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Distribution, Dissemination and Fate of Antibiotic Resistance Genes During Sewage Sludge Processing—a Review

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Abstract Sewage sludge, a common by-product of wastewater treatment plants, is one important repository of antibiotic resistance genes (ARGs). The growing demands of sewage sludge reclamation, such as land application, increase the possibility of introducing ARGs into the environment and even the further dissemination of antibiotic resistance. Previous studies have paid much attention to the removal efficiencies of conventional pollutants such as heavy metals and pathogenic microorganisms during the sludge treatment processes. However, the effects on the abundance and diversity of ARGs got great concerns only recently. This paper mainly focuses on the enrichment and transmission modes of ARGs in sludge and the effects of representative sludge treatment technologies on ARG distributions in sludge. It seems that most physical and chemical techniques such as microwave, alkali treatment, and coagulation are ineffective in ARG reduction. The impacts of biological sludge treatment technologies on ARGs are varied, probably because of the diverse microbial community structures, operational parameters, and even environmental factors such as rainwater. Therefore, the sensitivities of potential hosts of specific ARG to the sludge treatments should determine the abundance of ARG before and after treatment. In addition, a reasonable combination of different sludge process techniques is usually a better choice than the single one for ARGs' removal due to its better ability to efficiently damage the embedded cells and directly degrade the released ARGs. In summary, the appropriate treatment techniques should be applied on the excess sewage sludge to help mitigate the release of ARGs to the environment.

Keywords Antibiotic resistance genes · Cell destruction · Microbial community structure · Sewage sludge processing · Wastewater treatment plant

Abbreviations
WWTPs Wastewater treatment plants

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WAS	Waste activated sludge
ARG	Antibiotic resistance gene
AD	Anaerobic digestion
ARB	Antibiotic-resistant bacteria
CSTR	Continuous-stirred tank reactor
AMR	Antimicrobial resistance
NPs	Nanoparticles
VGT	Vertical gene transfer
HGT	Horizontal gene transfer
MW	Microwave
US	Ultrasound
MGEs	Mobile genetic elements
MDR	Multidrug resistance
ICEs	Integrative and conjugative elements
MWS	Municipal waste sludge
ROS	Reactive oxygen species
DNases	Deoxyribonucleases
SRT	Solids retention time
HT	Hydrothermal
DS	Dry sludge

1 Introduction

Antibiotics have been widely used in health care, livestock and poultry breeding, and aquaculture industries (Tan et al. 2019) since they can not only prevent and treat diseases caused by bacterial infections (Laws et al. 2019), but also be employed as animal growth promoters. Excessive and inappropriate application of multiple antibiotics has raised considerable concern recently since 30–90% of the ingested antibiotics to humans and animals are excreted by urine and feces (Du and Liu 2012), which eventually end up into the sewage. Selective pressure from antibiotics induces the emergence of antibiotic resistance genes (ARGs), and a group of antibiotic-resistant bacteria (ARB) occur which are resistant to antibiotics because of either genetic mutations or ARG acquirement (Amarasiri et al. 2020).

The transfer of ARGs into dangerous pathogens would cause the compromise of the antibiotics treatment efficiencies (McConnell et al. 2018), resulting in the impairment of bacterial infection. If no corresponding control measures are taken, 10 million people will die of antimicrobial resistance (AMR) each year by 2050 (Robinson and Carrique-Mas 2016). As a result, the World Health Organization has declared antibiotic resistance as one of the three major threats to public health in the twenty-first century (Shankar 2014).

Wastewater treatment plants (WWTPs) receive and enrich antibiotics and ARGs from the sewage of hospitals, residential areas, factories, and the animal husbandry industry. High microbial densities, nutrient contents, and sub-inhibitory concentrations of antibiotics in WWTPs provide a favorable environment for ARB survival and the transformation and distribution of ARGs (Osińska et al. 2019; Rizzo et al. 2013). In addition to antibiotics, selection pressure from co-exposure to biocides and heavy metals in sewage also exert the potential stress to increase the ARG mutations (Bengtsson-Palme et al. 2016). What's more, two main pathways by which ARGs are distributed among bacteria, namely, vertical gene transfer (VGT) and horizontal gene transfer (HGT) (Li et al. 2019), furtherly aggravate the dissemination of ARGs and multiplication of ARB in WWTPs. VGT means the transmission of modified genetic information located on bacterial chromosomes to subsequent generations of daughter cells (Pazda et al. 2019). HGT (Fig. 1) is a way of spreading resistance genes between different bacterial species through transformation, transduction, or conjugation (Pak et al. 2016). Given the fact that WWTPs are not designed to remove ARGs, the efficiencies of currently applied wastewater treatment technologies on ARG elimination are inconsistent and may even have contradictory results among different WWTPs, since complex factors such as influent sources, ARG types, treatment scales, salinity, and operating parameters will readily influence the removal effects (Liu et al. 2018). Therefore, various antibiotics and ARGs were commonly detected in the WWTPs' effluents and sludges.

Sewage sludge usually enriches antibiotics through electrostatic gravity and adsorption (Gao et al. 2012), allowing the microorganisms contained in the sludge flocs to be continuously exposed to the antibiotics with the sub-inhibitory concentrations (Osińska et al. 2019). As a result, ARGs are induced with a high probability under the selective pressure of antibiotics. Extracellular DNA carrying ARGs in wastewater would also be reduced through adsorption by activated sludge, leading to much higher ARG concentrations in excess sludge than that in wastewater (Wen et al. 2016). Besides, intimate cell contacts promote the HGTs of ARGs between different bacterial species. It has been reported that the total ARG levels in the discharge

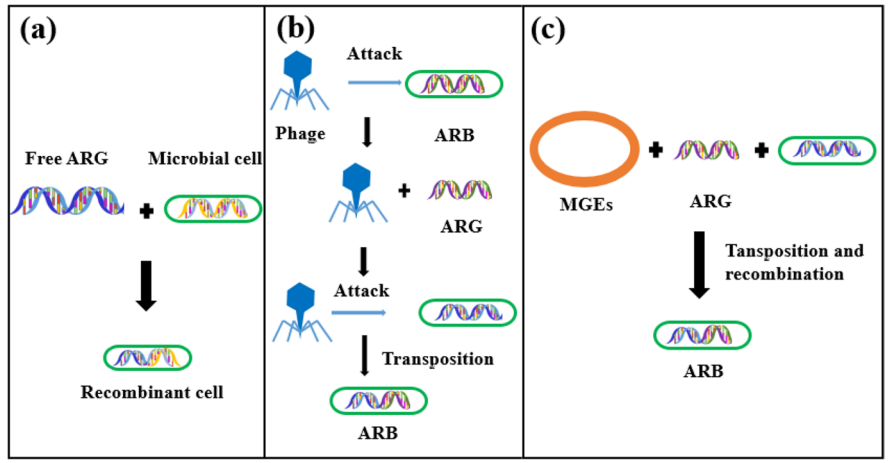

Fig. 1 Three forms of ARG HGT (**a** transformation; **b** transduction; **c** conjugation) (adapted from (Tan et al. 2019) with permission from Elsevier Inc.)

from the sludge dewatering process was 7–308-fold higher than those in the influents and 16- to 638-fold higher than those in the effluents (Qiao et al. 2018). Therefore, it is generally considered that sewage sludge contributed higher amounts of ARGs into the environment than the effluents from WWTPs. The appropriate sludge treatment and disposal are critical to minimize the potential risk of ARG causing "secondary pollution" in the downstream environment. There are numerous pieces of evidences that the application of sludge as the soil amendment is an important pathway for the introduction and further dissemination of ARGs into the soil environment (Sun et al. 2021). Considering the growing demands of reclaiming the sewage sludge for land application, extensive attention has been paid to the removal efficiencies of conventional pollutants such as heavy metals and pathogenic microorganisms during the sludge treatment. However, the variations of the abundance and diversity of ARGs during the sludge treatment process have not been carefully addressed yet. This review focuses on the impacts of different sludge treatment technologies on ARG existences in sewage sludge, reveals and compares the ARG removal effects among different technologies, and provides academic supports for the selection of appropriate sludge treatment process for their subsequent safe disposal and utilization.

2 Occurrence and Dissemination of ARGs in Sewage Sludge

2.1 Representative Types of ARGs

According to the different chemical structures and characteristics, the commonly detected antibiotics in WWTPs include five categories, i.e., quinolone, macrolide, β-lactam, tetracycline, and sulfonamide antibiotics, which were all up to the level of micrograms/liter in the raw sewages (Pazda et al. 2019). Their corresponding resistance genes are quinolone resistance genes (*gyr*A, *gyr*B, *par*C) (Lindberg et al. 2006), macrolide resistance genes (e.g., *mph*(A), *mph*(B), *mefA*, *msrA*) (Leclercq 2002), β-lactam resistance genes (e.g., *ampC*, *ampR*, bla_{TEM}, bla_{CIT}, bla_{FOX}, bla_{CTX-M}) (Li et al. 2007), tetracycline resistance genes (e.g., *tet*A, *tet*O, *tet*Q, *tet*S, *tet*T, *tet*W, *tet*X) (Aminov et al. 2001), and sulfonamide resistance genes (e.g., *sul*I, *sul*II, *sul*III) (Wang et al. 2014), respectively. Apart from these, multidrug resistance (MDR) genes are often observed (Pazda et al. 2019). In addition, the class 1 integron integrase gene (*intI*1) is considered a promising indicator for ARG horizontal migration potential assessment (Zheng et al. 2020), and is also commonly detected in WWTPs. Although the absolute abundance of ARGs in the

Table 1 Reported absolute abundance of total ARGs in sludge samples collected from different WWTPs around the world

Target	Concentration/ (copies/g DS)	Location	Reference
Total ARGs	10^{10}–10^{11}	Ontario, Canada	Lau et al. (2017)
	10^{9}–10^{12}	Southern Minnesota, USA	Burch et al. (2013b)
	10^{8}–10^{9}	Barcelona, Spain	Calero-Cáceres et al. (2014)
	10^{8}–10^{9}	UK	Redhead et al. (2020)
	10^{10}–10^{11}	Columbia, USA	Ma et al. (2011)
	10^{9}–10^{10}	Michigan, USA	Munir et al. (2011)
	10^{9}–10^{10}	Wisconsin, USA	Auerbach et al. (2007)
	10^{10}–10^{11}	Nevada, USA	Zhang et al. (2013)
	10^{9}–10^{11}	Minnesota, USA	Burch et al. (2013a)
	10^{11}–10^{16}	Gifu, Japan	Li et al. (2021)
	9.23×10^{12}	Gwangju, Korea	Jang et al. (2017)
	10^{9} -10^{13}	Hebei, China	Pei et al. (2016)
	10^{8}–10^{11}	Nanjing, China	Wang Chen et al. (2020)
	2.76×10^{10}	Wuxi, China	Lu et al. (2020)
	10^{8}–10^{10}	Hangzhou, China	Wu et al. (2016)
	10^{13}–10^{14}	Beijing, China	Zou et al. (2020)
	10^{11}–10^{13}	Northern China	Dong, Wang, et al. (2019)
	10^{7}–10^{10}	Chengdu, China	Wang, Deng, et al. (2020)

DS dry sludge

sewage sludge of WWTPs in different regions is varied in order of magnitude, in general, the concentration values of total ARGs detected is between 10^{8} and 10^{14} copies/g dry sludge (Table 1).

2.2 The Occurrence and Dissemination of ARGs

Antibiotic resistance genes are generally classified as intrinsic resistance genes and acquired resistance ones (Hiller et al. 2019). Long before the environmental selection pressure caused by modern clinical use of antibiotics, microorganisms generate intrinsic ARGs to compete against natural antibiotics. For example, a collection of DNA encoding the resistance to β-lactam and tetracycline antibiotics was identified from the 30,000-year-old Beringian permafrost sediment samples (D'Costa et al. 2011). Different from the intrinsic resistance genes, the acquired resistance genes are incorporated via HGT (McInnes et al. 2020). In transformation, free DNA is absorbed by microorganisms from the extracellular environment and inserted into their genomes (Rizzo et al. 2013). Transduction is the way through which genetic material is transferred between donor and recipient bacteria via bacteriophage intermediates (Chiang et al. 2019). Conjugation process is mediated by mobile genetic elements (MGEs), such as plasmids and integrative and conjugative elements (ICEs) (Ilangovan et al. 2015) via a pilus between bacteria in close proximity to each other (Cabezón et al. 2014). Due to the fact that HGT occurs across not only the same but also different species (McInnes et al. 2020), HGT is considered to significantly contribute to the global dissemination of ARGs compared with VGT (von Wintersdorff et al. 2016).

2.3 Main Mechanisms of Antibiotic Resistance

The general mechanisms that bacteria resist antibiotics through ARGs can be classified into the following four categories (Blair et al. 2015): (1) decrease in cell permeability—on the one hand, limiting the entry of antibiotics into bacterial cells can be achieved by reducing the number of channels for drugs to enter the cell, choosing more selective channels or creating an alternative metabolic pathway. Another mechanism is to modify the cell surface to limit its interaction with drugs and reduce the entry of antibiotics (Schaenzer and Wright 2020); (2) modification of antibiotic target (Fig. 2)—absorption of DNA can confer antibiotic resistance by target protein modification through the formation of "mosaic" genes (Blair et al. 2015).

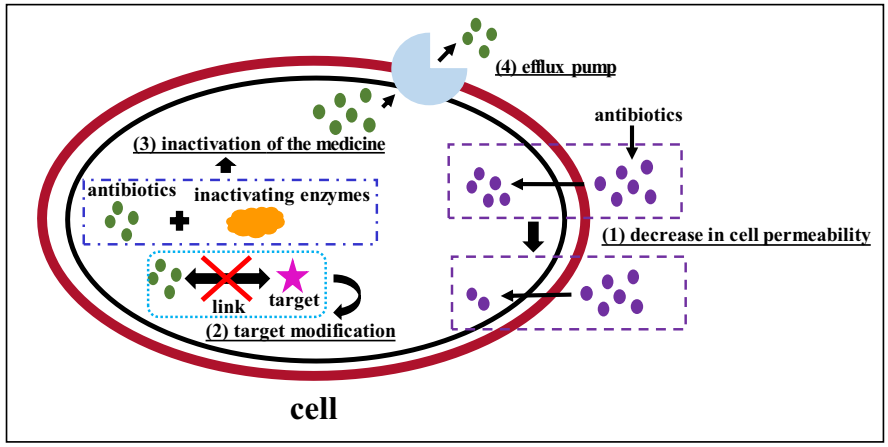

Fig. 2 Main mechanisms of antibiotic resistance: (1) decrease in cell permeability; (2) target modification; (3) inactivation of the medicine; (4) efflux pump (adapted from Pazda et al. (2019) with permission from Springer Verlag)

Another way to change target is the acquisition of genes homologous to the original target. Both of them would result in a functional target with reduced affinity for the antibiotic. In addition, modifying the target by adding chemical groups can also prevent antibiotic binding; (3) direct inactivation of the medicine (Munita and Arias 2016)—for the antibiotics that have entered the cell, the antibiotics can be inactivated by hydrolysis, or by the transfer of chemical groups; that is, the bacterial enzymes add chemical groups to the vulnerable sites on the antibiotic molecules to prevent the antibiotics binding to its target protein, causing antibiotic resistance; (4) active antibiotic removal from bacterial cell using an efflux pump—efflux pumps are found in almost all bacterial species which allows direct extrusion of various drugs from cytosol or periplasmic space to the outside of bacterial cells (Poole 2007). Efflux pumps can not only discharge a variety of antibiotics, but also drive the obtainment of additional resistance mechanisms by lowering intracellular antibiotic concentration and promoting mutation accumulation (Sun et al. 2014). In addition, it is worth mentioning that even in ARGs encoding the same type of antibiotic, the resistance mechanism between different ARG subtypes will also be different. For example, resistance mechanism of *tet*A is an efflux pump, while *tet*O is ribosomal protection.

3 Impacts of Sludge Treatments on ARGs in Sludge

Usually, there are two ways to characterize the abundance of resistance genes in sludge. One is absolute abundance, which is the copy number of resistance genes per gram of dry sludge, and the other is relative abundance with the value of absolute abundance normalized by the 16S rRNA gene, which can represent the average content of resistance genes in the entire microbial community. There are a variety of existing sludge treatment methods, including physical, chemical, biological, and combined technologies; each of them has its own advantages and disadvantages (Table 2).

3.1 Impacts of physical treatments on ARGs

Physical technologies such as thermal hydrolysis, microwave, and ultrasound have been extensively studied for improving sludge dewatering performances due to their superior cell destruction abilities. However, their impacts on ARGs in sludge are still lacking (Table 3).

3.1.1 Thermal hydrolysis

Thermal hydrolysis has been proven to be promising for reducing both absolute and relative abundances of ARGs in sewage sludge as the high temperature and pressure can not only cause cell destruction to release the intracellular degradable components, but also directly destroy DNA structure (Donoso-Bravo et al. 2011). For example, Pei et al. observed a significant reduction of absolute abundance on five *tet* genes in both pharmaceutical waste sludge and municipal waste sludge (MWS), among which *tet*(X) was 100% removed. The difference in removal efficiency between the sludge types and gene subtypes may be explained by the physical characteristics

Table 2 Summary of advantages and drawbacks of current sludge treatment technologies to reduce ARGs in sewage sludge

Technique		Advantages	Drawbacks
Physical treatments	Thermal hydrolysis	(1) Short-term and efficient; (2) Non-specific removal; (3) Direct destruction of DNA structure	(1) High cost of capital construction; (2) High energy consumption
	Microwave treatment	Effective inactivation of bacteria	(1) High energy consumption
	Ultrasound		
	Air-drying	(1) Simple operation; (2) Relatively low investment costs	(1) Occupation of a large physical footprint; (2) Production of strong odors; (3) Effective for specific ARG subtypes only
Chemical treatments	Coagulants	(1) Low investment in facilities; (2) Simple operation	(1) Unable to remove ARGs or effective for specific ARG subtypes only; (2) Need for secondary treatment caused by compound residues
	Acidification/alkaline treatment	(1) Low investment in facilities; (2) Simple operation; (3) Effective inactivation of bacteria	(1) Mass release of intracellular ARGs; (2) Need for secondary treatment caused by compound residues
	Advanced oxidation	(1) Non-specific removal; (2) Direct damage of DNA structure; (3) Simple operation	Need for secondary treatment caused by compound residues
Biological treatments	Anaerobic digestion	(1) Mature technology; (2) Be friendly to the environment; (3) Low investment in construction and operation	(1) Unstable removal effect; (2) Different removal rates according to ARG subtypes
	Aerobic composting		
	Sludge constructed wetlands		
	Vermicomposting		
Joint treatments		Higher removal efficiency when properly combined	Higher construction and operating costs

of the microbe hosts in two kinds of waste sludges (Pei et al. 2016). Some research revealed that thermal hydrolysis reduced the absolute abundance of all ARGs (including *bla-Imp*, *sul* 1, and *CTX-M-9*, etc.) tested and *intl* 1 by 10–12,000-fold (Redhead et al. 2020; Sun et al. 2019). Interestingly, *MLSB* and *ermF* resistance gene rebound and enrichment after thermal hydrolysis was also observed in that study (Redhead et al. 2020). Other study also confirmed the relative abundances of some *tet* genes were enriched after the thermal hydrolytic pretreatment probably because of the indiscriminate destruction of all DNA. Sun et al. (2019) found that the absolute abundance of more than 94% of all selected ARGs (245 unique ARGs and 12 MGEs) in the sewage sludge collected from a WWTP in Beijing, China, using anaerobic-anoxic–oxic process were effectively reduced after thermal hydrolysis at 160 °C and 0.6 MPa for 30 min.

3.1.2 Microwave treatment

The microwave treatment is well known to be capable of destroying the structures of microorganisms and lead to the leakage of intracellular substances including DNA. However, it might enhance the spread of ARGs in the sludge due to the release of intracellular ARG containing DNA. For instance, Wang, Deng, et al. (2020) found that after microwave radiation at 700 W for 180 s, the absolute abundance of 6 selected *tet* genes in the excess sludge mixture (domestic and industrial sewage) showed the same increasing trend by 7.7–11.2% (except *tet*A and *intl* 1, which decreased by 0.01–0.39 logs), with the final concentrations higher than those in the control group. In addition, Tong et al. (2016) observed that the total ARG absolute concentration of in sludge was removed by only 2.2% during the microwave pretreatment, but the total

Table 3 Some published literature on the effects of physical treatments on the absolute/relative abundance of typical ARGs

Treatment		Thermal hydrolysis[a]						Thermal hydrolysis[b]		MW[a]				MW[b]			US[a]		US[b]		Air-drying Beds[a]	Air-drying Beds[b]
Tetracycline	*tet* A	−	−	−	−			−	+	−	−			−	+		+	−		±		−	−
	tet C						−						±	+			−						
	tet G	−	−				−	−	+				+					−		±			
	tet M			−	−	−				−	−	±		−	−	±	−		−		±		
	tet O					+	−					±	−	+	+	±	−		−		±		
	tet Q	−	−					−	−					+					−		±		
	tet W	−	−				−	+	+				−	+					−		±	−	−
	tet X	ND	−	−	−		−	ND	−	−	−		−	+			−					−	−
Sulfonamide	*sul* 1			−	−		−			−	−		−					+	−	+	+	−	±
	sul 2			−	−		−			−	−		−						−		+		
β-Lactam	bla_{TEM}			−	−					−	−							−		−			
	bla_{CTX-M}			−	−					+	+						±						
	bla_{NDM-1}			−	−					−	−												
Quinolone	*qnr* A			−	−					−	−												
	qnr S			−	−					−	−												
Macrolide	*mef* A			−	−	−				+	+	±			+	±							
	ere A					−						−			−	−							
	erm B			−	−	−	−			−	−	−	+		−	−		−		−		−	−
	erm F			−	−	−	−			−	−	−	−		−	−							
	erm X					−						−			±	+							
Integronase	*IntI* 1	−	−	−	−	−	−			−	−	+	−	−	±	+	+	−		−		−	±
Reference		Ma et al. (2011), Pei et al. (2016), Tong et al. (2019), Tong et al. (2017), Tong et al. (2018)								Tong et al. (2016), Tong et al. (2017), Tong et al. (2018), Wang, Deng, et al. (2020)							Huang, Liang, et al. (2020), Wang, Chen, et al. (2020)				Burch et al. (2013b)	

[a]Absolute abundance

[b]Relative abundance

MW microwave, *US* ultrasound, *ND* none detected

+means abundance increased after treatment, − means abundance decreased after treatment, ± means no significant changes after treatment

relative abundance of the ARGs increased by 45.0%, among which *tet*A increased by 234.4–945.9%.

3.1.3 Other Physical Treatment Technologies

Ultrasound is currently extensively studied for improving sludge solubilization. The rupture of cavitation bubbles produced by ultrasonic radiation will produce mechanical forces and lead to the destruction of microbial cells. Recently, the effects of ultrasound on ARGs in sludge has received more and more attention. Wang, Chen, et al. (2020) revealed that there was a reduction of less than 0.06 log units in the absolute abundances of total ARGs after ultrasound treatment of 2.5 W/mL, and the relative abundances of bla_{TEM}, *erm*B, and *intI*1 were reduced by 67.6%, 21.7%, and 56.8%, respectively. Besides, the absolute abundance of *sul*1 hardly fluctuated, while the relative abundance value elevated. However, Huang, Liang, et al. (2020) found that ultrasound treatment with a lower power density of 0.08 W/mL had almost no effect on the absolute abundance of two *sul* genes and nine *tet* genes, and their relative abundances significantly increased. The difference in the removal effects of ARGs may be attributed to the strength of ultrasonic energy and the type of ARGs targeted. Nowadays, ultrasound treatment is given high hopes for ARG removal due to the fact that ultrasound can not only effectively inactivate the ARG hosts to release intracellular ARGs, but also generate free radicals to directly damage the DNA fragments carrying ARGs (Zhao et al. 2020). In addition, the mechanical shearing effects caused by ultrasound may destroy the integrity of genomic DNA, resulting in a low abundance of ARGs detected (Wang, Chen, et al. 2020). Burch et al. (2013b) studied the effects of air-drying on ARGs in MWS, and the results verified that the rate and extent of ARG reduction were closely related to specific genetic targets. The absolute concentrations of most target genes were found to decrease eventually, while *tet* (X) increased by nearly 2 orders within 27 days. In terms of relative abundance, the ratio of *intI*1 and *sul*1 showed no fluctuation throughout the experiment while the ratios of the rest tested ARGs decreased after the experiment.

Overall, thermal hydrolysis is a reliable method to eliminate ARGs in sludge due to its ability to directly destroy the sequence of released ARGs, but its capital construction and operational costs are high. Microwave treatment is suggested not efficacious in ARG reduction as this technology can only destroy the cell structure but not the ARG itself. In addition, there is no differential inactivation between antibiotic sensitive bacteria and resistance bacteria, which may cause a larger loss of sensitive cells, resulting in the enrichment of ARG in the microbial group. This also creates conditions for the emergence of multi-drug bacteria, which will make antibiotic therapy more difficult. Although ultrasound and air-drying bed have shown to be highly promising in removing ARGs from sludge, the relevant theoretical and application studies are still limiting.

4 Impacts of Chemical Treatments on ARGs

A variety of chemical conditioning approaches have been extensively applied for many years to enhance sludge dewatering performances, including advanced oxidation, acidification/alkaline treatment, and coagulant addition. Recently, increasing attention has been paid to the ARG removal potential of these sludge conditioning treatments (Table S1 and S2).

4.1 Advanced oxidation technologies

Advanced oxidation pretreatments are well known to be capable of causing changes in cell membrane permeability and fluidity (Griveau and Le Lannou 1997) and induce cell apoptosis (Alvarez et al. 1987). At the same time, reactive oxygen species (ROS) generated from oxidative metabolism, e.g., $O_2^{\cdot -}$ (superoxide radical), $OH^{\cdot}$ (hydroxyl radical), and H_2O_2 (hydrogen peroxide) (Slupphaug et al. 2003), cause more than 20 different types of base damage (Slupphaug et al. 2003). In addition, oxidative stress can also cause strand breaks in DNA. For instance, the Fenton reaction destroys the DNA chain by generating ·OH close to the DNA molecule (Karaolia et al. 2017) and thus normally shows the high ARG removal potential due to its strong oxidative ability (Wang, Deng, et al. 2020). However, the oxidation technology alone did not always show a good removal effect in terms of relative abundance (Sharma et al. 2016), which may be due to insufficient dosage of oxidant. Zhuang et al. (2015) observed that the inactivation efficiencies on the selected genes increased significantly with the enlargement of the input ozone concentration. The

0.1 g O_3/g TS dose used in Pei et al.'s (2016) study was proven to be insufficient in *tet* gene reduction probably because that ozone first reacted with the soluble organic matters such as fatty acids or cell structure before ARGs (Zeng et al. 2014). Another study of Oh et al. also found that adding catalysts (potassium persulfate or monopersulfate) in the process of ozone oxidation could synergistically reduce ARG abundance (Oh et al. 2014). In a word, oxidant dose, ARG type, and contact time play a key role in ARG removal via advanced chemical oxidation technology. Oxidation reagent development, operation parameter optimization, and decay mechanism clarification of different ARG subtypes in the advanced oxidation process are the focus of future research.

4.2 Acidification/Alkaline Treatment

Acidification/alkaline treatments have been proved to impose good effects on destructing the cell wall of microorganisms (Chen et al. 2007), and are used in improving sludge solubilization (Wei et al. 2019). Meanwhile, the acidification process has been identified to generally cause a significant reduction in the absolute abundance of total ARGs, but increased their relative abundances (Wang, Chen, et al. 2020; Zheng et al. 2019). However, the alkaline treatment has no effect on absolute or relative ARG gene abundance reduction, and may even enhance its concentration, thereby increasing the risk of ARG release into the environment (Wang et al. 2019). For instance, Wang, Deng, et al. (2020) adjusted the pH of the sludge to 12 and all *tet* ARG absolute abundances were elevated after 2 h relative to the control. This increase may be due to the released intracellular DNA, leading to higher detection of related ARGs (Chen et al. 2007). The possible reason for the difference in the reduction of ARGs after acidification or alkaline treatment may be that the helix stability of DNA under alkaline conditions is stronger than that under acidic ones (Williams et al. 2001).

4.3 Coagulants Addition

The use of different kinds of coagulants would result in inconsistent ARG treatment efficiencies. The chemical conditioning with PAM or $FeCl_3$ (conventional coagulants used in WWTPs) (Jang et al. 2017; Zheng et al. 2019) alone was reported to fail to remove any ARGs or even worsen the situation in the sewage sludge while the Fe[III]/CaO addition would reduce both the absolute (1.71–4.13 log units) and relative abundances (8.35–100%) of most ARGs (Zheng et al. 2019). Considering that the coagulant does not play the key role of inactivating cells and the works of literature on the ARG fates after coagulations were very few, their specific mechanism of action is not clarified yet.

4.4 Other Chemical Treatment Technologies

The methanol addition for chemical conditioning was reported to be ineffective for ARG reduction. Most of the resistance genes are accumulated after treatment (Wang, Chen, et al. 2020). The possible explanation is that although methanol can promote cell lysis (Xu et al. 2018), it preserves the integrity of genomic DNA. What's more, some scholars unexpectedly found that the presence of nanoparticles may reduce the expression of some ARG subtypes in the sludge (Zhang et al. 2020). The oxidative stress caused by nano materials would lead to the damage of cell membrane and result in the increase of the risks of ARG transfers. The use of nano alumina has been reported to significantly promote the horizontal conjugate transfer of ARGs mediated by plasmid cross genera (RP4, RK2, and pCF10) (Qiu et al. 2012). The binding transfer of RP4 plasmid from *E. coli* to s*almonella* was 200 times enhanced after nano alumina treatment. Therefore, the scholars believe that the application of nano materials in wastewater and sludge treatment should be carefully considered (Qiu et al. 2012; Sharma et al. 2016). In addition, more investigations are needed to draw more general conclusions.

In summary, not all chemical treatment technologies can effectively remove ARGs in sludge. There are two main reasons: (1) the release of intact intracellular DNA fragments caused by only disrupting the cell structure; (2) even if the DNA structure can be directly destroyed, the effects may be related to the dosage of the chemical conditioning agent used. Moreover, the residues of the added chemical reagents make it unpractical for the direct utilization of the treated sewage sludge because of the probable secondary pollution risks.

5 Impacts of Biological Sludge Treatments on ARGs

Due to the advantages of low energy consumption and no risk of secondary pollution, various biological treatment technologies have been widely utilized in the treatment of residual sludge because of their economical and environmentally friendly advantages. Anaerobic digestion, aerobic composting, and sludge constructed wetlands are among the most applied sludge treatment technologies (Mateo-Sagasta et al. 2015). The internal mechanism for ARG abundance reduction during sludge aerobic digestion is unclear due to the relatively few research and application (Xue et al. 2019). In recent years, vermicomposting of sludge together with other biological treatment technologies have also attracted lots of attention including on ARGs elimination.

5.1 Anaerobic Digestion

Anaerobic digestion is a commonly used sludge stabilization technology for its advantages of reducing sludge mass, inactivating pathogens, and generating volatile fatty acids and methane gas. The impacts of anaerobic digestion on ARGs in sludge have been extensively investigated (Table S3 and S4). Meanwhile, a sludge digester is believed to be capable of physically destroying extracellular DNA through hydrolysis, and the presence of deoxyribonucleases (DNases) would also greatly degrade extracellular DNA (Yuan et al. 2019) thus showing the potentials to attenuate ARGs in sludge (Ma et al. 2011). However, due to the high density and diversity of microbial communities in the anaerobic digestion system, ARGs may tend to increase via vertical or horizontal transfers among the relatively enriched ARG hosts. Previous researches have indeed confirmed these contradictory results. For example, Wu et al. (2016) investigated the effects of a two-phase anaerobic digestion process on the reduction of representative ARGs: the presence of *tet*A, *tet*G, *tet*X, *sul*1, *erm*B, *dfrA*1, and *dfrA*12 exhibited 0.1–0.72 log unit removal, while the abundance of *tet*O, *tet*W, *sul*3, *erm*F, and bla_{TEM} even increased compared with the feed, and *sul*2 showed almost no change at all.

5.2 Composting

Composting is commonly used for the recycling of sewage sludge to improve soil fertility (Su et al. 2015). Whether composting can reduce ARGs have also been widely investigated. Some researchers indicated that composting is an effective ARG control method (Liao et al. 2018), but others pointed out that conventional sludge composting is ineffective in removing ARGs because of the increased diversity and abundances of sludge bacterial communities during the composting processes. For example, Su et al. (2015) observed a significant increment in the abundance and diversity of ARGs during the lab-scale composting process, which is speculated to be strongly influenced by the bacterial phylogenetic compositions. Notably, the total ARG was reported to be enriched by 2.04 times during the sludge composting, while the addition of the natural zeolite or the nitrification inhibitor would inhibit the enrichment of total ARGs (Tong et al. 2016).

5.3 Constructed Wetlands

The constructed wetlands (Table S5) for wastewater treatment have been proven to be effective in ARG removal. ARGs in sludge are usually eliminated by the combined actions of declination of microbial density, the biodegradation, and the active absorption by plants (Larsen et al. 2017). In addition, the removal efficiencies of the target ARGs have been found to be closely affected by the different operation parameters (Ma et al. 2019). For example, Ma et al. (2019) built 3 sludge treatment beds (i.e., unit 1: sludge drying bed with aeration tube; unit 2: ventilation reed sludge drying reed bed; unit 3: sludge reed bed without aeration tube). The concentrations of the target ARGs in all three units decreased significantly during the 1-year operation, and the removal efficiencies of *msr*SA and *tet*C (82.2% and 81.0%, respectively) were the highest in unit 2, while those of *erm*B and *tet*A (26.5% and 86.2%, respectively) were the highest in unit 1. Overall, the removal efficiencies of the second and third units with reeds were higher than those in the first unit without reeds. Generally speaking, the removal efficiencies of the individual ARGs also depend on the flow patterns of the constructed wetlands. Compared with the subsurface flow constructed wetlands, the surface flow ones displayed

higher removal efficiencies (Sharma et al. 2016). The obvious reductions of the ARG levels may be due to the aerobic biodegradation and photolysis (Liu et al. 2014). In most cases, the ARGs were not significantly reduced in the constructed wetlands with the horizontal or vertical subsurface flow pattern due to the anaerobic environment generated in the wetlands (Liu et al. 2013, 2014). Nevertheless, a few studies had also stated that the traditional vertical flow constructed wetlands reduced the ARG abundances. For instance, Huang et al. reported that the tetracycline resistance genes were reduced by 45–99% in the vertical flow constructed wetlands (Huang et al. 2015).

5.4 Vermicomposting

Vermicomposting involves the joint actions of earthworms and microorganisms, and is a common recycling method for sludge treatment (Zeb et al. 2020). The intestinal behavior and non-nutritive behavior of worms during their digestion can directly or indirectly control the microbial community, thereby affecting the distribution of potential hosts for ARGs (Huang and Xia 2018). The ARG removal in vermicompost mainly relies on the changes in microbial community and the bio-degradation by earthworms (Huang, Xia, et al. 2020). Attenuation of ARGs in dewatered sludge during vermicomposting has been reported in recent studies (Cui et al. 2018; Huang et al. 2018; Xia et al. 2019). Xia et al. (2019) found that the absolute abundance of the *intl* 1 gene significantly decreased by 0.7-fold after vermicomposting. In Cui et al.'s (2018) research, the absolute abundances of quinolone resistance genes were significantly reduced with a ratio of 85.6–100%. Huang et al. (2018) observed that the absolute abundances of most tested ARGs dropped by more than 60.8% at the end of the vermicomposting. However, Dong, Hong, et al. (2019) revealed that ARGs would accumulate in earthworm gut microbiota. Therefore, the proper dealing with the cultured earthworm should be critical during the sludge vermicomposting.

5.5 Sludge Aerobic Digestion

The literatures on the effects of the sludge aerobic digestion on ARG abundances are limited. The removal efficiencies of *sul* and *IntI1* are relatively litter when the aerobic digester operates in semi continuous mode (22–55 °C and hydraulic retention time (HRT) of 4 days). However, the removal rates of *merB*, *sul 1*, *tetA,* and *tetW* were 85–98% under the conditions of 20 °C and the HRT of 14 days (Diehl and LaPara 2010). Interestingly, the number of all ARGs declined by approximately two orders of magnitude in the batch experiment compared with those achieved in the semi-continuous flow reactions (Burch et al. 2013a). It is worth noting that the *tetW* and *ermB* abundances decreased by 0.90–1.10 log units, but those of other ARGs (such as *intl 1*, *tetA*, *sul1*, and *tetX*) increased in the sludge aerobic digestion processes (Burch et al. 2017). The above studies indicated that the variations of ARG removal efficiencies during the sludge aerobic digestion should be related to the ARGs types, the operation parameters, and the reactor design although further mechanical studies are still needed (Xue et al. 2019).

5.6 Impact of Operation Parameters on ARG Reduction

Sludge source, reactor design, and operating parameters would all affect the microbial community composition in sludge, which in turn affects the potential hosts of ARGs, resulting in the distinction in ARG removal efficiencies.

Reactor Design and Temperature. Burch et al. (2016) built four laboratory-scale anaerobic digesters. The studies have indicated that the ARG abundances greatly (> 50.0%) decreased after the acid production stage of sludge anaerobic digestion (Wu et al. 2018). In addition, the reduction rates may vary greatly with respect to the reactor design (i.e., batch or continuous flow) and ARG subtypes. For example, the absolute abundance of *tet*(A), *tet*(W), and *erm*(B) decreased by more than 90%, while *tet*(X) increased in quantity by fivefold during a composting for more than 175 days fed on a weekly basis of untreated municipal wastewater solids (Burch et al. 2013a). Tang et al.'s (2020) study showed that the abundance of most ARGs decreased after composting except for *aad*A (increased by 414%). Wei et al. (2020) found that the copy numbers of specific ARG subtypes decreased at different degrees with different initial C/N ratios, and the higher initial C/N ratio was more helpful to reduce some target ARGs, while other ARGs were hardly removed. More efficient ARG removal

efficiencies were obtained by hyper thermophilic composting compared to conventional composting, with the lower half-lives and final quantities of ARGs as a result of a higher reduction in abundance and diversity of potential ARG hosts (Liao et al. 2018). The changes of bacterial community caused by the shift of physicochemical properties is the main driving factor for the shaping of ARG structures in the process of sludge composting, rather than HGT (Su et al. 2015). The temperature plays an important role in determining the fate of ARGs during the anaerobic digestion (Xue et al. 2019). They operated at 40, 56, 60, and 63 °C, respectively, using municipal wastewater solids and demonstrated that the absolute abundances of specific ARGs are statistically significantly reduced. Meanwhile, the anaerobic digesters operated at higher temperatures perform better than that operated at 40 °C in terms of ARG elimination. In contrast, Zhang, Yang, et al. (2015) found that more ARG subtypes achieved significant reductions in the mesophilic (35 ± 2 °C) anaerobic digestion than in the thermophilic (55 ± 2 °C) one in the bench-scale anaerobic digesters. At higher temperature, the effective reductions of the ARG host and the acceleration of the ARGs decay rate are considered to be a reasonable explanation (Miller et al. 2013).

PH and Solids Retention Times. Compared with anaerobic digestion without pH regulation, the ARG contents were reduced by 1.36 (*sulII*), 1.11 (*tetQ*), 1.04 (*tetX*), 0.87 (*sulI*), 0.79 (*tetC*), and 0.42 (*tetO*) log units, respectively, when the pH is maintained at 10, which suggested that alkaline fermentation would be more effective in the attenuation of ARGs (Huang et al. 2017). Huang et al. (2017) showed that alkaline pH led to the apoptosis of ARG host cells, which might obstruct the vertical transfer of the target gene in the selected host and cause the reduction of the corresponding ARG. Solids retention times (SRTs) was also pointed out to affect the fates of ARGs during sludge treatment (Zhang et al. 2019), with the removal efficiency that varies from − 79.3 to 92.7% when SRT was set from 15 to 20 days: Longer SRTs showed worse ARG removal efficiencies with the possible reason that ARBs might be suppressed under the oligotrophic condition (Sun et al. 2019).

ARG subtypes. Once the growth of certain hosts of a particular ARG is suppressed, the risk of VGT and HGT of the ARG will decline to a certain extent due to the decrease of the number of receptor bacteria. For VGT, the death of parent cells prevents the birth of daughter cells; for HGT, the reduction in the scope of potential host decreases the spread probability of ARGs between different microorganisms. Different ARG subtypes behaved distinctly in the anaerobic digesters. The resistance mechanism of different ARG subtypes and the selective survival of their potential hosts during the treatments may be the reasons. In two-phase thermophilic digestion, the presence of *tet*A, *tet*G, *tet*X, *sul*1, and *erm*B exhibited slight removal, while the abundance of *tet*O, *tet*W, *sul*3, *erm*F, and bla_{TEM} even increased compared with the feed, and *sul*2 showed almost no change at all, suggesting that the effects of sludge digestion on each type of ARGs may not be consistent (Wu et al. 2016). Some researchers speculate that it may be related to the different responses of microorganisms (such as the increase or decrease of specific bacteria at different temperatures) to anaerobic digestion (Jang et al. 2017; Xue et al. 2019). Moreover, we should distinguish extracellular and intracellular ARGs to achieve more accurate ARG quantification (Syafiuddin and Boopathy 2021).

5.7 Other Biological Sludge Treatment Technologies

Biological drying is mainly used to reduce sludge through aerobic degradation of organic matter and conversion of evaporated water (Zhang, Cai, et al. 2015). Also, compared with sludge composting (30–50 days), sludge biological drying takes much less time (10–25 days). It has been reported that biological drying would effectively reduce most ARGs (0.4–3.1 logarithms) and MGE (0.8–3.3 logarithms), while the maximum reduction rate of *tetM* reached 3. 09 logs (Zhang, Cai, et al. 2015). However, some ARG levels would also increase during the biological drying process. For example, the *tetX* were enriched by as high as 1.96 logarithms; meanwhile, *ermF* and *sul1* also increased by 0.5–2.0 logarithms. The fluctuation of ARG abundances is expected to be closely related to the succession of bacterial communities. The decay and decomposition of ARGs may be attributed to the death of their potential host bacteria (Xue et al. 2019; Zhang et al. 2016).

In summary, the biological methods are mainly to achieve the removal of ARG by reducing biomass.

The intracellular ARGs are released into the extracellular environment, and DNA hydrolase further degrades them. However, the reduction effect is usually unstable and non-specific under different operating conditions.

6 Impacts of Joint Treatment Technologies on ARGs

Considering that the ARG removal efficiencies of a single sludge treatment method is ordinarily unstable and unsatisfying, the joint treatment processes, which are usually composed of physical or chemical pretreatment technologies coupled with biological ones, have gradually gained great attention nowadays. The main purpose of joining the various sludge treatment technologies is to strengthen the degree of cell destruction to help release intracellular substances of the microorganisms including the released intracellular DNA and further degrade them as ARGs mainly exist in the microbial genome.

6.1 Physical/Chemical Technology Coupled with Chemical Ones

Advanced oxidation technologies (such as ozone and Fenton) are usually selected for the joint sludge treatment due to their outstanding performance in the removal of ARGs (Table S6), and they have indeed played a significant role in helping to destroy DNA structures (Wang, Chen, et al. 2020; Wang, Deng, et al. 2020). However, not all physical and chemical combinations are effective or better than a single treatment method. For example, when microwave and alkali treatment are combined, the effects would not get better since both of them can only release intracellular ARGs by cell destruction, but cannot directly damage them, which ultimately leads to higher detection of ARGs (Wang, Deng, et al. 2020).

6.2 Physical or Chemical Pretreatments Coupled with Biological Ones

The physical and chemical technologies are often used as pretreatment techniques to disturb the sludge and microbial cell structures and then combined with the biological one, mainly anaerobic digestion to enhance the subsequent ARG degradation efficiencies (Table S7, S8 and S9). Although some researchers did observe a significant reduction in ARG abundances after the combined reactions (Ma et al. 2011; Pei et al. 2016; Syafiuddin and Boopathy 2021; Tong et al. 2016; Wang, Chen, et al. 2020; Wang, Deng, et al. 2020; Zhao et al. 2020), disappointingly, the results are not always positive. Thermal hydrolysis was reported to reduce the absolute abundances of ARG and *intl*1 (Redhead et al. 2020). After the subsequent anaerobic digestion, many ARGs showed rebound effects. Meanwhile, anaerobic digestion after the thermal hydrolysis treatment significantly enriched several macrolide, tetracycline, glycopeptide, and aminoglycoside resistance genes (Redhead et al. 2020). Pei et al. (2016) compared the responses of five *tet* ARGs to anaerobic digestion combined with ozone and thermal hydrolysis pretreatments, respectively. Both of them enhanced the reduction rates of all ARGs after pretreatment. However, the diversities and abundances of ARGs rebounded during the subsequent anaerobic digestion process (Ma et al. 2011). In the study of Sun et al. (2019), the situation is even worse. After feeding the pilot-scale continuous-stirred tank reactor (CSTR) of the anaerobic digesters with the thermal hydrolysis pre-treated sewage sludge, the absolute abundance of some ARG subtypes under specific operation conditions were even higher than those in the feed sludge. Furthermore, it is worth mentioning that compared with the ozone oxidation, the auxiliary effect of thermal hydrolysis seems to be better; the finding is in agreement with Tong et al. (2017). The behind mechanism deserves further investigation. Therefore, not all integrated treatment processes will perform better than a single one. Specific care should be taken to choose a suitable combination for ARG treatment from sludge.

Different technologies would create different survival environments for the development of various microbial communities in the sludge, which would amplify or attenuate the abundances of certain bacteria-carrying ARGs (Yang et al. 2014). The elimination of ARGs is positively correlated with the decrease of the abundance and diversity of microorganisms (Zheng et al. 2017). The release of ARGs from the hosts would help reduce their absolute abundance. Su et al. (2015) and Wu et al. (2016) both observed that ARG abundances and microbial diversity increased significantly at the same time.

7 Summary of the Removal Mechanism of ARG

The main role of physical and chemical methods is to damage the microbial cell structure to release intracellular ARGs, then the DNA degrading enzyme in the external environment can further degrade ARGs. However, since there is no preference for the destruction of the cell structure, more serious inactivation of antibiotic sensitive cells may result in an improvement in the overall relative abundance of ARGs. The special case is that thermal hydrolysis and ultrasonic wave can damage the sequence of ARG because such techniques can directly damage the DNA structure, so that the ARG is destroyed on the source, preventing it from propagating to the outside environment. Therefore, such technologies are more reliable in ARG elimination. Biological technologies mainly achieve ARG removal by reducing biomass; the removal efficiency is different under different operating conditions, which is closely related to the composition of the microbial community structure. The purpose of joint processing is to strengthen the degree of cell destruction to help release intracellular substances of the microorganisms including the released intracellular DNA and further degrade them.

Since the current sludge treatment technologies rarely remove ARG itself, mainly by inactivating resistance bacteria, the microbial community structure of the sludge has a large effect on the final removal effect. After all, the removal of potential ARG hosts can reduce the further spreading of ARG to a certain extent, whether VGT or HGT. Previous reports showed *Firmicutes*, *Proteobacteria*, and *Actinobacteria* are the most likely hosts to carry and spread ARGs (Huang et al. 2018; Ma et al. 2011; Tang et al. 2020; Wang, Deng, et al. 2020). *Firmicutes* and *Actinobacteria* are both Gram-positive bacteria, while *Proteobacteria* are Gram-negative. The difference in the cell structure between Gram-positive and Gram-negative bacteria will affect the ARG reduction since Gram-negative bacteria with the lack of the peptidoglycan layer may be more susceptible to physical and oxidative attack while Gram-positive bacteria are more sensitive to lysozyme (Kaneko et al. 2004). However, the potential hosts for specific ARG are unfortunately not fixed. Huang, Liang, et al. (2020) found a positive correlation of the *tet* gene both with *Sphingomonas* (genus belongs to the *Proteobacteria*) and *Staphylococcus* (genus belong to the *Firmicutes*). As a result, different ARG subtypes' response varied even to the same treatment given the non-specificity of these techniques, with some genes increasing in concentration and others decreasing.

8 Outlook on Future Research

Based on the existing documented researches, the following suggestions are put forward for future research:

(1) A more detailed study is required on the underlying internal removal mechanism of ARGs using current sludge treatment technologies and for the explanations of the contradictory results between different studies.
(2) Current studies mainly paid attention to the ARG concentration in the solid phase of the sludge after conditioning and ignore the part released into the liquid phase, which causes the possible illusion of absolute abundance reduction and needs more investigation.
(3) The current research is mainly implemented on a laboratory scale, and the investigation in real field conditions should be encouraged. In addition, the fates of ARGs after subsequent application to soil should be paid more attention to.
(4) Future research needs to focus on the study of the HGT ratio between exotic and local bacteria, especially opportunistic pathogens, in order to assess the threat to human health.
(5) Considering that although some network and correlation analyses have been proposed to find potential hosts of ARGs, these analyses have failed to provide clear evidence that relevant microorganisms carry antibiotic resistance, indicating that more intuitive methods are needed to precisely determine the ARG hosts. At the same time, free ARF-bearing DNA or plasmids do not mean that they will be taken up by bacterial cells. The competence for plasmids by different bacteria shows huge differences. It is very necessary for host microorganisms carrying ARG to further verify their resistance effect with antibiotics.
(6) As a new treatment technology, phage therapy uses the specific virulent phage of pathogenic bacteria to hydrolyze and reconstruct the host DNA containing ARGs, which has been applied

in soil and achieved good results. Since it has not been applied in sludge, the attempts can be carried out in the near future.

9 Conclusions

Microwave, alkali treatment, and coagulation are inferred as ineffective sludge treatments for ARG removal because of the only available ability to lyse cells to only partially reduce the absolute abundance of ARG in the sludge. The sludge treatment techniques to simultaneously destroy the cells and destruct the ARGs through cutting DNA into small fragments or oxidative decomposition, such as Fenton, are more promising for ARG elimination. Considering the close relationship between ARG removal and microbial community structure, the ARG removal effects of numerous sludge treatment technologies are varied due to their different shaping of microbial community composition. Different operation parameters such as temperature, SRT, and reactor design in the biological sludge treatments can impact the final results by affecting the microbial activity and community structures. ARG subtype is also an important factor that cannot be ignored. On the whole, the treatment of sewage sludge may be an excellent opportunity to mitigate the release of ARGs into the environment, and it is of particular importance to choose the appropriate treatment method according to the local conditions and considering the advantages and disadvantages of each sludge treatment technologies. It is worth mentioning that the combined sludge treatment technologies may be a better choice.

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Declarations

Conflict of Interest The authors declare no competing interests.

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