the range of 5.35 and 7.26 log). The genes with lowest abundance were tetracycline resistance genes (*tet*A between 3.67 and 5.83 log and *tet*W between 2.9 and 5.72 log L, respectively) at Plant 1-5, with the exception of *erm*B in the effluent of Plant 4 (4.51 log). At Plant 6, *sul*2 gene was the lowest abundance among all target genes before (5.29 log) and after

(3.46 log).

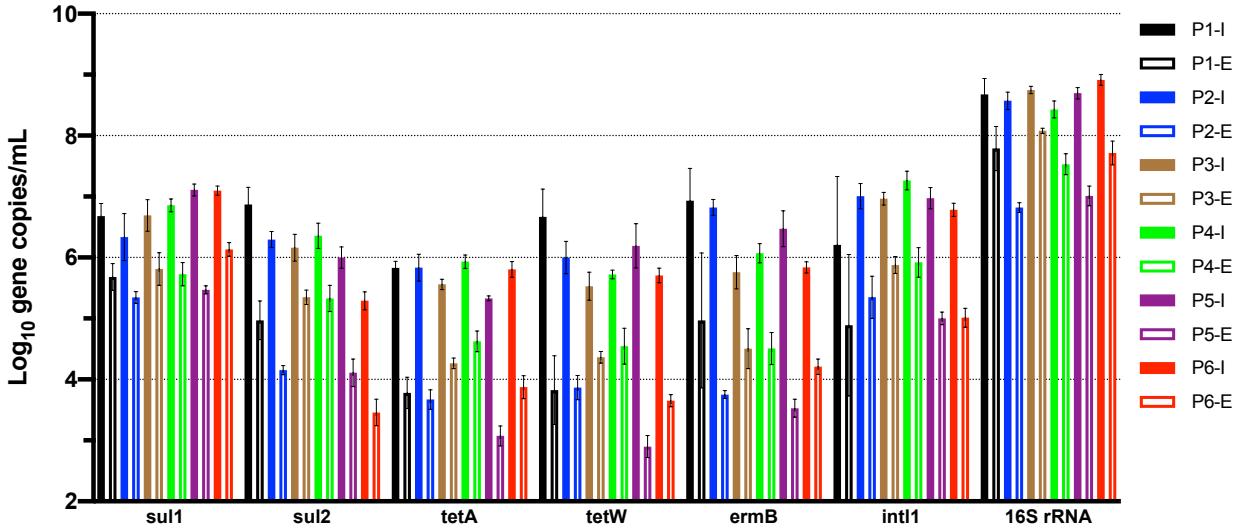


Figure 2-2. Absolute gene abundance of each target ARG at each of the six plants' influent and effluent.

The highest absolute abundances of all target ARGs were detected at the secondary influents of Plant 1, 4, and 5 between 5.93 (*tet*A) and 7.11 (*sul*1) log, but the lowest abundance of all target ARGs were detected at the effluent of Plant 1,2, and 5 between 2.90 (*tet*W) and 5.34 (*sul*1) log, except *sul*2 that was detected at the effluent of Plant 6 with 3.46 log. This indicates that long SRT plants may inactivate the abundance of ARGs better than short SRT plants.

Absolute Gene Abundance Changes of Target ARGs

ARG removals varied by plant and shown in Figure 2-3. Generally, the Plants 1, 2, and 5 (long SRT) more efficiently removed ARGs than Plants 3, 4, and 6 (short SRT). The average removal of 16S rRNA was higher at the three long SRT plants than at the three short SRT plants. Also, *sul*2, *tet*A, *tet*W, and *erm*B genes were removed more efficiently at long SRT but *sul*1 and *intI*1 genes were removed at the similar rates at long and short SRT plants.

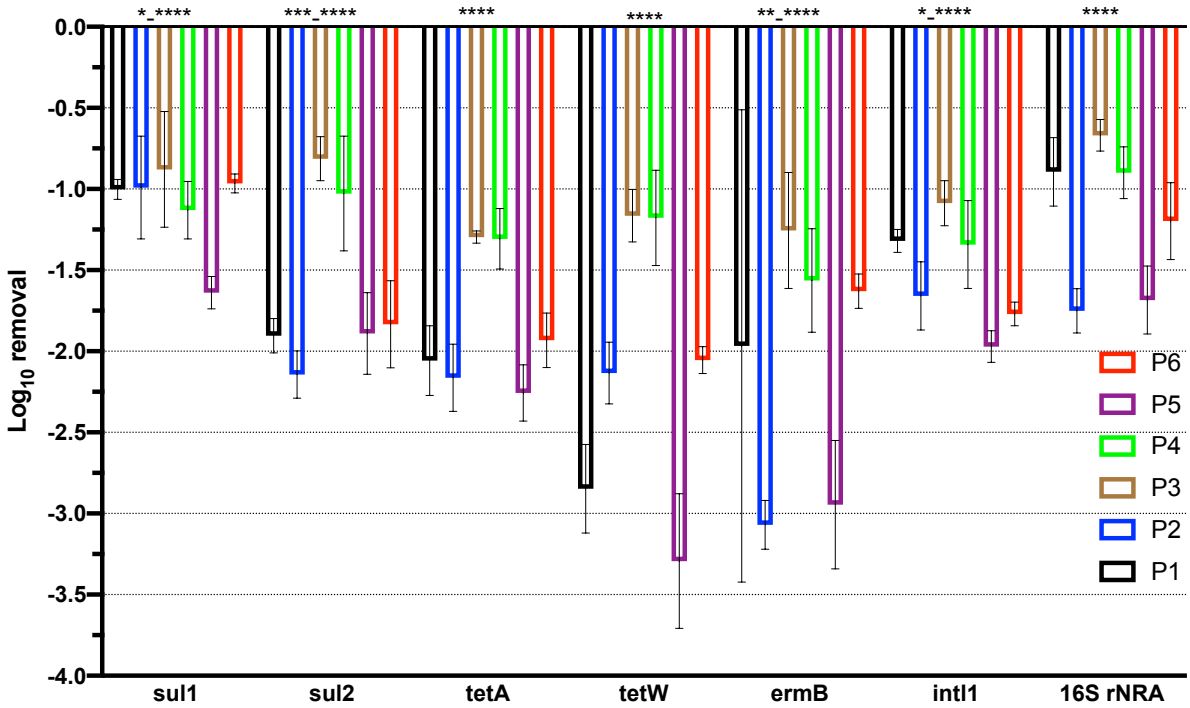


Figure 2-3. Absolute gene abundance removal of target ARGs at the six plants. Negative values indicate removal and positive values indicate increase. The results are expressed in log10 copies per mL. Significance differences of each plants' removal rates were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****)

At Plant 1, significant removals ($p < 0.05$) were observed for all of the target ARGs, from 1 log removal (*sul*1) to 2.85 log removals (*tet*W). Similar results were also observed at Plant 2 with all significant removals from 0.99 log removal (*sul*1) to 3.07 log removals (*erm*B) and at Plant 5 from 1.64 log removals (*sul*1) to 3.29 log removals (*tet*W). Previous studies also found lower removal efficiencies for *sul*1, and studies have suggested that sulfamethoxazole-resistant bacteria are more difficult to remove, which may explain these lowest removal efficiencies among all target ARGs (Gao et al., 2012; McConnell et al., 2018). In comparison to the long SRT plants, the overall removal rate of all the target genes was lower in the three short SRT plants, 3, 4, and 6, which had an average SRT of 1 to 2 days. Plant 3 achieved the log removals of 0.67 (16S rRNA) to 1.3 (*tet*A) log removals. Plant 4 achieved the log removals of 0.89 (16S rRNA) to 1.56 (*erm*B) log gene

removals. Plant 6 achieved the log removals of 0.97 (*sul*1) to 2.05 (*tet*W) log removals. In general, the average removal efficiencies of all target ARGs was 99% (1.98 log removals) at the three long SRT plants and 95% (1.29 log removals) at the three short SRT plants. Overall, our study is consistent with previous findings that the activated sludge processes can remove ARGs from wastewater (Huang et al., 2015; Pallares-Vega et al., 2019; Jilu Wang et al., 2015; Zhang et al., 2015).

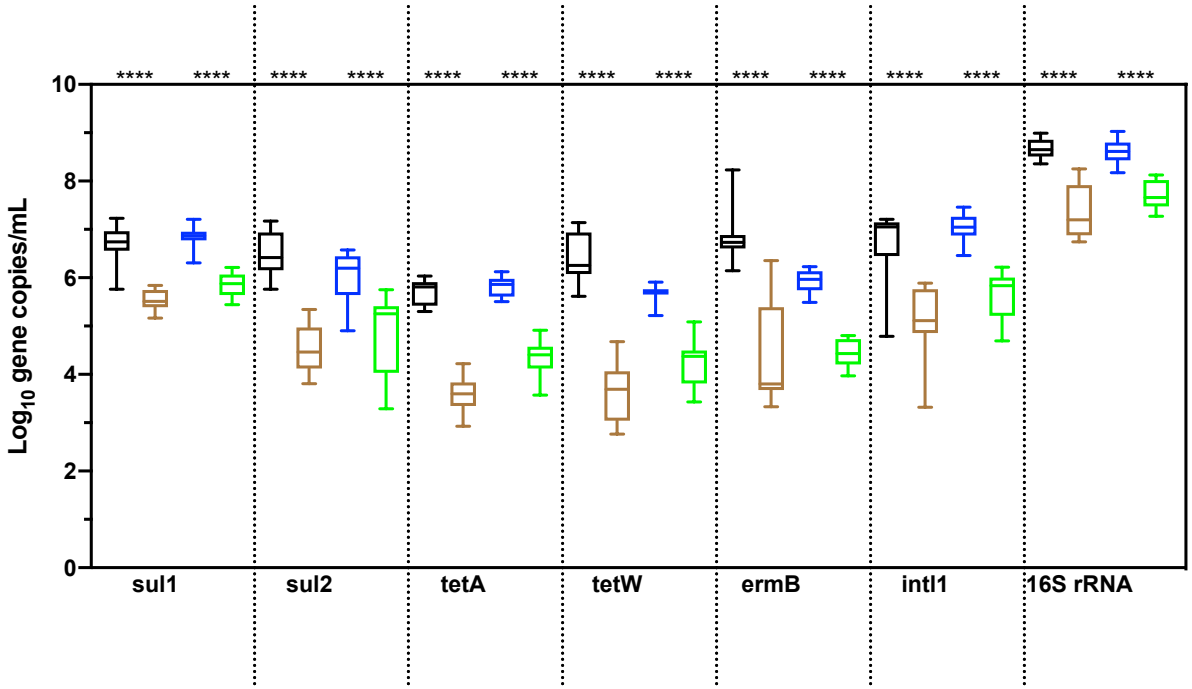


Figure 2-4. Absolute gene abundance of the target ARGs, in the three long SRT plants influent (black) and effluent (brown) samples and the three short SRT plants influent (dark blue) and effluent (green). Different genes are separated by vertical lines. The results are expressed in log10 copies per mL. The boxes represent the 1st and 3rd quartiles. The middle line represents the median, and the whiskers represent the min and max values. Significant differences in gene presence after treatment were assessed by a paired t test and values are indicated above each gene (****): $p < 0.0001$.

When we combined three long SRT plants together and three short SRT plants together and compared them, all of the three long SRT plants and three short SRT plants achieved significant ($p < 0.05$) removal rates between 0.9 and 2.8 log removals shown in Figure 2-4. Also, we found

significantly ($p < 0.05$) higher removal rates as much as two fold, for all of the target ARGs at long SRT plants than at short SRT plants shown except *sul*1 and *erm*B (Figure 2-5) This reinforces the generally held belief that activated sludge processes with longer SRT have higher removal rates for trace organics and pollutants of concern than short SRT processes (Leu et al., 2012).

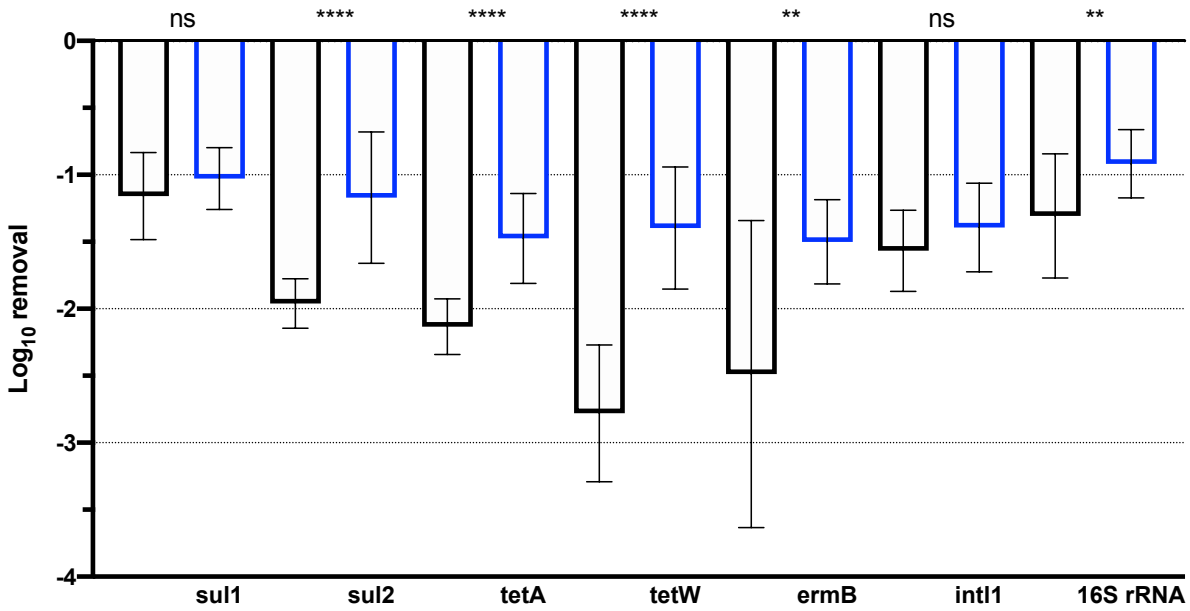


Figure 2-5. Absolute gene abundance removal of the target ARGs, in the three long SRT (black) samples and the three short SRT plants (blue). Negative values indicate removal and positive values indicate increase. The results are expressed in $\log_{10}$ copies per mL. Significant differences of removal rates in gene presence between long SRT plants and short SRT plants were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Potential correlations for ARG removals

The correlations of removal efficiencies of all target ARGs were studied and shown in Figure 2-6 at the three long SRT plants and the short SRT plants, respectively. At the three long SRT plants, significantly ($p < 0.05$) and moderately positive correlations ($0.5 < r < 0.7$) were observed between *sul*1 and *tet*W (0.64), *intI*1 (0.63), and 16S rRNA (0.52), respectively, and between *tet*A and *erm*B (0.67). Significantly and strongly positive correlation ($r > 0.7$) was observed between *intI*1 and 16S rRNA (0.77).

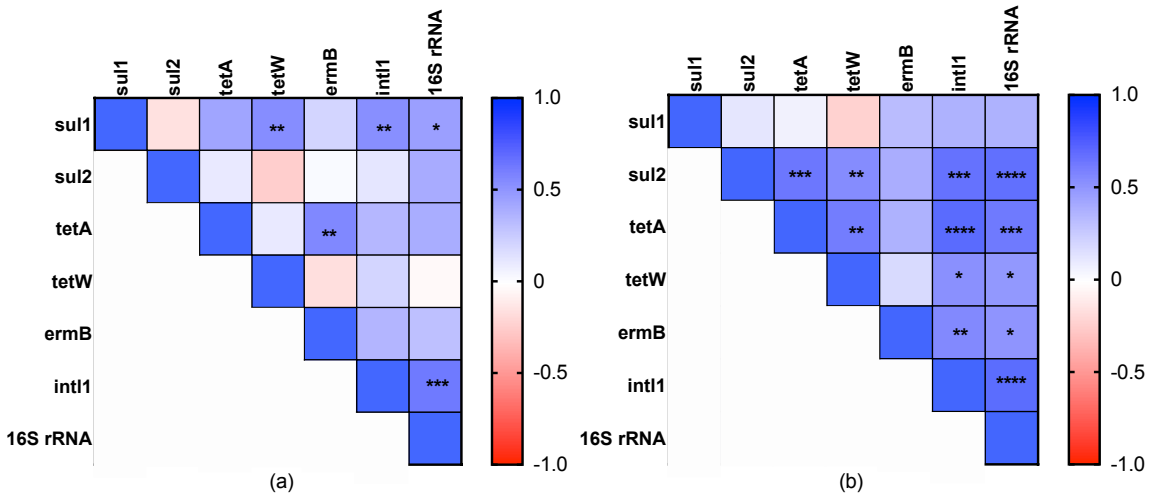


Figure 2-6. Correlation matrix (Pearson's correlation) of the absolute removal efficiency of the target ARGs for three long SRT plants (a) and three short SRT plants (b). Significant levels of correlation are indicated as follow $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

At the three short SRT plants, significant correlations ($p < 0.05$) were observed between all of the ARGs, with the exception between *sul*1 and all the other ARGs and between *erm*B and the two *sul* genes and two *tet* genes. Strongly positive correlations ($r > 0.7$) were observed between *sul*2 and *tet*A, *intI*1, and 16S rRNA, respectively, between *tet*A and *tet*W, *intI*1, and 16S rRNA, respectively, and between *intI*1 and 16S rRNA.

One possible explanation may be that different ARGs come from similar or same biological source or organisms. For example, bacteria including *E. coli* (Poppe et al., 2006), *Salmonella* (Dang et al., 2008), and *Vibrio* (Macauley et al., 2007) can develop resistance mechanisms to antibiotics and produce *tet*A and *sul*2. Treatment processes may inactivate these bacteria in wastewater so that both of these two genes are produced less, resulting in strongly decreasing correlations. Also, *sul*1 and *intI*1 proved to be significantly correlated in their persistence ($r = 0.63$) at the long SRT plants. This may be due to their association with diverse broad host range plasmids that can be disseminated and transferred horizontally within and between bacteria (Pallares-Vega et al., 2019;

Tennstedt et al., 2003).

The relative gene abundance (RGA) of ARGs per 16S rRNA at six activated sludge plants

16S sequences have been widely used to infer likely taxonomy based on similarity being clustered to generate operational taxonomic units and their representative sequences compared with reference databases (Johnson et al., 2019). 16S rRNA gene as a sequence-based bacterial analysis represents the bacterial DNA background value to evaluate likely antibiotic resistant taxonomy when being applied as per 16S rRNA (Liang et al., 2021). In this study, the 16S rRNA gene was selected as a proxy for the total bacteria. Relative gene abundance of target ARGs are shown in Figure 2-7. Relative gene abundance was described as gene copies/16S rRNA in base-10 logarithm. At plant 1, the wastewater flow before and after the ASP detected *erm*B and *sul*1 as the highest RGA for -1.75 and -2.11 log, respectively. *IntI*1 was detected as the highest RGA from the water flows at Plant 2,3, and 4, between -2.2 and -1.17 log. *Sul*1 was the highest RGA detected at Plant 5 and 6 between -1.81 and -1.54 log. Similar to absolute gene abundances, the two *tet* genes, *tet*A and *tet*W, were detected as the lowest RGA from Plant 1-5 between -4.01 and -2.71 log, except for the effluent of the activated sludge process at Plant 4 with the lowest RGA of *erm*B (-3.02 log) and the effluent at Plant 5 with *erm*B (-6.01 log). *Sul*2 was the least relatively abundant gene at Plant 6 (-3.62 log in the influent and -4.22 log in the effluent).

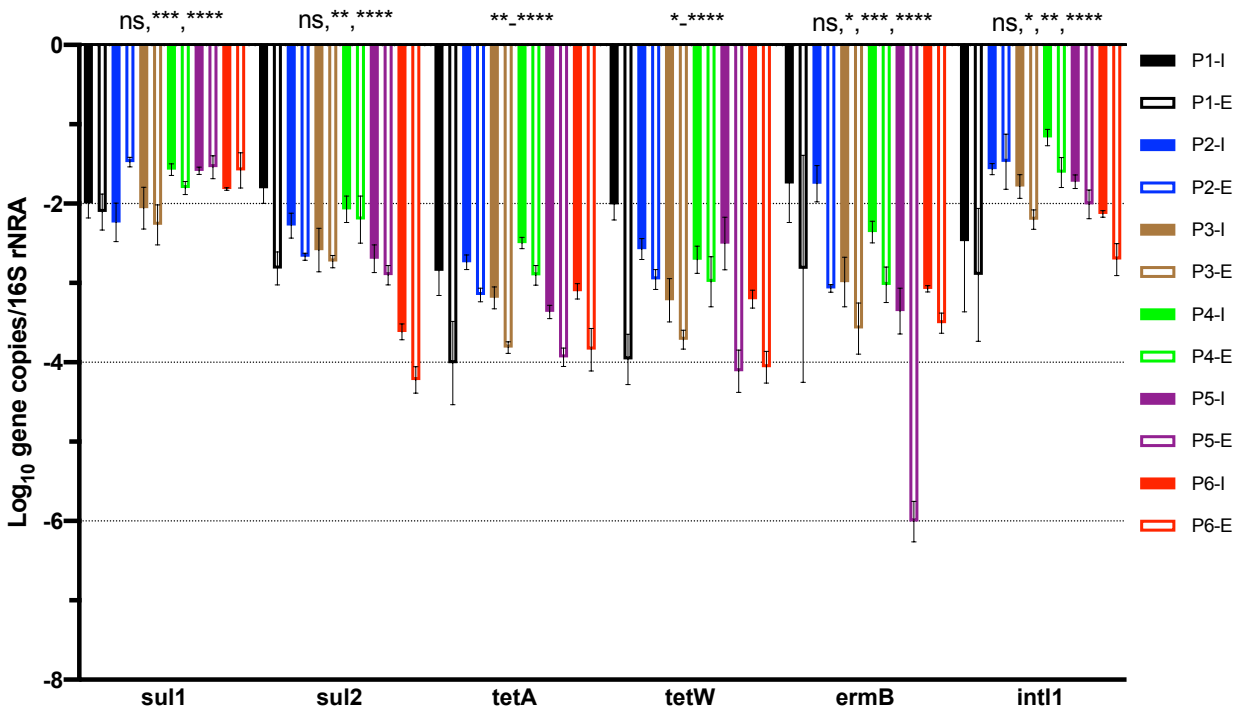


Figure 2-7. Relative gene abundance of target ARGs at each plant's influent and effluent. The results are expressed in log10 copies per 16S rRNA. Significance differences of each plant were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Different from the absolute gene abundance, the lowest RGA of target ARGs was not only detected at the long SRT plants but also at the short SRT plants, which may be due to lower RGA of ARGs at the influent of short SRT plants than long SRT plants. For example, the RGA of *sul*2 at the influent of Plant 6 was less than any other plants, which resulted in the lowest RGA at the effluent.

RGA Changes of ARGs

Relative gene abundance changes of ARGs are shown in Figure 2-7. At Plant 1, significant reductions ($p < 0.05$) of relative gene abundance per 16S rRNA were observed for all of the target ARGs, with exceptions of *sul*1 and *erm*B. At Plant 2, *sul*2, *tet*A, *tet*W, and *erm*B were significantly reduced in log gene copies/16S rRNA. However, significant increases of *sul*1 and *erm*B were observed at Plant 2. Previous studies reported that there was no significant removal during

treatment, indicating that the removal of *sul*2 may not be as efficient as other ARGs (Gao et al., 2012; Mao et al., 2015; Petrovich et al., 2020). At Plant 3, significant reductions of all relative ARG abundance were observed for *tet*A, *tet*W, and *intI*1. At Plant 4, significant reductions were observed for all the target ARGs except *sul*2 and *tet*W. RGA of two *tet* genes and *erm*B were observed to be significantly reduced at Plant 5. Finally, RGA of all target ARGs except *sul*1 were significantly reduced at Plant 6.

Generally, the RGA per 16S rRNA was more variable than the absolute gene abundance among six plants (Figure 2-8). A previous study theoretically predicted longer SRT reduced the bacterial diversity in the batch-scale reactors (Saikaly et al., 2005). They found that intermediate SRT (2.3 to 5.7 days) resulted the enhancement of species diversity. But when the SRT was above 5.7 days, competitive exclusion would dominate, and bacterial diversity was reduced (Huisman and Weissing, 2001, 1999; Saikaly and Oerther, 2004). This finding indicated that longer SRT reactors may better stabilize the antibiotic resistant communities. However, in the full-scale wastewater treatment systems, many other operational factors are present, making the microbial wastewater environment more complicated to predict and understand. There have been some studies as to whether viruses are associated with ARGs, and it plays an important role in horizontal gene transfer (Colomer-Lluch et al., 2014; Enault et al., 2017; Zhang and LeJeune, 2008). Studies have found that co-occurrence of ARGs and plasmid-associated genes may promote their mobilization between bacteria, especially under a high proportion of ARGs that likely associate with HGT in different wastewater backgrounds (Petrovich et al., 2020). Similar results were found in some other previous studies that treatment processes under longer SRT increased the RGA of some ARGs (Pallares-Vega et al., 2019; Sui et al., 2018; Zhang et al., 2018). Higher SRT might favor the

grazing of bacteria by protozoa, but it might also favor HGT events in the water environment and cause the proliferation of RGA of ARGs after a longer SRT treatment performance (Tsutsui et al., 2010). Nevertheless, in our study, we conclude that activated sludge processes still have the capability of reducing the absolute and relative levels of ARGs either at long or short SRT.

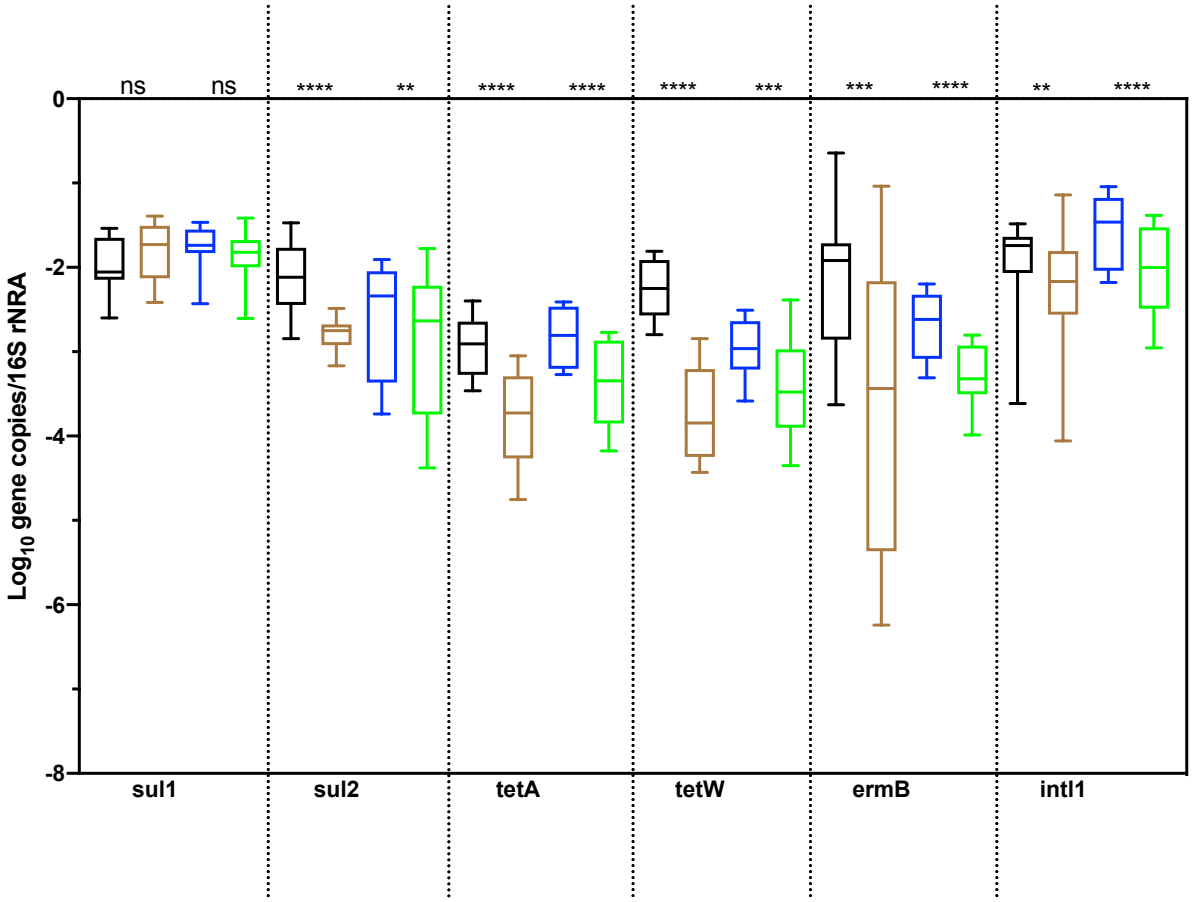


Figure 2-8. Relative gene abundance of the target ARGs per 16S rRNA, in the three long SRT plants influent (black) and effluent (brown) samples and the three short SRT plants influent (dark blue) and effluent (green). Different genes are separated by vertical lines. The results are expressed in log10 copies per 16S rRNA. The boxes represent the 1st and 3rd quartiles. The middle line represents the median, and the whiskers represent the min and max values. Significant differences in gene presence after treatment were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

The relative gene abundance (RGA_{VSS}) of ARGs per mg VSS during treatment at six activated sludge plants

Volatile suspended solids (VSS) are a critical performance factor to determining the operational behavior and biological concentration through the activated sludge process. VSS are generally biological solids generated during the aerobic/anaerobic treatment process and are a result of the organic material (BOD_5) in the wastewater being converted to biomass, water, and carbon dioxide. VSS are organic in nature. Determining the relative gene abundance of ARGs per mg VSS is believed to assist in understanding the correlations of the fate of ARGs and the performance factor in the activated sludge system.

The relative gene abundance per mg VSS for each plant was shown in Figure 2-9 and described as gene copies per mg VSS in base-10 logarithm. At Plant 1, the highest RGA_{VSS} was observed for *erm*B (8.16 log) before and *sul*1 (8.10 log) after activated sludge process. *IntI*1 gene was observed as the highest RGA_{VSS} at Plant 2, 3, and 4, with the range of 7.71 and 8.09 log. The RGA_{VSS} of *sul*1 was mostly highly observed at both of Plant 5 and Plant 6 between 7.99 and 8.59 log. Two target *tet* genes were observed as the lowest RGA_{VSS} at Plant 1 to 5 either before or after the activated sludge process between 5.42 and 7.06 log, except the wastewater after the activated sludge process at Plant 4 where *erm*B was observed as the lowest target ARGs of all in 6.48 log. At Plant 6, RGA_{VSS} of *sul*2 was least observed at Plant 6 before (6.47 log) and after (5.94 log) the ASP. Similar to the relative gene abundance per 16S rRNA, the lowest RGA_{VSS} of some ARGs was also observed at the short SRT plants.

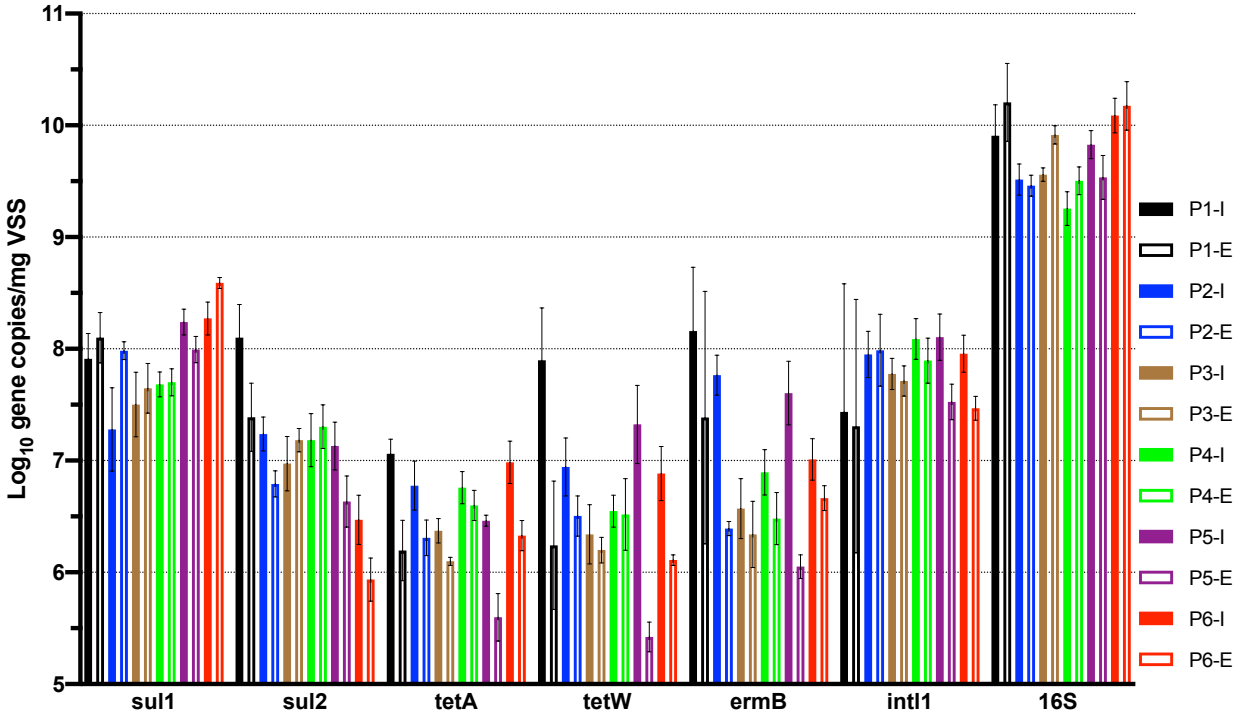


Figure 2-9. Relative gene abundance of target ARGs per mg VSS at each plant's influent and effluent. The results are expressed in log10 copies per mg VSS. Significance differences of each plant were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

RGA_{VSS} Changes of ARGs

The relative gene abundance per mg VSS removal of each plant was discussed and shown in Figure 2-10. At plant 1, significant ($p < 0.05$) reductions were detected for all the target ARGs, except for *sul*1 and 16S rRNA gene that were significantly increased. The highest reduction was *tet*W for 1.66 logs. At Plant 2, significant reductions were detected for *sul*2, *tet*A, *tet*W, and *erm*B in 0.45, 0.46, 0.43, and 1.37 log removals, respectively. No significant changes were detected for all of the target ARGs at Plant 3. But 16S rRNA was significantly increased by 0.35 log removal. At Plant 4, low but still significant reductions were detected for *tet*A (0.16 log removal) and *erm*B (0.41 log removal). All of the target ARGs were found to be significantly decreased up to 2 logs at Plant 5, with the exception of 16S rRNA. Finally, significant reductions of *sul*2 (0.53), *tet*A (0.65), *tet*W (0.77), and *erm*B (0.34) but significant increase of *sul*1 (0.32) was observed in log removal at Plant

6. In summary, RGA_{VSS} efficiencies of target ARGs were up to 99% at the six plants, although proliferations of some ARGs were also observed.

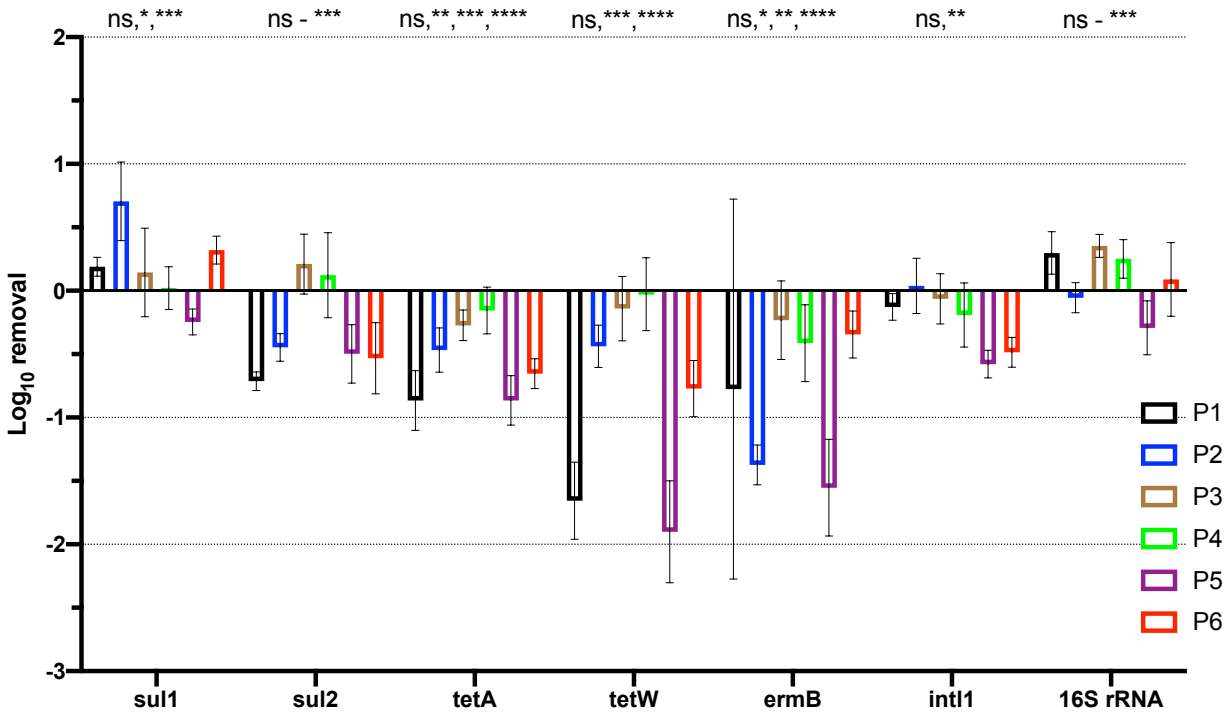


Figure 2-10. Relative gene abundance changes of target ARGs per mg VSS at the six plants. Negative values indicate removal and positive values indicate increase. The results are expressed in log10 copies per mg VSS. Significance differences of each plant's removal rates were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

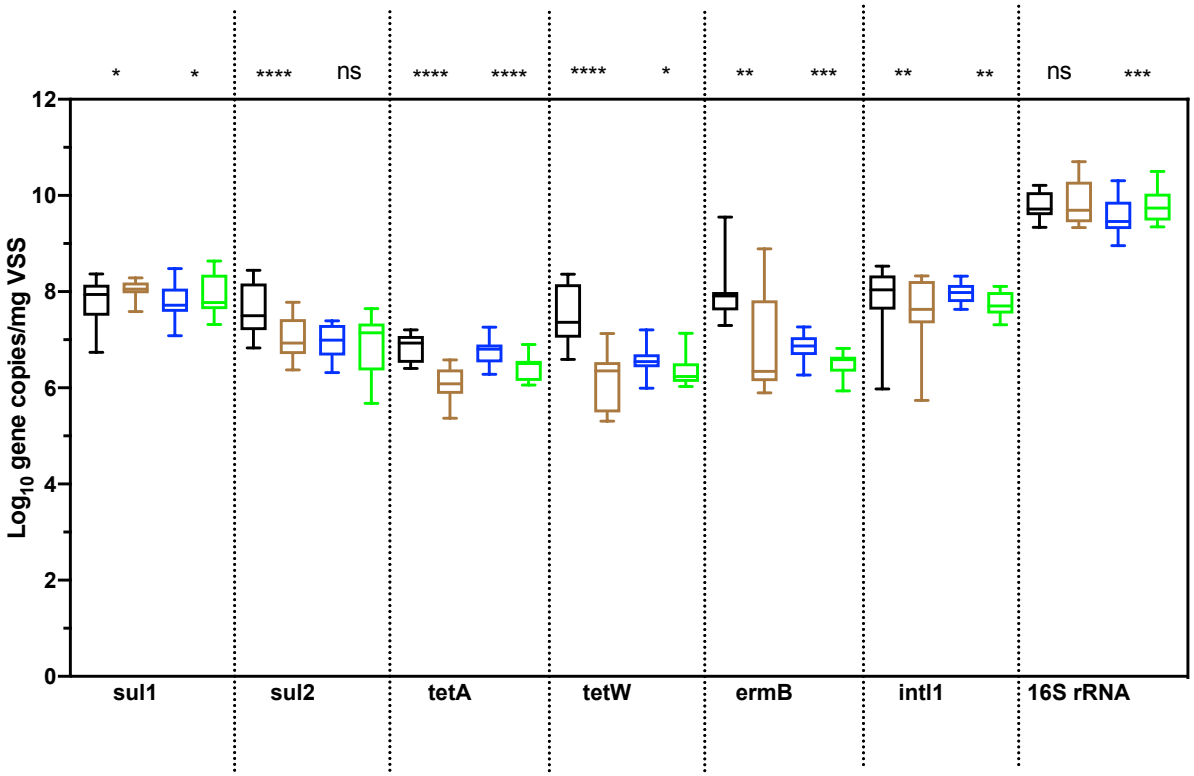


Figure 2-11. Relative gene abundance of the target ARGs per mg VSS, in the three long SRT plants influent (black) and effluent (brown) samples and the three short SRT plants influent (dark blue) and effluent (green). Different genes are separated by vertical lines. The results are expressed in log10 copies per mg VSS. The boxes represent the 1st and 3rd quartiles. The middle line represents the median, and the whiskers represent the min and max values. Significant differences in gene presence after treatment were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

In comparison between three long SRT plants together and three short SRT plants together shown in Figure 2-11 and Figure 2-12, long SRT plants significantly ($p < 0.05$) reduced the RGA of *sul*2, *tet*A, *tet*W, and *erm*B per mg VSS almost 5 times the maximum removal than at short SRT plants. However, RGA_{VSS} of 16S rRNA was little changed at three long SRT plants but there was significant increase at the three short SRT plants. The RGA_{VSS} was first introduced into the study of the fate of ARGs in wastewater treatment plants. VSS is one of the most important impact factors to the performance of activated sludge systems in microorganism communities (Haydar et al., 2007; Kumar et al., 2014). Absolute gene abundance provides one side of the fate of ARGs,

including both organic and inorganic materials into the ratio, but relative gene abundance per mg VSS excludes the possible influence of inorganic materials in the ratio to provide another side of the fate of ARGs in the wastewater treatment systems and is more concise and straightforward to people in the wastewater treatment field. In summary, previous discussions indicate that ASP at long SRT condition generally performed better than those at short SRT for the fate of ARGs in terms of VSS.

Table 2-3. TSS and VSS concentrations in average of the six plants.

Plant	Influent			Effluent		
	TSS	VSS	% VSS	TSS	VSS	% VSS
P1	71.8	59.2	83%	4.6	3.8	83%
P2	134.8	114.5	85%	2.7	2.3	85%
P3	182.3	154.9	85%	17.5	14.9	85%
P4	179.3	152.4	85%	12.7	10.8	85%
P5	83.3	74.2	89%	3.6	3.0	85%
P6	84.8	72.0	85%	4.3	3.6	82%

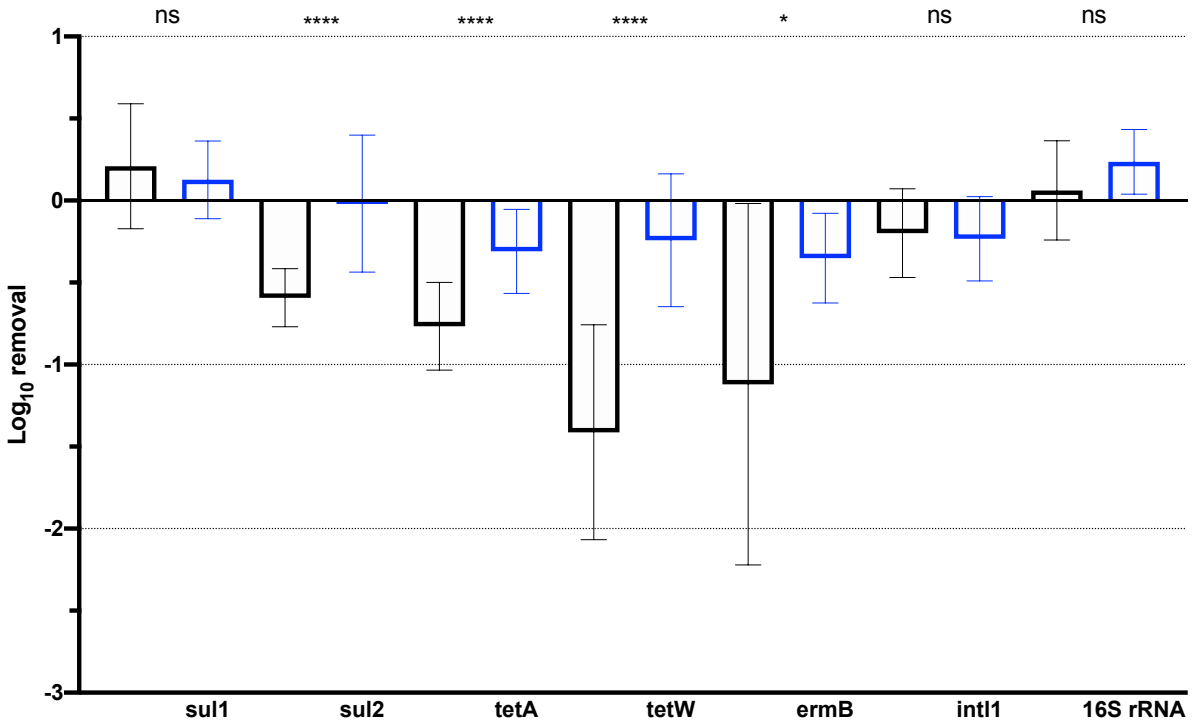
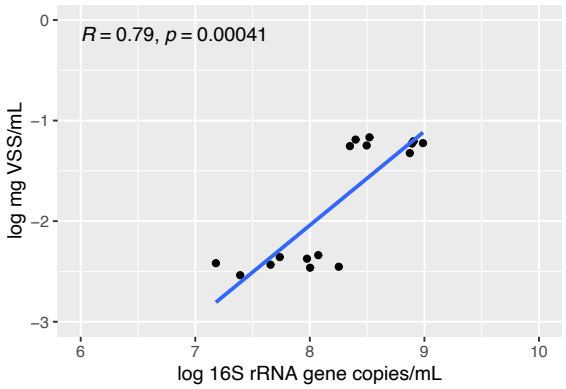


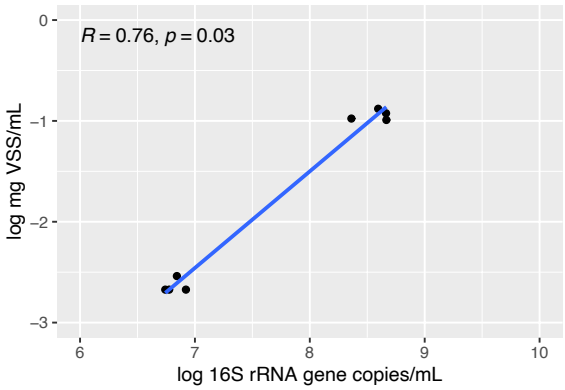
Figure 2-12. Relative gene abundance changes of the target ARGs per mg VSS, in the three long SRT (black) samples and the three short SRT plants (blue). Negative values indicate removal and positive values indicate increase. The results are expressed in log10 copies per mg VSS. Significant differences of removal rates in gene presence between long SRT plants and short SRT plants were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Correlation between 16S rRNA gene abundance and VSS concentration

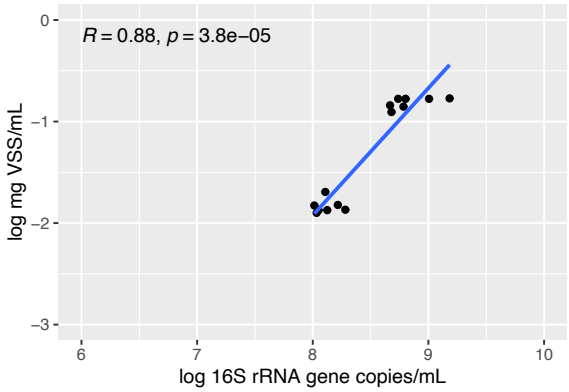
16S rRNA is widely used as a taxonomic marker for bacteria in the microbiological study and represents the total ARGs because it presents in almost all bacteria and it is large enough for informatics purposes (Earl et al., 2018; Janda and Abbott, 2007). Volatile suspended solids are important factors in activated sludge processes for biological and organic status. Average VSS concentrations of each plant were shown in Table 2-3. In our study, we found the significantly ($p < 0.05$) and strongly ($r > 0.7$) positive correlations between the concentrations of VSS and the 16S rRNA gene copies at each plant respectively (Figure 2-13). Besides, the VSS concentrations and the 16s rRNA gene copies in all the six plants together were also significantly, strongly, and positively correlated, indicating that the higher VSS concentration contains more 16S rRNA gene copies. Therefore, this result reinforces that long SRT plants may perform better than short SRT plants because the longer SRT ASP achieves lower VSS concentrations at the effluents (Chan et al., 2011; Leu et al., 2012; Li and Stenstrom, 2018). This is the first study introducing the correlation of these two parameters in the study of ARGs between the wastewater treatment and microbiological fields. Theoretically and ideally, if VSS can be totally removed, the total organic materials will be nearly zero, so the total ARGs will be fully removed.


R = 0.79, p = 0.00041
log mg VSS/mL
log 16S rRNA gene copies/mL

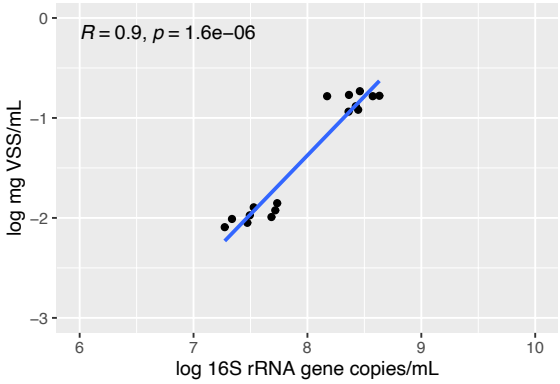
(a)


R = 0.76, p = 0.03
log mg VSS/mL
log 16S rRNA gene copies/mL

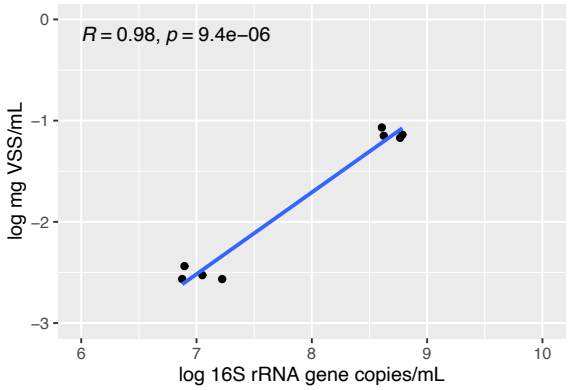
(b)


R = 0.88, p = 3.8e−05
log mg VSS/mL
log 16S rRNA gene copies/mL

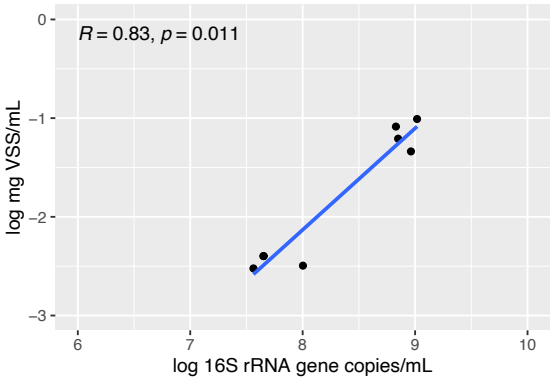
(c)


R = 0.9, p = 1.6e−06
log mg VSS/mL
log 16S rRNA gene copies/mL

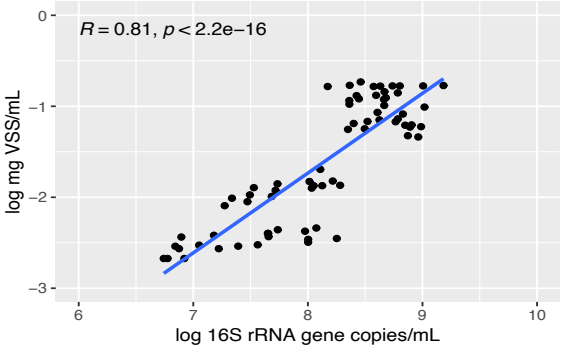
(d)


R = 0.98, p = 9.4e−06
log mg VSS/mL
log 16S rRNA gene copies/mL

(e)


R = 0.83, p = 0.011
log mg VSS/mL
log 16S rRNA gene copies/mL

(f)


R = 0.81, p < 2.2e−16
log mg VSS/mL
log 16S rRNA gene copies/mL

(g)

Figure 2-13. Correlations between 16S rRNA gene and VSS concentrations at Plant 1 (a), Plant 2 (b), Plant 3 (c), Plant 4 (d), Plant 5 (e), Plant 6 (f), and all of the six plants (g). The results are expressed in log 16S rRNA gene copies/mL for x-axis and log mg VSS/mL for y-axis. Correlation coefficient r (R) and p values are shown in each figure.

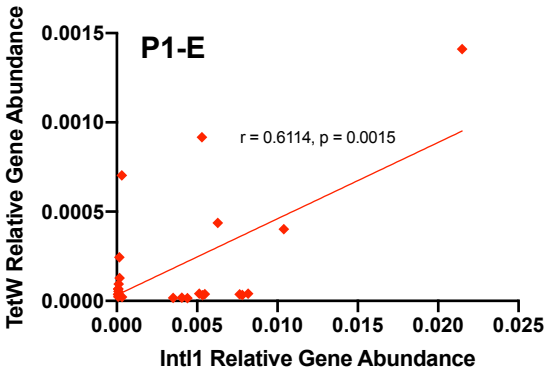
Potential HGT at Six Plants

Horizontal gene transfer (HGT) is widely recognized as the mechanism responsible for the widespread distribution of antibiotic resistance genes and previous studies have found the occurrences of HGT in the water environment (Chen and Zhang, 2013; De la Cruz and Davies, 2000; Jong et al., 2020; Zhou et al., 2021). Integrons, figured prominently in antibiotic resistance, serve as a genetic audition hall in capturing and allowing expression of random segments of DNA and affect HGT (Summers, 2006). Class 1 integrons (encoding by the *intI*1 gene) is most widely used as a marker to determine the HGT of ARGs because they carry most known cassettes (over 130 different ARG cassettes) via the exchange of integrons occurring during the gene transfer (Gaze et al., 2011; Gillings et al., 2015; Hsu et al., 2014; Summers, 2006). Major types of ARGs detected in different water environment and treatment processes have been reported to have significant correlations with *intI*1, such as *tet*, s*ul1*, *erm* genes. (Liao and Chen, 2018).

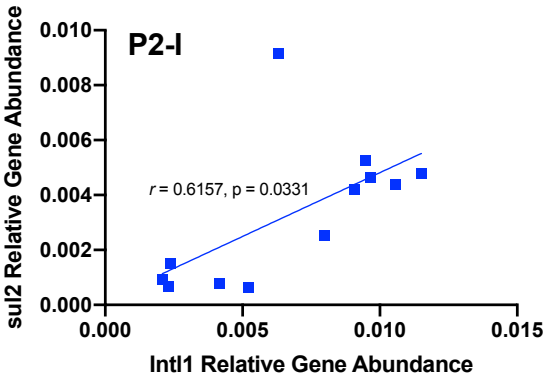
Figure 2-14 shows the significant correlations ($p < 0.05$) between relative gene abundance of *intI*1 and target ARGs. Significantly and moderately positive ($r = 0.61$) correlation between *tet*W and *intI*1 per 16S rRNA was found in the effluent of ASP at Plant 1 and significantly and moderately positive ($r = 0.62$) correlation between *sul*2 and *intI*1 per 16S rRNA was found in the influent of ASP at Plant 2. At Plant 3, both *sul*1 and two *tet* genes significantly, strongly and positively ($r > 0.7$) correlated to *intI*1 in the influent of ASP. *Tet*W per 16S rRNA significantly, strongly, and positively correlated to *intI*1 in both influent and effluent of the ASP. Significantly and moderately

positive (r = 0.62) correlations were only found between *sul*1 and *intI*1 in the effluent of ASP at Plant 4. At Plant 5, no significant ($p > 0.05$) correlations were found in the wastewater before the ASP but significant and moderate to strong correlations with *intI*1 were positively observed in the wastewater flow after the ASP for two *sul* genes (r = 0.88 and 0.60), two *tet* genes (r = 0.65 and 0.87), and *erm*B (r = 0.72). At Plant 6, *sul*1 was the only ARG observed to show significantly and moderately (r = 0.60) positive correlation for HGT in the influent of ASP, but significantly and strongly positive correlations were observed in the effluent of ASP for *sul*1, *tet*A, *tet*W, and *erm*B.

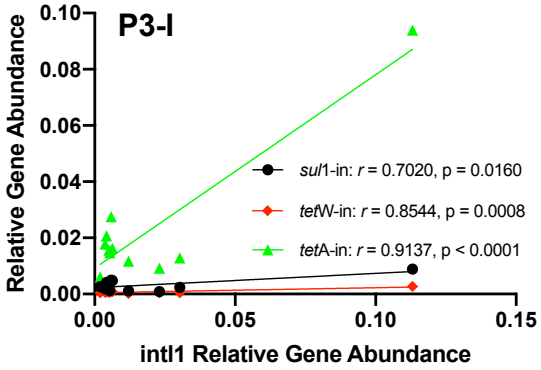
Previous studies also have found HGT in occurrence with strong correlations between *sul* and *tet* genes and *intI*1 gene (Ben et al., 2017; Boyd et al., 2000; Gillings, 2014)**,** because *tet* and *sul* genes have strong associations with mobile genetic elements to move between species (Li et al., 2015; Yoo and Lee, 2021)**.** Also, the *sul*1 gene has been previously found to be directly associated with class 1 integrons, which are widely distributed in indigenous bacteria and facilitate the HGT of ARGs (Luo et al., 2014, 2010; Mu et al., 2015; Jian Wang et al., 2015). Interestingly, in our study, we found that HGT determined by the correlations between *intI*1 and the target ARGs were found at the short-SRT ASP (Plant 3 and 4) more frequently than at the long-SRT ASP (Plant 1 and 2). Some previous study reported that shorter solids retention times were less likely to cause HGT to occur (Miller et al., 2016). However, it is very difficult to compare these results because it is still unknown if the occurrence of the ARG abundance in ASP processes is due to HGT or other factors such as organic or inorganic reactions during the process operations without further study. But one possible explanation may be either the different sources of wastewater or different operational types of ASP between long SRT plants and short SRT plants. Nevertheless, more studies are needed in the future research.


P1-E
r = 0.6114, p = 0.0015
TetW Relative Gene Abundance
Intl1 Relative Gene Abundance

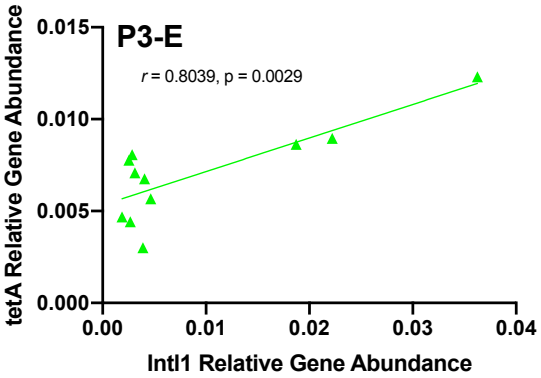
(a)


P2-I
r = 0.6157, p = 0.0331
sul2 Relative Gene Abundance
Intl1 Relative Gene Abundance

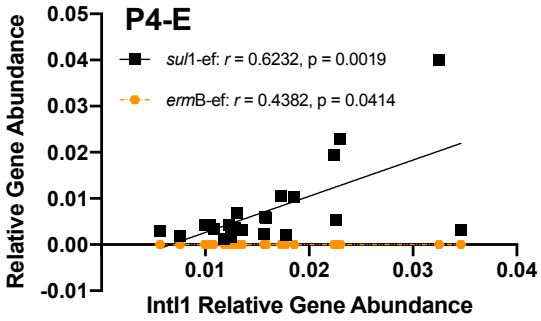
(b)


P3-I
sul1-in: r = 0.7020, p = 0.0160
tetW-in: r = 0.8544, p = 0.0008
tetA-in: r = 0.9137, p < 0.0001
Relative Gene Abundance
intl1 Relative Gene Abundance

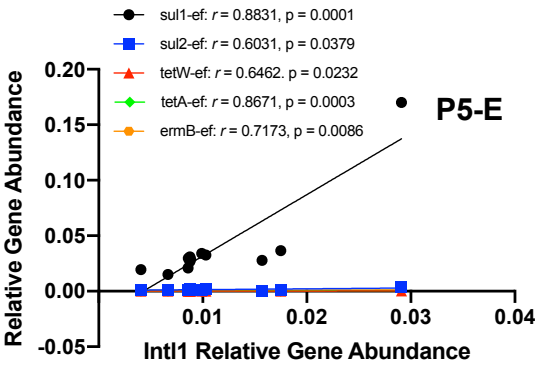
(c)


P3-E
r = 0.8039, p = 0.0029
tetA Relative Gene Abundance
Intl1 Relative Gene Abundance

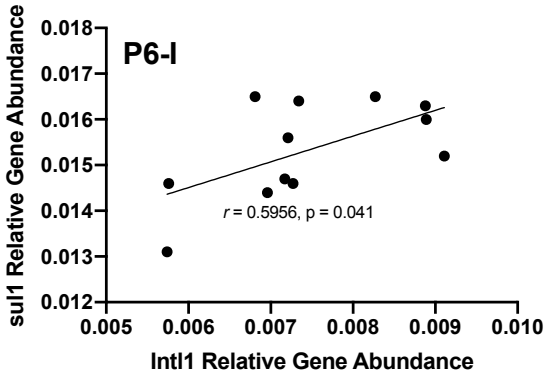
(d)


P4-E
sul1-ef: r = 0.6232, p = 0.0019
ermB-ef: r = 0.4382, p = 0.0414
Relative Gene Abundance
Intl1 Relative Gene Abundance

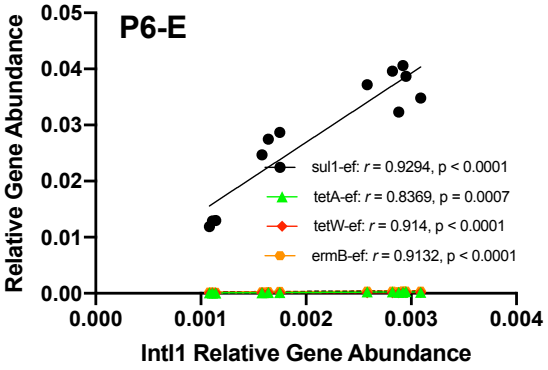
(e)


sul1-ef: r = 0.8831, p = 0.0001
sul2-ef: r = 0.6031, p = 0.0379
tetW-ef: r = 0.6462. p = 0.0232
tetA-ef: r = 0.8671, p = 0.0003
ermB-ef: r = 0.7173, p = 0.0086
P5-E
Relative Gene Abundance
Intl1 Relative Gene Abundance

(f)


P6-I
r = 0.5956, p = 0.041
sul1 Relative Gene Abundance
Intl1 Relative Gene Abundance

(g)


P6-E
sul1-ef: r = 0.9294, p < 0.0001
tetA-ef: r = 0.8369, p = 0.0007
tetW-ef: r = 0.914, p < 0.0001
ermB-ef: r = 0.9132, p < 0.0001
Relative Gene Abundance
Intl1 Relative Gene Abundance

(h)

Figure 2-14. Correlations between *intI*1 and ARG in gene copies/16S rRNA using a Pearson correlation at Plant 1 effluent (a), Plant 2 influent (b), Plant 3 Influent (c) and effluent (d), Plant 4 effluent (e), Plant 5 effluent (f), and Plant 6 influent(h) and effluent (i). Linear trendlines are only shown for correlations with significances $p \leq 0.05$. R and p values are shown.

2.4. Conclusion

ARGs are an increasing concern in wastewater treatment systems. In our study, six activated sludge treatment plants (three long SRT and three short SRT plants) near Los Angeles, CA, were studied for their ability to remove five ARGs (*sul*1, *sul*2, *tet*A, *tet*W, *erm*B, *intI*1, and 16S rRNA were analyzed for their abundances before and after the ASP in these six plants. From our study, we make the following conclusions:

1. All of the five ARGs, *intI*1, and 16S rRNA were detected before and after the ASP at the six plants. The absolute gene abundances of all the target ARGs, *intI*1, and 16S rRNA were significantly ($p < 0.05$) achieved between 0.67 and 3.3 log removal, which were between approximately 78.6% and 99.9% removal efficiencies. The three long SRT plants on average removed the absolute gene abundances of *sul*2, *tet*A, *tet*W, and *intI*1 two times greater than the three short SRT plants. The total 16S rRNA gene removal rate was also higher at long SRT plants than at short SRT plants. This indicates that long SRT and short SRT plants both can remove ARGs' abundance and long SRT plants achieved higher removal efficiencies than short SRT plants.

2. The relative gene abundance of target ARGs per 16S rRNA was more variable than the absolute gene abundance. Previous studies found that bacterial abundance was reduced at lab-scale activated sludge processes with long SRT. However, in the full-scale treatment

processes, other operational factors are present, so results can be more variable.

3. VSS represents an important factor for organic materials in wastewater treatment systems. Determining the relative gene abundance of ARGs per mg VSS was first introduced in the study of ARG abundance and was believed to better show the correlations of the fate of ARGs and performance factors in the activated sludge system. In our study, RGA_{VSS} removal efficiencies up to 99% were achieved by at six plants, except for *sul*1 and 16S rRNA. Significant increases were observed at Plant 1 and 6 for *sul*1 and at Plants 1, 3 and 4 for 16S rRNA. In total there were five significant increases, 21 significant decreases and 16 observations of no change. Generally, the RGA_{VSS} results reinforce the hypothesis that the activated sludge process can reduce RGA per mg VSS and that longer SRT plants achieve greater removals than short SRT plants.

4. Significantly and strongly ($r > 0.7$) positive correlations were found between the total 16S rRNA gene copies and VSS concentrations at all of the six plants. This is an important finding because it can better interpret wastewater treatment and microbiological fields with each other. Efforts to reduce VSS discharge from treatment plants will also reduce ARG discharges. Longer SRT operation and effluent filtration to reduce VSS are possible methods to reduce emissions.

5. Potential HGT was observed at all of the six WWTPs. Due to the correlations between the relative gene abundance of *intI*1 and the target genes, *sul1, sul2, tet*A, and *tet*W genes were found to be potentially horizontally transferred during the activated treatment processes at all of the plants except Plant 2. Also, the correlations between *intI*1 and the target ARGs

were more frequently found at the short-SRT ASP than at the long-SRT ASP. However, the actual relationship between the bacterial community and HGT in WWTPs should be further studied.

2.5. Appendix A

Table S2-1. Significant difference p value of absolute gene abundance between plants for each ARG.

*sul*1	P1	P2	P3	P4	P5	P6
P1	-	0.949	0.5453	0.0822	0.000251	0.3551
P2	-	-	0.6622	0.4621	0.02215	0.8873
P3	-	-	-	0.2589	0.000555	0.09389
P4	-	-	-	-	9.79E-05	0.03792
P5	-	-	-	-	-	0.000109
P6	-	-	-	-	-	-

*sul*2	P1	P2	P3	P4	P5	P6
P1	-	0.394	4.12E-05	0.00013	0.9153	0.6369
P2	-	-	1.25E-05	1.77E-05	0.1468	0.1034
P3	-	-	-	0.1601	8.57E-05	0.000157
P4	-	-	-	-	0.001126	0.00237
P5	-	-	-	-	-	0.7682
P6	-	-	-	-	-	-

*tet*A	P1	P2	P3	P4	P5	P6
P1	-	0.453	1.28E-05	3.46E-06	0.1311	0.3059
P2	-	-	0.003168	0.000667	0.5241	0.1433
P3	-	-	-	0.944	1.93E-05	0.000204
P4	-	-	-	-	8.23E-05	0.000709
P5	-	-	-	-	-	0.0384
P6	-	-	-	-	-	-

*tet*W	P1	P2	P3	P4	P5	P6
P1	-	0.000574	1.72E-07	1.35E-08	0.114	3.45E-05
P2	-	-	0.000255	7.46E-05	0.006102	0.4855
P3	-	-	-	0.9408	7.18E-05	0.005236
P4	-	-	-	-	0.000405	2.94E-05
P5	-	-	-	-	-	0.007878
P6	-	-	-	-	-	-

*erm*B	P1	P2	P3	P4	P5	P6
P1	-	0.6773	0.4279	0.4863	0.1107	0.5343
P2	-	-	0.02419	0.007773	0.5879	1.09E-05

P3	-		-	0.7281	7.53E-05	0.1248
P4	-	-	-	-	0.001718	0.6103
P5	-	-	-	-	-	0.005046
P6	-	-	-	-	-	-

intI1	P1	P2	P3	P4	P5	P6
P1	-	0.04652	0.03212	0.8344	0.00012	6.23E-05
P2	-	-	0.005707	0.05982	0.04873	0.363
P3	-	-	-	0.05186	9.24E-05	0.000503
P4	-	-	-	-	0.000179	0.002456
P5	-	-	-	-	-	4.19E-09
P6	-	-	-	-	-	0.01791

16S rRNA	P1	P2	P3	P4	P5	P6
P1	-	1.46E-05	0.0303	0.956	0.000783	0.07787
P2	-	-	2.84E-05	2.69E-05	0.6186	0.01086
P3	-	-	-	0.0124	0.000707	0.01473
P4	-	-	-	-	0.001405	0.07946
P5	-	-	-	-	-	0.02229
P6	-	-	-	-	-	-

Table S2-2. Significance p value of absolute gene abundance removals for each ARGs at each plant

	P1	P2	P3	P4	P5	P6
*sul*1	<0.000001	0.002472	<0.000001	<0.000001	<0.000001	0.000007
*sul*2	<0.000001	<0.000001	0.000002	<0.000001	0.000011	0.000009
*tet*A	<0.000001	0.000004	0.000016	<0.000001	<0.000001	0.000003
*tet*W	<0.000001	0.000014	0.000085	<0.000001	0.000003	<0.000001
*erm*B	0.000469	<0.000001	0.000629	<0.000001	0.000002	<0.000001
intI1	0.036526	0.000176	0.001072	<0.000001	0.000001	0.000002
16S rRNA	0.000059	<0.000001	0.003294	<0.000001	0.000002	0.000031

Table S2-3. Significant difference and p value of absolute gene abundance between three long and three short SRT plants

ARG	Plant	Log removal	P value	P Value
*sul*1	Long SRT	-1.159	<0.0001	0.2017
	Short SRT	-1.028	<0.0001	
*sul*2	Long SRT	-1.961	<0.0001	<0.0001
	Short SRT	-1.17	<0.0001	
*tet*A	Long SRT	-2.134	<0.0001	<0.0001
	Short SRT	-1.474	<0.0001	
*tet*W	Long SRT	-2.78	<0.0001	<0.0001
	Short SRT	-1.397	<0.0001	
*erm*B	Long SRT	-2.487	<0.0001	0.0024
	Short SRT	-1.5	<0.0001	
intI1	Long SRT	-1.567	<0.0001	0.1305
	Short SRT	-1.393	<0.0001	
16S rRNA	Long SRT	-1.306	<0.0001	0.0063
	Short SRT	-0.9174	<0.0001	

Table S2-4. Correlation matrix of absolute gene abundance removals for *r* and p value at three long SRT plants.

r	*sul*1	*sul*2	*tet*A	*tet*W	*erm*B	*intI1*	16S rRNA
*sul*1	1.00000	-0.15143	0.49706	0.63872	0.21330	0.63215	0.52207
*sul*2	-0.15143	1.00000	0.10707	-0.24313	0.02558	0.12346	0.45039
*tet*A	0.49706	0.10707	1.00000	0.10599	0.67255	0.38127	0.44193
*tet*W	0.63872	-0.24313	0.10599	1.00000	-0.16411	0.21654	-0.03265
*erm*B	0.21330	0.02558	0.67255	-0.16411	1.00000	0.39677	0.33420
intI1	0.63215	0.12346	0.38127	0.21654	0.39677	1.00000	0.76742
16S rRNA	0.52207	0.45039	0.44193	-0.03265	0.33420	0.76742	1.00000

p value	*sul*1	*sul*2	*tet*A	*tet*W	*erm*B	*intI1*	16S rRNA
*sul*1	-	0.57558	0.05013	0.00774	0.42766	0.00860	0.03804
*sul*2	0.57558	-	0.69309	0.36423	0.92509	0.64872	0.08000
*tet*A	0.05013	0.69309	-	0.69604	0.00431	0.14510	0.08655
*tet*W	0.00774	0.36423	0.69604	-	0.54363	0.42052	0.90446
*erm*B	0.42766	0.92509	0.00431	0.54363	-	0.12811	0.20583
intI1	0.00860	0.64872	0.14510	0.42052	0.12811	-	0.00052
16S rRNA	0.03804	0.08000	0.08655	0.90446	0.20583	0.00052	-

Table S2-5. Correlation matrix of absolute gene abundance removals for *r* and p value at three short SRT plants.

r	*sul*1	*sul*2	*tet*A	*tet*W	*erm*B	*intI1*	16S rRNA
*sul*1	1.00000	0.13020	0.06807	-0.22411	0.35162	0.41224	0.41692
*sul*2	0.13020	1.00000	0.77831	0.64292	0.43621	0.81633	0.83754
*tet*A	0.06807	0.77831	1.00000	0.73837	0.41664	0.87083	0.76920
*tet*W	-0.22411	0.64292	0.73837	1.00000	0.19004	0.62055	0.57078
*erm*B	0.35162	0.43621	0.41664	0.19004	1.00000	0.66031	0.59579
intI1	0.41224	0.81633	0.87083	0.62055	0.66031	1.00000	0.86080
16S rRNA	0.41692	0.83754	0.76920	0.57078	0.59579	0.86080	1.00000

p value	*sul*1	*sul*2	*tet*A	*tet*W	*erm*B	*intI1*	16S rRNA
*sul*1	-	0.63080	0.80221	0.40405	0.18171	0.11258	0.10815
*sul*2	0.63080	-	0.00038	0.00722	0.09120	0.00011	0.00005
*tet*A	0.80221	0.00038	-	0.00109	0.10841	0.00001	0.00050
*tet*W	0.40405	0.00722	0.00109	-	0.48083	0.01032	0.02094
*erm*B	0.18171	0.09120	0.10841	0.48083	-	0.00537	0.01488
intI1	0.11258	0.00011	0.00001	0.01032	0.00537	-	1.865E-05
16S rRNA	0.10815	0.00005	0.00050	0.02094	0.01488	0.00002	-

Table S2-6. Significant difference p value of relative gene abundance per 16S rRNA between plants for each ARG.

*sul*1	P1	P2	P3	P4	P5	P6
P1	-	0.00531	0.5412	0.102	0.1063	0.05252
P2	-	-	0.002043	0.0018	0.001874	0.01492
P3	-	-	-	0.9172	0.9069	0.4734
P4	-	-	-	-	0.01616	0.02782
P5	-	-	-	-	-	0.2326
P6	-	-	-	-	-	-

*sul*2	P1	P2	P3	P4	P5	P6
P1	-	0.000147	0.000438	4.98E-06	0.001229	0.004128
P2	-	-	0.09526	0.0469	0.2123	0.07452
P3	-	-	-	0.931	0.1139	0.005144
P4	-	-	-	-	0.6252	0.002762
P5	-	-	-	-	-	0.02914
P6	-	-	-	-	-	-

*tet*A	P1	P2	P3	P4	P5	P6
P1	-	0.000136	0.001483	4.98E-06	0.001051	0.02485
P2	-	-	0.01965	0.956	0.09887	0.05263
P3	-	-	-	0.006531	0.2241	0.06924
P4	-	-	-	-	0.07745	0.05157
P5	-	-	-	-	-	0.2715
P6	-	-	-	-	-	-

*tet*W	P1	P2	P3	P4	P5	P6
P1	-	1.61E-07	3.52E-05	5E-08	0.06745	0.00048
P2	-	-	0.4216	0.412	0.001594	0.0343
P3	-	-	-	0.2283	0.00267	0.1346
P4	-	-	-	-	5.91E-05	0.0124
P5	-	-	-	-	-	0.006447
P6	-	-	-	-	-	-

*erm*B	P1	P2	P3	P4	P5	P6
P1	-	0.07007	0.2255	0.4668	0.02445	0.2859
P2	-	-	0.0007	6.02E-07	0.000389	0.002872
P3	-	-	-	0.1991	0.000231	0.4967
P4	-	-	-	-	3.17E-05	0.05545

P5	-	-	-	-	-	4.7E-05
P6	-	-	-	-	-	-

intI1	P1	P2	P3	P4	P5	P6
P1	-	0.03893	0.93	0.8598	0.2188	0.2882
P2	-	-	0.04225	0.03731	0.09291	0.0166
P3	-	-	-	0.7846	0.9441	0.1257
P4	-	-	-	-	0.1513	0.3238
P5	-	-	-	-	-	0.07555
P6	-	-	-	-	-	-

Table S2-7. Significance p value of relative gene abundance per 16S rRNA changes for each ARGs at each plant

p value	P1	P2	P3	P4	P5	P6
*sul*1	0.310194	0.000901	0.293259	0.000037	0.568982	0.081726
*sul*2	<0.000001	0.002943	0.351073	0.305073	0.101803	0.000774
*tet*A	0.000095	0.000585	0.000201	0.000001	0.000203	0.002021
*tet*W	<0.000001	0.005612	0.015909	0.046322	0.000277	0.000294
*erm*B	0.06449	0.000028	0.040165	0.000005	0.000009	0.000646
intI1	0.341685	0.618057	0.004931	0.000046	0.028009	0.001382

Table S2-8. Significant difference and p value of relative gene abundance per 16S rRNA between three long and three short SRT plants

ARG	Plant	Log removal	P value
*sul*1	Long SRT	0.1473	0.1653
	Short SRT	-0.1096	0.1195
*sul*2	Long SRT	-0.6546	<0.0001
	Short SRT	-0.2519	0.0046
*tet*A	Long SRT	-0.8278	<0.0001
	Short SRT	-0.5447	<0.0001
*tet*W	Long SRT	-1.474	<0.0001
	Short SRT	-0.4775	0.0001
*erm*B	Long SRT	-1.53	0.0002
	Short SRT	-0.5869	<0.0001
intI1	Long SRT	-0.2613	0.0035
	Short SRT	-0.4696	<0.0001

Table S2-9. Significant difference p value of relative gene abundance per mg VSS between plants for each ARG.

*sul*1	P1	P2	P3	P4	P5	P6
P1	-	0.04228	0.8167	0.02762	0.0008975	0.009014
P2	-	-	0.05352	0.01505	0.005622	0.0832
P3	-	-	-	0.5413	0.1076	0.3965
P4	-	-	-	-	0.007527	0.004956
P5	-	-	-	-	-	0.000288
P6	-	-	-	-	-	-

*sul*2	P1	P2	P3	P4	P5	P6
P1	-	0.00836	0.00836	0.000931	0.1481	0.2902
P2	-	-	0.0006323	0.00404	0.7095	0.5982
P3	-	-	-	0.4606	0.005184	0.007071
P4	-	-	-	-	0.00404	0.0008486
P5	-	-	-	-	-	0.8524
P6	-	-	-	-	-	-

*tet*A	P1	P2	P3	P4	P5	P6
P1	-	0.01067	0.0001896	0.00001327	0.997	0.06429
P2	-	-	0.1232	0.02657	0.02301	0.1345
P3	-	-	-	0.2168	0.003544	0.004063
P4	-	-	-	-	0.001049	0.0003008
P5	-	-	-	-	-	0.1222
P6	-	-	-	-	-	-

*tet*W	P1	P2	P3	P4	P5	P6
P1	-	5.348E-06	3.055E-06	2.929E-08	0.3314	0.0004074
P2	-	-	0.1051	0.01155	0.00251	0.05623
P3	-	-	-	0.5058	0.0006656	0.0009793
P4	-	-	-	-	0.0005983	0.001209
P5	-	-	-	-	-	0.005236
P6	-	-	-	-	-	-

*erm*B	P1	P2	P3	P4	P5	P6
P1	-	0.2902	0.001176	0.01931	0.4707	0.01598
P2	-	-	0.001871	0.00003	0.4339	0.0001701
P3	-	-	-	0.3732	0.001944	0.5594
P4	-	-	-	-	0.03501	0.6414

P5	-	-	-	-	-	0.003666
P6	-	-	-	-	-	-

intI1	P1	P2	P3	P4	P5	P6
P1	-	0.2271	0.5761	0.5298	0.0005258	0.002798
P2	-	-	0.5157	0.1473	0.005429	0.009718
P3	-	-	-	0.3679	0.00714	0.01505
P4	-	-	-	-	0.00411	0.02036
P5	-	-	-	-	-	0.2907
P6	-	-	-	-	-	-

16S rRNA	P1	P2	P3	P4	P5	P6
P1	-	0.002691	0.4614	0.5701	0.004912	0.2527
P2	-	-	0.001907	0.005454	0.1138	0.4101
P3	-	-	-	0.1715	0.004941	0.1633
P4	-	-	-	-	0.007546	0.3556
P5	-	-	-	-	-	0.08267
P6	-	-	-	-	-	-

Table S2-10. Significance p value of relative gene abundance per mg VSS changes for each ARGs at each plant

p value	P1	P2	P3	P4	P5	P6
*sul*1	0.115992	0.01003	0.457451	0.730346	0.024452	0.00611
*sul*2	0.000311	0.003422	0.162998	0.277904	0.018962	0.010899
*tet*A	0.000001	0.013854	0.00317	0.041856	0.000209	0.001376
*tet*W	0.000019	0.03204	0.366614	0.830112	0.000052	0.000762
*erm*B	0.10524	0.000007	0.290183	0.002028	0.000051	0.018608
intI1	0.825643	0.850182	0.536265	0.066325	0.004467	0.00263
16S rRNA	0.079027	0.535155	0.000439	0.00265	0.045858	0.530379

Table S2-11. Significant difference and p value of relative gene abundance per mg VSS between three long and three short SRT plants

ARG	Plant	Difference	P value	P Value
*sul*1	Long SRT	0.2092	0.044	0.4661
	Short SRT	0.1265	0.0495	
*sul*2	Long SRT	-0.5927	<0.0001	<0.0001
	Short SRT	-0.01921	0.8564	
*tet*A	Long SRT	-0.7658	<0.0001	<0.0001
	Short SRT	-0.3097	0.0002	
*tet*W	Long SRT	-1.413	<0.0001	<0.0001
	Short SRT	-0.2418	0.0303	
*erm*B	Long SRT	-1.12	0.001	0.011
	Short SRT	-0.3508	0.0001	
intI1	Long SRT	-0.1993	0.01	0.7201
	Short SRT	-0.2331	0.0025	
16S rRNA	Long SRT	0.06192	0.4264	0.0631
	Short SRT	0.2364	0.0002	

Table S2-12. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 1: p > 0.05 (ns), p <0.05 (*), p < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****).

Wastewater Flow	Influent					Effluent				
ARG	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B
r	-0.269	-0.115	0.1973	-0.168	0.1629	0.2205	0.3477	0.6114	-0.477	-0.409
p value	0.2032	0.5923	0.3555	0.4332	0.447	0.3006	0.0959	0.0015	0.0184	0.047
significant at 0.05?	ns	ns	ns	ns	ns	ns	ns	**	*	*

Table S2-13. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 2: p > 0.05 (ns), p <0.05 (*), p < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****).

Wastewater Flow	In					Eff.				
ARG	sul1	sul2	tetA	tetW	*erm*B	sul1	sul2	tetA	tetW	*erm*B
r	-0.011	0.6157	0.6716	0.5709	-0.135	0.1978	-0.859	0.5707	0.5085	0.2978
p value	0.9727	0.0331	0.0568	0.0526	0.6766	0.5378	0.0003	0.0526	0.0914	0.3472
significant at 0.05?	ns	*	*	ns	ns	ns	***	ns	ns	ns

Table S2-14. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 3: p > 0.05 (ns), p <0.05 (*), p < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****).

Wastewater Flow	Influent					Effluent				
ARG	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B
r	0.702	0.5906	0.8544	0.9137	-0.003	-0.67	-0.161	0.4026	0.8039	0.3617
p value	0.016	0.0557	0.0008	<0.0001	0.9934	0.024	0.6358	0.2196	0.0029	0.2744
significant at 0.05?	*	ns	***	****	ns	*	ns	ns	**	ns

Table S2-15. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 4: p > 0.05 (ns), p <0.05 (*), p < 0.01 (**), p < 0.001 (***), and p < 0.0001 (****).

Wastewater Flow	Influent					Effluent				
ARG	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B
r	0.2972	0.0589	0.0578	0.0138	0.086	0.6232	0.0069	0.1172	0.332	0.4382
p value	0.1791	0.7945	0.7983	0.9513	0.7037	0.0019	0.9757	0.6034	0.1312	0.0414
significant at 0.05?	ns	ns	ns	ns	ns	**	ns	ns	ns	*

Table S2-16. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 5: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Wastewater Flow	Influent					Effluent				
ARG	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B
r	0.3384	0.1741	0.2785	-0.188	0.2967	0.8831	0.6031	0.6462	0.8671	0.7173
p value	0.282	0.5884	0.3807	0.5594	0.349	0.0001	0.0379	0.0232	0.0003	0.0086
significant at 0.05?	ns	ns	ns	ns	ns	***	*	*	***	**

Table S2-17. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 6: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Wastewater Flow	Influent					Effluent				
ARG	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B	*sul*1	s*ul*2	*tet*A	*tet*W	*erm*B
r	0.5956	-0.574	-0.057	0.2825	0.4663	0.9294	0.5374	0.8369	0.914	0.9132
p value	0.041	0.0511	0.8594	0.3736	0.1265	<0.0001	0.0716	0.0007	<0.0001	<0.0001
significant at 0.05?	*	ns	ns	ns	ns	****	ns	***	****	****

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Chapter 3: Fate of Antibiotic Resistance Genes in Four Full-Scale Anaerobic Digestion Processes

3.1. Introduction

Antibiotic resistance has been increasingly recognized as one of the significant emerging environmental pollutants in recent decades (Berendonk et al., 2015). Antibiotic resistance genes (ARGs) and their resistant bacteria (ARB) can find their ways back to humans via direct or indirect contact with water environment, such as drinking water from rivers, even after treatment or from the soil environment, via the food chain from the agricultural areas (Echeverria-Palencia et al., 2017; Wellington et al., 2013). Wastewater treatment plants (WWTPs) are a key technology for controlling or releasing ARGs and ARB into the environment (Pruden et al., 2013). WWTPs bring different sources of waste resulting in the accumulation of large amounts of biological wastes and provide a potential environment for horizontal gene transfer (HGT) between mobile genetic elements (MGEs) (Pallares-Vega et al., 2019; Schlueter et al., 2007). The fate of ARGs in WWTPs is a concern although studies have shown that WWTPs can reduce ARGs through different treatment processes (Xue et al., 2019).

In our previous study, we found that six full-scale activated sludge treatment plants all decreased six different ARGs on average of 67% to 99% removal efficiencies (Li et al. 2021). Many other previous studies also have documented the removal efficiencies for different ARGs and ARB at WWTPs, although some other studies have found proliferation of some ARGs after the treatments (An et al., 2018; Hayward et al., 2019; Ju et al., 2019; Korzeniewska and Harnisz, 2018; Tang et

al., 2016; Yang et al., 2014). However, sewage sludge is the other potential source of ARGs and ARB aside from wastewater flow in WWTPs (Auerbach et al., 2007; Munck et al., 2015).

Biosolids or sludge are a byproduct of wastewater treatment and are generally separated from the wastewater flow during primary and secondary settling processes. Biosolids from different treatment plant sources are usually combined for further treatment, dewatering and disposal. A previous study estimated that more than 90% of antibiotic resistance in WWTPs was associated with sewage sludge (Trust. et al., 2018). The treated sludge can be either landfilled or recycled for agricultural usage, with differing rules and regulations in different countries. One common treatment process of sewage sludge treatment is anaerobic digestion (AD). It involves the degradation and stabilization of organic materials under anaerobic conditions by microbial organisms and leads to the formation of biogas (a mixture of carbon dioxide and methane) and microbial biomass (Kelleher et al., 2002). Anaerobic digestion has numerous advantages, such as low sludge production, low energy requirements, pasteurization and energy recovery (Chernicharo et al., 2015; Ghosh and Pohland, 1974).

The fate of ARGs during anaerobic digestion has been an increasing concern. The effectiveness of AD of biosolids from treatment plants has been previously studied (Appels et al., 2008) for solids reduction and impacts on dewatering, but few studies have addressed ARGs. Two types and efficiencies of anaerobic digestion are generally used in the industry, including mesophilic (35 °C) and thermophilic (55 °C) digestion (Vindis et al., 2009). Studies found different removal efficiencies under different types of anaerobic digestion and that better reduction was observed for three tetracycline resistance genes and class 1 integron at thermophilic digestion in full-scale and

lab-scale (Diehl and LaPara, 2010; Ghosh et al., 2009). However, other researchers have found no removals or even increases in ARG abundance (Ma et al., 2011; Zhang et al., 2015). The behavior and fate of ARGs during anaerobic digestion remains unclear.

In this work, four full-scale anaerobic digestion processes at four WWTPs were studied for four weeks. Influent and effluent samples were collected weekly. Two tetracycline resistance genes (*tet*A and *tet*W), two sulfonamide resistance genes (*sul*1 and *sul*2), one macrolide resistance genes (*erm*B), class 1 integron (*intI*1), and 16S rRNA were targeted and observed. This study helps to understand the fates of ARGs in the anaerobic digestion and anaerobic digestion removal efficiencies for different ARGs. This is an important subject because most treatment plants dispose of digested, dewatered sludge in ways that may introduce ARGs back into the environment.

3.2. Materials and methods

Sample Collection

Four wastewater treatment plants, located near Los Angeles were selected for the collection of sludge samples before and after the anaerobic digestion, including one thermophilic anaerobic digestion process (AD3) and three mesophilic anaerobic digestion processes (AD4, AD5, and AD6). The digesters from AD4, AD5, and AD6 are continually mixed with gas mixing and are heated up to 35 °C. AD3 are thermophilic anaerobic digesters with the temperature of approximately 55 °C. The schematic layout of the four anaerobic digestions is shown in Figure 3-1 and information is detailed in Table 3-1. One-liter digester feed (DF) and one-liter digested sludge (DS) samples were collected in sterile containers at each plant (Figure 3-1). Digester feed

at plants AD3, AD5, and AD6 were composed of primary and waste activated sludge. Digester feed at AD4 is separated. One-liter influent samples were collected at AD3, AD5 and AD6. At AD4 0.5-liter primary sludge and 0.5-liter waste activated sludge were collected and mixed to form a represented feed sample. Digester sludge samples were collected before dewatering, except for AD5 where dewater samples were collected. Samples were prepared by the treatment plant operators for collection and then kept on ice during transport and stored at 4 °C until processing, which was always less than 24 hours. Samples were transferred into a 50-mL conical centrifuge tubes (Fisher Scientific Co LLC, Waltham, MA). A pellet size of 50-100 mg wet sludge was then transferred into a Lysing Matrix A tube (MP Biomedicals, LLC, Irvine, CA) and subsequently stored at -20 °C, awaiting DNA extraction. All processing was performed in triplicate and all samples were processed within 24 hours of collection.

Table 3-1. Four anaerobic digestion processes information

Plant	Anaerobic digestion	Type	Temperature	Sampling Time
3	AD3	Thermophilic	55-57 °C	Feb & Mar 2021
4	AD4	Mesophilic	35-37 °C	Feb & Mar 2021
5	AD5	Mesophilic	35-37 °C	Nov 2020
6	AD6	Mesophilic	35-37 °C	Feb & Mar 2021

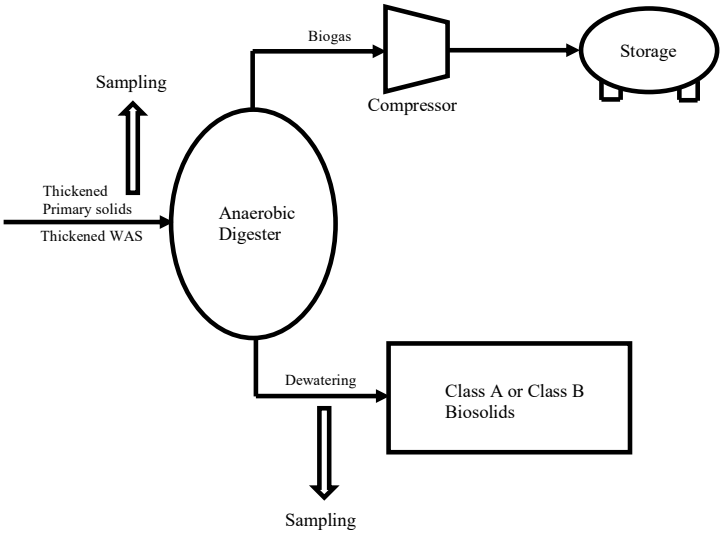


Figure 3-1. Schematic layout of the anaerobic digestion processes at the four WWTPs and sampling sites

DNA Extraction

DNA was extracted from sludge samples using MP Biomedicals FastDNA SPIN Kit (MP Biomedicals, LLC, Irvine, CA). Extractions proceeded as per the manufacturer's guidelines except for homogenizing samples that were performed with a Mini-Beatbeater (BioSpec Products, Inc., Bartlesville, OK) running for two, one-minute intervals. In order to meet the requirement of the purity of the DNA, samples were purified by adding prepared SEWS-M solutions to purify DNA three times instead of one time suggested by the protocols. Eluted DNA was determined using a Nanodrop 2000C spectrophotometer (Thermal Scientific, Waltham, MA) at 260/280 nm absorbance ratios above 1.80. Gene recoveries were calculated for each sample via a matrix spike and found to be within 80-120% (Armstrong et al., 2007).

Quantitative Polymerase Chain Reaction (qPCR)

Five antibiotic resistance genes, integron 1 class gene, and 16S rRNA were selected for quantitative detection using SYBR Green Master Mix qPCR, including two tetracycline resistance genes (*tet*A and *tet*W), two sulfonamide resistance genes (*sul*1 and *sul*2), one macrolide resistance gene (*erm*B), class 1 integron gene (*intI*1). These ARGs were chosen according to our previous study focusing on the fate of these ARGs in activated sludge processes (ASP) from six WWTPs, four AD processes of which belong to the WWTPs (Plant 3, 4, 5, and 6). The bacterial 16S rRNA gene was determined by qPCR to enumerate the total bacterial community and to calculate the relative abundance of ARG relative to the 16S rRNA copy numbers.

Table 3-2. Primer sequences used for qPCR

Target ARG	Primer	Concentration (nM)	Sequence (5'-3')	Annealing Temperature (°C)	References
*sul*1	*sul*1-F	200	CGCACCGGAAACATCGCTGCAC	65 °C/30s	(Pei et al., 2006)
	*sul*1-R		TGAAGTTCCGCCGCAAGGCTCG		
*sul*2	*sul*2-F	200	CTCCGATGGAGGCCGGTAT	60 °C/30s	(Luo et al., 2010)
	*sul*2-R		GGGAATGCCATCTGCCTTGA		
*tet*A	*tet*A-F	200	GCTACATCCTGCTTGCCTTC	55 °C/30s	(Ng et al., 2001)
	*tet*A-R		CATAGATCGCCGTGAAGAGG		
*tet*W	*tet*W-F	200	GAGAGCCTGCTATATGCCAGC	60 °C/30s	(Aminov et al., 2001)
	*tet*W-R		GGGCGTATCCACAATGTTAAC		
*erm*B	*erm*B-F	500	AAAACTTACCCGCCATACCA	60 °C/30s	(Knapp et al., 2010)
	*erm*B-R		TTTGGCGTGTTTCATTGCTT		
intI1	*intI1*-F	200	CCTCCCGCACGATGATC	55 °C/30s	(Goldstein et al., 2001)
	intI1-R		TCCACGCATCGTCAGGC		
16S rRNA	16S rRNA-F	250	CCTACGGGAGGCAGCAG	56 °C/30s	(Ji et al., 2012)
	16S rRNA-R		ATTACCGCGGCTGCTGG		

All assays used a 20-µL reaction volume consisting of 10-µL of SYBR™ Select Master Mix (Thermo Fisher Scientific, Waltham, MA), 1-µL of forward primer working concentrations, 1-µL of reverse primer working concentrations, 4-µL of molecular grade RNAse-free molecular biological grade water (Fisher Scientific, Pittsburgh, PA), and 4-µL of diluted DNA template. DNA template was diluted before qPCR to exclude inhibition effects, as confirmed and clarified previously. All assays were performed in 96-well reaction plates using an Mx3000P Real-Time PCR system (Agilent Technologies, Santa Clara, CA). Reaction conditions, including cycling conditions, primer sequences, and concentrations are shown in Table 3-2. Each assay run included a 5-point standard curve positive control, all applicable extraction samples, and a negative control of molecular grade RNAse-free molecular biological grade water in triplicate. Target-containing DNA fragments served as positive controls and analyzed using Geneious coupled with NCBI

Database information and ordered through IDT Technologies (Coralville, IA). Melt curves were used to further verify correct target gene quantification. Efficiencies ranged from 90 to 110 % and R2 values were > 0.99 for all standard curves. Similar techniques have been used previously by our group (Echeverria-Palencia et al., 2017).

Statistical Analysis

In order to understand the gene abundance in sludge-only samples, dry sludge content (η) was determined by drying the sludge samples in an oven at 100 °C for 24 hours. The dry sludge content was calculated by dry weight (w_d) divided by total weight (w_t) shown in Equation 1.

$$\eta = \frac{w_d}{w_t} \times 100\% \quad (1)$$

The dry-sludge absolute gene abundance (AGA_{dry}) was calculated and shown in Equation 2 by absolute gene abundance tested via qPCR divided by dry sludge content (η).

$$AGA_{dry} = \frac{AGA}{\eta} \quad (2)$$

The change in absolute gene abundance (Δ AGA_{dry}) was calculated and shown in Equation 3 by subtracting absolute gene abundance of DS ($AGA_{dry|D}$) with absolute gene abundance of digester feed ($AGA_{dry|F}$).

$$\Delta AGA_{dry} = AGA_{dry|D} - AGA_{dry|F} \quad (3)$$

The change in relative gene abundance (Δ RGA) was calculated by normalizing to the bacterial 16S rRNA gene as shown in Equation 4 by subtracting relative gene abundance of secondary influents (RGA_{DS}) with relative gene abundance of secondary influents (RGA_{DF}).

$$\Delta \text{ RGA} = RGA_{DS} - RGA_{DF} \quad (4)$$

Log gene abundance and log removal values were calculated using base-10 logarithms for the evaluation of removal efficiencies of the individual treatment plants. Relative *intI*1 abundance was correlated to all other ARG relative abundances. Correlations were calculated using Pearson's bivariate correlation coefficient (Galvin et al., 2010; Narciso-Da-Rocha et al., 2014).

Statistical analyses including two-tailed t-test were performed with GraphPad Prism version 8 (San Diego, CA) and Rstudio (Boston, MA). The differences at $p < 0.05$ level among samples were considered statistically significant. Correlation analysis using Pearson correlation between the removal of ARGs were studied and the strength of correlations were defined as strong ($r > 0.7$), moderate ($0.5 < r \leq 0.7$), weak ($0.3 < r \leq 0.5$), none or very weak ($r \leq 0.3$) (Moore and Kirkland, 2007).

3.3. Results and discussion

Absolute gene abundance of ARGs at the four anaerobic digestion processes

The average absolute gene abundance of each ARG is shown in Figure 3-2 for the four anaerobic digestion processes. The highest and lowest absolute gene abundance among the six target ARGs

were compared at each plant. At AD3, the highest absolute gene abundance from the DF was 9.34 log gene copies/g dw sludge (*sul*1) but the lowest absolute gene abundance was 7.49 log gene copies/g dw sludge (*erm*B). The highest absolute gene abundance from the DS at Plant 3 was 8.61 log gene copies/g dw sludge (*sul*1) but the lowest absolute gene abundance was 6.3 log gene copies/g dw sludge (*tet*A). At AD4, the highest and lowest absolute gene abundance from DF and DS was also detected for the same ARGs, which were *sul*1 (10.79) and *erm*B (8) for DF and *sul*1 (10.2) and *tet*A (7.63) in log gene copies/g dw sludge. At AD5, *sul*1 was detected at the highest absolute gene abundance from the sludge before and after the anaerobic digestion (10.36 and 10.04 log gene copies/g dw sludge) and *tet*A was detected at the lowest absolute gene abundance (8.21 log gene copies/g dw sludge from DF and 8 log gene copies/g dw sludge from DS). At AD6, *sul*1 was also detected at the highest absolute gene abundance before and after the anaerobic digestion for 11.19 and 10.07 log gene copies/g dw sludge, respectively, but *erm*B was detected at the lowest absolute gene abundance before and after the anaerobic digestion for 9.34 and 8.34 log gene copies/g dw sludge, respectively. Among the four AD sludge samples tested, 16S rRNA displayed the absolute gene abundance with more than 11.1 log gene copies/g dw sludge before AD and 10.26 log gene copies/g dw sludge after AD.

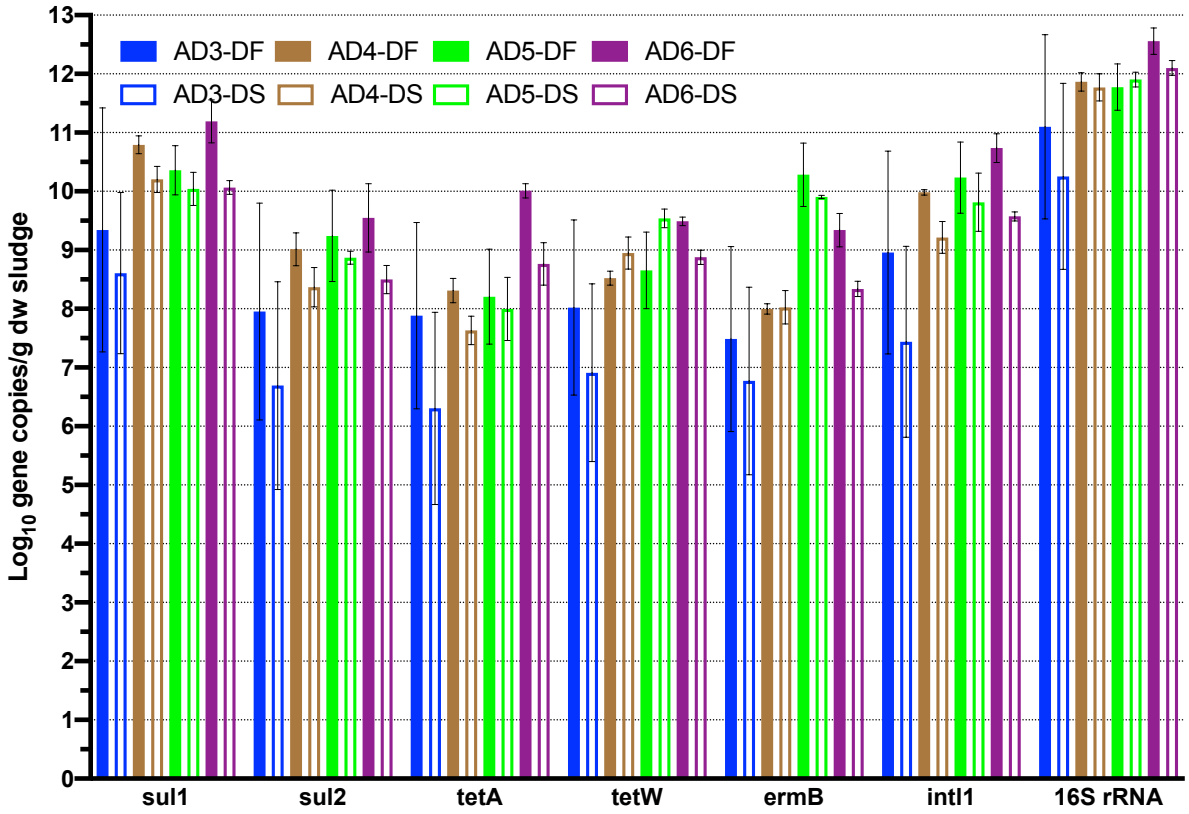


Figure 3-2. Absolute gene abundance of each target ARG at each of the four plants' anaerobic digester. The results are expressed in $\log_{10}$ copies per g dw sludge.

The absolute gene abundance of each ARGs was compared among the four AD. The highest absolute gene abundance of *sul*1 was observed at AD6's DF (11.19 log gene copies/g dw sludge) and AD4's DS (10.2 log gene copies/g dw sludge). The highest absolute gene abundance of *sul*2, *tet*W, and *intI*1 was observed at AD6's DF between 9.49 and 10.74 log gene copies/g dw sludge and AD5's DS between 8.87 and 9.81 log gene copies/g dw sludge. Sludge before and after the anaerobic digestion processes at AD6 was observed for the highest absolute gene abundance of *tet*A at 10.01 and 8.77 log gene copies/g dw sludge, respectively. Sludge before and after the anerobic digester at AD5 was observed for the highest absolute gene abundance of *erm*B at 10.28 and 9.9 log gene copies/g dw sludge, respectively. The 16S rRNA was most highly observed at the AD6's DF and DS among the four AD processes for 12.56 and 12.10 log gene copies/g dw

sludge. However, the lowest absolute gene abundance of all the six ARGs and 16S rRNA was all observed from the DF and DS at AD3, the only thermophilic anaerobic digestion process, between 6.3 and 11.1 log gene copies/g dw sludge. Sun et al. (2016) found thermophilic AD controlled the abundance of most of the ARGs better than mesophilic AD. In addition, previous studies also found similar abundance levels of these ARGs as high as 13.16 log gene copies/g dw sludge (Dong et al., 2019; Zou et al., 2020). Nevertheless, we did not find highly abundant gene copies of *tet*A comparing to other ARGs in our study, which was different from previous studies that *tet*A was mostly highly detected in their sludge (Huang et al., 2015; Zhang and Zhang, 2011). Generally, the absolute abundance of all target ARGs from the sludge was all higher than from wastewater in our previous study (Li et al. 2021).

Absolute gene abundance changes of ARGs at the four AD processes

The AGA changes of each ARG at the four AD processes was studied and shown in Figure 3-3 by averaging the four weekly samples. At Plant 3, significant removals ($p < 0.05$) were observed for all the ARGs between 0.71 and 1.58 log log removals, except *sul*1. At Plant 4, significant removals were observed for *sul*1 (0.59), *sul*2 (0.65), *tet*A (0.68), and *intI*1 (0.77), but significant increase of *tet*W was also observed for 0.43 log. *Erm*B was not significantly ($p > 0.05$) changed after the AD treatment. No significant changes were observed at Plant 5 for any of the ARGs. In addition, significant removals were observed at Plant 6 for all the ARGs between 0.61 and 1.13 log removals. The total 16S rRNA changes were observed at Plant 3, 4, and 6 for up to 0.84 log removal, but no significant change was observed at Plant 5 for 16S rRNA. The AD processes significantly ($p < 0.05$) achieved higher removal rates at Plant 3 than at Plant 4 for *sul*1, *sul*2, and *tet*A. Significantly higher removal rate for *tet*W was achieved at Plant 3 than at Plant 6 but significant increase was

observed at Plant 4. No significant differences ($p < 0.05$) were observed among the four AD processes for *erm*B. The AD at Plant 3 achieved approximately two times higher log removal rates of *intI*1 than at Plant 4. The total 16S rRNA was also removed at the higher rate at Plant 3 than at Plant 6 for approximately two times.

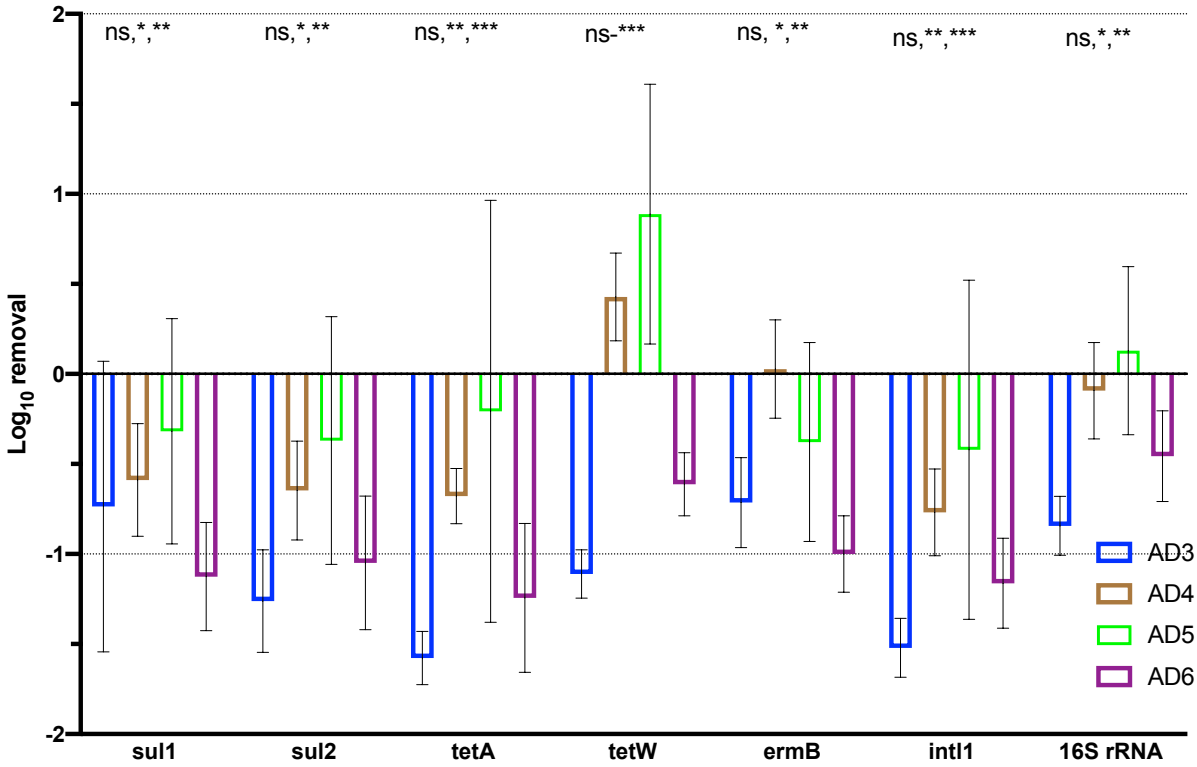


Figure 3-3. Absolute gene abundance changes of target ARGs at the four anaerobic digesters. Negative values indicate removal and positive values indicate increase. The results are expressed in $\log_{10}$ copies per g dw sludge. Significance differences of each AD' removal rates were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Generally, the AD3 achieved the removal efficiencies between 80.7% and 97.4%; AD4 the removal efficiencies of all ARGs were between 74.2 % and 83%; AD6 achieved the removal efficiencies between 65.1% and 94.3%. However, no significant removal efficiencies were observed at Plant 5 for any of the ARGs.

The removal rates of each ARG at the four AD processes together are shown in Figure 3-4 and were compared by averaging all the four weekly samples at all of the four plants. Significant removal rates were observed for all the target ARGs with the range of 0.1 and 1 log. The average removal efficiencies were observed for up to 90%. Previous studies also found removal rates in different AD processes either in lab or full scale (Diehl and LaPara, 2010; Ghosh et al., 2009; Han and Yoo, 2020; Ma et al., 2011; Tian et al., 2016; Zhang and Zhang, 2011). However, one study found that the abundance of *tet*W was significantly increased while *sul*2 was no measurably different using their lab-scale reactors, which was not in accordance with this study (Wu et al., 2016). One possible reason for the reduction has been speculated to be associated with the blockage of ARGs' horizontal gene pathways and higher temperatures in AD may prohibit more horizontal transfer potential for ARGs (Diehl and LaPara, 2010; Ma et al., 2011; Tian et al., 2016). AD might also directly impact the vertical transfer of ARGs in bacterial community during the treatment (Tian et al., 2016). Generally, in this study, most of ARGs were significantly reduced by more than 90%, although they were still at a higher abundance after the anaerobic digestion treatments than wastewater effluents because of higher ARG abundance at digester feed sludge, indicating that AD has capability of removing ARGs.

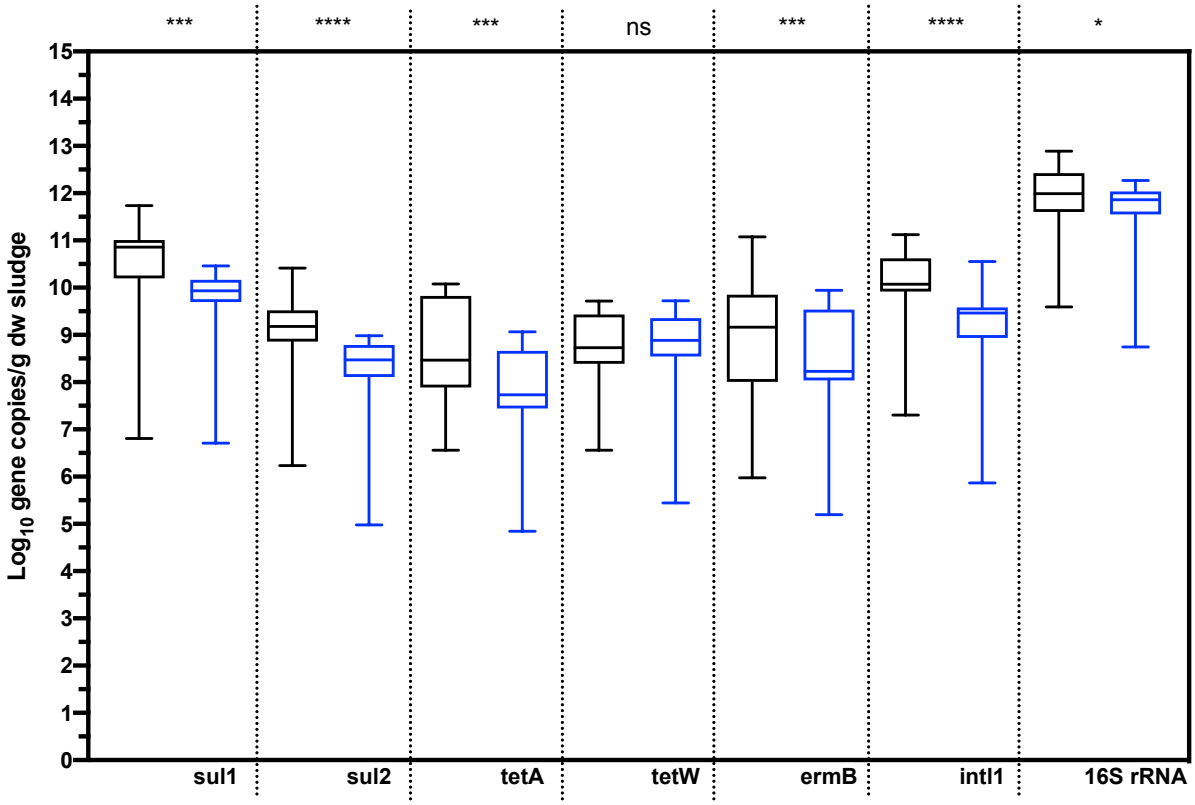


Figure 3-4. Absolute gene abundance of the target ARGs, in the four anaerobic digesters' digester feed (black) and digested sludge (blue) samples. Different genes are separated by vertical lines. The results are expressed in $\log_{10}$ copies per g dw sludge. The boxes represent the 1st and 3rd quartiles. The middle line represents the median, and the whiskers represent the min and max values. Significant differences in gene presence after treatment were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Potential correlations for ARG removals at the four AD processes

The potential correlations between ARG removals at the four AD processes together were studied and shown in Figure 3-5. Significant and positive correlations ($p < 0.05$, $r > 0$) were observed among all the ARGs and 16S rRNA. For *sul*1, strong correlations ($r > 0.7$) were observed between *sul*1 and *intI*1 while moderate ($0.5 < r \leq 0.7$) correlations were observed between *sul*1 and other genes other than *intI*1. Strong correlations were observed between *sul*2 and two *tet* genes, *erm*B, and *intI*1. Strong correlations were also observed between the two *tet* genes, between *tet*A and *intI*1, between *erm*B and *intI*1, and between *tet*W and *intI*1, and between *erm*B and *intI*1.

Correlations were strong between the total 16S rRNA and all the other ARGs, except *sul*1. Other than the strong correlations, the rest of correlations were tested moderate ($0.5 < r \leq 0.7$).

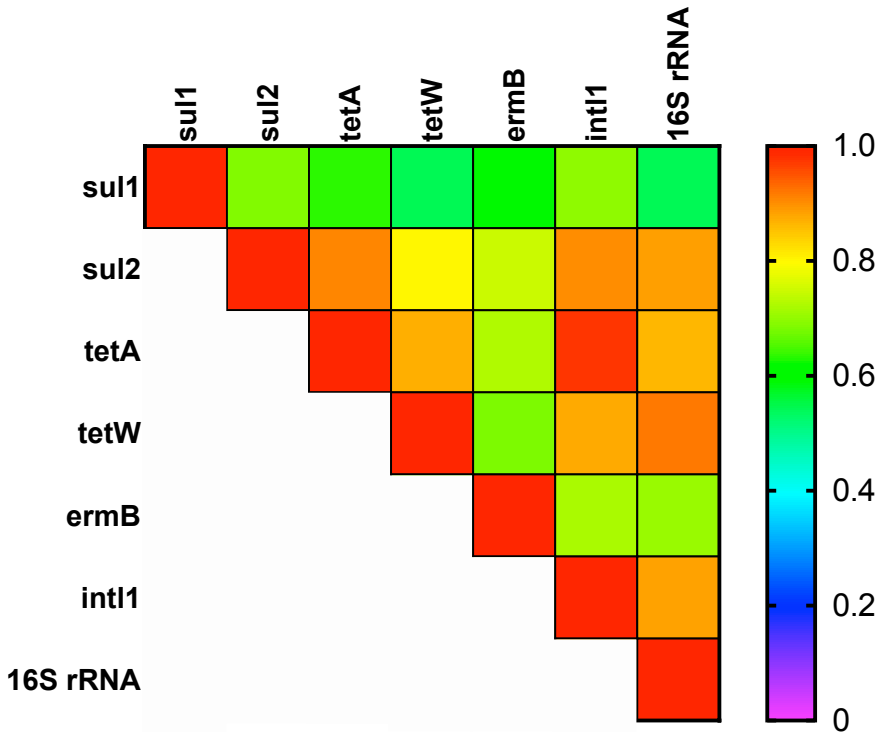


Figure 3-5. Correlation matrix (Pearson's correlation) of the absolute gene abundance changes of the target ARGs for the four anaerobic digesters. Significant correlations are tested for all the genes for $p < 0.05$.

In our previous study, we hypothesized that the correlations among different ARG removals were possibly caused by the similar or same biological organisms or bacteria. Treatment processes may not only remove the abundance of ARGs but also inactivate bacterial growth so that lower abundance of ARGs is produced. Bacteria, including *Escherichia*, *Bacteroides*, and *Clostridium* were most frequently identified at the ARGs host in the environment (Zeng et al., 2019). For example, *Escherichia* harbored most of ARG types such as tetracycline, aminoglycoside, and macrolides, lincosamides and streptogramins (MLS). MLS is frequently carried by *Bacteroides* that was found to be the dominant fermentative bacteria in sludge (Tian et al., 2016). To better

understand if the correlations are related to the bacterial community in the sludge environment, more studies are needed in the future.

Relative gene abundance of ARGs at the four AD processes

DNA-DNA hybridization is the gold standard for identifying bacterial species and it is complex (Brenner et al., 1967). 16S rRNA gene sequencing is used as a tool to identify bacteria at the species level and assist with differentiating closely related bacterial species, because of its wide presentation in all bacterial species (Maiden et al., 1998; X. Wang et al., 2015). The relative gene abundance of each ARG, which was normalized to 16S rRNA is shown in Figure 3-6. At Plant 3, the RGA for DF was between -3.61 (*erm*B) and -1.76 (*sul*1) log gene copies/16S rRNA and the RGA for DS was between -3.95 (*tet*A) and -1.65 (*sul*1) log gene copies/16S rRNA. The RGA before and after the AD process at Plant 4 was between -3.87 (*erm*B) and -1.07 (*sul*1) log gene copies/16S rRNA and between -4.14 (*tet*A) and -1.57 (*sul*1) log gene copies/16S rRNA. At Plant 5, the RGA of all ARGs from the sludge before the AD was between -3.57 (*tet*A) and -1.41 (*sul1*) log gene copies/16S rRNA and the RGA of all ARGs after the AD was between -3.91 (*tet*A) and -1.86 (*sul*1) log gene copies/16S rRNA. In addition, the RGA in log gene copies/16S rRNA at Plant 6's AD was between -3.22 (*erm*B) and -1.37 (*sul*1) for DF and between -3.76 (*erm*B) and -2.04 (*sul*1) for DS. The most abundant gene per 16S rRNA before and after the AD from all the four AD processes was s*ul*1 while the least abundant genes per 16S rRNA before and after the AD was either *tet*A or *erm*B. The distribution of the highest and lowest relative gene abundance was the same as the that of the highest and lowest absolute gene abundance at the four plants, indicating that *sul*1 was the most dominant ARG at all of the four AD processes while *tet*A and *erm*B were the least dominant ARGs.

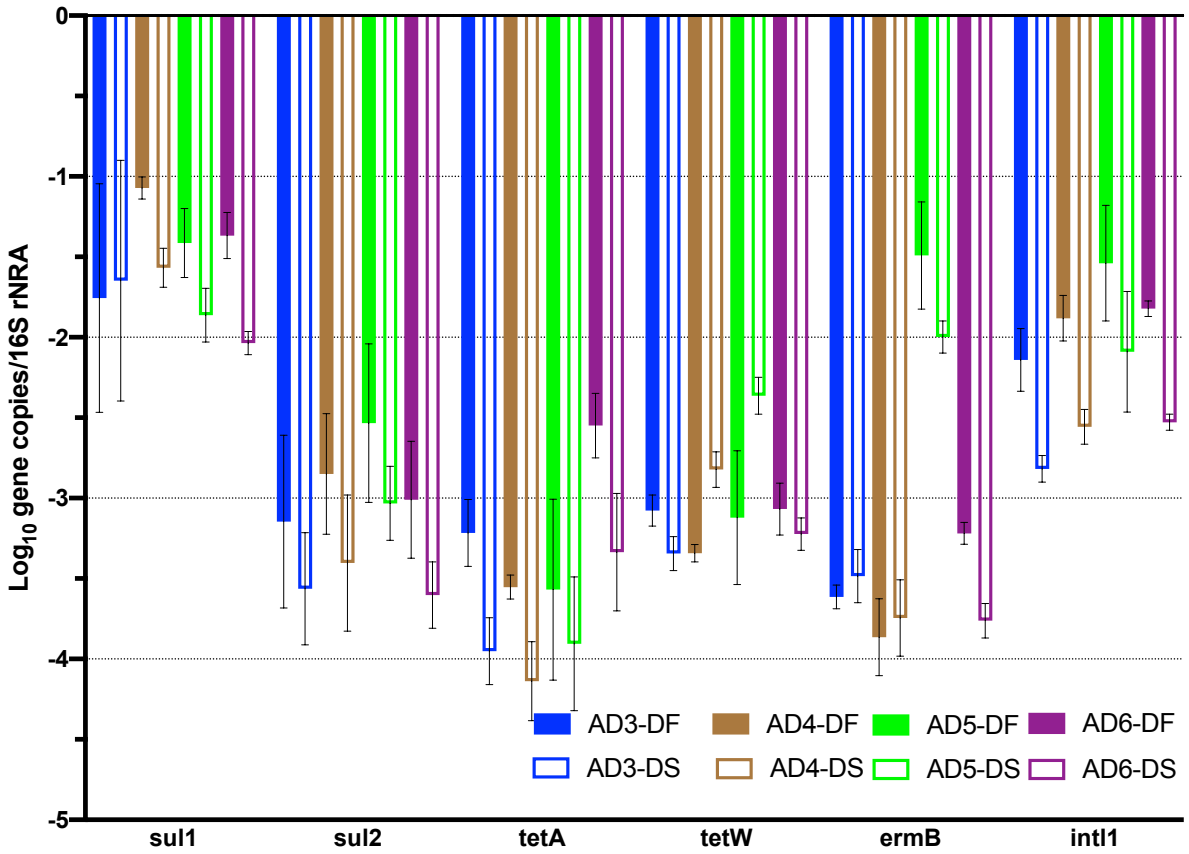


Figure 3-6. Relative gene abundance of each target ARG at each of the four plants' anaerobic digester. The results are expressed in $\log_{10}$ copies per 16S rRNA.

The relative gene abundance of each ARG was compared among the four plants. For *sul*1, the highest RGA before and after the AD was both detected at Plant 4 for -1.07 and -1.57 log gene copies/16S rRNA, respectively, but the lowest RGA was detected for -1.76 log gene copies/16S rRNA from Plant 3's DF and for -2.04 log gene copies/16S rRNA from Plant 6's DS. For *sul*2, the highest RGA was both detected at Plant 5 for -2.53 and -3.03 log gene copies/16S rRNA, but the lowest RGA was detected from Plant 3's DF for -3.15 log gene copies/16S rRNA and from Plant 6's DS for -3.6 log gene copies/16S rRNA. The highest RGA of *tet*A was -2.55 log gene copies/16S rRNA from the DF and -3.34 log gene copies/16S rRNA from the DS at Plant 6 while the lowest RGA was -3.57 log gene copies/16S rRNA from Plant 5's DF and -4.14 log gene copies/16S rRNA from Plant 4's DS. For the RGA of *tet*W in log gene copies/16S rRNA, the highest abundance was

detected from Plant 6's DF for -3.07 while the lowest abundance was detected from Plant 4's DF for -3.34; among the DS among the four plants, the highest abundance was -1.49 at AD5 while the lowest abundance was -3.34 at AD3. For *erm*B, the highest RGA in log gene copies/16S rRNA was -1.49 and -2 at Plant 5 before and after the AD but the lowest abundance was -3.87 before AD4 and -3.76 after AD6. Finally, the highest RGA of *intI*1 was at Plant 5's DF (-1.54) and DS (-2.09) but the lowest RGA was at Plant 3's DF (-2.14) and DS (-2.82) in log gene copies/16S rRNA. In summary, most of the ARGs were detected at the highest relative gene abundance at Plant 5 but the lowest absolute gene abundance of all the ARGs was detected at Plant 3. This indicates that the gene abundance of *sul*1 may not be very high, but its fraction and activity was the most among all the ARGs.

RGA changes of ARGs at the four AD processes

Five ARGs belonging to three ARG types and *intI*1 were studied for their RGA changes during different AD at the four plants. The results were shown in Figure 3-7. At AD3, the relative gene abundance of *sul*2, *tet*A, *tet*W, and *intI*1 was significantly ($p < 0.05$) decreased by 46% - 82% while that of *sul*1 and *erm*B was not significantly ($p > 0.05$) changed. At AD4, the RGA of two *sul* genes, *tet*A, and *intI*1 was significantly and similarly decreased by approximately 68 – 79% while the RGA of *tet*W was significantly increased. The RGA of *erm*B was not significantly changed. There were no significant changes for the RGA of any ARGs at AD5. Nevertheless, all of ARGs' RGA was significantly decreased by 30.14 - 80.32% at AD6.

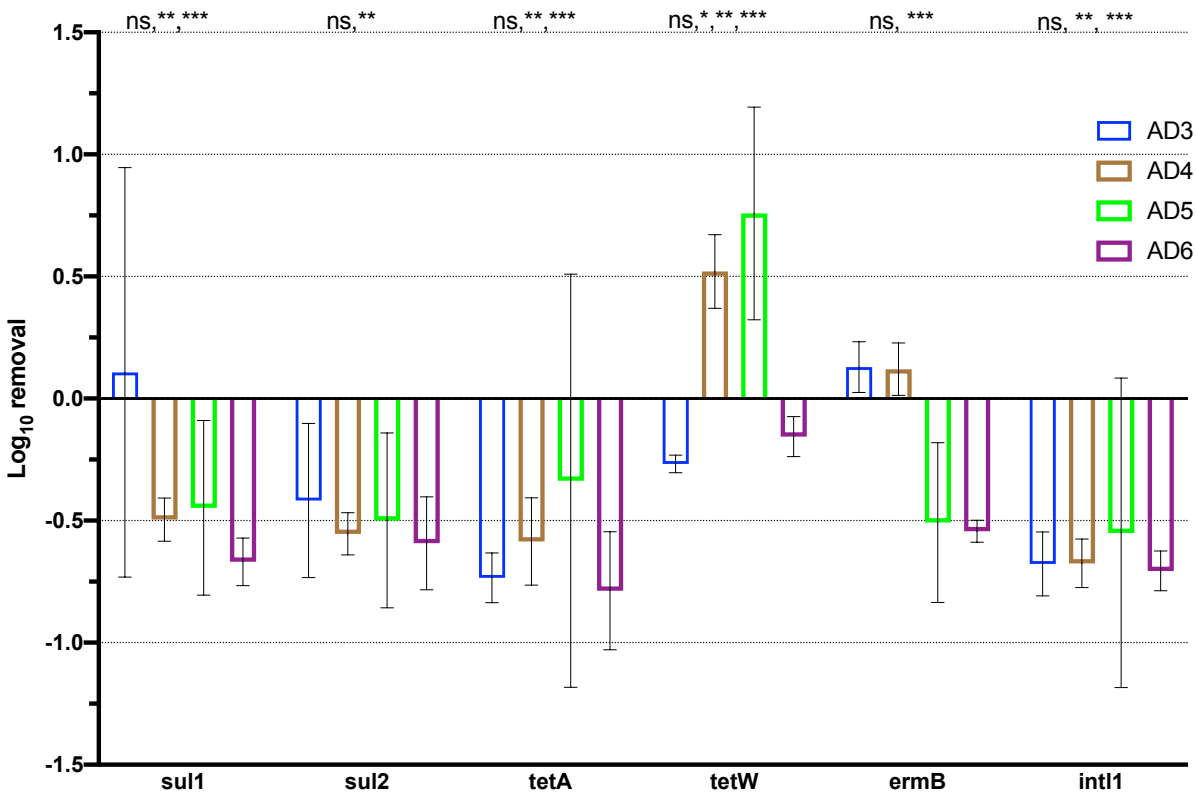


Figure 3-7. Relative gene abundance changes of target ARGs at the four anaerobic digesters. Negative values indicate removal and positive values indicate increase. The results are expressed in $\log_{10}$ copies per 16S rRNA. Significance differences of each AD' removal rates were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

On average of the four-AD RGA of each target ARG (16 samples in total), significant decreases were observed for all the ARGs, except *tet*W, which did not significantly change (shown in Figure 3-8). However, no significant differences between each AD's RGA changes were observed (Table S4). Interestingly, previous studies found little similar observations with ours that most of ARGs' RGA was decreased after the AD, but their studies found either consistent or increased RGA of different ARGs after the AD (Sun et al., 2016; Xin-rong et al., 2021). Although it is difficult to speculate the drivers that may favor certain resistance types over others, one possible explanation might be that the microbial communities carrying different resistance mechanisms (including efflux pump, drug inactivation, target modification, and target bypass) reacted differently in

different environment, such as loading rate or the operating temperature of the anaerobic digesters (Mulvey and Simor, 2009; Zhang et al., 2015). In addition, Rysz et al., 2013 has reported that dissolved oxygen concentrations in aerobic processes potentially play an important role in the cost of resistance and the maintenance of the organism tendency or the trend of potential horizontal gene transfer.

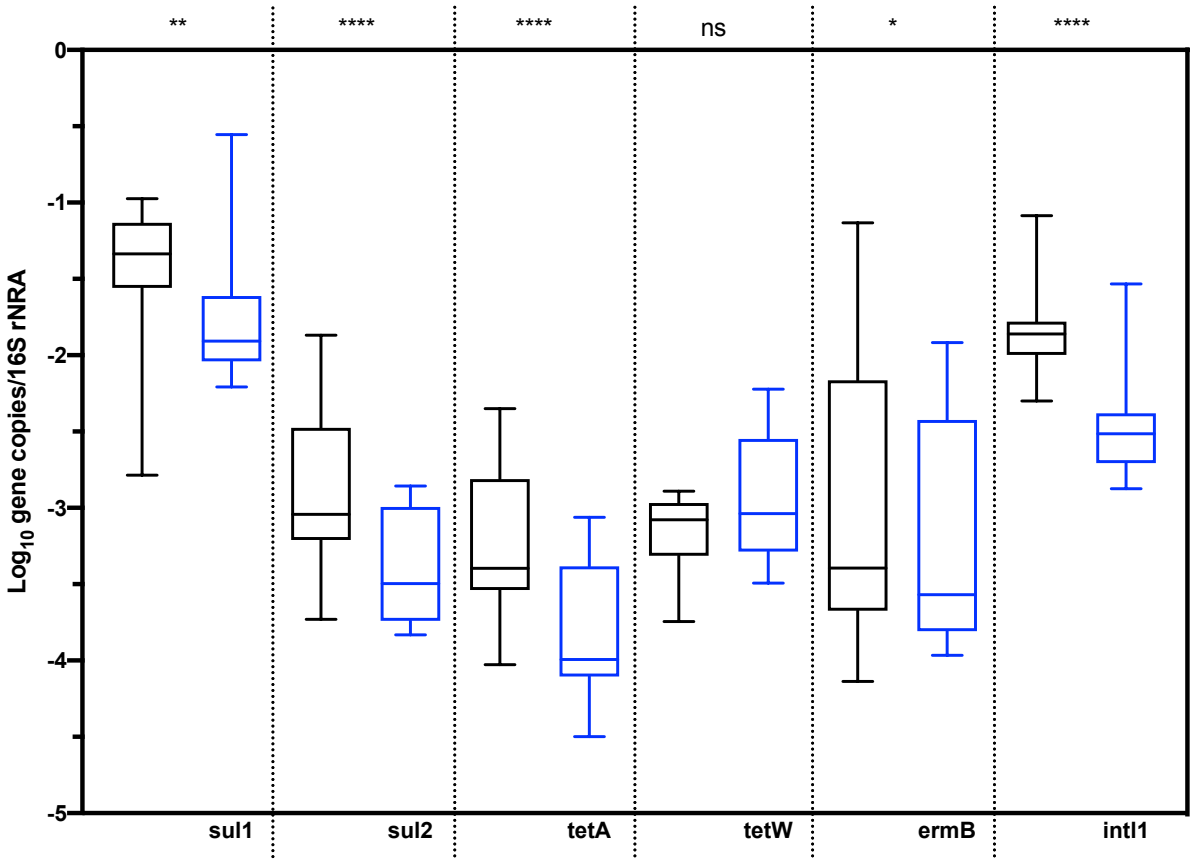


Figure 3-8. Relative gene abundance of the target ARGs, in the four anaerobic digesters' digester feed (black) and digested sludge (blue) samples. Different genes are separated by vertical lines. The results are expressed in $\log_{10}$ copies per 16S rRNA. The boxes represent the 1st and 3rd quartiles. The middle line represents the median, and the whiskers represent the min and max values. Significant differences in gene presence after treatment were assessed by a paired t test and values are indicated above each gene: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

Potential correlations for RGA changes of ARGs at the four AD

Correlations with different ARGs' relative gene abundance changes were examined by calculating the Pearson correlation coefficient between each ARG among the four AD processes together and

are shown in Figure 3-9. The number of all ARG copies was normalized to the basal level of 16S rRNA gene to minimize the variance caused by differences in background bacterial abundances (Resende et al., 2014). Significant ($p < 0.05$) and positive ($r > 0$) correlations were observed between *sul*2 and *tet*A, *sul*2 and *intI*1, *tet*A and *tet*W, and *tet*A and *intI*1. The strongest correlation ($r = 0.9$) was observed between *tet*A and *intI*1 among the four AD processes together.

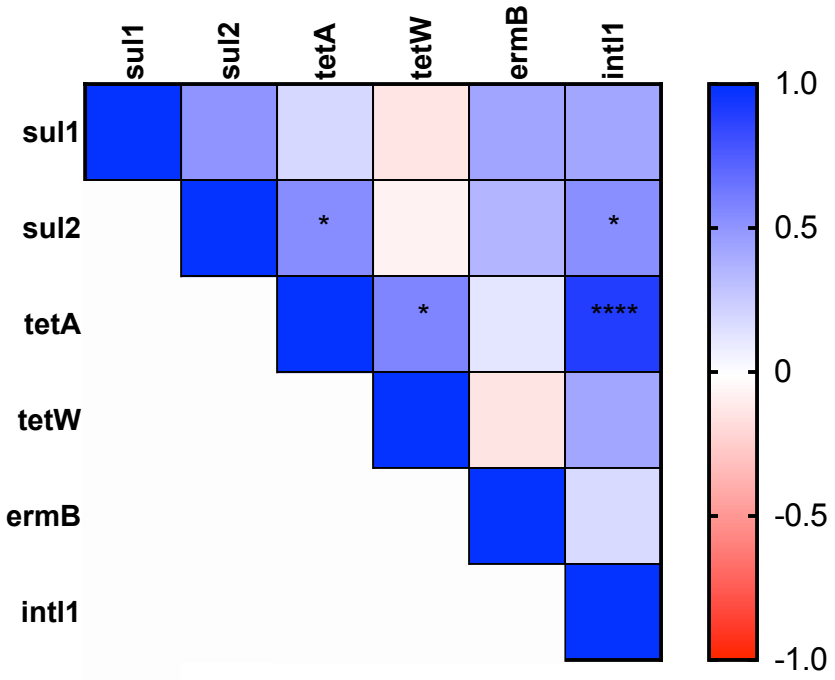


Figure 3-9. Correlation matrix (Pearson's correlation) of the relative gene abundance changes of the target ARGs for the four anaerobic digesters. Significant levels of correlation are indicated as follow $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

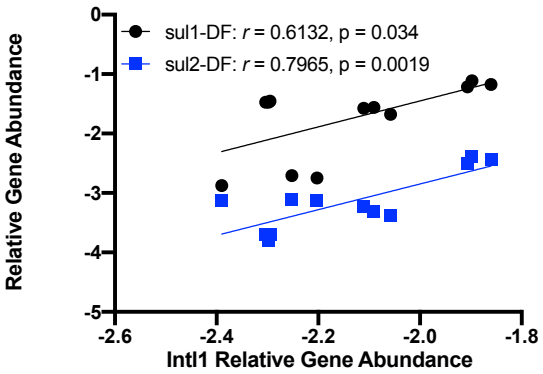
Potential horizontal gene transfer at the four AD processes

Horizontal gene transfer has been an increasing concern of ARG transfer in different environment. Besides vertical gene transmission, where parent cells transfer their genetic information to the offspring, HGT occurs when cells containing no ARGs originally obtain ARGs from other cells or free DNA via conjugation (DNA passing through direct cell-to-cell contact), transduction (bacteriophage introducing ARGs into microbial cells), or transformation (uptake of partial,

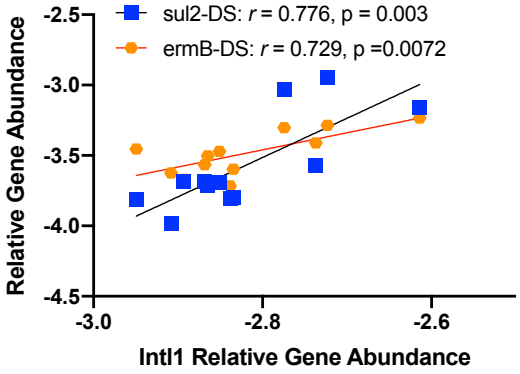
extracellular DNA by naturally transformable bacteria) (Levy and Marshall, 2004; Thomas and Nielsen, 2005). In the microbial world, HGT is a common gene transfer form that contributes significantly to genome evolution and structure (Jain et al., 1999; Koonin et al., 2001; Ochman et al., 2000). Therefore, understanding and limiting the spread of ARGs via HGT is important. Class 1 integron (*intI*1), the predominate integron that is an important form of mobile gene elements transfer ARGs through HGT in various environment, is treated as a marker of HGT and shows significant correlations with ARGs in different environment in terms of the recombination between the *intI*1 and gene cassettes (Conza and Gutkind, 2010; Gaze et al., 2011; Mazel, 2006; Ndi and Barton, 2011; Partridge et al., 2009). Studies have shown that *intI*1 changes play an important role in ARG changes by developing correlations between specific ARGs and *intI*1 via pairwise Pearson's correlation coefficients (r) among microbes to study whether there is the same trend such as increasing or decreasing, indicating that the treatment can limit the role of *intI*1 (the spread of ARGs via *intI*1) (Liao and Chen, 2018).

Potential HGT was studied at the four AD processes before and after the processes and shown in Figure 3-10. All the correlations discussed were significant ($p < 0.05$) and positive ($r > 0$) between different ARGs and *intI*1 in gene copies/16S rRNA. In AD3, strong correlations were found between *sul*2 and *intI*1 from the DF and DS. Strong ($r > 0.7$) correlation was also found between *erm*B and *intI*1 from the DS. Only moderate ($0.5 < r \leq 0.7$) correlation was found from the DF between the relative gene abundance of *sul*1 and *intI*1. In AD4's digester feed, strong correlation was found between *erm*B and *intI*1 while moderate correlation was found between *sul*2 and *intI*1. Moderate correlations were found between *sul*1 and *intI*1 and between *erm*B and *intI*1 at AD4's digested sludge. In AD5, strong correlations were found between all the ARGs and *intI*1 from the

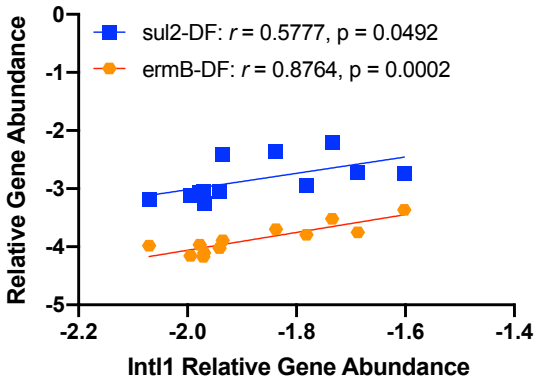
digester feed, respectively, except *tet*W, but strong correlations were only found between *sul*1 and *intI*1 and *tet*A and *intI*1 from the digested sludge. Finally, strong correlation was found between *erm*B and *intI*1 and moderate correlation was found between *sul*1 and *intI*1 from the AD6's digester feed.



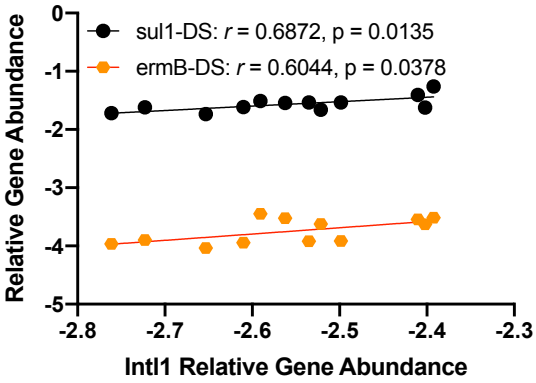
(a)



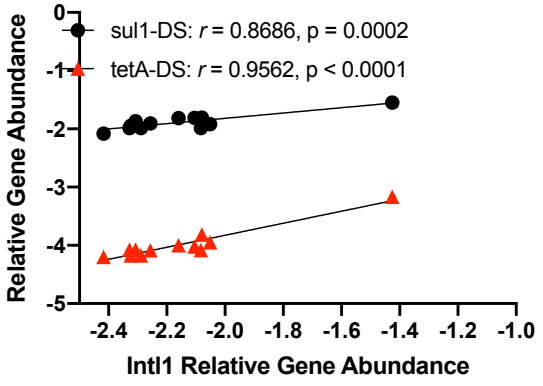
(b)



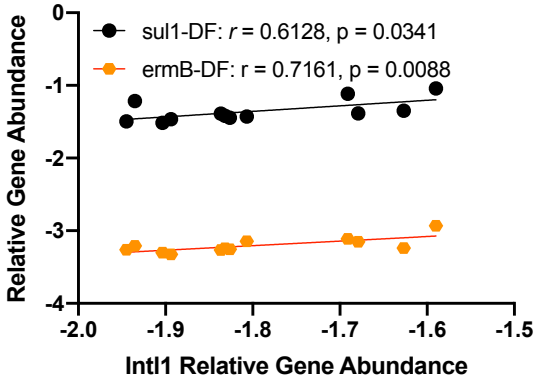
(c)



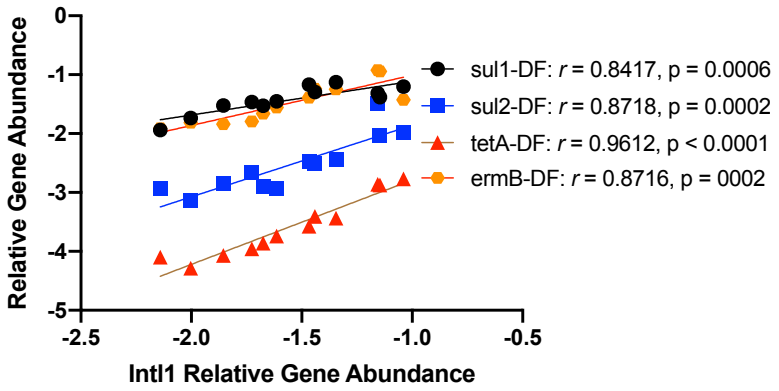
(d)



(e)



(f)



(g)

Figure 3-10. Correlations between *intI*1 and ARG in gene copies/16S rRNA using a Pearson correlation at AD3 digester feed (a) and digested sludge (b), AD4 digester feed (c) and digested sludge (d), AD5 digester feed (g) and digested sludge (e), and AD6 digester feed (f). Linear trendlines are only shown for correlations with significances $p \leq 0.05$. *r* and p values are shown.

Significant, strong or moderate, and positive correlations were found between the two *sul* genes and *intI*1 in gene copies/16S rRNA at all the four AD processes, indicating that potential horizontal gene transfer may have occurred for them. This observation was consistent with previous studies either in batch-scale anaerobic digestion in China or in other fields such as estuary, landfill and WWTPs (Chen et al., 2015, 2019; Di Cesare et al., 2016; Lu et al., 2015; Yu et al., 2016). Previous studies have also confirmed that *sul*1 is genetically linked to *intI*1 (Liao and Chen, 2018). Because integrons themselves are not mobile, the transfer of integrons mainly reply on the associated MGEs such as plasmids (Boucher et al., 2007). *Sul*1 and *sul*2 are usually found in small plasmids, and *sul*1 has been confirmed its presence in the gene cassettes of integrons carried on plasmid metagenome and it has been found to be directly associated with class 1 integrons (Luo et al., 2014, 2010; Mu et al., 2015; J. Wang et al., 2015; Zhang et al., 2011). As to *sul*2, despite no information about its presence in the gene sequence of *intI*1, the co-occurrence of s*ul*2 and *intI*1 has been reported in genera such as *Enterobacteriaceae* and *E. coli*, indicating the possible MGEs sharing between them (A. et al., 2002; C. et al., 2012).

Moderate to strong correlations between *erm*B and *intI*1 were observed at all the AD processes, indicating potential HGT may have occurred in the AD processes. This is in accordance with Zhang et al. (2020). *Erm*B was enriched in the broad host range plasmid, pAMbeta1, which could transfer frequently in various environment and between commensal microbes (Luo et al., 2005; Zhang et al., 2020). Therefore, pAMbeta1 could mainly attribute to the HGT with the enrichment of *erm*B (Zhang et al., 2020). Nevertheless, *tet*W was the only ARG that was not found to be correlated with *intI*1 in RGA, which was consistent with Miller et al., (2016), who also found no correlation between *tet*W and *intI*1 in their lab-scale mesophilic or thermophilic digested sludge. This may be explained that *tet*W is found on plasmid and chromosomes, but has not been found within integron cassettes (Roberts, 2005). Generally, correlations between target ARGs and *intI*1 have been observed during the anaerobic digestion processes either in mesophilic or thermophilic conditions and it indicates potential HGT may occur. HGT has been shown to cause a lot of attention in the wastewater treatment systems and AD processes should be specially more focused.

3.4. Conclusion

This paper selected four full-scale anaerobic digestion processes from four wastewater treatment plants located near Los Angeles to study the fate of six antibiotic resistance genes and 16S rRNA during the anaerobic digestion processes. From the study, we made the following conclusions:

1. All the six ARGs were detected before and after the four AD processes. Significant removal efficiencies were achieved as high as 97.4%. The total 16S rRNA was removed significantly at AD3 and AD 6 for -0.84 and 0.46 log. The lowest absolute gene abundance

of all the ARGs was found at AD3, which is the only thermophilic anaerobic digestion process. But the absolute gene abundance of all the ARGs and 16S rRNA was more highly observed from the digested sludge than from the secondary effluent at the four WWTPs. In addition, potential and significant correlations of six ARGs and 16S rRNA in absolute gene abundance were found among each other.

2. The relative gene abundance of six ARGs was more variable at the four AD processes than the absolute gene abundance. Most of the ARG's relative gene abundance per 16S rRNA was significantly decreased up to 82%, although some were also significantly increased. Our study found more frequent decreases of different ARGs than some previous studies.

3. Significant, strong or moderate, and positive correlations were found between *sul*1, *sul*2, *tet*A, and *erm*B and *intI*1 in gene copies/16S rRNA at the four AD processes, respectively, indicating the potential HGT occurrences. In addition, potential HGT of two *sul* genes and *erm*B was found and shown with the significant and positive correlations with *intI*1 due to the either direct or indirect co-occurrence of plasmids. Generally, potential HGT may have occurred in the anaerobic digestion processes.

3.5. Appendix B

Table S3-1. Significant difference p value of absolute gene abundance between anaerobic digesters for each ARG.

sul1	AD3	AD4	AD5	AD6
AD3	-	0.02093	0.07468	0.4018
AD4	-	-	0.4956	0.1361
AD5	-	-	-	0.1473
AD6	-	-	-	-

sul2	AD3	AD4	AD5	AD6
AD3	-	0.0001506	0.1003	0.2073
AD4	-	-	0.4817	0.06515
AD5	-	-	-	0.1755
AD6	-	-	-	-

tetA	AD3	AD4	AD5	AD6
AD3	-	0.0001506	0.1003	0.2072
AD4	-	-	0.4817	0.06515
AD5	-	-	-	0.1755
AD6	-	-	-	-

tetW	AD3	AD4	AD5	AD6
AD3	-	0.0001162	0.0102	0.004694
AD4	-	-	0.2991	0.0006822
AD5	-	-	-	0.02208
AD6	-	-	-	-

ermB	AD3	AD4	AD5	AD6
AD3	-	0.007192	0.3274	0.1333
AD4	-	-	0.2529	0.001254
AD5	-	-	-	0.1058
AD6	-	-	-	-

intI1	AD3	AD4	AD5	AD6
AD3	-	0.003019	0.09982	0.05989
AD4	-	-	0.5208	0.06335
AD5		-	-	0.2142
AD6	-	-	-	-

16S rRNA	AD3	AD4	AD5	AD6
AD3	-	0.005021	0.01956	0.04838
AD4	-	-	0.4476	0.09522
AD5		-	-	0.08271
AD6	-	-	-	-

Table S3-2. Significance p value of absolute gene abundance removals for each ARGs at each anaerobic digester and all the four AD together.

ARG	AD3	AD4	AD5	AD6	AD3-6
sul1	0.1656	0.0327	0.3837	0.0049	0.0002
sul2	0.003	0.018	0.3612	0.0109	<0.0001
tetA	0.0002	0.003	0.7473	0.0092	0.0003
tetW	0.0005	0.0394	0.0909	0.006	0.655
ermB	0.0106	0.8556	0.2643	0.0025	0.001
intI1	0.0003	0.0078	0.4374	0.0026	<0.0001
16S rRNA	0.0019	0.5368	0.6178	0.0361	0.017

Table S3-3. Correlation matrix of absolute gene abundance changes for *r* and p value at four anaerobic digesters

r	sul1	sul2	tetA	tetW	ermB	intI1	16S rRNA
sul1	1	0.6909	0.6376	0.5472	0.6100	0.7008	0.5458
sul2	0.6909	1	0.9103	0.8035	0.7511	0.9048	0.8892
tetA	0.6376	0.9103	1	0.8733	0.7283	0.9780	0.8657
tetW	0.5472	0.8035	0.8733	1	0.6873	0.8789	0.9222
ermB	0.6100	0.7511	0.7283	0.6873	1	0.7228	0.7092
intI1	0.7008	0.9048	0.9780	0.8789	0.7228	1.0000	0.8850
16S rRNA	0.5458	0.8892	0.8657	0.9222	0.7092	0.8850	1

P value	sul1	sul2	tetA	tetW	ermB	intI1	16S rRNA
sul1	-	0.003	0.0079	0.0283	0.0121	0.0025	0.0287523
sul2	0.003	-	1E-06	0.0002	0.0008	1E-06	4.082E-06
tetA	0.0079	1E-06	-	1E-05	0.0014	6E-11	1.47E-05
tetW	0.0283	0.0002	1E-05	-	0.0033	7E-06	3.758E-07
ermB	0.0121	0.0008	0.0014	0.0033	-	0.0016	0.0020963
intI1	0.0025	1E-06	6E-11	7E-06	0.0016	-	5.241E-06
16S rRNA	0.0288	4E-06	1E-05	4E-07	0.0021	5E-06	-

Table S3-4. Significant difference p value of relative gene abundance per 16S rRNA between anaerobic digesters for each ARG.

sul1-RGA	AD3	AD4	AD5	AD6
AD3	-	0.2461	0.2895	0.161
AD4	-	-	0.8091	0.0397
AD5	-	-	-	0.3093
AD6	-	-	-	-

sul2-RGA	AD3	AD4	AD5	AD6
AD3	-	0.4583	0.7453	0.3867
AD4	-	-	0.7821	0.7304
AD5	-	-	-	0.6652
AD6	-	-	-	-

tetA-RGA	AD3	AD4	AD5	AD6
AD3	-	0.2107	0.418	0.7072
AD4	-	-	0.6027	0.2323
AD5	-	-	-	0.3717
AD6	-	-	-	-

tetW-RGA	AD3	AD4	AD5	AD6
AD3	-	0.001258	0.01774	0.06474
AD4	-	-	0.3655	0.0007548
AD5	-	-	-	0.02264
AD6	-	-	-	-

ermB-RGA	AD3	AD4	AD5	AD6
AD3	-	0.9061	0.02477	0.0002567
AD4	-	-	0.02563	0.003291
AD5	-	-	-	0.8426
AD6	-	-	-	-

intI1-RGA	AD3	AD4	AD5	AD6
AD3	-	0.9796	0.7186	0.7273
AD4	-	-	0.7217	0.6509
AD5		-	-	0.6586
AD6	-	-	-	-

Table S3-5. Significance p value of relative gene abundance per 16S rRNA changes for each ARGs at each anaerobic digester and all the four AD together.

ARG	AD3	AD4	AD5	AD6	AD3-6
sul1	0.8145	0.0015	0.0876	0.0008	0.0099
sul2	0.0771	0.001	0.0685	0.0083	<0.0001
tetA	0.0007	0.0073	0.4846	0.0074	<0.0001
tetW	0.0007	0.0062	0.04	0.0317	0.1053
ermB	0.0885	0.111	0.053	0.0002	0.0483
intI1	0.0019	0.0009	0.1809	0.0004	<0.0001

Table S3-6. Correlation matrix of relative gene abundance changes for *r* and p value at four anaerobic digesters

r	sul1	sul2	tetA	tetW	ermB	intI1
sul1	1	0.5103	0.1910	-0.1262	0.4345	0.4311
sul2	0.5103	1	0.5441	-0.0628	0.3549	0.5339
tetA	0.1910	0.5441	1	0.5731	0.1315	0.8998
tetW	-0.1262	-0.0628	0.5731	1	-0.1362	0.4270
ermB	0.4345	0.3549	0.1315	-0.1362	1	0.1843
intI1	0.4311	0.5339	0.8998	0.4270	0.1843	1

r	sul1	sul2	tetA	tetW	ermB	intI1
sul1	-	0.0434	0.4786	0.6413	0.0926	0.0954
sul2	0.0434	-	0.0293	0.8173	0.1774	0.0331
tetA	0.4786	0.0293	-	0.0203	0.6274	0.0000
tetW	0.6413	0.8173	0.0203	-	0.6150	0.0990
ermB	0.0926	0.1774	0.6274	0.6150	-	0.4945
intI1	0.0954	0.0331	0.0000	0.0990	0.4945	-

Table S3-7. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 3's anaerobic digester: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

AD3	Digester Feed					Digested Sludge				
ARG	sul1	sul2	tetA	tetW	ermB	sul1	sul2	tetA	tetW	ermB
r	0.6132	0.7965	-0.3479	-0.9072	0.4912	0.0076	0.776	-0.5451	-0.5032	0.7292
p value	0.0349	0.0018	0.2678	<0.0001	0.1049	0.9814	0.003	0.0668	0.0953	0.0072
p value summary	*	**	ns	****	ns	ns	**	ns	ns	**

Table S3-8. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 4's anaerobic digester: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

AD4	Digester Feed					Digested Sludge				
ARG	sul1	sul2	tetA	tetW	ermB	sul1	sul2	tetA	tetW	ermB
r	-0.2332	0.5777	-0.1098	0.2067	0.8764	0.6872	0.4452	-0.4812	-0.3173	0.6033
p value	0.4657	0.0492	0.7342	0.5191	0.0002	0.0135	0.147	0.1132	0.3154	0.0378
p value summary	ns	*	ns	ns	***	*	ns	ns	ns	*

Table S3-9. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 6's anaerobic digester: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

AD5	Digester Feed					Digested Sludge				
ARG	sul1	sul2	tetA	tetW	ermB	sul1	sul2	tetA	tetW	ermB
r	0.8417	0.8718	0.9612	0.4164	0.8716	0.8686	-0.4284	0.9562	-0.1914	-0.4832
p value	0.0006	0.0002	<0.0001	0.1782	0.0002	0.0002	0.1644	<0.0001	0.5513	0.1116
p value summary	***	***	****	ns	***	***	ns	****	ns	ns

Table S3-10. Correlation and significant p Value for HGT between target ARG and *intI1* in relative gene per 16S rRNA at Plant 6's anaerobic digester: $p > 0.05$ (ns), $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****).

AD6	Digester Feed					Digested Sludge				
ARG	sul1	sul2	tetA	tetW	ermB	sul1	sul2	tetA	tetW	ermB

r	0.6128	0.5503	-0.0997	-0.109	0.7161	0.4424	0.2574	0.2292	0.4186	0.4129
p value	0.0341	0.0638	0.7579	0.736	0.0088	0.1502	0.4192	0.4736	0.1757	0.1821
p value summary	*	ns	ns	ns	**	ns	ns	ns	ns	ns

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Chapter 4: Accumulation of Selected Heavy Metals and Their Influence on The Fate of Antibiotic Resistance Genes in Three Full-Scale Anaerobic Digestion Processes

4.1. Introduction

Heavy metals typically precipitate with hydrogen sulfide and have a density of 5 g/cm^3 or greater (Nies, 1999). The toxicity of heavy metals above certain concentrations affect human health, and are toxic to other organisms, microorganisms, and plants, and is well documented in many previous studies (Brown and Lester, 1979; Ehrlich, 1997; Nies, 1999). Wastewater treatment plants (WWTPs) receive heavy metals from a variety of sources such as industries, stormwaters and illegal disposals or discharges. Accumulation of heavy metals in WWTPs is impacted by many factors, such as type of industrial dischargers, type of treatment plant, combined vs separate sewers, and local laws regulating products such as batteries. Strict regulations and legislation aim at protecting the environment to restrict discharges to wastewater treatment facilities to comply with different heavy metal limits in effluents and sludge. Numerous studies have reported the concentrations of heavy metals that inhibit treatment functions, such as nitrification and denitrification processes, reduce microbial oxidation of organic compounds and produce toxicity in effluents. (Dahle and Birkeland, 2006; Ng and Stenstrom, 1987; Waara, 1992). Therefore, heavy metals are of great importance to not only WWTPs but also human health.

Sewage sludge, also known as biosolids, is a waste organic material generated in wastewater treatment plants as a by-product of wastewater treatment. Due to rapid urbanization and industrialization, the amount of sludge has been increasingly. In 2019, approximate 4.75 million dry metric tons (dmt) of biosolids were generated in the United Sates, according to US

Environmental Protection Agency (EPA) (US EPA 2016). Among the amount of biosolids, approximate 1.4 million dmt biosolids were applied to agricultural land use while approximate 1 million dmt biosolids were landfilled. A fraction of the heavy metals present in the influent wastewater will accumulate in sewage (Gawdzik and Gawdzik, 2012). The amount and bioavailability of the metals depends on the metal, type of process, and environmental conditions such as pH and dissolved oxygen concentration. However, WWTPs do not remove or detoxify all heavy metals, which in turn may lead to secondary environmental pollution (Cantinho et al., 2016). For instance, in 2019 in the United States, approximately 30% of biosolids from WWTPs were used in agricultural (Figure 4-1). High concentrations of heavy metals in the biosolids may cause contamination of soil, surface and ground water, and crops, resulting a negative impact on living organisms (Spanos et al., 2016). The speciation of chemical form determines the mobility, bioavailability, and toxicity of heavy metals and are influenced by their leaching and interactions with different components of natural ecosystems (Turek et al., 2019; YANG et al., 2017). Therefore, biosolids is an important concern of heavy metal source.

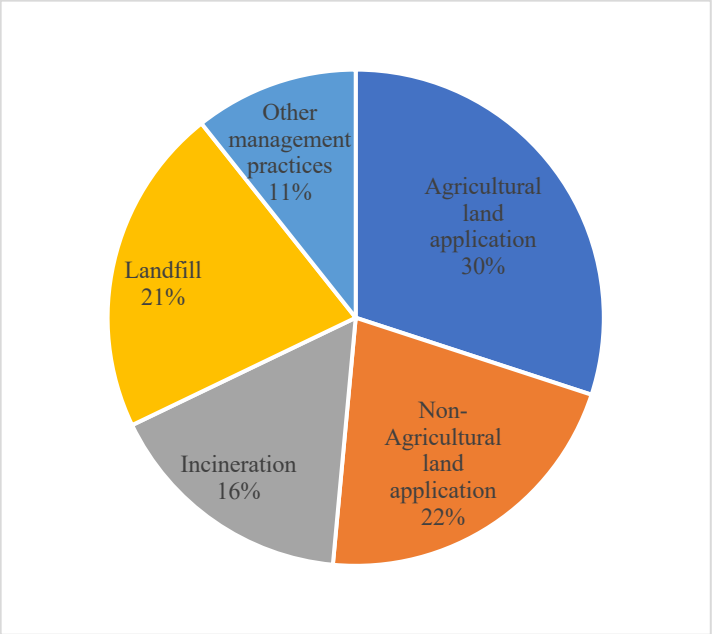


Figure 4-1. Distribution of biosolids use and disposal from major publicly-owned treatment works (US EPA, 2019)

Anaerobic digestion (AD) is the major processes in many WWTPs treating biosolids. Anaerobic digestion involves the degradation and stabilization of organic materials under anaerobic conditions by microbial organisms and leads to the formation of biogas and biosolids (Kelleher et al., 2002). The general advantages of AD include low energy input, low waste sludge production, improved dewaterability with less odor production, biogas for energy reuse, especially for gas power station with heat recovery and disinfection (Abdel-Shafy and Mansour, 2014). The heavy metal concentrations are an important factor to identify the quality of the biosolids and its compliance with permissible limits for biosolids reuse (ABDEL-SHAFY and ABDEL-SABOUR, 2006; Wiegant, 2001). Different types and concentrations of heavy metals have different effects on AD processes (Lee et al., 2017; Xie et al., 2015). Studies have found the impact of different heavy metals on microbial community, enzymatic activity, organic degradation, and process stability of digesters (Guo et al., 2019). Certain heavy metals such as Ni, Co, Mn, and Fe are found to potentially enhance biogas in the digesters with small amount to stimulate bacterial activities

(Basiliko and Yavitt, 2001; Ilangovan et al., 1990; Yue et al., 2007). On the contrary, some other heavy metals such as Cu, Pb, Cr and Zn can inhibit the performance of anaerobic digestion (Nasr and Abdel Shafy, 1992; Zandvoort et al., 2006). The toxic effect of these heavy metals can inactivate enzymes, resulting in inhibiting the growth of bacteria (Cadillo-Quiroz et al., 2006; Selling et al., 2008). Therefore, different heavy metals can either benefit or inhibit the performance of AD process.

In addition, it is believed that heavy metals can also select for antibiotic resistance genes (ARGs) (Baker-Austin et al., 2006; Seiler and Berendonk, 2012). Many researchers have found the closely linked relationships between ARGs and heavy metals by the cross-resistance and co-resistance of co-selection mechanisms (Cheng et al., 2013). However, the relationship of heavy metals on ARGs have limitedly been studied in previously in AD systems, so the relationship of different ARGs and heavy metal concentrations is not clear (Yin et al., 2017; Yu et al., 2017).

This work presents the results of the accumulation and removal of selected heavy metals (copper, zinc, titanium, lead, manganese, iron, and barium) by three full-scale anaerobic digestion processes from three municipal WWTPs in southern California. The aims of this study were: (1) to examine heavy metal concentrations before and after the anaerobic digestion; (2) to compare heavy metal concentrations among these three AD processes; (3) to study the potential correlations between these heavy metal concentrations and five ARGs (*sul*1, *sul*2, *tet*A, *tet*W, and *erm*B), *intI*1, and 16S rRNA.

4.2. Materials and Methods

Sample collection

Three wastewater treatment plants located in southern California were selected, and their anaerobic digestion processes were selected for biosolids sampling, including one thermophilic (AD3) and two mesophilic anaerobic digestors (AD4 and AD6). Plant 3 (AD3) produced approximately 264,000 metric tons (wet weight) of Class B biosolids per year, all of which were used for land application. Plant 4 (AD4) produced approximately 488,516 metric tons (wet weight) of biosolids per year, 10% of which were used for land application while 90% of which were used for either landfill or compost. Plant 6 (AD6) produced approximately 126,000 metric tons (wet weight) of biosolids in 2015, which were Class B and were used for land application. Full information is listed in Table 4-1. AD3 is thermophilic anaerobic digesters with the temperature of approximately 55 °C while AD4 and AD6 are mesophilic anaerobic digesters with the temperature of approximately 35 °C. The schematic layout the anaerobic digestion is shown in Figure 4-2. One-liter biosolids samples were collected from digester feed (DF) and one-liter from digested sludge (DS) in sterile containers at each plant (Figure 4-2). Digester feed at plants AD3 and AD6 were composed of primary and waste activated sludge. Digester feed in AD4 is separated. One-liter influent samples were collected in AD3, AD5 and AD6. In AD4 0.5-liter primary sludge and 0.5-liter waste activated sludge were collected and mixed to create a representative feed sample. Samples were prepared by the facility workers and then kept on ice during transport and stored at 4 °C until the time of processing. All processing was performed as early as possible but always in less than one week.

Table 4-1. Three anaerobic digestion processes information and sludge production

Plant	Anaerobic digestion	Type	Temperature (°C)	Size (mtww*)
3	AD3	Thermophilic	55-57	264000
4	AD4	Mesophilic	35-37	488516
6	AD6	Mesophilic	35-37	126000

Note: *mtww = metric tons (wet weight per year). Samples collected Feb and March 2021.

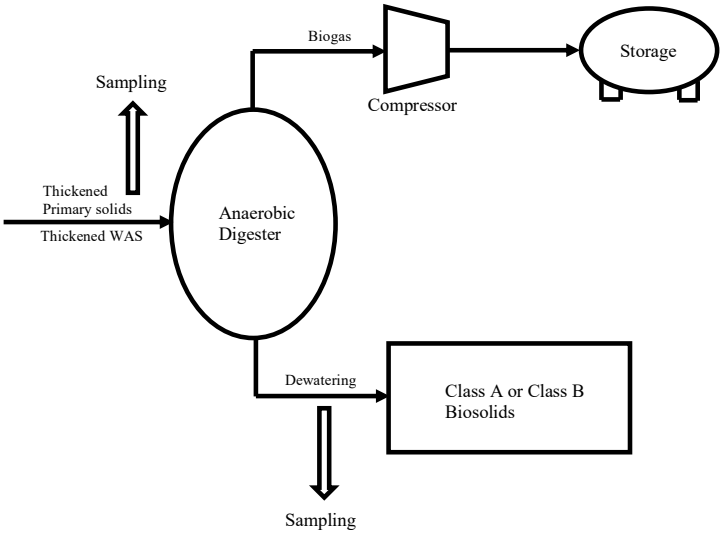


Figure 4-2. Schematic layout of the anaerobic digestion processes at the four WWTPs and sampling sites

Sample preparation and analysis

Samples were transferred into 50-mL glass bottles pre-washed with 10% hydrochloric acid solutions for washing off heavy metal concentrations. Samples were then evaporated to dryness at 100 °C for 24 hours. After cooling, dry samples were transferred into XRF sample cups (Chemplex Industries, Inc, Palm City, FL) at full and sealed with SpectroMembrane Prolene Thin-Film (Chemplex Industries, Inc, Palm City, FL). All processing was performed in duplicate. The prepared sludge samples were analyzed using S1 TITAN Handheld XRF 600 Analyzer (Billerica, MA) following its protocols. The concentration of heavy metals in sludge samples was selected and determined, including barium (Ba), copper (Cu), iron (Fe), manganese (Mn), lead (Pb), rubidium (Rb), strontium (Sr), titanium (Ti), zinc (Zn), and zirconium (Zr). The phosphorus (P) concentration was also determined. All tests were carried out in triplicates and their mean value was considered as the final result.

Statistical analysis

The methodology for measuring the dry-sludge ARG abundance was reported previously in Chapter 3. These data were used to determine the correlation between the ARG abundance and the concentration of metal metals. Statistical analyses including two-tailed t-test were performed with GraphPad Prism version 8 (San Diego, CA). The differences at $p < 0.05$ level among samples were considered statistically significant. Correlation analysis using Pearson' correlation between the removal of ARGs were performed and the strength of correlations were defined as strong ($r > 0.7$), moderate ($0.5 < r \leq 0.7$), weak ($0.3 < r \leq 0.5$), none or very weak ($r \leq 0.3$) (Moore and Kirkland, 2007). Redundancy analysis (RDA) was performed through CANOCO 4.5 to explore the relationship between the environmental factors (heavy metals and P) and the ARG community in

the three AD processes. Eleven environmental variables included Ba, Cu, Fe, Mn, Pb, Rb, Sr, Ti, Zn, Zr, and Phosphorus (P). Relative abundance of five ARGs included *sul*1, *sul*2, *tet*A, *tet*W, *erm*B, and integron 1 class (*intI*1). A Monte Carlo permutation test based on 499 random permutations was conducted to test the significance of the eigenvalues of the first canonical axis. Inter-set correlations from the ordination analysis were used to assess the importance of the environmental variables (Heino, 2001).

4.3. Results and discussion

Heavy metal concentrations in digester feed and digested sludge in three AD processes

Table 4-2 shows the detection limits for the heavy metals in our analysis. Heavy metals were detected and the concentrations are shown in Figure 4-3, including Barium (Ba), copper (Cu), iron (Fe), manganese (Mn), lead (Pb), rubidium (Rb), strontium (Sr), titanium (Ti), zinc (Zn), and zirconium (Zr). However, heavy metals such as cadmium (Cd), chromium (Cr), mercury (Hg), and nickel (Ni) were not detected in any of the three AD processes, although some of them are present in significant levels in other waste activated sludge (Xu et al., 2017). This may be explained by the fact that these heavy metals exist in these WWTPs' biosolids but their concentrations were below the limit of detection of XRF analyzer, which is shown in Table 4-2.

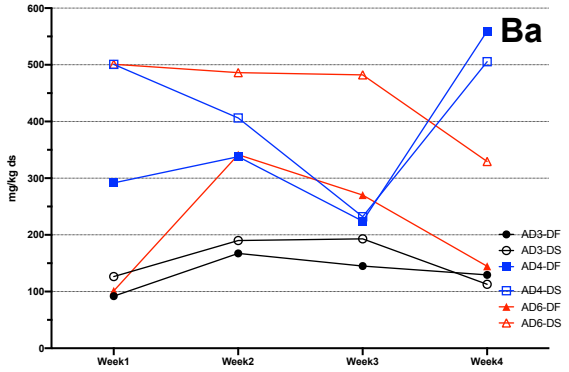
Figure 4-3 shows the results of seven heavy metal concentrations in both digester feed and digested sludge at the three AD processes: Ba concentrations were between 92 (AD3's DF) and 559.5 (AD4' DF) mg/kg ds; Cu concentrations were between 109.2 (AD6's DF) and 835.4 (AD3's DS) mg/kg ds; Fe concentrations were the highest concentrations among all heavy metals between 26110.5

and 87184.7 mg/kg ds in AD6's DF; Mn concentrations were between 24.5 (AD's DS) and 269.2 (AD6's DF) mg/kg ds; Pb concentrations were between 18.7 (AD4's DF) and 43.1 (AD3's DS) mg/kg ds; Rb concentrations were the lowest concentrations among all heavy metals between 5.2 (AD4's DF) and 29.9 (AD3's DS) mg/kg ds; Sr concentrations were between 98 (AD3's DF) and 476.3 (AD6's DS) mg/kg ds; Ti concentrations were between 151.5 (AD6's DS) and 2374.3 (AD3's DF) mg/kg ds; Zr concentrations were between 45.3 (AD4's DF) and 111.8 (AD3's DS) mg/kg ds. Generally, the highest concentrations of Cu, Pb, Rb, Ti, Zn, and Zr were detected in AD3's biosolid samples and the highest concentrations of Fe, Mn, and Sr were detected in AD6's biosolid samples. However, all the heavy metals' lowest concentrations were detected in either AD4's or AD6's biosolid samples, except Ba and Sr that were detected in AD3.

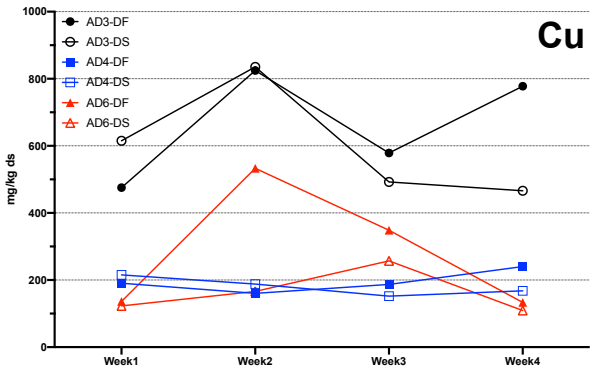
Table 4-2. Limit of detection for selected heavy metals and nutrients according to S1 TITAN Handheld XRF 600 Analyzer

Heavy metals & Nutrients	Ba	Cd	Cr	Cu	Fe	Hg	Mn
Calibration Range (%)	0-0.26	0-0.045	0.1-1.08	0-3.08	0-24.8	0-0.008	0-0.21
LOD* in pure SiO2 (ppm)	160	20	5	<5	15	10	15
Heavy metals & Nutrients	Ni	Pb	Rb	Sr	Ti	Zn	Zr
Calibration Range (%)	0-0.27	0-2.1	0-0.012	0-0.054	0-0.011	0-9.9	0-0.033
LOD* in pure SiO2 (ppm)	5	9	<5	<5	6	<5	5

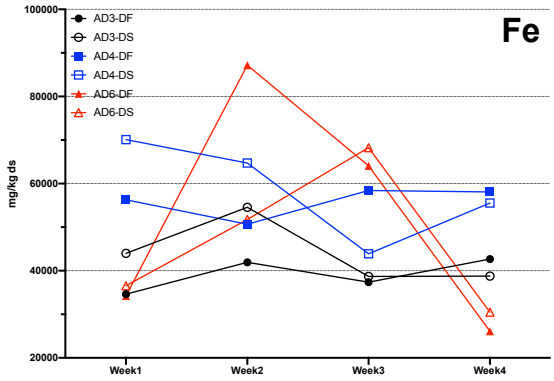
Note: Note: 1. Calibration range: Concentration range covered by reference samples in the calibration of the application. In practice, the minimum concentration that can be reliable analyzed is determined by Limit of Quantification (LOQ); 2. LOD = limit of detection.


Ba
mg/kg ds
AD3-DF
AD3-DS
AD4-DF
AD4-DS
AD6-DF
AD6-DS
Week1
Week2
Week3
Week4

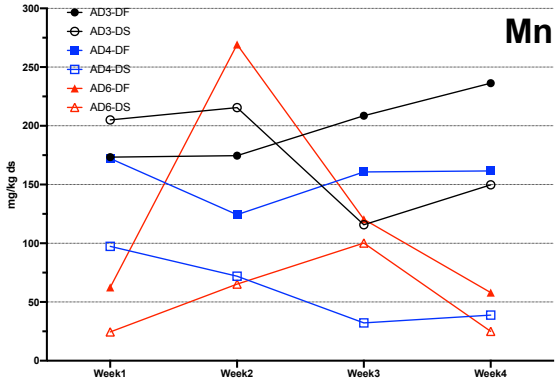
(a)


Cu
mg/kg ds
AD3-DF
AD3-DS
AD4-DF
AD4-DS
AD6-DF
AD6-DS
Week1
Week2
Week3
Week4

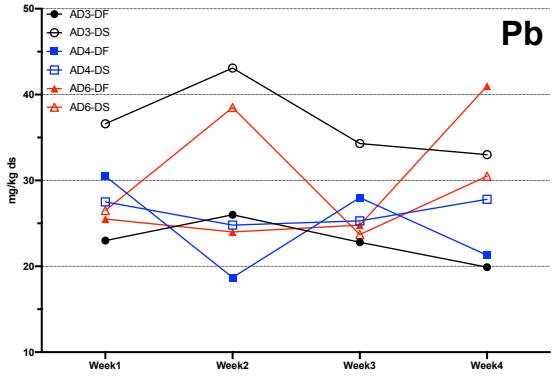
(b)


Fe
mg/kg ds
AD3-DF
AD3-DS
AD4-DF
AD4-DS
AD6-DF
AD6-DS
Week1
Week2
Week3
Week4

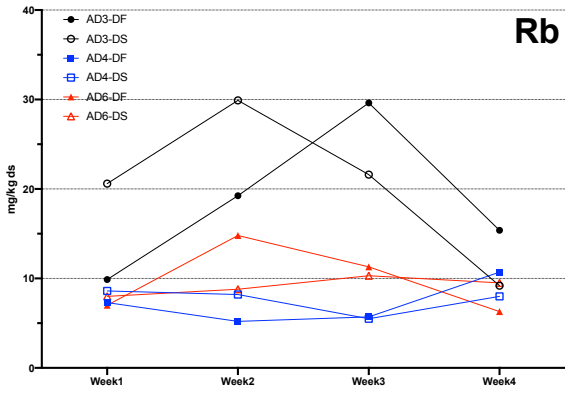
(c)


Mn
mg/kg ds
AD3-DF
AD3-DS
AD4-DF
AD4-DS
AD6-DF
AD6-DS
Week1
Week2
Week3
Week4

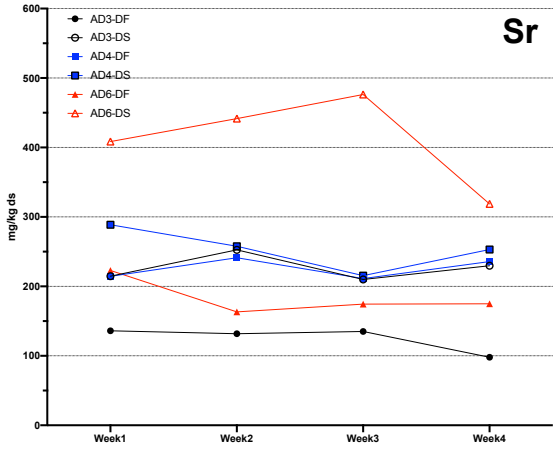
(d)

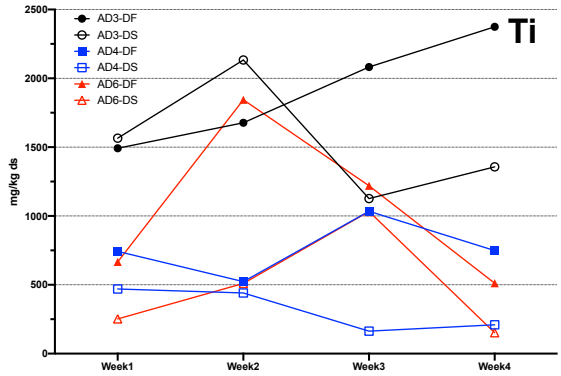

Pb
mg/kg ds
AD3-DF
AD3-DS
AD4-DF
AD4-DS
AD6-DF
AD6-DS
Week1
Week2
Week3
Week4

(e)


Rb
mg/kg ds
AD3-DF
AD3-DS
AD4-DF
AD4-DS
AD6-DF
AD6-DS
Week1
Week2
Week3
Week4

(f)


Sr
mg/kg ds
AD3-DF
AD3-DS
AD4-DF
AD4-DS
AD6-DF
AD6-DS
Week1
Week2
Week3
Week4


Ti
mg/kg ds
AD3-DF
AD3-DS
AD4-DF
AD4-DS
AD6-DF
AD6-DS
Week1
Week2
Week3
Week4

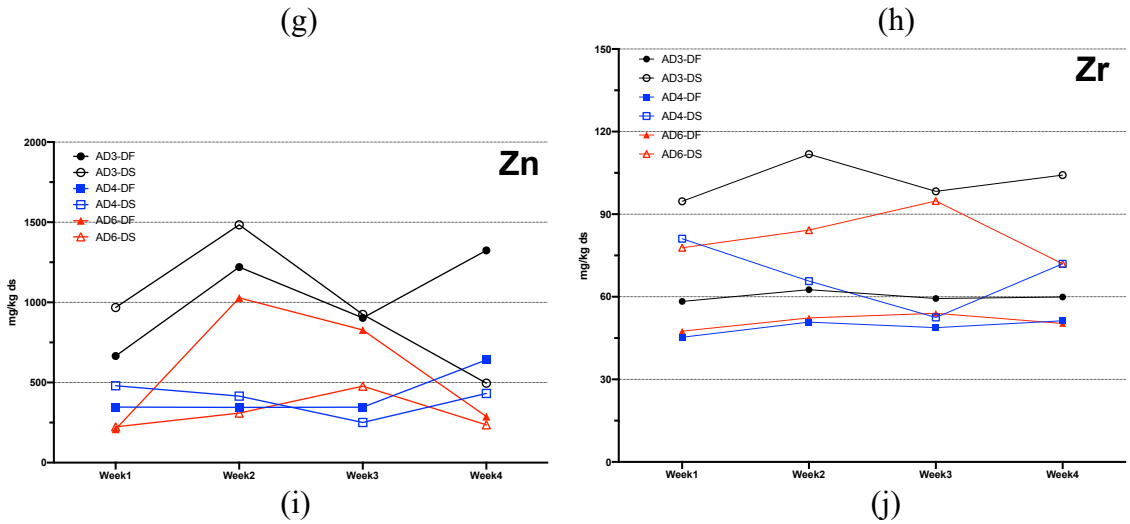


Figure 4-3. Variation in selected heavy metal contents in digester feed and digested sludge in the three AD processes.

The average log heavy metal concentrations of each AD process are shown in Figure 4-4 to 4-6 for easier interpretation. On average, the highest heavy metal concentrations were all detected in AD3 except Ba, Fe, Pb, and Sr. The results were expectable because AD3's digester feed includes not only its own untreated primary and secondary sludge but also biosolids from three plants, resulting in higher chance of high heavy metal concentrations. The highest Ba and Sr concentrations were detected in AD4's DF and AD6's DS. The highest Fe concentrations were detected both in AD4's DF and DS. High Fe concentration is to be expected since it used to control hydrogen sulfide concentrations as well as improve primary clarifier performance. The highest Pb concentration was detected in AD6's DF. On the contrary, The lowest heavy metal concentrations on average were detected and distributed more variably at the three AD processes. The lowest heavy metal concentrations of Cu, Zn, Ti, Rb, and Zr were detected in AD4's DF and the lowest Pb, Rb, Ti, and Zr were detected in AD4's DS. The lowest Mn concentration was detected in AD6's DF and the lowest Cu, Mn, and Zn concentrations were detected in AD6's DS. In AD3, the concentrations of Ba, Fe, Pb, and Sr were least in the DF and the concentrations of Ba, Fe, and Sr

were least in the DS. In the order of maximum to minimum concentrations among all the heavy metal concentrations: the orders were Fe > Ti > Zn > Cu > Mn > Ba > Sr > Zr > Pb > Rb in AD3's digester feed and Fe > Ti > Zn > Cu > Sr > Mn > Ba > Zr > Pb > Rb in AD3' digested sludge; the order of heavy metal concentrations in AD4's DF were Fe > TI > Zn > Ba > Sr > Cu > Mn > Zr > Pb > Rb, which was different from the DS (Fe > Ba > Zn > Ti > Sr > Cu > Zr > Mn > Pb > Rb); in AD6, the order in DF (Fe > Ti > Zn > Cu > Ba > Sr > Mn > Zr > Pb > Rb) is also different from that in DS (Fe > Ti > Ba > Sr > Zn > Cu > Zr > Mn > Pb > Rb). Generally, the Fe concentrations were the highest, but Rb concentrations were the lowest at all the three AD processes. The heavy metals found at these three treatment plants are typical of heavy metals at many domestic wastewater treatment plants.

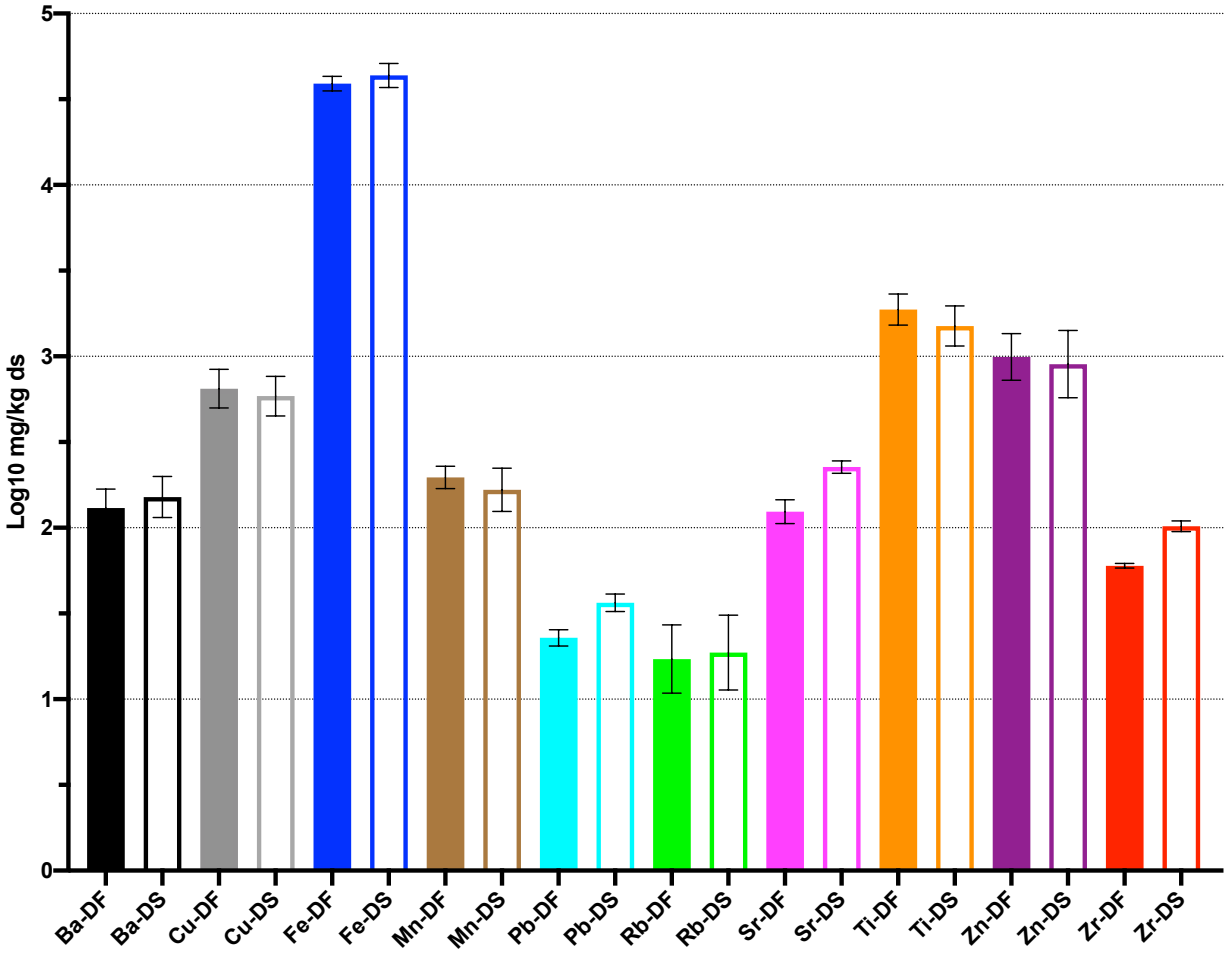


Figure 4-4. Average heavy metal contents in $\log_{10}$ mg/kg ds in digester feed (DF) and digested sludge (DS) at AD3.

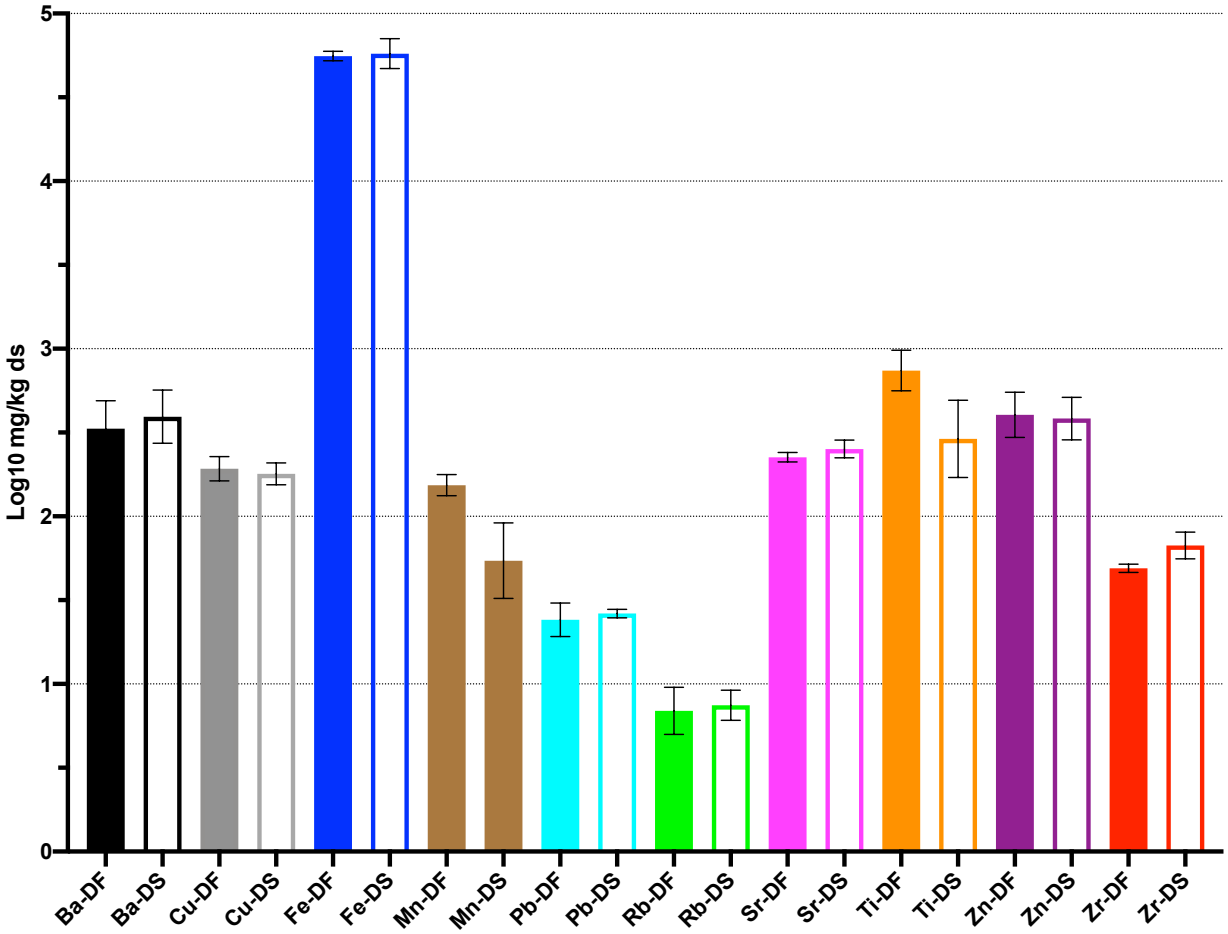


Figure 4-5. Average heavy metal contents in $\log_{10}$ mg/kg ds in digester feed (DF) and digested sludge (DS) at AD4.

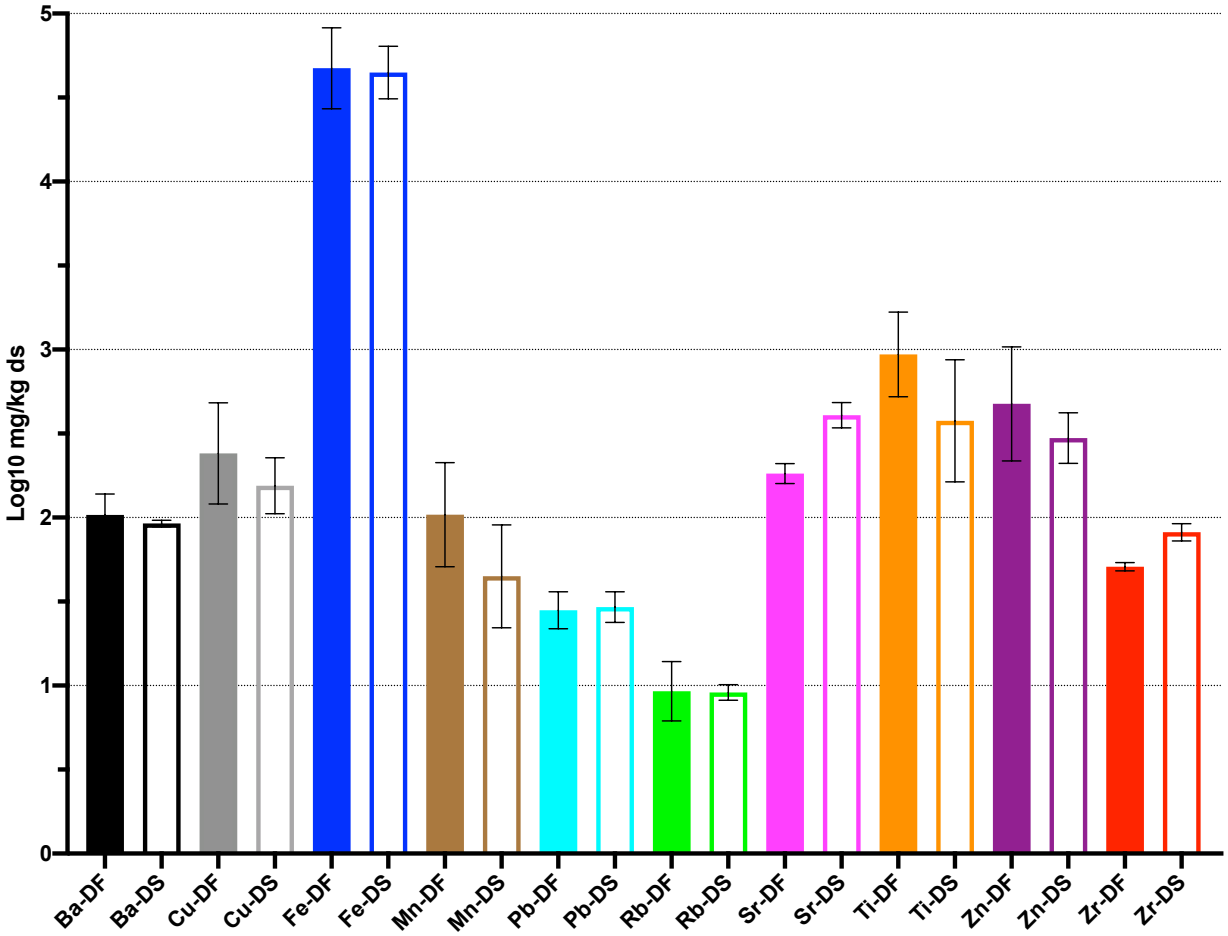


Figure 4-6. Average heavy metal contents in $\log_{10}$ mg/kg ds in digester feed (DF) and digested sludge (DS) at AD6.

Changes of heavy metal concentrations between digester feed and digested sludge in three AD processes

To examine the relationship between heavy metal concentrations in DF and DS at the three AD processes, the changes of heavy metal concentrations on average were studied and showed variable results at different AD processes. The results in base-10 logarithm are shown in Figure 4-7. The heavy metal concentrations of Cu, Zn, Ti, and Mn decreased at all the three AD processes, between 13.7 and 573.6 mg/kg ds. The Fe concentration was also decreased by 6137.2 mg/kg ds in AD6. However, all the other heavy metal concentrations were increased between 1.8 and 4850.7 mg/kg ds. The percentage of changes on average for each selected heavy metal are shown in Table 4-3 at

the three AD processes. The highest removal rate was 61% for Mn in AD4 but the highest increase rate was 124% for Sr in AD6. Previous studies reported that the increases in heavy metal concentrations after digestion processes was expected in dry weight basis because biodegradable organic and inorganic matter were decomposed to end products (CH_4, CO_2, N2, H_2S, etc.) during the process, resulting the observed increases in conservative constituents such as heavy metals (Stronach et al., 1987; Tchobanoglous and Burton, 1991). Because heavy metals are not biodegradable, they can accumulate in the biosolids (Sterritt and Lester, 1980). An undocumented aspect of these AD processes is the recycle of supernatants. Supernatants are typically recycled in anaerobic digestion so there is an opportunity for soluble and suspended material to remove from the digesters.

Table 4-3. Heavy metal changes in percentage at the three AD processes.

Heavy Metal	AD3	AD4	AD6
Ba	17%	16%	110%
Cu	-9%	-7%	-43%
Fe	12%	5%	-12%
Mn	-13%	-61%	-58%
Pb	60%	7%	3%
Rb	10%	5%	-7%
Sr	81%	12%	124%
Ti	-19%	-58%	-54%
Zn	-6%	-6%	-47%
Zr	70%	38%	61%

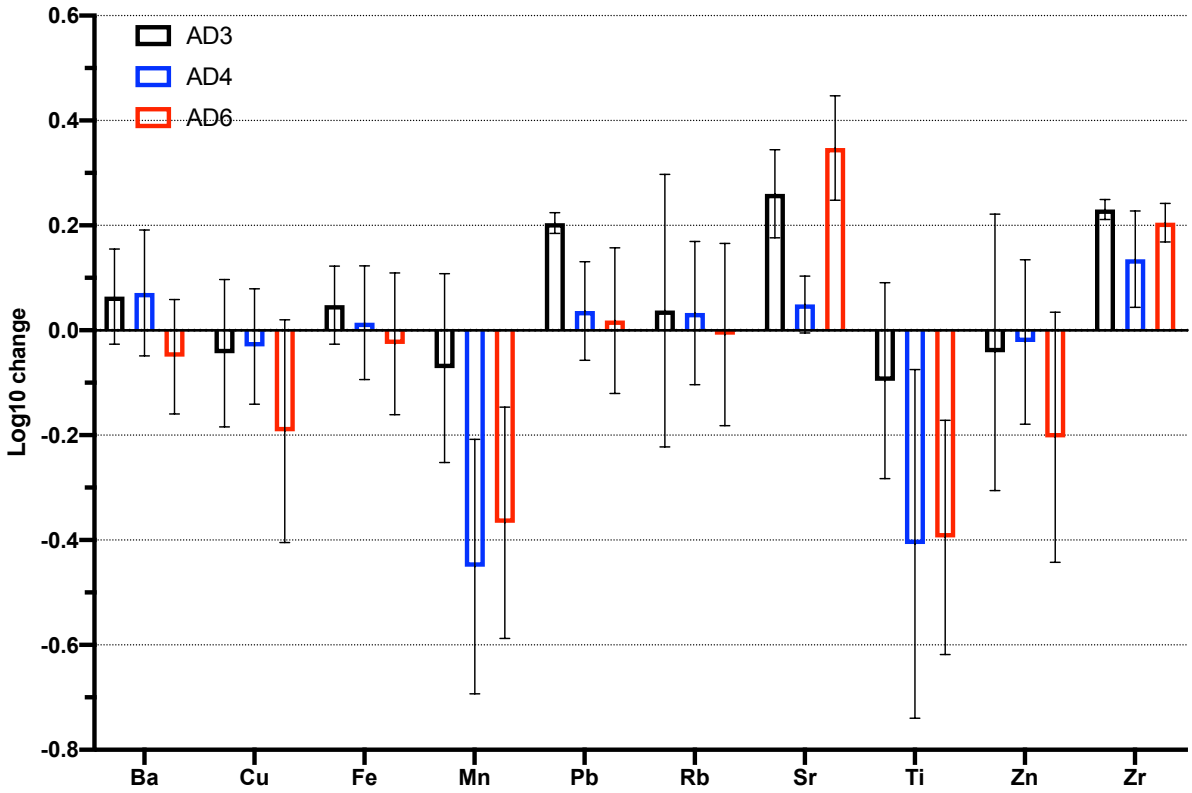


Figure 4-7. Log changes of heavy metal contents at three AD processes.

The heavy metals would be stimulatory or inhibitory to anaerobic microorganisms depending on the total metal concentrations, chemical forms of the metal, and process-related factors such as pH and redox potential (Lin and Chen, 1999; Mudhoo and Kumar, 2013; Zayed and Winter, 2000). Many heavy metals are part of the essential enzymes that drive numerous anaerobic reactions, such as Cu, Zn, and Fe (Guo et al., 2019). For example, a previous study reported that cellulase activity showed 16.33% higher levels than in the control group when the Cu^{2+} concentration was 150 mg/kg, but the microbial community activities were completely inhibited when the Cu^{2+} concentration exceeded 300 mg/kg (Guo et al., 2012; Wu et al., 2011). Low Cu^{2+} concentration may promote enzyme activity as a cofactor and maintain microbial physiological activity (Guo et al., 2012; Kim et al., 2010). Nevertheless, high Cu^{2+} concentrations are toxic to microbes and

enzymes by impacting the spatial structure of the enzyme and reducing cellulase activity, and may prevent microorganisms from degrading complex organic macromolecules (Guo et al., 2012).

In the AD process, Zn is also an essential trace element for various enzymes, which could stimulate fermentation process (Wang et al., 2018). Appropriate Zn^{2+} concentration can promote accumulated biogas yield in the AD process (Altaş, 2009; Chan et al., 2018; Choong et al., 2016; Lo et al., 2012). Previous studies reported that 5 mg/L of Zn^{2+} promoted accumulated biogas yield but did not promote methane production (Chan et al., 2018; Lo et al., 2012). However, when the concentration of Zn^{2+} increased to 100 mg/L, they found that the biogas production was inhibited by approximately 34% compared to the control group but methane production was neither further decreased nor increased. Therefore, it indicates that trace amounts of Zn^{2+} are necessary to maintain AD operations for biogas production.

Iron is an important heavy metal and its Fe^{2+} form can reduce sulfide interference and stabilize the AD process (Moestedt et al., 2016; Nordell et al., 2016). A previous study found that low concentration of Fe^{2+} increased cellulase activity and biogas yield in the early stage of AD process but started to inhibit cellulase activity in the later stage, when the fermentation substrates contained 1545 mg/kg ds Fe^{2+} (Zhang et al., 2016). However, the Fe^{2+} concentrations as high as 4000 mg/L significantly increased the accumulated biogas production and methanogens (Andriamanohiarisoamanana et al., 2018; Khatri et al., 2015). Studies also found that Fe was necessary for metabolic enzymes and was important in stimulating the formation of cytochromes and ferredoxin that are critical for election transportation (Choong et al., 2016; Zhang and Jahng, 2012). In our study, high Fe concentrations (39148 – 58558 mg/kg ds) were observed in the three

AD processes, which exceeds the maximum concentrations of Fe that can contribute to the operations of AD. However, the Fe concentrations detected were the total Fe, but the Fe^{2+} was unknown. Therefore, it is difficult to conclude if these high Fe concentrations can benefit or inhibit the ARG abundance.

Other heavy metals including titanium, barium, lead, rubidium, strontium, and zirconium were detected. The Ti concentrations were noticed in the effluent sludge of WWTPs in the form of TiO_2 for approximately 100 – 1000 mg/kg sludge (Tou et al., 2017). Previous research found that high Ti concentrations as high as 500 mg/L reduced the methane yield by 18.6% and inhibited the abundance of methanogenic archaea in the sludge fermentation system while some others found contradictory results with positive influences on the methane yield, although these researches were based at lab scale (Cervantes-Avilés et al., 2018; Chen et al., 2014; Farghali et al., 2021; García et al., 2012). In addition, previous studies found that the Ti concentrations reduced the activities of biomass growing in illuminated conditions but had no adverse impact on them under dark conditions (Adams et al., 2006; Braydich-Stolle et al., 2009; Ge et al., 2011; Liu and Hurt, 2010). Because of its strong photocatalytic activity and powerful oxidative stress inducer, TiO_2 enables the synthesis of reactive oxygen species under wavelength light (Kubo et al., 2005). In our study, the total Ti concentrations were decreased after the AD processes by up to 50%. One possible explanation may be the reactive operations between Ti and/or its electronic Ti^{2+} and microorganisms.

The Ba concentrations were detected much lower than other heavy metal concentrations such as Ti and Cu. Barium, considered a non-essential trace elements, has been reported that its presence

could stimulate hydrolytic enzymes and enhance sulphate removal by precipitation such as Barite (Navamani Kartic et al., 2018; Zandvoort et al., 2006). Nevertheless, the addition of Ba may negatively affect the substrate availability of the process as it may promote carbonate formation (Wyman et al., 2019). Wyman et al., (2019) reported that the Ba concentrations higher than 200 mg/L affected hydrolytic activity and the addition of Ba inhibited the methane production. Therefore, the Ti and Ba concentrations both can affect the AD operations. In our study, the lead concentration was much lower than any other heavy metal concentrations and it was consistent during the anaerobic digestion processes. Previous studies also showed lower detection of Pb concentrations in the AD processes and relative stability than other heavy metals (Zheng et al., 2020). Also, the Pb concentrations detected in all the AD processes were lower than the Pb limit for land applied sewage sludge regulated by US EPA (840 mg/kg) (US EPA 2020).

Rubidium is an alkali metal and is used in specific fields such as fiber optic telecommunication and laser technology (Naidu et al., 2016). Rb concentrations were the lowest among all the heavy metals and is reported to be present at low concentrations with other dominant ions such as sodium, calcium, magnesium, and potassium (Naidu et al., 2018). Strontium is also an alkaline earth metal and has been found in some WWTPs (Echeverria-Palencia et al., 2017; Kamei-Ishikawa et al., 2013; Sasmaz et al., 2021). In our study, Sr concentrations were above 100 mg/kg ds and its concentrations were all increased after three AD processes. However, there are little research on other AD processes. Therefore, more studies are needed.

In general, most of the heavy metals can stimulate the performance of anaerobic digestion operations under appropriate concentrations. However, if the heavy metal concentrations are

higher than the limits, the anaerobic digestion performance would be inhibited. Other heavy metals such as Rb, Sr, and Zr are still unknown for their influences on the AD operation.

Relationships between ARGs and heavy metal variables

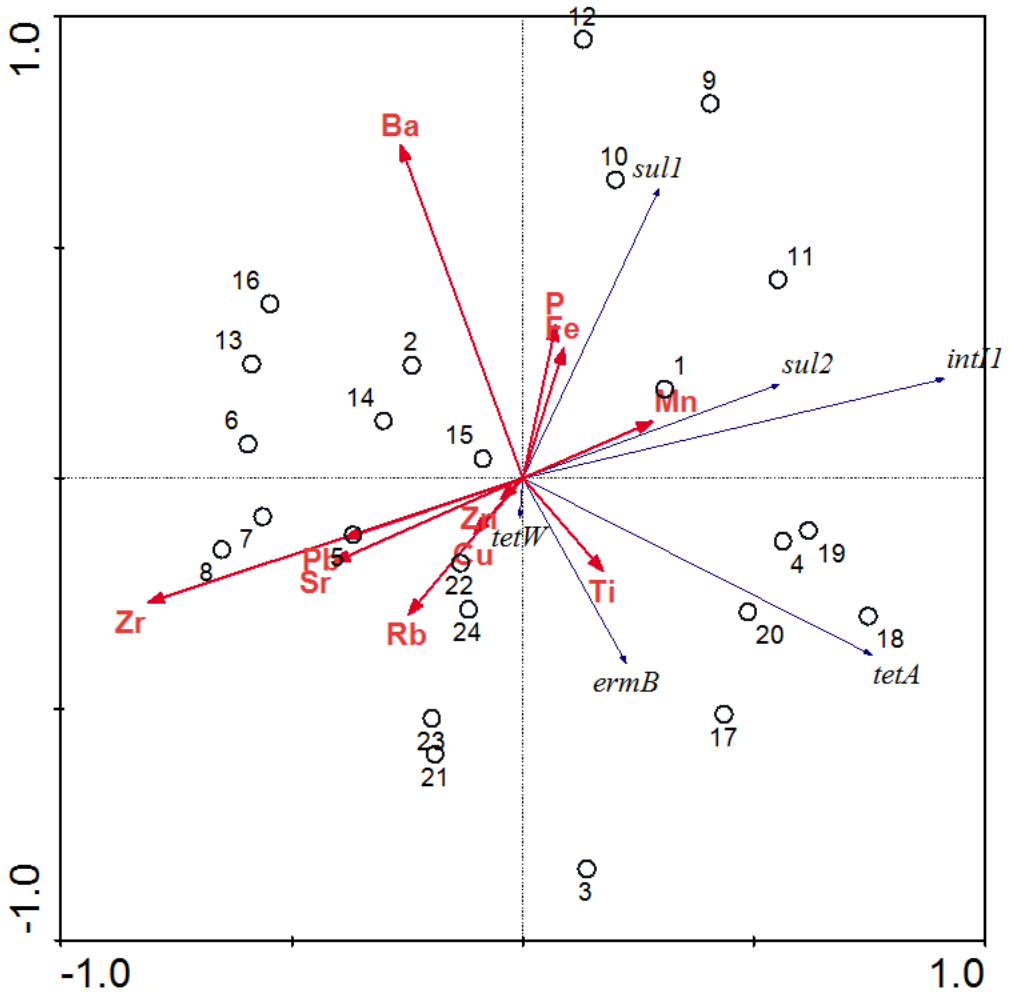


Figure 4-8. Ordination diagram showing the results of Redundancy analysis of the relationships between environmental factors (red arrows), and target ARGs and *intI*1 (black arrows) during anaerobic digestion. Circles represent each sample.

The relationships between the environmental factors and ARGs have been analyzed by RDA and are shown in Figure 4-8 and Table 4-4. The RDA results showed the cumulative percentage

variance of species-environment datof the first and second axis is 57.3% and 81.3%, respectively. The cumulative percentage variance of species occurrence data explained on the first four axes of the RDA was 65.8%. There was a strong correlation between the ARG abundance and environmental factors with species-environment correlations of 0.904 on the first axis and 0.769 on the second axis. The Monte Carlo permutation test indicated that the environmental variables significantly ($p < 0.05$) explained the variation along the first ordination axis ($p = 0.012$) and the total variance ($p = 0.006$). This indicated that both axes were highly correlated with the set of variables.

Table 4-4. Redundancy analysis of target ARG abundance and heavy metals

Axis	1	2	3	4	Total Variance
RDA					
Eigenvalues	0.387	0.162	0.077	0.032	1.000
Species-environmental Correlation	0.904	0.769	0.849	0.887	
Cumulative percentage variance of species data	38.7	54.9	62.6	65.8	
Cumulative percentage variance of species-environment data	57.3	81.3	92.7	97.4	
Sum of all	Eigenvalues				1.000
Sum of all canonical	Eigenvalues				0.675
Test of significance of first canonical axis	Trace	F-Ratio	P-Value		
	0.387	7.587	0.0120		
Test of significance of all canonical axes	0.675	2.27	0.00600		

Figure 4-8 illustrates the relations among the probabilities for ARG abundance and heavy metals. The first ordination axis represented the heavy metal concentrations' impact on ARG abundance. Zirconium was the best explanatory variable with the highest scores, followed by barium. Few previous studies studied the correlation between Zr concentration and ARG abundance. But previous studies reported that bacterial resistance to barium occurred in rivers (Barancheshme and Munir, 2019; Icgen and Yilmaz, 2014). The study of 79% of resistance to barium out of 290 river

isolates was reported by them. Phosphorus did not significantly correlated with selected ARG abundance as much as other heavy metals. But the nutrient P has been found to have a major influence on the microbial community (Schäfer et al., 2001). Previous studies observed that the P level was strongly correlated with the emergence of antibiotic-resistant phenotypes in different environment (Ali et al., 2016; Cheng et al., 2016; Cui et al., 2018; Yu et al., 2012). The presence of P may induce the development of resistance to antibiotics in *E. faecalis* strains that interfere with ribosomal binding (Clancy et al., 1996). In addition, the Pb concentration was also found to be collectively associated with ARGs (N. et al., 2012; Rosewarne et al., 2010) but did not significantly correlate with ARGs in our study.

In Figure 4-8, heavy metals including Zr, Pb, Sr, Cu, and Rb were strongly and positively correlated with each other. Similarly, P, Fe, and Mn were strongly and positively correlated with each other. Ba and Ti, however, were strongly and negatively correlated with each other. The correlations between heavy metals and ARGs were evaluated: P and Fe concentrations was strongly and positively correlated with *sul*1 gene; Mn concentration was strongly and positively correlated with *sul*2 and *intI*1; Ti concentration was strongly and positively correlated with *tet*A and *erm*B. On the contrary, Ba concentration was negatively correlated with *tet*A and *erm*B. The concentrations of Zr, Pb, and Sr were negatively correlated with *sul*2 and *intI*1 and Rb concentration negatively correlated with *sul*1. The impact of heavy metals on the fate of some ARGs in this study was consistent with previous studies (Cui et al., 2016; Ji et al., 2012; Riaz et al., 2020; Zhu et al., 2013). These results can possibly be explained that heavy metals are considered a co-selection factors for antibiotic resistance (Baker-Austin et al., 2006; Knapp et al., 2010). Heavy metals may drive the selection for antibiotic resistant bacteria and promote their

proliferation when ARGs and their host bacteria directly contacted with heavy metals (Becerra-Castro et al., 2015; Stepanauskas et al., 2006). In addition, cross-resistance, the mechanism that bacteria are resistant to both one antibiotic and a metal simultaneously, and co-regulation, the mechanism that the expression of resistance systems to metals and antibiotics are controlled by a common regulator, may also lead to the changes in ARGs (Baker-Austin et al., 2006; Chapman, 2003; Li et al., 2017; Pal et al., 2017). Furthermore, previous studies found that some heavy metals, such as Zinc and Copper, were more correlated with the fate of ARGs in the bio-available forms than the total heavy metals (Cui et al., 2016; Zhang et al., 2018, 2017). The bio-available metals could penetrate cell envelopes and exert biological effects, resulting the impact on the microbial community or imposition of selective pressure on microbes (Zhang et al., 2018). Thus, the influence of bio-available heavy metals in the abundance of ARGs should be considered. In general, the fate of ARGs and heavy metal concentrations were significantly correlated, which needs further study as anthropogenic levels of heavy metals are currently several orders of magnitude greater than levels of antibiotics and metals are not subject to degradation and therefore can subsequently represent a long-term selection pressure (Stepanauskas et al., 2005).

4.4. Conclusion

Heavy metal concentrations and their correlations with five ARGs and *IntI*1 were studied in three full-scale anaerobic digestion processes at three WWTPs near Los Angeles. AD3 detected the highest concentrations of Cu, Mn, Rb, Ti, Zn, and Zr both before and after the anaerobic digestion process. The Fe concentrations were the highest while the Rb concentrations were the lowest among all the heavy metals. There were 30 observations in total for ten heavy metal concentrations at three AD processes. There were 14 decreased observations where the highest decrease was 61%

in AD4 for Mn. However, 16 observations increased where the highest increase was 124% in AD6 for Sr. In addition, ARG abundance was correlated with environmental variables. Zr and Ba were the best explanatory variables to the ARG changes. heavy metals including Zr, Pb, Sr, Cu, and Rb were strongly and positively correlated with each other while P, Fe, and Mn were strongly and positively correlated with each other, although Ba and Ti were strongly and negatively correlated. In addition, correlations between environmental variables and ARGs were found between *sul*1 and P and Fe, between Mn and *sul*2 and *intI*1, and between Ti and *tet*A as well as *erm*B. Although, the anaerobic digestion process may not remove all the heavy metals, it provides an environment for reactions and relationships among the heavy metals and other environmental variables and ARG abundance.

4.5. References

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