Advancements in detection and removal of antibiotic resistance genes in sludge digestion: a state-of-art review

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Abstract:

Sludge from wastewater treatment plants can act as a repository and crucial environmental provider of antibiotic resistance genes (ARGs). Over the past few years, people's knowledge regarding the occurrence and removal of ARGs in sludge has broadened remarkably with advancements in molecular biological techniques. Anaerobic and aerobic digestion were found to effectively achieve sludge reduction and ARGs removal. This review summarized advanced detection and removal techniques of ARGs, in the last decade, in the sludge digestion field. The fate of ARGs due to different sludge digestion strategies (i.e., anaerobic and aerobic digestion under mesophilic or thermophilic conditions, and in combination with relevant pretreatment technologies (e.g., thermal hydrolysis pretreatment, microwave pretreatment and alkaline pretreatment) and additives (e.g., ferric chloride and zero-valent iron) were systematically summarized and compared in this review. To date, this is the first review that provides a comprehensive assessment of the state-of-the-art technologies and future recommendations.

Keywords: Antibiotic resistance genes; Anaerobic sludge digestion; Aerobic sludge digestion; Wastewater treatment plants.

1. Introduction

Antibiotics have been produced and applied in medical care to promote human health and animal farming and aquaculture for agricultural production for more than 70 years (Xue et al., 2019). There is no doubt that the invention and application of antibiotics have not only saved countless lives clinically but also helped the economic prosperity. However, the widespread antibiotic resistance is posing new risks to public health, which might turn this into a Pyrrhic victory. It is estimated that the death of more than 35,000 people in America each year is due to antibiotic resistance (Centers for Disease Control and Prevention, 2019). Antibiotic resistance will become one of the biggest threats to public health and the economy worldwide by 2030 if no action is taken (WHO, 2021). Most antibiotics (30%-90%) used as human and veterinary medicine are excreted in non-metabolized forms via urines and feces and eventually enter the sewer (Wang et al., 2020; García et al., 2020; Nguyen et al., 2021). Even at low concentrations, these non-metabolized antibiotics still posed a constant selection pressure on the microbial community, leading to the occurrence and dissemination of antibiotic resistant genes (ARGs) in the various microbial community in wastewater treatment plants (WWTPs) (Aminov, 2011; Chow et al., 2015; Karkman et al., 2018). More importantly, more than 99% of ARGs eventually accumulate in sludge from WWTPs, which becomes a major environmental source of ARGs (Xue et al., 2019; Nguyen et al., 2021).

Conventionally, for the detection of ARGs in sludge, isolating pure cultures have played the most crucial role among the microbial communities (Pazda et al., 2019; Karkman et al., 2018). However, sludge contains a wide variety of environmental microorganisms and most of them cannot be isolated and purely cultured in the laboratory (Pazda et al., 2019; Karkman et al., 2018). This greatly hindered the detection and understanding of ARGs in sludge from WWTPs. In past decades, the advancement of molecular biological techniques greatly improved the detection accuracy and efficiency of ARGs in sludge, which paved the way for the understanding and management of ARGs in sludge. Large amounts of diverse ARGs that represent the resistance to nearly all major classes of antibiotics have been detected in sludge using advanced detection techniques (Karkman et al., 2018; Martinez et al.,

2015). To date, various studies have reviewed the occurrence of ARGs in wastewater or during wastewater treatment in WWTPs in terms of their toxicity and detection approach (Hiller et al., 2019; Pazda et al., 2019; Wang et al., 2020). Although some detection techniques (such as RT-qPCR) were shared between wastewater and sludge, due to the different characteristics of wastewater and sludge, the performance and application of detection techniques varied between wastewater and sludge for ARGs detections (Tamminen et al., 2015; Bouki et al., 2013; Karkman et al., 2018).

Sludge from WWTPs can not only act as a repository but also a major environmental source of ARGs due to its reuse and disposal (Heuer et al., 2011; Zhu et al., 2013). Due to the presence of valuable nutrients and organic matter, sludge is commonly used as a fertiliser and for soil remediation purposes (Zhang et al., 2021a; Kube et al., 2019). For instance, more than 67% of sludge is reused in agriculture in Australia (Australian and New Zealand Biosolids Partnership, 2019). This may lead to the spread of ARGs from sludge to the local environment, elevating the health risk of sludge reuse. To minimize the health risk, the efficient removal of ARGs during sludge treatment before the land application became critical. In recent years, the fate of ARGs during sludge treatment with various sludge reduction technologies has become a research hotspot. Sludge treatment techniques, including anaerobic and aerobic digestion under thermophilic and mesophilic conditions, exhibited different removal efficiency of ARGs (Guo et al., 2017; Miller et al., 2016; Diehl & LaPara. 2010). Previous reviews stated that anaerobic and aerobic digestion could reduce the abundance of ARGs in sludge (Xue et al., 2019). However, the recent developments of pretreatment methods (i.e. thermal hydrolysis, microwave-based treatment, free ammonia treatment, etc.) and additives (i.e. zero-valent iron, FeCl3, Fe3O4) applied commonly altered the fate of ARGs during the digestion (Pei et al., 2016; Tong et al., 2018; Zhang et al., 2020c; Zhang et al., 2021b; Zhou et al., 2021a; Jang et al., 2017). The summary of and the discussion of impacts of sludge digestion strategies with different pretreatment techniques and additives are still lacking.

This review summarized and discussed up-to-date literature of ARGs in the sludge from WWTPs

with a broader perspective for the first time on the following aspects:1) the occurrence and detection techniques of ARGs in sludge from WWTPs; 2) the fate of ARGs during the sludge treatment with various digestion techniques, pretreatment methods and additives.

2. Occurrence of ARGs in sludge from WWTPs

Antibiotics have been discovered and used for disease treatment for more than 90 years (Dougherty & Pucci, 2011). Due to incomplete metabolism or waste of unused antibiotics, a large amount of wastewater from hospitals, households, pharmaceutical plants, livestock, *etc*. containing antibiotics is discharged into WWTPs through sewage pipes (Bouki et al., 2013; Rodriguez-Mozaz et al., 2015; Wang et al., 2015; Yuan et al., 2018; Syafiuddin & Boopathy, 2021) (Figure 1). Inevitably, these antibiotics become the culprits behind the occurrence of ARGs in WWTPs. For instance, tetracyclines, a type of spectrum antibiotics that are commonly used in humans, livestock and aquaculture, have caused the occurrence of significant amounts of tetracycline resistance genes in WWTPs sludge (Auerbach et al., 2007; Martinez, 2009). It was reported that the abundance of *tetA* and *tetQ* (two types of tetracycline resistance genes) in sludge could reach 10^8-10^9 and 10^4-10^7 copies/g-TS (TS: total solids TS), respectively (Auerbach et al., 2007).

(Position for Figure 1)

WWTPs are also hot spots for the proliferation of ARGs (Figure 1). ARGs widely disseminate in bacterial communities through both vertical gene transfer (VGT) and horizontal gene transfer (HGT) (Shao et al., 2018; Xue et al., 2019; Zhang et al., 2018; Cheng et al., 2020). By VGT, antibiotic resistance bacteria (ARB) pass on ARGs through cell reproduction (Shao et al., 2018). The HGT is mediated by mobile genetic elements (MGEs), which consist of plasmids, transposons, phages, insertion sequences, and integrons (Guo et al., 2017; Nguyen et al., 2021). Through HGT, non-ARB obtain one or multiple ARGs from ARB or environments (Shao et al., 2018; Xue et al., 2019; Zhang et al., 2018). Moreover, different kinds of pollution compounds such as pharmaceuticals and personal

care products (PPCPs), bactericide, heavy metals, and even artificial sweeteners, also produce selection stress on bacteria, thus promoting the HGT of ARGs (Qiu et al., 2021). With a high bacterial density and a large number of biofilms, WWTPs undoubtedly increased opportunities for VGT and HGT of ARGs (Karkman et al., 2018; Li et al., 2020).

Currently, almost all known ARGs could be found in sludge from WWTPs (Ju et al., 2019). An et al., (2018) applied high-throughput real-time quantitative reverse transcription (HT-qPCR) to test 285 ARGs of sludge samples from 11 sewage WWTPs in China. The results of this study indicate that each sample contains 95 ± 46 subtypes of ARGs, the absolute abundance of ARGs is approximately 2.0×10^{13} copies/L, and the relative abundance is approximately 0.4 copies/16S rRNA. Similarly, Yang et al., (2014) investigated ARGs of sludge in Shatin WWTP from Hong Kong using a metagenomics-based approach and found 102 different subtypes of ARGs with a relative abundance of 47.41%.

Eventually, the ARGs in WWTPs enter the natural environment by effluent discharge and sludge application (Figure 1). Burch et al., (2013) reported that the abundance of ARGs in sludge was much greater than that of effluent (>100 times). The application of sludge will inevitably lead to the spread of ARG from the sludge to the soil, and further spread in nature, and ultimately harm human health (Riber et al., 2014; Xie et al., 2016; Chen et al., 2016). Therefore, the detection and reduction of the ARGs in the sludge become an urgent research need.

3. Techniques for ARGs detection in sludge

Hitherto, isolation of pure cultures for the detection of antibiotic resistance has been the most important method clinically (Karkman et al., 2018). This method was also applicable to the detection of antibiotic resistance in wastewater or sludge and has played an important role in identifying antibiotic resistance in WWTPs (Karkman et al., 2018). However, cultures and drug sensitivity tests have limitations for environmental bacteria, since only a small proportion of environmental bacteria (such as *Pseudomonas* and *Enterococci*) can be grown under laboratory conditions (Riber et al., 2014;

Tong et al., 2017). Therefore, culture-independent molecular biology techniques are widely applied in the detection of antibiotic resistance in sludge. The presence and identification of ARGs in microorganisms commonly rely on the genetic information in DNA extracted from samples (Guo et al., 2017; Nguyen et al., 2021). However, recent studies revealed the presence of ARGs and MGEs are also in the form of RNA in several microorganisms such as *M. tuberculosis*, *M. aviumcomplex* (Cockerill III et al., 1999), etc. Thus, for ARGs detection using culture-independent molecular biology techniques, DNA and/or RNA from sludge samples were extracted using corresponding extraction kits, i.e. Fast DNA™ Spin Kit for Soil (MP Biomedicals, USA) for DNA and RNeasy Mini Kit (QIAGEN®, Germany) for RNA (Guo et al., 2017; Xu et al., 2020a; Wang et al., 2019c). It is worth noting that due to the instability of RNA, the extracted RNA is generally synthesized to cDNA and then used for the ARGs detection through DNA microarray, metagenomics or RT-qPCR (Aydin et al., 2015; Guo et al., 2017; Cockerill III et al., 1999.) The currently available molecular biology techniques applied for ARGs detection in sludge are summarized in Table 1.

(Position for Table 1.)

3.1 *Real-time quantitative reverse transcription (RT-qPCR)*

RT-qPCR is the most widely used culture-independent approach in the determination of targeted genes for various ARGs (Monpoeho et a., 2000; Karkman et al., 2018). This technique could determine the absolute abundance and relative abundance of the ARGs by monitoring the amplification reaction using fluorescence, the resistance genes to common types of antibiotics such as tetracycline, beta-lactam and sulfonamide have been detected in sludge from WWTPs (Auerbach et al., 2007; Ju et al., 2019). The major benefits of this technique is: 1) high specificity; 2) rapid examination (short handling time, i.e. within 24 h); 3) providing absolute abundance; 4) low limit of detection (LOD) and limit of quantitation (LOQ) which is about 3 copies/μL (Table 1) (Rizzo et al., 2013; Bouki et al., 2013; Karkman et al., 2018).

RT-qPCR detection relies on primers, which need to be uniquely designed and will act only for each target gene in the sample (Takenaka et al., 2018). Thus this technique has high specificity. With the availability of primers for the target gene, RT-qPCR results can be obtained within 24 hours (contact with technicians). In RT-qPCR, absolute abundance is determined by the standard curve method. The standard curves are built based on the quantification of a known quantity of target genes in the sample. The units commonly used in the relative research are gene copies/g-DW (DW: dry weight), gene copies/g-TS, or gene copies/ml. Another unit that is commonly used for ARGs quantification is relative abundance. Relative abundance represents the percentage of target gene abundance in the total DNA reads of a given sample. For the convenience of comparison, the relative abundance of ARGs is normalized to the abundance of 16S rRNA as gene copies/16s rRNA. 16S rRNA is normally expressed as biomass in related articles (Tong et al., 2017). Absolute abundance can be used to quantify the risk of each sludge sample, and relative abundance can be used to quantify the risk of each microbial community. The microbial biomass in different sludge samples may vary greatly, so absolute abundance is considered more representative of the potential hazard of sludge. Furthermore, the LOD of RT-qPCR (3 copies/reaction) normally is much lower than the sequencing approach (Table 1), which allows the detection of ARGs under sensitive and low abundance environments (Forootan et al., 2017).

The major drawback of RT-qPCR is that knowledge is required prior to the detection, to design the primers for the target ARGs. This limits the detection of unknown or unexpected ARGs (Karkman et al., 2018; Zhu et al., 2013). In addition, some errors in the amplification process may cause the number of target genes to be amplified (Ruijter et al., 2013). This may be due to editing errors that occur during enzyme replication catalyzed by DNA polymerase or errors caused by thermal damage to DNA (Pienaar et al., 2006). Furthermore, flux is another major disadvantage of this approach. For RT-qPCR, only one target ARG can be detected at a time, which greatly limits its throughput (Stedtfeld et al., 2008). To quantify multiple ARGs in the same sample, the test steps need to be repeated for each target gene. In addition, there is a growing demand for information about the host

of ARGs as a key to finding the potential way to reduce the abundance of ARGs. However, RT-qPCR lacks the capability to directly locate the hosts of the ARGs because the goal of a primer is to find and bind the target gene without knowing where the target gene is (Li et al., 2015) (Table 1).

3.2 *HT-qPCR*

Recently, HT-qPCR becomes more popular for the determination of the abundance of ARGs in sludge (Table 1). HT-qPCR is a platform for miniaturizing traditional RT-qPCR and processing large numbers of samples (Lamas et al., 2016). With quality control, it has achieved the same accuracy as conventional RT-qPCR (Wang et al., 2014). HT-qPCR has all the advantages of RT-qPCR (Table 1). Compared with traditional RT-qPCR, HT-qPCR undoubtedly breaks the limitation of detection flux (Waseem et al., 2019). Depending on the experimental equipment, HT-qPCR could measure hundreds of ARGs simultaneously in sludge, thus covering more antibiotic classes at one test (An et al., 2018). For example, Chen et al., (2016) used HT-qPCR (WaferGen SmartChip, USA) to measure the abundance of 108 ARGs in sludge with one run which greatly reduces the measurement time compared to traditional RT-qPCR. However, as this method is still based on RT-qPCR, it has similar disadvantages as RT-qPCR, such as the need to design primers prior to the detection and the inability to provide the host information (Table 1).

3.3 *Shotgun high-throughput sequencing (next-generation sequencing)*

Unlike RT-qPCR and HT-qPCR, which measure the abundance of targeted ARGs only, metagenomics collects the genetic information of an entire microbial community. Currently, the shotgun high-throughput sequencing platform is the most widely used sequencing platform and has been widely applied in detecting ARGs in the diverse environment including sludge (Hu et al., 2013; Li et al., 2015) (Table 1). The major advantages of this method include: 1) obtaining the information of all ARGs in the sample, 2) providing the host information of ARGs by network analysis (Wang et al., 2013; Yang et al., 2014) (Table 1). Since metagenomics collects all the genetic information in the sample, in theory, it could provide the relative abundance of all the species and ARGs in a sample

(Hugenholtz & Tyson, 2008). With the assist of network analysis, potential hosts for ARGs in the sample can be further identified (Guo et al., 2017). However, in practice, the annotation of ARGs heavily relies on ARG databases (Karkman et al., 2018), such as the Comprehensive Antibiotic Resistance Database (CARD) and Antibiotic Resistance Genes Database (ARDB). Thus, only the known resistance genes can be annotated. It is worth mentioning that ARDB has stopped updating in 2009 and CARD is a monthly updating database. Furthermore, the shotgun high-throughput sequencing platform generates short reads which require to be assembled into longer and overlapping DNA segments (i.e. contigs) to provide more information about ARGs. However, this method strongly relies on powerful computational resources. In addition, the detection limit of target genes is closely related to the sequencing depth, which greatly increases the size of data. For instance, Liu et al. (2019) obtained more than 77GB of data per sludge sample to detect the low abundant ARGs, in which the lowest relative abundance of ARGs detected was 1.20×10^{-4} (nitroimidazole resistance genes). Although this improves the accuracy, it also increases the handling time, cost, and consumption of computational resources. More importantly, metagenomics sequencing normally provides the relative abundance of target ARGs. In recent studies, the relative abundance obtained by metagenomic analysis was also transformed into absolute abundance by adding the Internal Standard (Crossette et al., 2021). However, the Internal Standard need to be measured and calibrated using RT-qPCR (Crossette et al., 2021), which adds additional effort for the methodological optimization and may introduce biases.

3.4 *Emerging methods*

Traditional molecular biology techniques provide an accurate approach for the identification and quantification of ARGs in sludge. However, the drawback of these techniques is that they cannot directly show the real host of the ARGs in sludge due to the read length limitation. There is an urgent need to understand the real host of ARGs, which may help uncover the diversity of the hosts of ARGs and implemented interventions to reduce the spread of ARGs in sludge. Thus, some emerging methods have been developed recently, including Emulsion paired isolation and concatenation

(Epic)PCR, the third-generation sequencing platforms, and single-cell genome sequencing.

EpicPCR connects functional genes and phylogenetic markers in uncultured single cells, which can be obtained by measuring hundreds of thousands of cells (Tamminen et al., 2015). EpicPCR combines the advantages of RT-qPCR and 16S rRNA sequencing. It first linked the target gene to the 16S rRNA gene, then amplified the target gene and sequenced the 16S rRNA gene to determine the host of ARGs and the abundance of ARGs (Spencer et al., 2016) (Table 1). Hultman et al., (2018) used EpicPCR to detect three ARGs in influent and effluent samples and identified their hosts in different samples. However, this method also does not overcome the problems of primer design and detection flux which is the same as the RT-qPCR approach (Table 1).

Recently, the third-generation sequencing platforms (e.g., Oxford Nanopore and Pacific Biosciences Single Molecule Real-Time (SMRT)) have been considered as promising technologies to identify ARGs in sludge (Che et al., 2019). The platforms also aim to collect all the genes of the entire microbial community. Compared to the shotgun high-throughput sequencing platform, the third-generation sequencing platform could produce long reads (>500 bp) to find the authentic hosts of ARGs in sludge, which undoubtedly provides more accurate host information and countermeasures to reduce the transmission of ARGs (Che et al., 2019; Ashton et al., 2015; Peterson et al., 2019) (Table 1). The major drawback of third-generation sequencing platforms is low accuracy, which is normally higher than second generation sequencing platforms (Wee et al., 2018). Currently, the error rate is 10-15% in the SMRT and 5-20% in the Oxford Nanopore (the error rate of Shotgun high-throughput sequencing is 0.1-1%) (Petrackova et al., 2019; Xiao & Zhou, 2020). Although there have been many methods to correct raw data of third-generation sequencing by software to reduce the error rate, due to the lack of authoritative certification, this method is not ideal to detect ARGs in sludge at present (Amarasinghe et al., 2020) (Table 1).

DNA Microarray technique is another method that has been used in clinical antibiotic resistance

298 detection. The main advantages of this method are short detection time (several hours), high detection
299 flux and high accuracy (Zhu et al., 2007, Zhang et al., 2009) (Table 1). Take Zhu et al. (2007)'s study
300 as an example, a multiplex asymmetric PCR (MAPCR)-based DNA microarray was applied to detect
301 six types of ARGs in different bacteria species clinically. However, the disadvantages of this method
302 are obvious (Table 1). This method has a high LOD of only 10^3 copies/μL and can only detect the
303 presence or absence of ARGs, but not their abundance (Zhang et al., 2009; Ma et al., 2020).
304
305 Droplet Digital PCR (ddPCR) is a novel PCR technique. Compared with RT-qPCR, the absolute
306 abundance of target ARGs can be obtained without running the standard curve. Cave et al., (2016)
307 measured two ARGs (*qnrB* and *sul1*) in soil and organic residues by RT-qPCR and ddPCR and found
308 that the LOD of ddPCR could achieve 0.5 copies/μL, which is more sensitive than RT-qPCR (Cave
309 et al., 2016; Campomenosi et al., 2016; Villamil et al., 2020; Kojabad et al., 2021) (Table 1). The
310 technique of ddPCR also has similar drawbacks to RT-qPCR, such as limited detection flux and the
311 need for primer design (Table 1). Currently, ddPCR has been used in several studies to quantify the
312 abundance of ARGs in water, soil and organic residues (Cao et al., 2015; Cave et al., 2016). To date
313 it has not been applied to sludge, however, due to the much lower detection limit, it could potentially
314 help to quantify ARGs at low abundance in sludge in the future.
315
316 Single-cell genome sequencing requires efficient separation of cells from the environment into single
317 cells, extraction of DNA, and sequencing of DNA (Gawad et al., 2016). To date, only one study has
318 applied this new technology to identify the ARGs as well as their hosts by measuring more than
319 50,000 cells from a sample of ocean beaches in the USA (Lan et al., 2017). Similarly, compared to
320 shotgun high-throughput sequencing, hosts of ARGs could be directly obtained by this technique
321 (Table 1), which shows great potential for future application in ARGs detection in sludge.
322
323 **4. Fate of ARGs in anaerobic and aerobic sludge digestions**
324 It is well known that sludge is a nutrient-rich resource that can be used, especially in agriculture

(Singh and Agrawal, 2008). However, the presence of ARGs in sludge leads to the potential release of ARGs from sludge to the natural environment during sludge reuse. Recent studies have observed the increase of ARGs in the adjacent local environment of sludge reuse (Rahube et al., 2014; Chen et al., 2016). Taking Chen et al., (2016)'s study as an example, long-term use of sludge as a soil conditioner resulted in a significant enrichment of 108 ARGs and MGEs, including a 3,845-fold increase in *mexF*, over a 10-year follow-up. This undoubtedly shows that the application of sludge brings huge potential risks to the environment and human health. Therefore, removal or reduction of ARGs abundance in sludge becomes critical to minimize the risks during sludge reuse.

Anaerobic and aerobic digestion are mature sludge treatment methods that are widely used globally. Anaerobic digestion has been extensively studied for its benefits of sludge reduction, stabilization and bioenergy recovery (Batstone et al., 2002; Pei et al., 2016; Wang et al., 2017; Xie et al., 2018). Both mesophilic and thermophilic anaerobic digestion showed promising results in ARGs removal (Pei et al., 2016; Xu et al., 2020b), although some studies indicated that thermophilic anaerobic digestion is superior to mesophilic anaerobic digestion in terms of ARGs reduction (Xu et al., 2020b). In addition, additives and sludge pretreatment technologies could also affect the fate of ARGs during anaerobic digestion, which is to be discussed in section 4.1.

Aside from anaerobic digestion, aerobic digestion also plays an important role in sludge reduction especially in small WWTPs (Yu et al., 2008; Semblante et al., 2017; Wang et al., 2018). Recent studies indicated that both mesophilic aerobic digestion and thermophilic anaerobic digestion are effective in the removal of ARGs in sludge (Burch et al., 2013; Jang et al., 2018). In addition, post thermophilic aerobic digestion for anaerobically digested sludge was also observed to further reduce ARGs (Jang et al., 2019). These findings will be discussed in section 4.2.

4.1 *The fate of ARGs in anaerobic digestion of sludge*

4.1.1 *Fate of ARGs in mesophilic and thermophilic anaerobic sludge digestion*

Mesophilic (35-40 ℃) anaerobic digestion plays a significant role in waste minimization, pathogen removal and energy recovery (Appels et al., 2008). Recently, a few studies proved that anaerobic digestion could reduce the abundance of ARGs in the sludge (Table 2). According to Yang et al. (2014), mesophilic anaerobic digestion reduced the abundance of ARGs by above 20% in relative abundance using metagenomics. Xu et al., (2020) also observed a reduction of 29.7-32.3% using mesophilic anaerobic digestion. However, recent studies revealed that certain types of ARGs were not eliminated but increased in abundance during mesophilic anaerobic digestion. For example, Jang et al., (2017) found that the absolute abundance of *tetD* enriched by 1.43 times during mesophilic anaerobic digestion compared with the initial. Mesophilic anaerobic digestion may even result in the occurrence of new ARGs in digested sludge. As an example, Guo et al., (2017) found 42 ARGs subtypes belonging to 10 ARGs types in the secondary sludge but identified 51 ARGs subtypes belonging to 9 ARGs types in the anaerobically digested sludge.

(Position for Table 2.)

Compared with mesophilic (35-40 ℃) anaerobic digestion, thermophilic (55-60 ℃) anaerobic digestion could produce higher biomethane yields and reducing pathogens (Buhr and Andrews, 1977). However, due to the energy consumption, fewer thermophilic anaerobic sludge digesters were operated in the full-scale WWTPs. Recent studies suggested that thermophilic sludge anaerobic digestion outperformed mesophilic sludge anaerobic digestion with a higher ARGs removal efficiency. Tian et al., (2016) found mesophilic and thermophilic anaerobic digestion reduced the relative abundance of target ARGs by 38.8% to 65.0%, respectively (Table 3). However, another study using the metagenomic approach found that both mesophilic anaerobic digestion and thermophilic anaerobic digestion did not reduce some types of ARGs and even led to the proliferation of certain ARGs such as *aadA*, *macB* and *sul1* (Zhang et al., 2015). This may be due to the fact that the hosts

of these ARGs reproduce under thermophilic conditions.

4.1.2 *Effect of sludge pretreatment technologies on the fate of ARGs during anaerobic digestion*

To improve sludge minimization and methane production of sludge, some sludge pretreatment technologies have been studied and even successfully commercialized. Recent studies have shown that these pretreatment technologies may also facilitate the removal of ARGs during anaerobic digestion.

(Position for Table 3.)

Thermal hydrolysis (TH) pretreatment is a commercialized pretreatment method during anaerobic digestion (Abelleira-Pereira et al., 2015). Typically, the TH pretreatment heats sludge at 120-170℃ for 30-60 min to improve the substrate degradation rate and extent (Wang et al., 2017). In terms of the ARGs removal, TH pretreatment significantly removed most ARGs after pretreatment and further enhanced ARGs reduction in anaerobic digestion (Table 3). Compared with the control group, TH pretreatment could promote tested ARGs reduction by 0.5 to 3 log10 copies/g-TS after anaerobic digestion (Pei et al., 2016, Tong et al., 2019; Wang et al., 2019a; Sun et al., 2019). The enhanced removal of ARGs due to the TH pretreatment is likely related to the high temperature during the pretreatment process, which directly kills most of the ARB in the sludge. This is considered as the main way of TH pretreatment to reduce the abundance of ARGs in the anaerobic sludge digestion (Pei et al., 2016).

Microwave-based pretreatment is another effective pretreatment method in anaerobic digestion, which includes microwave pretreatment, microwave-heat pretreatment, and microwave-H_2O_2 pretreatment. Microwave pretreatment uses 600 W microwave irradiation to heat the sludge from 20 ℃ to 100 ℃ for 5 min and showed promising results in increasing methane production by up to 84% (Tong et al., 2016; Tong et al., 2018; Zhang et al., 2016). More importantly, microwave pretreatment

reduced the abundance of most tested ARGs abundance during anaerobic digestion by 0.05 to 0.70 log10 copies/g-TS compared with the control group (Table 3). It is evident that microwaves could directly kill ARB in the sludge (Qiao et al., 2020) and destroy extracellular genes include ARGs (Yang & Hang, 2013). In addition, microwave pretreatment can change the microbial community and reduce the frequency of HGT during anaerobic sludge digestion, which may also be the other two reasons for reducing the abundance of ARGs (Tong et al., 2018).

For microwave-heat, the pH of sludge is adjusted to 2.5 and heated by microwave irradiation at 600w from 20℃ to 100℃ (Tong et al., 2016; Zhang et al., 2017). Microwave-heat pretreatment significantly decreased the absolute abundances of *tetC, tetM, tetO, tetX, blaSHV, blaCTX-M* and *ampC* during anaerobic digestion by 0.1 to 0.7 log10 copies/g-TS. The gene of *tetA* is the only exception which increased by 0.2 log10 copies/g-TS (Table 3). In terms of microwave-H_2O_2 pretreatment, the pH of sludge was adjusted to 10 and heated by microwave irradiation at 600 w from 20℃ to 100℃. Then 30% (w/w) of H_2O_2 was added into the sludge for several mins. The batch-scale results indicated that compared with the control group, microwave-H_2O_2 pretreatment slightly enriched *ampC, blaCTX-, blaSHV, ermB, mefA,tetM, tetX* and *pcoA* abundance during anaerobic digestion by less than 0.5 log10 copies/g-TS. On the contrary, compared with the control group, the abundances of *blaOXA-1*, *blaTEM*, *ereA*, *ermF*, *sulI*, *sulII* and *tetG* decreased by 0.1 to 0.8 log10 copies/g-TS during anaerobic digestion (Table 3). Similar to microwave pretreatment, death of ARB and destruction of ARGs during the pretreatment stage, as well as changes in the microbial community during anaerobic digestion, are major reasons for the decrease of ARGs (Tong et al., 2016; Zhang et al., 2017). Instead, changes in the microbial community may have facilitated the proliferation of hosts of some ARGs, leading to the enrichment of these ARGs (Tong et al., 2016; Zhang et al., 2017).

Ozone pretreatment is another widely used method in anaerobic digestion (Wang et al., 2017). Ozone pretreatment is typically operated at 0.1 g-O_3/g-TS with a gas flow rate controlled at 2 L/min for one

day (Pei et al., 2016; Tong et al., 2017). Compared with the control group, ozone pretreatment promoted ARG removal rates for all detected ARGs (Tong et al., 2017) (Table 3). As a strong oxidant, ozone can effectively kill ARB during the pretreatment stage (Zheng et al.,2017; Wu et al., 2021). Ozone pretreatment changes the microbial community and reduces the frequency of HGT during anaerobic digestion, which might be another reason for the enhanced ARGs removal (Tong et al., 2018). However, it is worth noting that ozone pretreatment has limited performance in ARGs reduction during the pretreatment stage and could decrease all tested ARGs after anaerobic digestion by 0.2 to 0.5 log10 copies/g-TS (Pei et al., 2016; Tong et al., 2017).

Ultrasonic pretreatment, another common sludge pretreatment method, is usually operated at 20 kHz for several hours (frequently less than 1h) (Wang et al., 2017). Ultrasonic pretreatment does not affect the abundance and diversity of ARGs in the pretreatment stage but significantly enhanced ARGs reduction by 0.05 to 1.02 log10 copies/g-TS during anaerobic digestion compared with the control group (Table 3). The gene of *tetB* is the only exception which increased by 0.2 log10 copies/g-TS in anaerobic digestion after ultrasonic pretreatment (Table 3). It is evident that ultrasound could kill bacteria including ARB but has not been shown to have a direct effect on ARGs (Muqbil et al., 2005; Chen et al., 2021). In addition, ultrasonic pretreatment also changed the microbial community and reduce the frequency of HGT during anaerobic digestion, affecting the fate of ARGs during anaerobic digestion (Wang et al., 2019a).

Alkaline pretreatment is a kind of sludge pretreatment technology that is still in the laboratory research stage. Generally, alkaline pretreatment was performed at pH 10 under 110℃-130℃ for 0.5 to 1 hr or under pH 10 at room temperature for 1 or 2 days (Wang et al., 2019a). A recent study revealed that alkaline pretreatment does not affect the abundance and diversity of ARGs in the pretreatment stage but significantly enhances ARGs reduction by 0.03 to 0.53 log10 copies/g-TS during anaerobic digestion compared with the control group (Table 3) (Wang et al., 2019a). According to Xiao et al., (2015)'s study, alkaline pretreatment could effectively kill bacteria including ARB but

had no effect on extracellular DNA. Therefore, the change of microbial community during anaerobic digestion by alkali pretreatment may be the main reason affecting the fate of ARGs during anaerobic digestion (Wang et al., 2019a).

The use of free ammonia pretreatment to increase biomethane production during anaerobic sludge digestion is a novel method (Wei et al., 2017a,b; Wang et al., 2019b; Liu et al., 2021). Free ammonia is the by-product of WWTPs and can be extracted directly, making this technique quite attractive (Wang, 2017; Liu et al., 2021). Recently, Zhang et al.,(2021a) reported that free ammonia pretreatment at 420 mg NH_3-N/L for 24h increased the removal efficiency of the total tested ARGs by approximately 15% compared to the anaerobic digestion without pretreatment. The gene of *tetG* is the only exception which rebounded during anaerobic digestion with free ammonia pretreatment (Table 3). Zhang et al., (2021a) attributed this result to the removal of ARB and ARGs during the pretreatment stage and the change of microbial community during anaerobic digestion.

Two-phase anaerobic sludge digestion (TPAD) is considered to be an effective optimization of anaerobic sludge digestion. TPAD includes two phases, where the first phase is for hydrolytic acidification, i.e. pretreatment stage, and the second phase is majorly operated for the methanogensis (Zhang et al., 2020a). TPAD not only improves the methane production of sludge in anaerobic digestion but also effectively reduces the abundance of pathogens in sludge (Wu et al., 2016; Zhang et al., 2020a). However, conflicting observations have been reported regarding the fate of ARGs during TPAD. In a lab-scale continuous TPAD, negligible changes in the abundance of all targeted ARGs were observed due to the TPAD (Wu et al., 2016) using RT-qPCR analysis. In contrast, a recent lab-scale study found that TPAD increased the total abundance of ARGs in sludge from 229.1 ppm (ppm, 1 read in a million reads) to 355.7 ppm through metagenomics (Wu et al., 2018). These conflicting observations might be related to the detection approaches and operational conditions of the TPAD. The RT-qPCR analysis provided the absolute abundance of targeted ARGs (i.e. 14 ARGs in total) per gram DW of sludge (Wu et al., 2016) but metagenomics analysis revealed the relative

abundance of a broad-spectrum of ARGs (i.e. around 30) in the total reads of DNA sequence (Wu et al., 2018). Furthermore, the operational conditions and sludge properties in these two studies were not identical, where their impacts on the fate of ARGs during TPAD remain unclear to date. Thus, comprehensive evaluations of ARGs abundance (both absolute and relative abundance) during TPAD with various operational conditions are highly recommended.

4.1.3 *Effect of additives on the fate of ARGs* in anaerobic sludge digestion

In addition to pretreatment technologies, some additives have also been added to the anaerobic sludge digestion to enhance biogas production, including zero-valent iron, ferric chloride, and magnetite. The impacts of these additives on the removal of ARGs during anaerobic sludge digestion are summarized in Table 4.

(Position for Table 4.)

Zero-valent iron (ZVI, Fe^0) is a low-cost reductive metallic material that has the ability to increase methane production during anaerobic digestion (Feng et al., 2014; Wei et al., 2018). The impact of ZVI on the removal of ARGs during anaerobic digestion was found to be closely linked to its dosage (Table 4). Compared with the control, ZVI reduced the absolute abundance of the tested ARGs by 28.27–100.00% during anaerobic digestion (Zhang et al., 2020c; Zhou et al., 2021). Both Zhang et al., (2020) and Zhou et al., (2021) attributed this to changes in the microbial community during anaerobic digestion with the addition of ZVI. In addition, the physical and chemical properties of ZVI itself also influenced the fate of ARGs. For example, Fang et al., (2011) found that ZVI could effectively remove some antibiotics, such as metronidazole, which also helped to reduce the selection pressure for the occurrence of ARGs.

$FeCl_3$, a common coagulant, has been used to enhance sludge dewatering properties and methane production in anaerobic digestion recently (Jang et al., 2017; Ju et al., 2016). A dosage of $FeCl_3$ in

anaerobic digestion at 1.53 to 100 mg/L increased methane production by 20% (Jang et al., 2017). However, $FeCl_3$ addition increased the abundance of almost all tested ARGs from 1.3×10^7 copies/g-VS in the control digester to 3.3×10^{10} copies/g-VS in the digester with $FeCl_3$ addition, except *tetH*, *aac(6')-Lb-Cr* and *blaTEM* during anaerobic sludge digestion (Table 4). The increased abundance of ARGs is likely caused by the increase of hosts of ARGs in the microbial community due to $FeCl_3$ (Jang et al., 2017). Furthermore, $FeCl_3$ also significantly increased the abundance of *intI1* by 1.19×10^{12} copies/g VS, indicating a higher frequency of HGT and proliferation of ARGs. As a result, the addition of $FeCl_3$ may not be ideal for ARG reduction in anaerobic sludge digestion.

Magnetite is an iron-based conductive material that has been applied in anaerobic digestion to promote methane production recently (Liu et al., 2015; Wang et al., 2019a; Zhang et al., 2019; Xie et al., 2020). Although promising results have been achieved with the methane production, the addition of magnetite from 0.5 g/L to 4 g/L did not affect the abundance of all tested ARGs during anaerobic sludge digestion compared with the control group (Table 4) (Wang et al., 2019a).

4.2 *Fate of ARGs in aerobic sludge digestion*

Aerobic sludge digestion is a sludge treatment technique that achieves sludge reduction and stabilization through aeration and is usually used in small WWTPs (Yu et al., 2008; Semblante et al., 2017; Wang et al., 2018). Recent studies revealed that aerobic digestion also effectively reduced the abundance of ARGs in sludge (Table 2). Diehl & LaPara (2010) firstly found that aerobic digestion which operated at 22 °C with a mean SRT of 15 days in a semi-continuous mode, significantly reduced the abundance of *tetA, tetL, tetO, tetW, tetX* by up to three orders in sludge. Similar results were confirmed in Zhang et al., (2021b)'s study, where the abundance of nine detected ARGs was reduced by approximately 15%. The removal of ARGs achieved by aerobic digestion was attributed to endogenous digestion, which kills ARB and reduced the abundance of potential hosts of ARGs in the microbial community (Zhang et al., 2021c). Interestingly, Burch et al. (2017) found that ARGs of *tetA*, *tetX* and *sulI* increased in the full-scale sludge aerobic digestor. This may be related to changes in the

host of ARGs, which require further investigations.

Thermophilic aerobic digestion is a sludge treatment method that uses aeration and high temperature to achieve sludge reduction, stabilization and pathogen removal (Roš and Zupančič, 2002; Jang et al., 2014). Compared with aerobic digestion, thermophilic aerobic digestion shortened SRT even though it requires higher energy consumption (Layden et al., 2007). Jang et al., (2018) found that thermophilic aerobic digestion could effectively decrease the abundance of ARGs from sludge. In this study, the abundance of 20 different types of ARGs decreased by 20.39% to 99.00% and *tetB, tetE, tetBP* and *blaCTX* were completely removed in sludge after the treatment.

In addition, thermophilic aerobic digestion has also been used as a post-treatment for anaerobically digested sludge to achieve further sludge reduction and stabilization (Jang et al., 2019). During this process, *tetB, tetD, tetH, tetM, tetX, tetBP, blaCTX* and *floR* were completely removed in sludge and the abundance of the remaining 11 types of ARGs dropped between 16% and 99%. This post-treatment showed the potential to further reduce the abundance of ARGs in sludge.

4.3 *The mechanism behind the fate of ARGs due to sludge digestion and in combination with pretreatments or additives*

The mechanism behind the fate of ARGs during sludge treatment is related to 1) the changes of microbial communities; 2) the frequency of HGT; 3) selection pressure posed by the environment. The changes of microbial communities weres closely related to changes of the ARGs during anaerobic digestion (Guo et al., 2017). The proliferation of ARGs is highly reliant on VGT. During anaerobic and aerobic digestion, the microbial community has undergone tremendous changes. In general, anaerobic digestion is not conducive to the reproduction of most ARGs host bacteria (Yang et al., 2014; Zhang et al., 2020b). The decay of the host bacteria thereby reduced the ARGs during the anaerobic digestion. Similarly, changes in the microbial community also occur during aerobic digestion, which is considered to be one of the main reasons for the decrease of ARGs in aerobic

digestion (Burch et al., 2013). However, both aerobic and anaerobic digestion does not always hinder the reproduction of the hosts of ARGs (Burch et al., 2013; Guo et al., 2017). Thus, certain types of ARGs were enriched during the digestion process, leading to an increase of certain types of ARGs during the digestion process (Table 3).

Moreover, both aerobic and anaerobic digestors provide a relatively stable environment such as stable temperature and pH for microorganisms activities, thus reducing the frequency of HGT (Xue et al., 2019; Sardar et al., 2021). A reduced HGT could facilitate the ARGs removal in sludge digestion. For instance, thermophilic anaerobic digestion showed a higher removal ratio of ARGs because the higher temperature limited microbial diversity in an anaerobic digester, reducing the horizontal gene transfer (Miller et al., 2016; Shin et al., 2020; Zhang et al., 2021a). Recent research development also revealed the importance of extracellular ARGs (eARGs) and intracellular ARGs (iARGs) in the HGT of ARGs (Sui et al., 2019). Both eARGs and iARGs are mutually transmissible and directly involved in HGT (Zhou et al., 2019). Natural transformation (a form of HGT) is thought to be the only mechanism for eARGs to enter living cells, by which eARGs become iARGs and non-ARB become ARB (Jin et al., 2020). However, compared to iARGs, eARGs are relatively easy to be removed in the sludge digestion process. Both aerobic and anaerobic sludge digestion has been shown to physically destroy eARGs (Ma et al., 2011; Zou et al., 2020; Zhang et al., 2020c). Hence, the eARG released by dead ARB might have less opportunity to be absorbed by other cells during sludge digestion, reducing the natural transformation. In addition, thermophilic anaerobic digestion, TH pretreatment and FA pretreatment have proved to be effective methods to destroy eARGs (Miller et al., 2016; Pei et al., 2016; Zhang et al., 2021b). The removal of iARGs is mainly achieved by the shift of the microbial community, reduction of the hosts of ARGs during sludge digestion or death of ARB. Notably, iARGs in ARB are released into the nearby environment after the ARB dies and becomes eARGs. Most of iARGs were located on MGEs and could achieve HGT through transduction and conjugation, turning non-ARBs into ARBs (Zarei-Baygi and Smith, 2021). To date, the understanding of eARGs and iARGs on the fate of ARGs during sludge digestion remains unclear. For future research, it is highly

recommended to extract iARGs and eARGs separately to investigate their fate and mechanism in the process of sludge digestion. Besides, environmental elements also promote the proliferation of ARG, such as antibiotics, PPCPs (e.g., triclosan and triclocarban) and heavy metals (Qiu et al., 2021). These compounds are degraded and passivated during anaerobic digestion and aerobic digestion (Guerra et al., 2015), which also facilitates the reduction of ARGs.

5. Future perspectives

With the advancement of detection techniques, the knowledge of the occurrence, and removal of ARGs in the sludge from WWTPs were improved substantially in the last decades. Although recent progress in metagenomics and other emerging molecular-based methods has enabled a comprehensive assessment of ARGs in the sludge, due to the cost, accuracy, and handling time, RT-qPCR is still the most commonly used method for ARGs detection. Most studies applied RT-qPCR to quantify the abundance of 5-25 ARGs. These ARGs are chosen based on the availability of primer and probes, the variety of antibiotic classes, and resistance mechanisms (e.g., efflux pump and ribosomal protection protein). However, these target ARGs can not represent all of the ARGs in the sample, the results of which may not completely reveal the total abundance and distribution of ARGs in the sludge sample. Thus, further developments in the detection approach to accurately, and efficiently quantify a wide spectrum of ARGs in sludge are still crucial.

In addition, recent advancements in the detection techniques allow the identification of the hosts for ARGs directly (i.e. third-generation sequencing) or through network analysis (i.e. shotgun high-throughput sequencing). This provides the potential of eliminating the ARGs in the community by removing or reducing the hosts accordingly. In recycled water systems, removing the natural hosts (i.e. *Amoebae*, *Protozoa* and *Legionella spp*.) through membrane ultrafiltration significantly reduced the ARGs abundance (Drigo et al., 2021). However, to date, in the wastewater and sludge system, the understanding and application of eliminating ARGs by controlling the host are still largely lacking. This is likely related to 1) lacking specific treatment in removing a certain or a series of microbial

hosts for ARGs; 2) the contributions of HGT in ARGs spreading. Growing evidence suggests that many ARGs are harbored and expressed by a broad range of microorganisms due to the frequent HGT and VGT (Salyers & Amabile-Cuevas et al., 1997; Guo et al., 2017). This requires an effective treatment to remove or reduce a series of microbial hosts for ARGs otherwise the residual hosts might still spread ARGs through frequent HGT and VGT. More importantly, some ARGs were harbored in the functional microorganisms of WWTPs. For instance, network analysis based on shotgun high-throughput sequencing revealed that 5 ARG subtypes (*penA, oqxBgb, vanHAc2, vanR-F* and *dfrK*) and 4 ARGs (*peb_EC, SFO-1, vanR-B* and *vanR-C*) were potentially harbored by *Nitrosomonas* (a typical ammonia-oxidizing bacteria), and *Candidatus 'Accumulibacter'* (a typical organism for phosphorus removal) (Guo et al., 2017). The removal of these functional microorganisms might eliminate the ARGs at the cost of undermined treatment efficiency. Furthermore, the effective removal of ARGs hosts may not indicate the elimination of antibiotic resistance in the microbial community (Pruden et al., 2006). As discussed in section 4.3, iARGs in ARB are released into the nearby environment after the death of ARB and become eARGs, which are transmissible and directly involved in HGT (Zarei-Baygi & Smith, 2021). This might lead to the occurrence of new hosts of ARGs. Thus, so far, there is a lack of comprehensive understanding and effective methods in ARGs elimination by hosts control, which requires future investigations.

Anaerobic digestion and aerobic digestion are considered to be effective methods to remove ARGs from sludge. Various sludge digestion strategies (i.e., anaerobic and aerobic digestion under mesophilic or thermophilic conditions, and in combination with relevant pretreatment technologies (e.g., thermal hydrolysis pretreatment, microwave pretreatment and alkaline pretreatment) and additives (e.g., ferric chloride and zero-valent iron) have been applied for sludge treatment. Thermophilic anaerobic digestion, mesophilic anaerobic digestion combined with different pretreatments and post aerobic digestion for anaerobically digested sludge showed superior performance in the reduction of ARGs in the sludge compared with mesophilic anaerobic digestion. The conducive effect of sludge treatment showed promising results in reducing the spread of ARGs.

However, in current studies, there is a lack of uniform units for the measurement and comparison of the absolute abundance of ARGs in the sludge. Due to the lack of standards or commonly adopted methods and protocols, various units have been applied in different studies. Generally, gene copies/g-TS and gene copies/g-DW are two common absolute abundance units, but some studies have also used gene copies/μl-DNA, gene copies/μg-DNA, gene copies/L, gene copies/g-VS and gene copies/g-dry solid. Although the choice of units does not affect the trends of ARGs during sludge digestion in each study, it creates a barrier for the comparison between different studies. This largely limited the evaluation of different sludge treatment methods and the management of risks due to sludge reuse. Therefore, it is recommended to apply uniform units such as gene copies/g-TS, of absolute abundance of ARGs in sludge for future research development.

Furthermore, the scale of sludge digestion in currently available studies is generally limited. Unlike those commercialized techniques (i.e. mesophilic anaerobic digestion, thermophilic anaerobic digestion, and aerobic sludge digestion), many of the methods that summarized are still operated in the bench-scale or batch scale (Table 3, 4 & 5). Although these bench-scale or batch scale methods have shown satisfactory results in the ARGs removal, there is still a lack of evidence for equally satisfactory results in commercialization. Future research is recommended to scale up the experiment, such as pilot-scale or semi-continuous scale, to provide a more comprehensive evaluation of different sludge digestion techniques in ARGs removal.

In addition, the effectiveness of sludge digestion technologies in removing ARG is currently evaluated based on the change in the abundance of ARGs before and after sludge digestion. The risks of sludge reuse for the transfer of ARGs in the natural environment are thus based on the abundance of ARGs in digested sludge. It is clear that ARGs can be transferred from sludge to the natural environment due to sludge reuse, leading to the proliferation of ARGs (Rahube et al., 2014; Chen et al., 2016). However, along with the changes of ARGs, different sludge digestion methods also altered the microbial community in the sludge. The impact of the microbial community in sludge and the

subsequent ARGs transfer into the natural environment is virtually unknown. Future research focusing on the change in soil ARGs caused by the application of these treated sludge is thus recommended.

6. Conclusion

This study summarized the state-of-the-art technologies on ARGs detection and removal in sludge from WWTPs, and proposed future perspectives. It leads to the following conclusions:

- Despite the inherent bias of RT-qPCR, it is still the most commonly used method for ARGs detection. Further development on improving the throughput of RT-qPCR and or the accuracy and cost of other emerging techiniques are recommended.
- Various sludge digestion strategies in combination with relevant pretreatment technologies and additives showed promising results in ARGs removal. However, the scale of testing and the understanding of risks of ARGs due to sludge reuse are limited.

CRediT authorship contribution statement:

Zehao Zhang: Conceptualization, Visualization, Data curation, Writing-original draft, Writing - review & editing. **Xuan Li**: Supervision, Conceptualization, Writing - review & editing, **Huan Liu**: Visualization, Writing - review & editing, Resources, **Arash Zamyadi**: Writing - review & editing. **Wenshan Guo:** Writing - review & editing. **Haiting Wen:** Data curation, Writing - review & editing. **Li Gao:** Writing - review & editing. **Long D. Nghiem:** Writing - review & editing. **Qilin Wang**: Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

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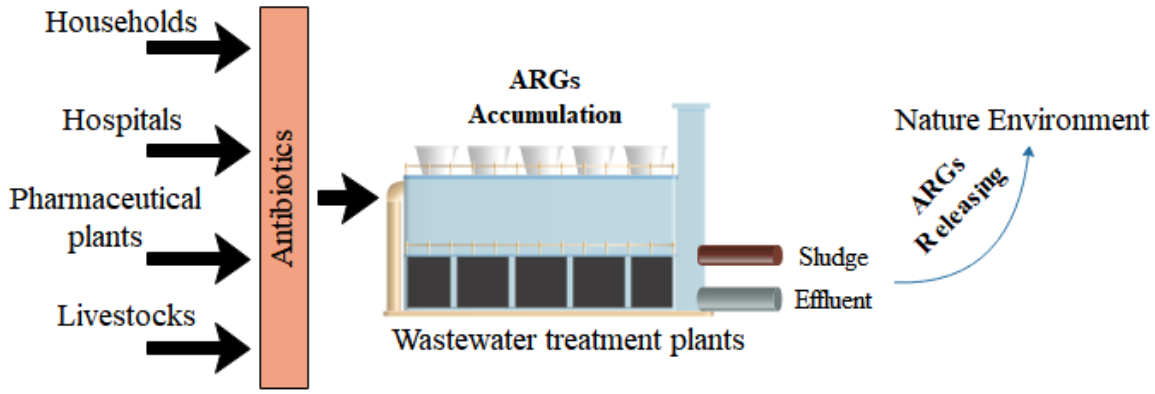

Figure 1. Occurrence of ARGs in WWTPs.

Table 1. Detection technology and emerging technology of ARG in sludge

Detection Techniques	Absolute abundance	Amplicatio n	Throughput	LOD or LOQ	Accuracy	Primer design	Database require	Host information	Other	Reference
Isolation of pure cultures	No	-	-	High	High	-	-	Directly	-	(Riber et al., 2014; Tong et al., 2017; Karkman et al., 2018)
RT-qPCR	Yes	Yes	One	High, 3 copies/μL	High, >99%	Yes	No	Yes, by calculation	-	(Rizzo et al., 2013; Bouki et al., 2013; Karkman et al., 2018; Zhu et al., 2013; Ruijter et al., 2013; Li et al., 2015)
HT-qPCR	Yes	Yes	Hundreds	High, 3 copies/μL	High, >99%	Yes	No	Yes, by calculation	-	(Lamas et al., 2014; Wang et al., 2016 Chen et al., 2016; Waseem et al., 2019; An et al., 2018)
Shotgun high-throughput sequencing	No	Usually not	All ARGs	Depends on data size	High, >99%	No	Yes	Yes, by calculation	Computing resources requires	(Hugenholtz & Tyson, 2008; Guo et al., 2017; Liu et al., 2019; Yang et al., 2014)
Emerging Techniques										
EpicPCR	Yes	Yes	One	High, 3 copies/μL	High, >99%	Yes	No	Yes, by calculation	-	(Tamminen et al., 2015; Spencer et al., 2016)
The third-generation sequencing	No	No	All ARGs	Depends on data size	80-95%	No	Yes	Directly	Computing resources requires; High error rate	(Che et al., 2019; Petrackova et al., 2019; Ashton et al., 2015; Wee et al., 2018; Xiao & Zhou, 2020)
DNA Microarray	No	Usually not	Dozens	Low, 10^3 copies/μL	High	No	No	Directly	-	(Zhang et al., 2009; Gilbride et al. 2006)
ddPCR	Yes	Yes	One	High, 10 times lower than RT-qPCR	High	Yes	No	Yes, by calculation	-	(Cave et al., 2016; Campomenosi et al., 2016; Kojabad et al., 2021)
Single-cell genome sequencing	No	No	All ARGs	-	-	No	Yes	Directly	Computing resources requires	(Gawad et al., 2016)

Table 2. Main findings of the ARGs in different sludge digestion methods.

Digestion methods	Digestion conditions	ARGs detection methods	Target ARGs	Findings		Reference
				Increase/No effect	Decrease	
Mesophilic anaerobic digestion	Bench-scale, full-scale; Mesophlic; SRT=15-30 days	Metagenomics	All known ARGs	Nine new subtypes of ARGs subtypes were found after sludge treatment	Decrease the relative abundance of ARGs by above 20%	(Guo et al. 2017; Yang et al., 2014)
Thermophilic anaerobic digestion	Bench-scale, full-scale; Thermophlic; SRT=15 days	RT-qPCR; Metagenomics	24; All known ARGs	*AadE, aadA, aacA4, mef(A), bl2d_oxa10* and *catb3* increased by 2.86–130.92%	Decrease the total absolute abundance of ARGs by 65.0%	(Miller et al., 2016; Zhang et al., 2015; Tian et al., 2016)
Aerobic digestion	Bench-scale, semi-continuous scale; 20-25 ℃; SRT=15-25 days	RT-qPCR	13	*aac(6')-Ib-cr, ermB, ermF, dfrA1, sul1* and *sul2* increased up to 93.8%	*tetA, tetL, tetO, tetW, tetX* decreased by up to three orders;	(Diehl & LaPara 2010; Burch et al., 2013; Burch et al., 2017; Zhang et al., 2021c)
Thermophilic aerobic digestion	Bench-scale, Thermophlic, SRT=15 days	RT-qPCR	23	-	All ARGs by 20.39% to 99.00%; *tetB, tetE, tetBP* and *blaCTX* are fully removed	(Jang et al., 2018)
Thermophilic aerobic digestion of anaerobically digested sludge	Batch, mesophilic, SRT=12 days	RT-qPCR	19	-	All ARGs by 16% to 99%; *tetB, tetD, tetH, tetM, tetX, tetBP, blaCTX* and *floR*are fully removed	(Jang et al., 2019)

Table 3. Main findings of pretreatment on the fate of ARGs in anaerobic sludge digestion

Pretreatment methods	Pretreatment conditions	Digestion conditions	Target ARGs	Findings		Reference
				Increase/No effect	Decrease	

Thermal hydrolysis	120–170 °C; 30–60 mins	Batch-scale, mesophilic; SRT= 30 days	16	-	Decrease all tested ARGs after anaerobic digestion by 0.5 to 3 logs during anaerobic digestion compared with the control group.	(Tong et al., 2019; Wang et al., 2019a; Pei et al., 2016 ; Sun et al., 2019)
Microwave	heated by microwave irradiation at 600w from 20℃ to 100℃	Batch-scale and semi-continuous scale; mesophilic; SRT=15-30 days	20	-	Decrease rest of ARGs by 0.05 to 0.70 log10 copies/g-TS during anaerobic digestion compared with the control group.	(Tong et al., 2017; Tong et al., 2018; Zhang et al., 2019; Tong et al., 2016)
Microwave-heat	Sludge adjusted to pH = 2.5 and heated by microwave irradiation at 600w from 20℃ to 100℃.	Batch-scale; mesophilic; SRT=30 days	8	Enrich *tetA*by 0.2 log10 copies/g-TS during anaerobic digestion compared with the control group.	Siginificantly decrease *tetC, tetM, tetO, tetX, blaSHV, blaCTX-M* and *ampC* by 0.1 to 0.7 log10 copies/g-TS during anaerobic digestion compared with the control group.	(Tong et al., 2016)
Microwave-H_2O_2	Sludge adjusted to pH = 10, heated by microwave irradiation at 600w from 20℃ to 80℃, dosed with H_2O_2 (30%, w/w)	Batch-scale; mesophilic; SRT=20-30 days	13	Enrich *mefA/E , ermB, ermF, tetG* and *tetM* less than 0.5 log10 copies/g-TS *during anaerobic digestion* compared with the control group.	Decrease *blaOXA-1, blaTEM, ereA, ermF, sulI, sulII* and *tetG* by 0.1 to 0.8 log10 copies/g-TS during anaerobic digestion compared with the control group.	(Zhang et al., 2017; Zhang et al., 2019)
Ozone	0.1 g O3/g TS	Batch-scale; mesophilic; SRT=30 days	5	-	Decrease all tested ARGs after anaerobic digestion by 0.2 to 0.5 log10 copies/g-TS during anaerobic digestion compared with the control group.	(Pei et al., 2016; Tong et al., 2018)
Alkaline	0.04g NaOH/g-TS; 1 day	Batch, mesophilic, 30 days	9	-	Decrease all tested ARGs after anaerobic digestion by 0.03 to 0.53 log10 copies/g-TS during anaerobic digestion compared with the control group.	(Wang et al., 2019a)
Ultrasonic	20kHZ; 30 mins	Batch-scale; mesophilic; SRT= 30 days	9	Enrich *tetB* by 0.2 log10 copies/g-TS during anaerobic digestion compared with the control group.	Decrease rest of ARGs by 0.05 logs to 1.02 log10 copies/g-TS during anaerobic digestion compared with the control group.	(Wang et al., 2019a)

Free ammonia	20 mg NH_3-N/L; 1 day	Batch-scale; mesophilic; SRT= 45 days	9	Slightly increased *tetG* by 14%; Has no effect on the fate of *sul1* and *tetM* during anaerobic digestion compared with the control group.	Decreased *aac(6')-Ib-cr, sul1, sul2, tetA, tetB, tetG* and *tetX* by 22~89% during anaerobic digestion compared with the control group	(Zhang et al., 2021b)
Two-phase anaerobic digestion	acidogenic phase; pH=6; 3 days	Lab-scale; continuous; mesophilic; SRT=13 days	All known ARGs	Increased the total ARGs from 229.8 ppm to 355.7 ppm during the process compared to the beginning.6	-	(Wu et al., 2016; Wu et al., 2018)

Table 4. Main findings of additives on the fate of ARGs in anaerobic sludge digestion

Additives	**Dosage**	**Digestion conditions**	**Target ARGs**	**Findings**		**Reference**
				Increase/No effect	**Decrease**	
Zero-valent iron (ZVI)	0.5 g/L to 4 g/L	Batch-scale and semi-continuous scale, mesophilic; SRT= 10-20 days	17	-	Decrease all tested ARGs after anaerobic digestion by 28.27–100.00% during anaerobic digestion compared with the control group.	(Zhang et al., 2020b; Zhou et al., 2021)
Ferric chloride ($FeCl_3$)	1.53 to 100 mg/L	Bench-scale; mesophilic; SRT=35days	19	Increased the abundance of almost all tested ARGs by 1.3×10^7 to 3.3×10^{10} copies/g-VS, Has limited impact on *AAC (6') -LB-Cr* and *blaTEM* during anaerobic digestion compared with the control group	*tetH* was completely removed during anaerobic digestion compared with the control group	(Jang et al., 2016)
Magnetite (Fe_3O_4)	0.5 g/L to 4 g/L	semi-continuous scale; mesophilic; SRT=20 days	11	No effect on ARG removal during anaerobic digestion compared with the control group.	-	(Zhang et al., 2020c)