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Role of anaerobic sludge digestion in handling antibiotic resistant bacteria and antibiotic resistance genes – A review

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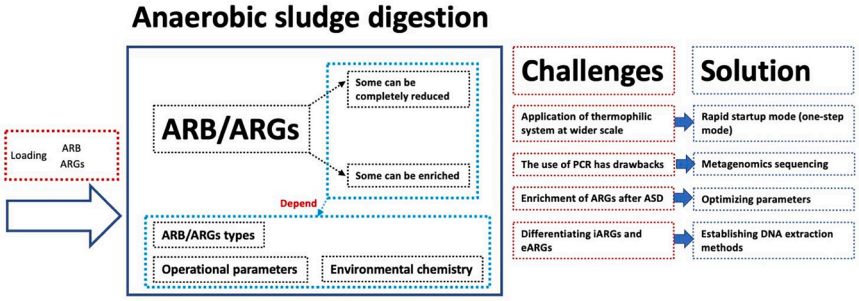
HIGHLIGHTS

- Abundance of ARB and ARGs in anaerobic sludge digestion (ASD) are reviewed.
- ASD can remove ARGs depending on operational and environmental parameters.
- Some ARGs can be enriched after ASD and *Firmicutes* are commonly found.
- Differentiating eARGs from iARGs is critical and should be explored in the future.

GRAPHICAL ABSTRACT



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ABSTRACT

Currently, anaerobic sludge digestion (ASD) is considered not only for treating residual sewage sludge and energy recovery but also for the reduction of antibiotic resistance genes (ARGs). The current review highlights the reasons why antibiotic resistant bacteria (ARB) and ARGs exist in ASD and how ASD performs in the reduction of ARB and ARGs. ARGs and ARB have been detected in ASD with some reports indicating some of the ARGs can be completely removed during the ASD process, while other studies reported the enrichment of ARB and ARGs after ASD. This paper reviews the performance of ASD based on operational parameters as well as environmental chemistry. More studies are needed to improve the performance of ASD in reducing ARGs that are difficult to handle and also differentiate between extracellular (eARGs) and intracellular ARGs (iARGs) to achieve more accurate quantification of the ARGs.

1. Introduction

The widespread usage of antibiotics to treat and prevent bacterial infection in health-care systems leads to the increasing environmental problems such as the generation of antibiotic resistance. Antibiotic resistance can occur when an organism becomes resistance to an antibiotic and the existence of antibiotic resistance genes (ARGs) is the main trigger. It is noted that several mechanisms are commonly adopted for the explanation of antibiotic resistance, which are modification of efflux pumps, drug inactivation, target modification, and permeability barriers. Thailand reported that more than 38,000 deaths per year can occur because of antibiotic resistance while the United States and

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European Union reported slightly lower deaths, which are 23,000 and 25,000 per year, respectively (Berglund, 2015). This calls for better approaches for reducing the spread of antibiotic resistance in the environment. Wastewater treatment plants (WWTPs) can be a good tool to manage the presence of ARGs because they are central collection points for various wastewater sources such as pharmaceutical and household activities. In addition, WWTPs have been recognized to have capability to reduce antibiotic resistant bacteria (ARB) via disinfection processes such as chlorination, ultraviolet (UV) light, and ozone treatment. A greater contribution to the release of ARGs detected in WWTPs comes from sludge, which contributes about 1000 times higher compared to the discharging from wastewater effluent (Munir et al., 2011). Thus, a proper sludge treatment is needed to control their release in the environment.

Anaerobic sludge digestion (ASD) has the capability as effective technology for the treatment of residual sewage sludge and energy generation as well as has been a promising treatment technology for ARGs reduction (He et al., 2020, Li et al., 2019, Lin et al., 2021). Intensive efforts have been carried out to improve the capability of ASD and have been continuously revised to achieve better and efficient system. For instance, ASD can eliminate ARGs and the removal efficiency can be improved by increasing the temperature of digestion (35 °C to 55 °C) (Tian et al., 2016). Alternatively, the use of appropriate dose of zero valent iron (Fe^0) can improve the performance and increase the removal of ARGs (Wei et al., 2017). Another performance improvement can also be achieved by using a rapid startup mode of thermophilic ASD which showed more effective for the removal tetracycline resistance genes ARGs (*sul*I and *sul*II) and sulfonamides resistance genes ARGs (*tet*A, *tet*W, and *tet*X) compared to conventional mode (step-wise) (Xu et al., 2018). Although some detected ARGs can rebound after ASD, their concentration in the thermophilic ASD can be minor ($2.79 \pm 1.19 \times 10^6$ copies/μL) than in the mesophilic ASD ($1.94 \pm 0.42 \times 10^7$ copies/μL) (Sun et al., 2019). Similar observation also proved that thermophilic ASD outperformed compared to mesophilic ASD (Xu et al., 2020). Although the performance of ASD in handling ARB and ARGs has been pronounced, the performance is not universal and there are remaining challenges such as its incapability in handling several ARGs such as *erm*F, *qnr*S, and bla_{NDM-1} that were difficult to be reduced and were prone to propagation during anaerobic digestion as reported by Li et al. (2021). The above-mentioned studies noticed that the ASD performance is closely related to operating and environmental parameters.

Although many review papers related to ARB and ARGs such as highlighting their presence in wastewaters (Sorinolu et al., 2021, Xue et al., 2019), groundwaters (Zainab et al., 2020), surface waters (O'Flaherty and Cummins, 2017, Singh et al., 2019), and the performance of anaerobic membrane reactor (Cheng et al., 2018) have been reported in the literature, a comprehensive review focusing on ARB and ARGs in ASD has never been conducted. Aligning the research gap, the current paper comprehensively reviews the fate and abundance of ARB and ARGs in ASD. The novelty of this review is to show the reason why ARB and ARGs exist in ASD and how ASD performs the elimination of ARB and ARGs. This may provide an insight into the capabilities of ASD in handling ARB and ARGs and also may be useful in designing advanced ASD in the future.

2. Abundance and removal of ARGs

Sewage sludge from WWTPs is recognized as a central reservoir for ARGs (Boopathy, 2017, Everage et al., 2014, García et al., 2020, Grabert et al., 2018, Naquin et al., 2014, Naquin et al., 2015, Naquin et al., 2020, Naquin et al., 2017). Currently, ASD has been proposed and found to be suitable for reducing ARGs. Table 1 shows the recent studies in investigating ARGs and ARB in ASD. For instance, identification of ARGs in the sample collected from ASD of Shatin STP in Hongkong was carried out via high-throughput sequencing-based metagenomic approach (Yang et al., 2014). The average abundance of ARGs in the ASD was 47.41% with a total of 102 ARGs subtypes. Among these, ARGs of tetracycline became the major ARG types detected in ASD (27.6%) and followed by macrolide-lincosamide-streptogramin (MLS) (22.9%) and acriflavine (10.1%) genes. The study found that the detected ARGs in ASD were speculated to be coming from the influent as the majority of ARGs are found in the influent. It is noted that the study investigated the presence of ARGs in the influent, activated sludge, ASD and effluent of the wastewater treatment plant. The study found that 78 ARGs subtypes belonging to 12 ARGs types in influent were also shared by all of the other samples (activated sludge, ASD, and effluent). This seems to be logic since the wastewater treatment plant receives wastewater from households and hospitals where antibiotics are applied.

The detection of ARGs in ASD of Stanley WWTP, China confirmed high level with 133 subtypes (Ma et al., 2014). The distribution of ARGs and the bacterial profiles of waste sludge from WWTP with A^2O and A^2O-MBR processes during anaerobic digestion were investigated (Li et al., 2021). ASD had a conformity effect on the fate and profiles of ARGs in the digested sludge. Among detected genes, *erm*F, *qnr*S, and bla_{NDM-1} were the most difficult to be reduced and were prone to propagation during anaerobic digestion.

The capability of ASD in reducing ARGs is another major concern. Several detected ARGs can be removed up to 100% but the average removal efficiency was about 20.70% as reported by Yang et al. (2014). The study also confirmed that several ARGs, including MLS, polymyxin, tetracycline, and vancomycin can be rebounded after ASD as indicated by increasing their abundance. This is partly due to incapability of ASD to eliminate bacteria hosting the ARGs. Moreover, *sul*I, *sul*II, and *int*I1 genes can be removed by 1- to 2-log reductions using the thermophilic and mesophilic sludge digestion and their performance were comparable (Miller et al., 2013).

A comparison of thermophilic and mesophilic ASD in handling ARGs and ARB was examined and found that the thermophilic system was better than mesophilic system. Interestingly, the presence of *Chloroflexi* (20.29%–2.64%) and *Proteobacteria* (36.39% to 6.80%) declined, but *Firmicutes* increased from 18.22% to 74.89% after thermophilic ASD (Xu et al., 2020). Similar observation was also reported by Sun et al. (2019) who observed ARGs can be increased but their concentration was still lower in the thermophilic system compared to the mesophilic system, which are $2.79 \pm 1.19 \times 10^6$ copies/μL and $1.94 \pm 0.42 \times 10^7$ copies/μL, respectively. A lower ARGs removal by using the mesophilic system was also reported by Gros et al. (2019) who observed that the system has limited capability for reducing ARGs. In general, the above mentioned findings show similarities with those previously reported by using mesophilic ASD (Chen et al., 2010, Cheng et al., 2016, Sui et al., 2016, Wu et al., 2016). Moreover, some ARGs can also be rebounded after ASD treatment (Zhang et al., 2016).

Recently, the presence of ARGs in two-stage anaerobic digestion was evaluated and quantified for better removal and compared with one-stage system (M) (Shi et al., 2021). The concept of two-stage ASD proposed in the study was as follows: (i) thermophilic alkaline fermentation was adopted before applying mesophilic ASD (TM) or mesophilic alkaline fermentation was adopted before applying mesophilic ASD (MM). The study found that the proposed TM or MM performed better removal efficiency compared to M. The results are in agreement with those reported by Zhang et al. (2017), exhibiting that two-stage system provided some improvements in the removal of ARGs. Moreover, Shi et al. (2021) confirmed that MM or TM provided similar ARGs reduction. It has been well known that the microbial community compositions can be correlated with ARGs compositions. Thermophilic and mesophilic alkaline fermentations can affect microbial community compositions in ASD and this may have significant impacts on the constitution of ARGs. The study found that the hydrogen and methane production stages of two-stage ASD processes were observed to have dissimilar impact on the fate of ARGs. MLS resistance genes can be enriched, especially in the hydrogen production reactors of TM and MM processes.

Classification of ARGs resistance mechanism is commonly carried

Table 1
General description and main findings of ARB and ARGs studies.

Study design	ARGs identification method	Total detected ARG subtypes	Dominant ARGs resistance mechanism	ASD parameters	Main finding	Reference
Lab-scale	RT-qPCR	11	Not studied	SRT = 10 and 20 days Temperature = 35 °C Two-stage system	The ASD effectively removed ARGs particularly for AAC (6′)-IB-CR and *tet*B genes. At the optimum ASD condition, the dominant organisms were *Erysipelotrichia*, *Verrucomicrobia*, *Clostridia*, *Caldiserica*, and *Alphaproteobacteria*	Zhou et al. (2021)
Lab-scale	Metagenomic sequencing by the Illumina Hiseq 2500	95–111 at two-stage ASD and 158 at one-stage ASD	Efflux pump, inactivation, and target alteration	HRT = 5 and 10 days Temperature = 37 °C to 55 °C One and two-stage system	Two-stage ASD performed better removal efficiency than one-stage ASD	Shi et al. (2021)
Lab-scale	Metagenomic sequencing by the Illumina HiSeq PE150	> 1360	Efflux pump, target alteration, inactivation	HRT = 15 days Temperature = 37 °C to 55 °C Two-stage system	Thermophilic ASD performed better removal efficiency than mesophilic ASD	Xu et al. (2020)
Full-scale, Shanghai, China	qPCR	10 targetted	Not studied	One-stage system	Some detected ARGs can be enriched (27.43–43.71%) after ASD and these can be correlated with Bacteroidetes	Wang et al. (2020)
Lab-scale	qPCR	18	Not studied	HRT = 20 days Temperature = 37 °C to 55 °C One-stage system	Thermophilic ASD can reduce by 86% of the ARGs	Shin et al. (2020)
Lab-scale	qPCR	11	Not studied	HRT = 20 days Temperature = 37 °C Two-stage system	Iron nanoparticles reduced microbial diversity and changed microbial community structure in ASD sludge	Zhang et al. (2020)
Lab-scale	qPCR	2	Not studied	HRT = 10 days Temperature = 37 °C to 55 °C Two-stage system	Ultrasound irradiation combined with ozone pretreatment can remove bacteria that harboring resistance genes	Zhao et al. (2020)
Lab-scale	qPCR	1	Not studied	SRT = 30 days Temperature = 35 °C Two-stage system	ASD can remove *cmy*-2 even under the presence of ceftiofur	Flores-Orozco et al. (2020)
Lab-scale	qPCR	15	Not studied	SRT = 20 days Temperature = 35 °C Two-stage system	Roxithromycin increased antibiotic resistance genes in digested sludge	Ni et al. (2020)
Full-scale, Seoul, South Korea	Metagenomic sequencing by the Illumina Hiseq 2000	181	Not studied	–	The abundance of multidrug and β-lactam resistance genes can be reduced after ASD	Yoo et al. (2020)
Full-scale, UK	qPCR	13	Not studied	SRT = 12–25 days Temperature = 32.6 to 38 °C Two-stage system	Thermal hydrolysis can reduce all ARGs by 10–12,000 fold but after subsequent ASD, some detected ARGs can be rebounder with the highest increase was observed for *ermF*, which increased from 1.2×10^4 to 1.2×10^8 copies/g dry wt)	Redhead et al. (2020)
Lab-scale	PCR	4	Not studied	Temperature = 22 °C One-stage system	The identified *L. mesenteroides* was observed to have the *sul*1 gene to resist the toxicity of trimethoprim and sulfamethoxazole	Naquin et al. (2020)
Full-scale, Shanghai, China				SRT = 18 days Temperature = 39 to 40 °C	Archaea biomass and community affect the fate of ARGs. There is shifting in the microbial community after the thermal hydrolysis (spore-forming bacteria) and ASD (fermentative bacteria)	Tong et al. (2019)

(*continued on next page*)

Table 1 (*continued*)

Study design	ARGs identification method	Total detected ARG subtypes	Dominant ARGs resistance mechanism	ASD parameters	Main finding	Reference
Lab-scale	qPCR	5	Not studied	Two-stage system HRT = 20 days Temperature = 35 °C Two-stage system	ASD can reduce aac(6′)-IB and blaOXA-1 by 94% and 44.82%, respectively, but three of six macrolide resistance genes can be increased	Xiang et al. (2019)
Full-scale, Catalonia, Spain	qPCR	5	Not studied	HRT = 75–80 days Two-stage system	ASD exhibited moderate to low ARGs reduction	Gros et al. (2019)
Lab-scale	qPCR	11	Not studied	SRT = 15–20 days Two-stage system	Shorter SRT was needed for the removal of ARGs using two-stage ASD with microwave pretreatment while one-stage ASD with microwave pretreatment needed longer SRT	Zhang et al. (2019)
Lab-scale	qPCR	10	Not studied	Temperature = 37 °C One-stage system	ARGs removal efficiency of ASD without pretreatment was 50.77%, alkaline-ASD was 66.38%, thermal hydrolysis-ASD was 52.50%, and ultrasonic pretreatments-ASD was 75.07%	Wang et al. (2019)

out according to the CARD database (Jia et al., 2017). Several mechanisms were proposed for clarification of ARGs resistance mechanism in one-stage and two-stage ASD (Shi et al., 2021). It can be observed that the resistance gene of aminoglycoside became the dominant in the two-stage ASD while the beta-lactam resistance gene became the dominant in the one-stage ASD. Formation of inactive derivatives can be an option in controlling enzymatic inactivation of antibiotics (Davies, 1994). Shi et al. (2021) confirmed that antibiotic inactivation and efflux pump became dominant mechanisms in the two-stage ASD. Another study found that efflux pump and inactivation mechanisms can be enhanced during ASD (Christgen et al., 2015). Alternatively, efflux pump and target alteration can also be the main mechanisms in thermophilic and mesophilic ASD (Zhang et al., 2015). In general, efflux pump mechanism is still becoming the main antibiotic resistance mechanism since it has the capability to gather defense against several inhibitory constituents (Christgen et al., 2015).

Another study investigated the resistance mechanisms of ARGs detected in ASD under thermophilic and mesophilic conditions (Xu et al., 2020). The study observed that efflux pump mechanism was the main mechanism, which accounts up to 70% while target alteration and inactivation mechanisms were in lower percentages by 15% and 5%, respectively. In thermophilic system, target alteration and efflux pump mechanism can be decreased by 30.68% and 32.86%, respectively. The above-mentioned findings indicated that ASD can perform different resistance mechanisms and this seems to be logic since the mechanism depends highly on various operational conditions and reactor configurations.

The spread of ARGs in environment is mainly by two processes, which are vertical gene transfer attributed to the reproduction of bacterial hosts and horizontal gene transfer attributed to the transfer of ARGs between different bacterial cells via mobile elements. It has been widely acceptable that class 1 integron (*intI*1) is used as an indicator for horizontal ARGs transfer. For example, the reduction of ARGs during ASD can be correlated with the decrease in *intI*1, which is speculated because of the blockage of horizontal transfer pathways (Ma et al., 2011). In addition, the total abundance of 18 bacterial genera suggested as the possible hosts for 13 ARGs decreased from 23.27% in mesophilic ASD to 11.92% in thermophilic ASD and this is speculated because of the decrease of both the horizontal and vertical transferability of ARGs (Tian et al., 2016).

3. Performance of ASD

It is noted from the above discussion that the performance of ASD for the removal of ARGs depends highly on several parameters such as solids retention time (SRT), temperature, as well as environmental chemistry, which include the presence of other pollutants such as heavy metals. There is a possibility to increase the removal efficiency by performing ASD at a longer SRT. The hypothesis was proven by Ma et al. (2011) who found that increase in the removal performance at 20 day SRT by using the mesophilic ASD compared to 10 day SRT mesophilic anaerobic digester. The finding seems to be logic since the SRT can affect the capabilities and performance of biological treatment systems by influencing the dominant microbial species (Xia et al., 2012). Another study also confirmed the effect of the total hydraulic retention time (tHRT) on the performance of mesophilic two-phase ASD to remove ARGs (Wu et al., 2019). The study exhibited that the reactor performance was similar at tHRT of 16 and 20 d but specifically for the sulfonamide ARGs, their removal was lower at the tHRT of 16 d compared to the tHRT of 20 d.

Temperature is also a critical environmental parameter that can affect the performance of ASD and control the diversity of microorganisms. ASD can be performed by using mesophilic (30–40 °C) and thermophilic (50–60 °C) conditions and the selection of these systems can determine the successful removal of ARGs. A study observed that thermophilic ASD at 47 °C, 52 °C, and 59 °C was better compared to mesophilic system operated at 35 °C (Ma et al., 2011). This finding was consistent with previous work by Ghosh et al. (2009) and Diehl and LaPara (2010) particularly in the context of *tet*(O) and *tet*(W) genes. It is believed that the temperature is an important parameter that can control reaction rates. Following this logic, increase in temperature can facilitate the enhancement of biological and chemical reaction rates, which can enhance the removal of ARGs during ASD. Destroying extracellular DNA can be achieved through hydrolysis and biodegradation, which generally are expected to occur at higher rates with increased temperature (Ma et al., 2011). It is also interesting to note that the thermophilic ASD exhibited lower bacterial variety compared to the mesophilic ASD as indicated by the analysis using denaturing gradient gel electrophoresis (DGGE) (Ma et al., 2011). Similar finding can also be seen that the thermophilic ASD performed better removal efficiency particularly for *tet*(O) and *tet*(W) genes compared to the mesophilic ASD (Miller et al., 2013). Sun et al. (2016) reported that thermophilic ASD can remove

ARGs by 80%, which is higher compared to mesophilic ASD, which is only 50%. Lab-scale thermophilic microcosm study confirmed better removal particularly for *sul*I, *sul*II, *tet*A, *tet*O, and *tet*X (Zou et al., 2020b). Some ARGs can be removed by 50%–97% using thermophilic after 57 days operation (Tian et al., 2016). For a comprehensive overview, Table 2 lists the comparison between thermophilic and mesophilic ASD for the removal of selected ARG subtypes. In general, the thermophilic ASD provided better removal efficiency compared to mesophilic ASD. For instance in the case of *tet*A, Ghosh et al. (2009), Diehl and LaPara (2010), Jang et al. (2017), Xu et al. (2020), Zou et al. (2020a) consistently reported better performance of the thermophilic ASD than mesophilic ASD. Inconsistency can be observed in the case of *erm*B and *tet*G. Ma et al. (2011) found that the removal of *erm*B by using thermophilic ASD was better than mesophilic ASD but Zhang et al. (2015) reported the opposite performance. For the *tet*G, Ma et al. (2011) and Jang et al. (2017) were in similar finding but Zhang et al. (2015) found the opposite removal performance.

Pretreatment cannot only facilitate the subsequent anaerobic digestion of excess sludge but also may eliminate the abundance of ARGs and reduce the risk of ARGs spreading during ASD. Several pretreatment techniques were proposed and examined by Wang et al. (2019). The study exhibited that the ASD without the pretreatment method had the capability to remove ARGs by only 50.77%. However, an improvement in the removal capability was observed when the ASD was combined with the pretreatment methods, which performed ARGs removal efficiency by ranging from 52.50% to 75.07% with the use of the ultrasonic pretreatment became more superior compared to alkaline and thermal hydrolysis methods. More importantly, ARGs rebound can be observed after the subsequent ASD when the thermal hydrolysis method was applied. Currently, the results by Mao et al. (2020) showed that the removal of ARGs by thermo-alkaline, thermal hydrolysis, and microwave pretreatments were 3.32 logs, 3.13 logs, and 2.95 logs, respectively. As a comparison, the ultrasonic wave pretreatment only removed 0.58 logs of the ARGs. It is noted that the ultrasonic wave pretreatment radiation was observed to increase cell membrane permeability by rupturing the spore coat and inner membrane, leading to the increased leakage of protein and DNA (Fang et al., 2011). Most ARGs carrying bacteria have little resistance with thermo-alkaline, thermal hydrolysis, and microwave pretreatments. A proposed strategy can be designed by extending the pretreatment time to effectively reduce the ARGs in sludge.

The presence of heavy metals in sludge of WWTPs have been reported in many studies (Boopathy, 2019, Oubre and Boopathy, 2020) and they have the potential to affect the fate of ARGs during ASD. Following this hypothesis, Huang et al. (2019) confirmed that the presence of CuO or ZnO nanoparticles (NPs) can increase the concentration of ARGs although the resistance mechanism was not significantly changed. The study proposed a mechanism on how dissemination of ARGs can be affected by the presence of the heavy metals via the signal transduction. The study proposed that the existence of the NPs can activate cell signaling (pili synthesis, metal tolerance, and quorum sensing). As a result, the bacterial profiles can be shifted and can promote gene transfer potential and the stimulation of co-selection. The mechanism has the potential to affect the fate and behaviors of ARGs during ASD.

Alternative study evaluated the impacts of zero valent iron (Fe^0) on the abundance of ARGs during thermophilic ASD (Wei et al., 2017). The use of an appropriate dose of Fe^0 (0.10 g/g VSS) can improve the capability of the ASD and interestingly, the abundance of tetracycline ARGs and *int*I1 genes can also be decreased, suggesting the capability of ASD in handling the ARGs. Although the presence of Fe^0 has the potential for ARGs removal, this finding is not universal as the use of higher dosage (1.17 g/g VSS) cannot enhance the removal capability, exhibiting the lower capability of ASD to remove ARGs at higher dosage of Fe^0. Similar findings by Gao et al. (2017) found an increase in ARGs reduction with the addition of Fe^0 but the increase in the dosage did not significantly enhance the ARGs reduction. The consequences of different doses of Fe^0 on the fate and distribution of tetracycline ARGs were investigated (Yang et al., 2018). The study found that the plasmid conjugation was proposed as the possible mechanism to promote the horizontal gene transfer of the ARGs. Previous studies have demonstrated that Fe^0 can improve anaerobic biological processes and alter the phylogenetic distribution of dominant bacteria in anaerobic digesters (Feng et al., 2014). Beside declining the oxidation–reduction potential (ORP) in anaerobic systems, Fe^0 can be an electron-donor for participating microbial metabolism (Karri et al., 2005)

4. Abundance of ARB and their correlation with ARGs

It is believed that bacteria are important carriers of ARGs, thus, changing by reduction of bacteria may be responsible for controlling the abundance of ARGs since the variation and abundance of ARGs can occur via the vertical gene transfer (VGT) mechanism because of the reproduction of bacterial hosts. Study on the abundance of ARB profiles present in ASD of Shatin WWTP in Hongkong was carried out by Yang et al. (2014). *Proteobacteria* was one of the dominant phyla detected in the ASD with a percentage of 48% followed by *Bacteroidetes* (18.4%), *Firmicutes* (17.3%), and *Actinobacteria* (12.9%). Similarly, Ma et al. (2011) also confirmed that *Firmicutes* were the dominant bacteria found in the ASD. The detection of these bacteria seems to be logic since the majority of these can be commonly observed in anaerobic setting (Kim et al., 2012, Swithers et al., 2011).

The successful ASD in the removal of ARB has also been reported in many studies. For instance, the detection of tetracycline and sulfonamide resistant bacteria was reported in ASD of full-scale WWTP in Michigan, USA (Munir et al., 2011). The study found that the use of ASD can reduce significantly the identified ARB compared to the conventional dewatering and gravity thickening methods. Another study also confirmed that the mesophilic ASD can reduce ARB by 1.48–1.64 log unit ($P < 0.05$) as reported by Tong et al. (2014). The study also

Table 2
Comparison between thermophilic and mesophilic ASD for the removal of ARG subtypes.

ARG subtypes	Ghosh et al. (2009)	Diehl and LaPara (2010)	Ma et al. (2011)	Zhang et al. (2015)	Jang et al. (2017)	Xu et al. (2020)	Zou et al. (2020a)
*erm*B	–	–	TM	MM	–	–	–
*erm*F	–	–	TM	–	–	–	–
*tet*A	TM	TM	–	–	TM	TM	TM
*tet*C	–	–	TM	–	–	–	–
*tet*G	–	–	TM	MM	TM	–	–
*tet*L	–	TM	–	–	–	–	–
*tet*O	TM	TM	TM	MM	–	–	TM
*tet*W	–	TM	TM	MM	–	–	–
tetX	TM	TM	MM	–	TM	–	TM
*sul*1	–	–	Si	TM	TM	–	TM
*sul*2	–	–	Si	Enr	TM	–	TM

It is noted that TM is the thermophilic, MM is the mesophilic, Enr is the enriched, and Si is the similar.

confirmed that the fate and distribution of ARB in the sludge varied based on seasonal changes with the higher abundance of ARB in the sludge can be observed in cold season than the warm season ($P < 0.05$). Moreover, mesophilic and thermophilic ASD can perform differently in controlling the presence of ARB during the ASD process as indicated by the relative abundance analysis (Xu et al., 2020). At the mesophilic ASD, the top 3 dominant bacterial members were *Proteobacteria* (39.26%), *Chloroflexi* (20.32%), and *Firmicutes* (14.78%), which were shifted at the thermophilic ASD. At the thermophilic ASD, *Firmicutes* became the dominant and their abundance can be enriched from 18.22% to 74.89% while *Proteobacteria* and *Chloroflexi* were found to be decreased by ranging from 36.39% to 6.80% and 20.29%–2.64%, respectively. The presence of *Methanosaeta* (2.39%–4.30%) and *Syntrophomonas* (0.93%–5.12%) was the dominant genera in the mesophilic system while *Coprothermobacter* were the dominant genera in the thermophilic system. The dominance of these bacteria was in line with the previous study on the thermophilic ASD (Pervin et al., 2013).

Microbial community is believed to affect the fate and distribution of ARGs in WWTPs. However, evaluation of the effect of microbial community on ARGs abundance particularly in ASD is still inadequate because the correlation between them is very complicated. Several methods have been proposed to analyze the correlation between ARB and ARGs profiles, which become the initial steps to further analyze more complex relations. Redundancy analysis (RDA) becomes more popular and has been used for the analysis of correlation between ARB and ARGs profiles. For example, RDA analysis carried out by Yang et al. (2014) exhibited that six genera including G1-G5 showed significant correlation with ARGs ($P = 0.002$). It is noted that G1, G2, G3, G4, G5, and G5 refer to *Flavobacterium*, *Poriferibacter*, *Bacteroides*, *Acinetobacter*, *Actinobaculum*, and *Streptococcus*, respectively. In detail, the abundance of ARGs in Cluster 1 can be correlated only with G1 and G2, Cluster 2 can be correlated with all six genera, and Cluster 3 can be correlated only with G3, G4, G5, and G6. Zhang et al. (2017) by using RDA showed that Firmicutes and intI1 can control the distribution and abundance of tetX, tetM, mefA/E, and ereA. As a comparison, Bacteroidetes were believed to be the main source for the ermB and ermF. Procrustes analysis can be an alternative approach to analyze the correlation between ARGs and microbial communities as carried out by Shi et al. (2021). However, this method has the limitation since it only provides the basic correlation information. Hence, the detailed correlation information by using network analysis is crucial and currently commonly used as carried out by Shi et al. (2021). By using the analysis, the study found that *Candidatus Nitrospira defluvii* is the potential host for multidrug resistance genes (*ceo*B, *cmo*B) and fosmidomycin resistance genes (*ros*B). The correlation and network analysis only suggest the potential host for ARGs and do not provide the clear evidence that the correlated microorganisms are carrying antibiotic resistance (Sun et al., 2020). The correlation between ARGs and bacterial community was also reported by Wang et al. (2019) who found that the presence of *Proteobacteria* reduced after ASD process while *Bacteroidetes* and *Firmicutes* increased after ASD. The Pearson's bivariate correlation indicated that *tet*M and *tet*Q can be enriched after ASD, hence, increase in *tet*M and *tet*Q was possibly because of increase in *Bacteroidetes* and *Firmicutes* (Diehl and LaPara, 2010).

5. Perspectives and challenges

It has been well known that temperature is a critical parameter in controlling the performance of ASD. The above discussion has mentioned that increase in temperature can facilitate the enhancement of ARB and ARGs removal in sludge. For instance, several studies have confirmed that thermophilic ASD performs better than mesophilic ASD. A challenge can be faced since most of WWTPs implementing ASD technology in full-scale adopt mesophilic digestion. This is particularly because the application of thermophilic system needs a higher operational and maintenance costs compared to mesophilic system. Another major limitation to implementing thermophilic digestion at wider scale is the longer startup time. To maintain the stability of reactor and save startup time, an innovation by using a rapid startup mode (one-step mode) of thermophilic ASD was proposed (Xu et al., 2018). In the study, the two systems were first operated at 35 °C for 30 days to achieve a stable biogas production. Reactor SW (step-wise mode) was setup by increasing the temperature (35 °C to 55 °C) gradually for 20 days. The innovation of the study was proposed via the one-step mode (OS) reactor by increasing temperature (35 °C to 55 °C) directly. Next, the performance of both reactors was compared and evaluated for 80 days in order to achieve a steady condition. In general, the study found that the OS design performed better in the removal of *sul*I, *sul*II, *tet*A, *tet*W, and *tet*X compared to SW design. This suggests that OS can be an alternative strategy to implement thermophilic ASD in full-scale since this innovation can diminish the long startup time and more importantly can improve the reduction of ARGs.

Previous studies mainly explored the quantitative PCR for the identification of ARGs in ASD. However, the approach seems to be not practically convenient particularly because of the limited primer among highly diverse ARGs, suggesting that the quantitative PCR cannot comprehensively identify the diversity of ARGs in environmental samples. In closing the gap, metagenomics sequencing-based approaches have been proposed and become popular for the identification of ARGs since they can provide the broad profile of ARGs. Numerous studies have been conducted to implement the approaches and have been successful for the identification of 271 (Yang et al., 2014), 158 (Shi et al., 2021), 181 (Yoo et al., 2020), and >1360 of ARGs subtypes (Xu et al., 2020). The use of metagenomics approaches can eliminate the limitations as provided by amplification-based methods such as the limited availability of primers, the possible bias in the amplification process, and the possible false-negative results triggered by the enzyme inhibitor (Volkmann et al., 2007).

Enrichment of ARGs during ASD process because of rebound phenomenon has been observed in many studies and more importantly, there are still remaining challenges that some ARGs have been difficult to remove. A study reported that some ARGs types were observed to be enriched after ASD process (Yang et al., 2014). In addition, increase in bacterial pathogens such as *Clostridium* and *Mycobacterium* after ASD becomes another concern since they were reported to be the potential host bacteria for 23 and 8 resistance gene types, respectively (Zhang et al., 2017). Another study reported that *tet*M can be enhanced after ASD up to 5.0×10^{-2} copies/16S rDNA copies (Xu et al., 2018). Shi et al. (2021) also observed that the thermophilic and mesophilic ASD can enhance MLS resistance genes and this exhibits that the proposed ASD cannot handle the abundance of the ARGs type. Moreover, concentration of *tet*L and *tet*W genes did not show statistical significance in both reactors, indicating the incapability to eliminate these detected ARGs. WWTP with A^2O and A^2O-MBR processes during anaerobic digestion was observed to be difficult to remove *erm*F, *qnr*S, and bla_{NDM-1} (Li et al., 2021). Increase of *tet*M and *tet*Q after ASD was also reported and it was speculated because of the increase in the population of *Bacteroidetes* and *Firmicutes* in the ASD (Wang et al., 2019). It is noted that MLS ARGs becomes the most challenged ARGs to be removed using ASD (Shi et al., 2021, Yang et al., 2014). Therefore, other strategies can be carried out by changing the operational parameters of ASD that are possible to improve the removal efficiency of ARGs. Future studies should focus on the ASD performance improvement and the investigation of MLS resistance genes in complex samples. This will be highly useful to investigate main mechanism of ARGs dissemination.

It is noted that ARGs can be present in the form of extracellular (eARGs) and intracellular ARGs (iARGs). Both eARGs and iARGs are involved in horizontal gene transfer and have been believed to play key roles in controlling the fate of ARGs. Their involvement is in different mechanisms, which are (i) iARGs are disseminated by conjugation and transduction and (ii) eARGs are disseminated via accumulation in competent bacteria by natural transformation. However, many recent

studies have not differentiated between eARGs and iARGs. It has been well known that eARGs indicated the chemical features of the DNA and the iARGs exhibited the biological features (Wang et al., 2016). The eARGs can be present as iARGs via transformation while the iARGs can be turned to eARGs after the death of bacteria. Hence, the removal of eARGs becomes important for the ARGs removal (Sommer et al., 2017). Future studies are recommended to do the differentiation between iARGs and eARGs when determining and quantifying ARGs in ASD. Distinguishing between iARGs and eARGs can be a good way to achieve more accurate determination and quantification of the ARGs. Zou et al. (2020a) can be a good reference as a benchmark to do this since they proposed an extraction method that can identify iARGs and eARGs from sludge. The study estimated iARGs and eARGs as the function of eDNA and iDNA quantified by using qPCR. For the eDNA extraction, two methods were utilized, which are regular extraction (Dong et al., 2019, Zhang et al., 2013) and SDS addition extraction while the iDNA extraction was carried out from the residual sludge pellet using a FastDNA Spin Kit.

The differentiation of iARGs and eARGs in ADS samples was carried out by Zou et al. (2020a) who found that the absolute abundances of all investigated eARGs ranged from 2.9×10^7 to 3.4×10^{11} copies/g dry weight (dw) sludge and higher absolute abundances of iARGs were also detected ranging from 6.5×10^7 to 1.4×10^{13} copies/g dw sludge. Another study by Dong et al. (2019) reported lower absolute abundances of iARGs and eARGs compared to reported by Zou et al. (2020a). Specifically, Dong et al. (2019) found that the absolute abundances of eARGs can be detected in the range of 7.3×10^3 to 1.2×10^{10} copies/g dw sludge while eARGs ranged from 1.0×10^5 to 2.7×10^{12} copies/g dw sludge. Higher and more concentrated ARGs reported by Zou et al. (2020a) are possible because the study carried out optimization for the eDNA and iDNA extractions, which can be adopted for future study to more accurate estimation of ARGs in environmental samples.

6. Conclusion

This paper has highlighted abundance of ARB and ARGs in ASD and presented the role of ASD in the reduction of ARB and ARGs. The major implication of this work is to highlight several aspects of ASD in enhancing its capability to control the spread of ARB and ARGs to environment that can be achieved via optimized ASD parameters and pretreatment promotion. In addition, by highlighting some detected ARB and ARGs that are difficult to handle by ASD, new innovations are still needed to be proposed. Although some correlation and network analyses have been proposed and used for suggesting the potential host for ARGs, these analyses do not provide the clear evidence that the correlated microorganisms are carrying antibiotic resistance, suggesting the need for more deeper analysis in the future.

CRediT authorship contribution statement

Achmad Syafiuddin: Writing - original draft. **Raj Boopathy:** Conceptualization, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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