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Temperature-phased anaerobic sludge digestion effectively removes antibiotic resistance genes in a full-scale wastewater treatment plant

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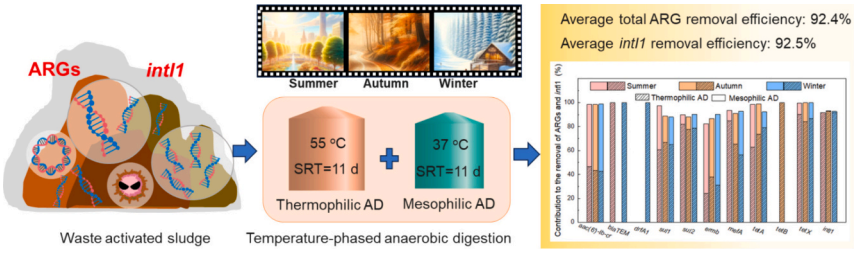
HIGHLIGHTS

- TPAD reduced the average absolute abundance of targeted ARGs and *intI1*.
- Thermophilic AD plays a major role in the ARGs and *intI1* removals.
- The removal of ARGs is related to the decreased abundance of ARB and *intI1*.
- The abundance of targeted ARGs was higher in winter than in summer and autumn.

GRAPHICAL ABSTRACT



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ABSTRACT

Sludge is a major by-product and the final reservoir of antibiotic resistance genes (ARGs) in wastewater treatment plants (WWTPs). Temperature-phased anaerobic digestion (TPAD), consisting of thermophilic anaerobic digestion (AD) (55 °C) and mesophilic AD processes (37 °C), has been implemented in WWTPs for sludge reduction while improving the biomethane production. However, the impact of TPAD on the ARGs' fate is still undiscovered in lab-scale experiments and full-scale WWTPs. This study, for the first time, investigated the fate of ARGs during the TPAD process across three seasons in a full-size WWTP. Ten typical ARGs and one integrase gene of class 1 integron (*intI1*) involving ARGs horizontal gene transfer were examined in sludge before and after each step of the TPAD process. TPAD reduced *aac(6')-Ib-cr*, *blaTEM*, *drfA1*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, *tetB* and *tetX* by 87.3–100.0 %. TPAD reduced the overall average absolute abundance of targeted ARGs and *intI1* by 92.39 % and 92.50 %, respectively. The abundance of targeted ARGs in sludge was higher in winter than in summer and autumn before and after TPAD. During the TPAD processes, thermophilic AD played a major role in the removal of ARGs, contributing to >60 % removal of ARGs, while the subsequent mesophilic AD contributed to a further 31 % removal of ARGs. The microbial community analysis revealed that thermophilic AD reduced the absolute abundance of ARGs hosts, antibiotic resistant bacteria. In addition, thermophilic AD reduced the abundance of the *intI1*, while the *intI1* did not reproduce during the mesophilic AD, also contributing to a decline in the absolute abundance of ARGs in TPAD. This study demonstrates that TPAD can effectively reduce the abundance of ARGs in sludge, which will suppress the transmission of ARGs from sludge into the natural environment and deliver environmental and health benefits to our society.

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1. Introduction

Antibiotics have delivered extensive benefits to human and animal well-being. However, it has also inevitably led to the pervasive emergence of antibiotic resistance and antibiotic resistance genes (ARGs) in the environment, posing a rising threat to global ecosystems and human society (Su et al., 2013a; Zhang et al., 2022; Zhou et al., 2024). Antibiotic resistance reduces the effectiveness of antibiotics and increases the risk of treatment failure, complications, and mortality associated with bacterial infections (Woolhouse et al., 2016). The annual number of deaths due to antibiotic resistance is >700,000 globally and continues to rise (Lorenzo et al., 2018; Robinson et al., 2016). Projections suggest that the worldwide annual fatalities due to antibiotic resistance will exceed 10 million by 2050, accompanied by >100 trillion dollars of healthcare-related expenses (Robinson et al., 2016). The mortality is initially attributed to widespread ARGs (Amarasiri et al., 2020).

Wastewater treatment plants (WWTPs) are regarded as the reservoir of ARGs and antibiotic-resistant bacteria (ARB) because ARGs inducers, such as pathogenic bacteria and antibiotic residues, are received and accumulated in WWTPs (Pazda et al., 2019). ARB can carry ARGs and have resistance to antibiotics (Rizzo et al., 2013). Furthermore, over 99 % of ARGs in WWTPs are captured or adhered to the sludge, which leads to over 10^{11} copies/g TS (total solids) of ARGs commonly detected in sewage sludge (Zhang et al., 2022). The sludge from WWTPs eventually ends up in the land through sludge reuse or disposal, which poses a considerable risk of ARGs migration into surface waters, sediment, and soils, as well as the subsequent adverse impact on human health (Brown et al., 2020; Su et al., 2013b; Wang et al., 2020; Wang et al., 2024). Hence, it is crucial for WWTPs to understand and find ways to diminish or eliminate ARGs in sludge treatment.

Over the past two decades, temperature-phased anaerobic digestion (TPAD) has been widely industrialized for sludge treatment in WWTPs across various nations, such as Australia and Europe (Bungay and Abdelwahab, 2008; Wang et al., 2020). TPAD consists of the thermophilic anaerobic digestion (AD) process at a high temperature (~ 55 °C) and the mesophilic AD process at a mesophilic temperature (~ 37 °C) (Ge et al., 2010, 2011). Previous research has uncovered many advantages of TPAD compared to traditional mesophilic anaerobic digestion, which include improved biomethane generation, decreased sludge volume, less investment, and better pathogen control (Wang et al., 2017; Rajendran et al., 2020). However, the effect of TPAD on the fate of ARGs has never been investigated. Therefore, it is necessary for WWTPs to evaluate the ARGs' fate in the TPAD process for its wide implementation and the safe disposal of anaerobically digested sludge (ADS).

This study focuses on the ARGs' fate in sludge during the TPAD process in a real WWTP. The abundance of ARGs during the TPAD process in a full-scale WWTP (Australia) was investigated across three seasons (summer, autumn, and winter). Ten typical ARGs from various antibiotic classes were quantified by real-time quantitative polymerase chain reaction (RT-qPCR). Mobile genetic elements (MGE) are DNA segments that can move within or between genomes and cause ARGs transfer (Guo et al., 2017). The integrase gene of class 1 integron (*intI1*) as the representative mobile genetic elements (MGE) was quantified as well. Changes in the abundance of ARB (potential ARGs' hosts) were revealed through microbial community analysis to further reveal the potential mechanism of the TPAD process on ARGs removal. The effects of TPAD on the abundance of ARGs, removal efficiency of ARGs, and elimination mechanisms in real-application scenarios are investigated in this study.

2. Materials and methods

2.1. Sludge source and properties

The sludge was sampled from a full-scale WWTP with a TPAD unit in Melbourne, Australia. TPAD unit consists of sequential thermophilic (55 °C) AD and mesophilic (37 °C) AD, with a sludge retention time (SRT) of 11 days each.

To evaluate the fate of ARGs in the TPAD process, mixed sludge (MS, i.e. secondary sludge mixed with primary sludge), thermophilic anaerobically digested sludge (ADS), and mesophilic ADS samples, were collected from three sampling sites (Fig. 1), i.e., the influent of thermophilic anaerobic digestor, the effluent of thermophilic anaerobic digestor (the influent of mesophilic anaerobic digestor) and the effluent of mesophilic anaerobic digestor, respectively. Three sludge samples were obtained on three sequential and rainless days (i.e., Monday, Wednesday, and Friday) within one week in each season (summer, autumn, and winter) at each sampling site (Fig. 1). The average temperature of summer, autumn, and winter in the sampling area were 16.0–26.2 °C, 12.7–20.7 °C, and 7.6–14.6 °C, respectively. For sample collection and preparation, 50 mL of sludge samples obtained at the same site from each season were stored at −80 °C and then used for DNA extraction.

2.2. DNA extraction and ARGs quantification

DNA extraction of sludge samples was conducted by using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) following the manufacturer's instructions. The concentration of extracted DNA was detected by the Nanodrop ND-1000 (Nanodrop, USA). DNA integrity and purity were checked by the electrophoresis on a 1 % agarose gel.

12 ARGs representing the resistance to various antibiotics were selected in this study. This includes one aminoglycoside and fluoroquinolone resistance genes (*aac(6′)-Ib-cr*), one beta-lactamase resistance gene (*blaTEM*), two trimethoprim resistance gene (*drfA1* and *drfA7*), two sulfonamide resistance genes (*sul1* and *sul2*), two macrolides resistance genes (*mefA* and *ermb*), and six tetracycline resistance genes (*tetA*, *tetB*, *tetG*, and *tetX*) (Zhang et al., 2021 and 2022). Horizontal gene transfer (HGT) indicates the exchange of genetic material containing ARGs between bacteria in the same environment. The integrase gene (*intI1*) representing the MGE change involved in ARGs HGT was also quantified (Amos et al., 2018).

The abundance of the target genes was quantified by RT-qPCR in this study. The abundance of the 16S rRNA gene representing the total microbial biomass in the sludge was quantified (Burch et al., 2013). The absolute abundances of the targeted genes were standardized to gene copies/g TS. Every DNA sample was amplified in triplicate. The detailed information on annealing temperatures, primers, and q-PCR reaction matrix of target genes is listed in the supporting information (Tables S1 and Text S1, Supporting Information).

2.3. Microbial community analysis

For each DNA sample, the V3–V4 regions of bacterial 16S rRNA genes were amplified by using polymerase chain reaction (PCR) primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) with details provided in the supplementary information. The size of the amplicons was confirmed using agarose gel electrophoresis. The paired-end sequencing for the amplicons was performed by the Illumina MiSeq sequencing platform (Illumina, USA).

The raw sequences were demultiplexed using QIIME2. After demultiplexing, the resultant sequences were combined with FLASH (v1.2.11), and their quality was filtered with Fastp (0.19.0). The high-quality sequences were then de-noised using the DADA2 plugin in the Qiime2 (version 2020.2) pipeline using the suggested parameters, yielding a single nucleotide resolution based on sample error patterns. DADA2 denoised sequences were dubbed based on amplicon sequence variations (ASVs). To limit the impact of sequencing depth on alpha and beta diversity measures, the number of sequences from each sample was normalized to 4000, resulting in an average coverage of 97.90 %. ASV taxonomy was determined using the Blasts consensus taxonomy classifier implemented in Qiime2 and the SILVA 16S rRNA database (v138).

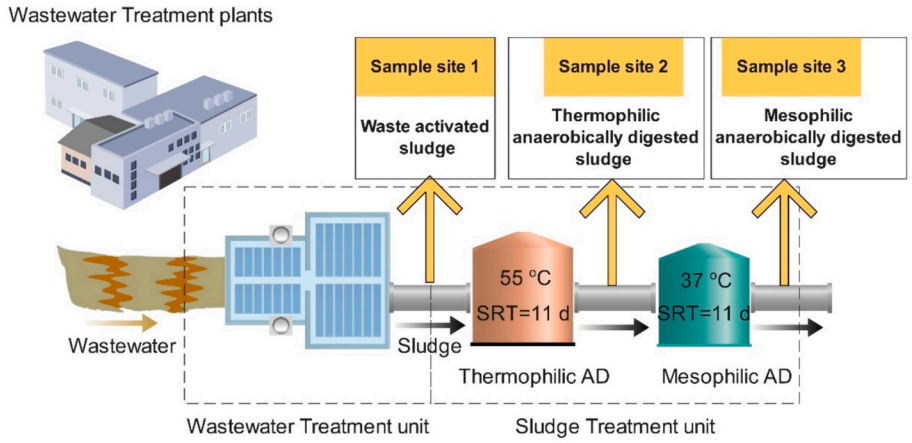

Fig. 1. Sampling sites and TPAD process in the full-scale WWTP.

2.4. Data analysis

Spearman's rank correlation coefficient (R) was used to determine the relationship between ARGs and *intI1*/microbial community (Zhang et al., 2021). Potential microbial hosts for targeted ARGs were selected based on the connection between the absolute abundance of a microbial genus and an ARG with $R > 0.8$ and $p < 0.05$ (Li et al., 2015). The correlation between ARGs and the *intI1*/microbial community was calculated and visualized using MATLAB R2022a (MathWorks, USA). The absolute abundance of bacteria was represented by numbers per gram TS, displayed using Hel's heatmap (v1.0). The absolute abundance of each microorganism was determined by multiplying the percentage of each microorganism by the abundance of 16S rRNA.

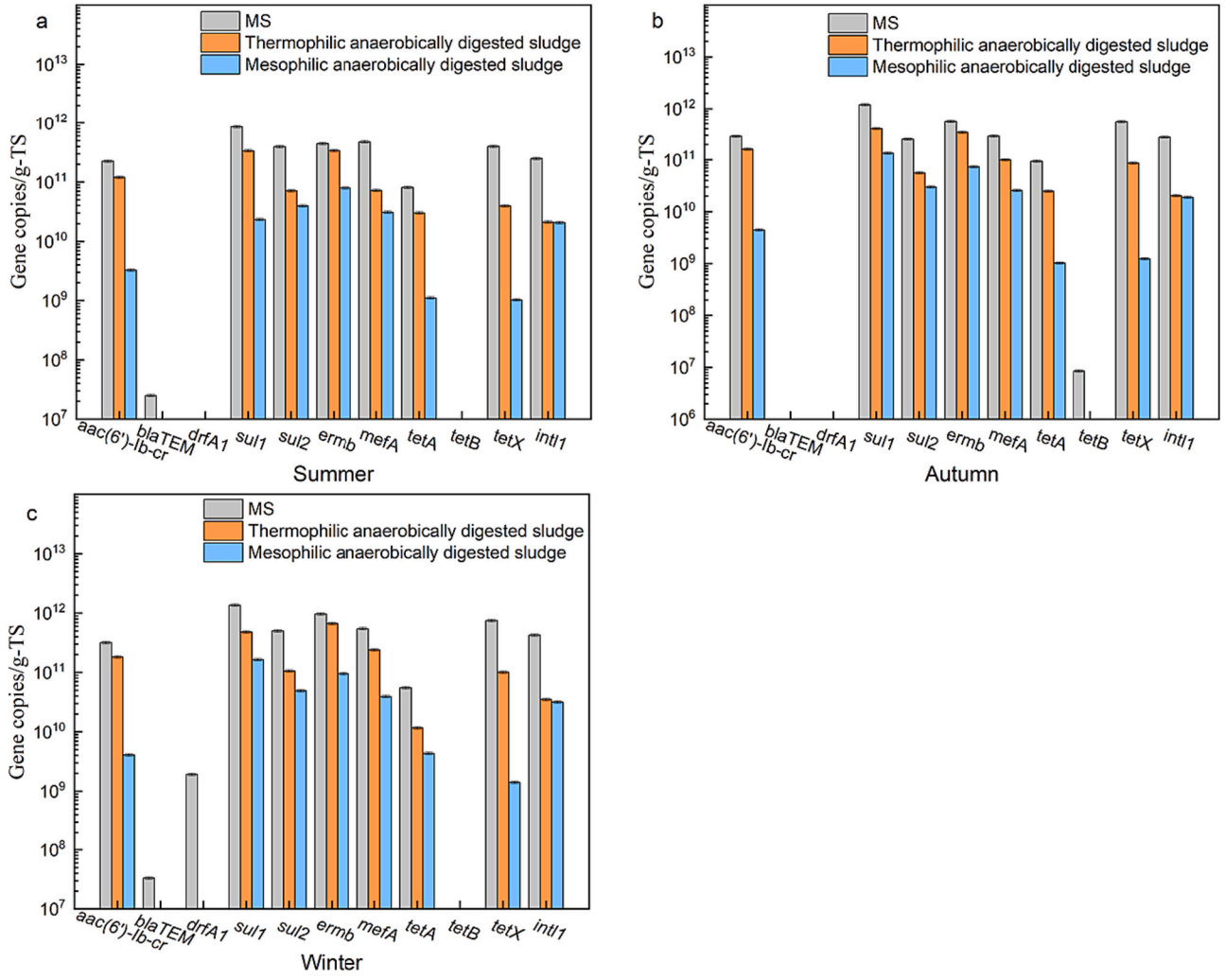

Fig. 2. Absolute abundances of ARGs and *intI1* in summer (a), autumn (b), and winter (c). The unshown bars indicate the ARGs were not detected in the season.

3. Results

3.1. Effects of thermophilic AD on the fate of ARGs and intI1 in the sludge

The absolute abundances of 12 ARGs (i.e. *aac(6′)-Ib-cr*, *blaTEM*, *drfA1*, *drfA7*, *sul1*, *sul2*, *mefA*, *ermb*, *tetA*, *tetB*, *tetG*, and *tetX*) and *intI1* in sludge before (i.e. MS) and after thermophilic AD across three seasons (i.e. summer, autumn, and winter) were quantified to evaluate the effect of thermophilic AD on the fate of ARGs and *intI1* (Fig. 2). Generally, *aac(6′)-Ib-cr*, *sul1*, *sul2*, *mefA*, *ermb*, *tetA*, *tetB*, and *tetX* were detected in all the samples across three seasons, ranging from 1.02×10^9 to 1.70×10^{13} gene copies/g TS. Two ARGs, *drfA7* and *tetG*, were not detected in all samples and thus not shown in the figures. *BlaTEM* was only detected in the summer and winter MS samples among all the sludge samples. In contrast, *drfA1* was only detected in the winter MS samples, and *tetB* was only detected in the autumn MS sample. Three ARGs (i.e., *BlaTEM*, *drfA1*, *tetB*) were entirely removed during thermophilic AD.

In summer, eight ARGs including *aac(6′)-Ib-cr*, *blaTEM*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* were detected in MS, which were 2.25×10^{11}, 2.50×10^{7}, 8.56×10^{11}, 3.95×10^{11}, 4.46×10^{11}, 4.77×10^{11}, 8.11×10^{11} and 3.98×10^{11} gene copies/g TS, while the absolute abundance of *intI1* was 2.49×10^{11} gene copies/g TS (Fig. 2a). Two ARGs, *drfA1* and *tetB*, were not detected in all sludge samples. Completed removal of *blaTEM* was achieved by thermophilic AD. Thermophilic AD reduced the absolute abundances of *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* to 1.20×10^{11}, 3.37×10^{11}, 7.11×10^{10}, 3.38×10^{11}, 7.22×10^{10}, 3.01×10^{10}, and 3.95×10^{10} gene copies/g TS, respectively, which were decreased by 46.7 %, 60.6 %, 82.0 %, 24.2 %, 84.9 %, 62.9 %, and 90.1 % compared to those in MS, respectively (Figs. 2 & 3). Thermophilic AD reduced the total absolute abundance of targeted ARGs by 65.0 % from 2.88×10^{12} to 1.01×10^{12} gene copies/g TS. Apart from that, thermophilic AD reduced the absolute abundance of *intI1* by 91.5 % and achieved 2.11×10^{10} gene copies/g TS in the thermophilic ADS (Figs. 2a & 3).

In autumn, eight ARGs were detected in MS, i.e. *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, *tetB*, and *tetX*, which were 2.83×10^{11}, 1.20×10^{12}, 2.50×10^{11}, 5.48×10^{11}, 2.87×10^{11}, 9.46×10^{10}, 8.50×10^{6} and 5.44×10^{11} gene copies/g TS, respectively. The *intI1* in MS was 2.74×10^{11} gene copies/g TS (Fig. 2b). Two ARGs, *blaTEM* and *drfA1*, were not detected in autumn sludge samples. Among the ARGs, *tetB* was entirely removed by thermophilic AD. Thermophilic AD decreased the absolute abundances of *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* by 43.5 %, 66.8 %, 77.6 %, 38.0 %, 65.2 %, 73.7 %, and 84.1 %, respectively. The absolute abundance of *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* in the thermophilic ADS were 1.60×10^{11}, 3.99×10^{11}, 5.59×10^{10}, 3.40×10^{11}, 9.98×10^{10}, 2.49×10^{10}, and 8.66×10^{10} gene copies/g TS (Figs. 2b & 3), respectively. The MGE (*intI1*) was decreased by 92.6 %, which was 2.04×10^{10} gene copies/g TS in the thermophilic ADS. Thermophilic AD reduced the total absolute abundance of the targeted ARGs by 63.6 % from 3.21×10^{12} in MS to 1.17×10^{12} gene copies/g TS in the thermophilic ADS. For *intI1*, thermophilic AD reduced the absolute abundance of *intI1* to 2.04×10^{10} gene copies/g TS in the thermophilic ADS, which was decreased by 92.6 % compared to that in MS (Figs. 2b & 3).

In winter, nine ARGs including *aac(6′)-Ib-cr*, *blaTEM*, *drfA1*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* were detected in MS, which were 3.17×10^{11}, 3.30×10^{17}, 1.91×10^{9}, 1.36×10^{12}, 5.01×10^{11}, 9.65×10^{11}, 5.47×10^{11}, 5.53×10^{10}, and 7.47×10^{11} gene copies/g TS. The MGE (*intI1*) was 4.25×10^{11} gene copies/g TS (Fig. 2c). The *tetG* was not detected in all the sludge samples. Two ARGs, *blaTEM* and *drfA1*, were completely removed during the thermophilic AD. Thermophilic AD also decreased the absolute abundances of *aac(6′)-Ib-cr sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* by 42.6 %, 64.8 %, 78.8 %, 31.0 %, 56.3 %, 79.0 %, and 86.5 %, respectively, which achieved 1.82×10^{11}, 4.79×10^{11}, 1.06×10^{11}, 6.66×10^{11}, 2.39×10^{11}, 1.16×10^{10}, and 1.01×10^{11} gene copies/g TS in thermophilic ADS (Figs. 2c & 3). Thermophilic AD reduced the total absolute abundance of the tested ARGs by 60.3 % (from 4.49×10^{12} to 1.78×10^{12} gene copies/g TS) in the sludge. For *intI1*, thermophilic AD can reduce the absolute abundance of *intI1* by 91.8 % to 3.49×10^{10} gene copies/g TS in the sludge (Figs. 2c & 3).

In comparison to the MS, thermophilic AD reduced the total targeted ARGs by 60.3 % - 65.0 % in the thermophilic ADS in three seasons. The average ARGs abundance across three seasons decreased from 3.53×10^{12} gene copies/g TS in MS to 1.32×10^{12} gene copies/g TS in thermophilic ADS with an average ARGs removal efficiency of 62.6 %. For *intI1*, thermophilic AD reduced the average absolute abundance of *intI1* by 91.9 % from 3.16×10^{11} to 2.55×10^{10} gene copies/g TS in the sludge.

3.2. Effects of mesophilic AD on the fate of ARGs and intI1 in the sludge

To evaluate the effect of mesophilic AD on the fate of ARGs and *intI1*, the absolute abundances of ARGs and *intI1* before (thermophilic ADS) and after mesophilic AD (mesophilic ADS) were investigated. The abundances of 7 ARGs (i.e. *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX*) were investigated during mesophilic AD because *drfA7* and *tetG* were not detected in all the samples and *blaTEM*, *drfA1*, *and tetB* were eradicated by thermophilic AD.

In summer, mesophilic AD further decreased the absolute abundance of *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA* and *tetX* by 97.3 %, 93.1 %, 44.3 %, 76.5 %, 57.2 %, 96.3 %, and 97.4 %, respectively, resulted to 3.26×10^{9}, 2.33×10^{10}, 3.96×10^{10}, 7.94×10^{10}, 3.09×10^{10}, 1.11×10^{9}, and 1.03×10^{9} gene copies/g TS in mesophilic ADS (Figs. 2 & 4). For *intI1*, mesophilic AD further decreased the absolute abundance by

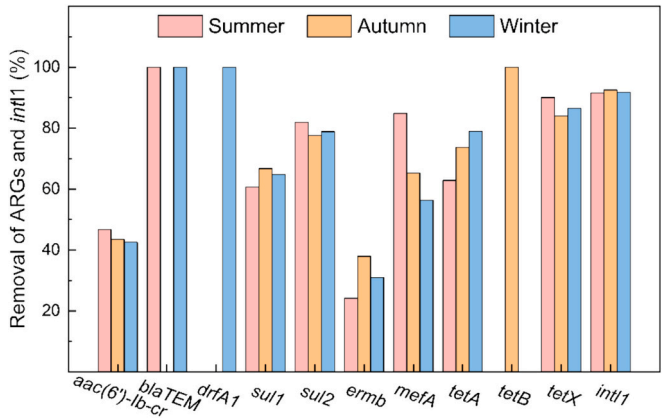

Fig. 3. Removal of ARGs and *intI1* in thermophilic AD. The unshown bars indicate the ARGs were not detected in the season.

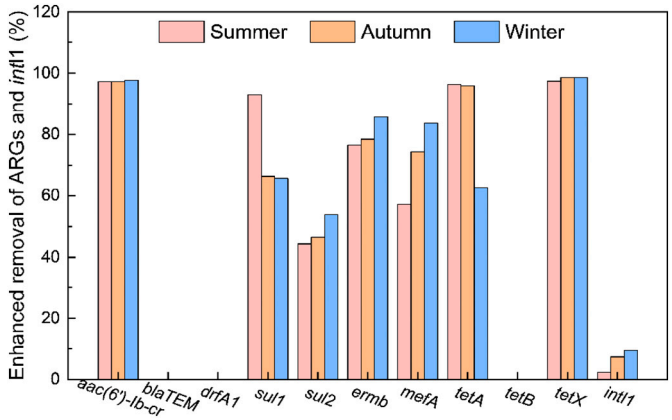

Fig. 4. Enhanced removal of ARGs and *intI1* in mesophilic AD. The unshown bars indicate the ARGs were not detected in the season.

2.4 % and achieved 2.06 × 10^{10} gene copies/g TS in the mesophilic ADS (Figs. 2 & 4). Additionally, mesophilic AD resulted in a further reduction of 82.3 % in the abundance of ARGs, achieving the total absolute abundance of ARGs of 1.79 × 10^{11} gene copies/g TS in the mesophilic ADS.

In autumn, mesophilic AD further decreased the absolute abundances of sludge *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* to 4.46 × 10^{9}, 1.34 × 10^{11}, 2.99 × 10^{10}, 7.33 × 10^{10}, 2.56 × 10^{10}, 1.02 × 10^{9}, and 1.23 × 10^{9} gene copies/g TS in the mesophilic ADS, respectively, which were decreased by 97.2 %, 66.4 %, 46.5 %, 78.4 %, 74.3 %, 95.9 %, and 98.6 %, respectively (Figs. 2 & 4). For *intI1*, mesophilic AD further decreased its absolute abundance to 1.89 × 10^{10} gene copies/g TS in the mesophilic ADS, which was only reduced by 7.4 % (Figs. 2 & 4). Additionally, mesophilic AD contributed to a further reduction in the total absolute abundance of ARGs of 76.9 %, resulting in the total absolute abundance of 2.70 × 10^{11} gene copies/g TS in the mesophilic ADS.

In winter, mesophilic AD decreased the absolute abundance of *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* to 4.07 × 10^{9}, 1.64 × 10^{11}, 4.90 × 10^{10}, 9.54 × 10^{10}, 3.92 × 10^{10}, 4.32 × 10^{9}, 1.40 × 10^{9} gene copies/g TS in the mesophilic ADS (Fig. 2), respectively, resulting in removal efficiencies of 97.8 %, 65.8 %, 53.8 %, 85.7 %, 83.6 %, 62.8 %, and 98.6 %, respectively (Figs. 4 & 5). For *intI1*, mesophilic AD decreased its absolute abundance by 9.5 % to 3.16 × 10^{10} gene copies/g TS in the mesophilic ADS (Figs. 2 & 4–5). In addition, mesophilic AD promoted a further reduction of the total absolute abundance of ARGs to 3.57 × 10^{11} gene copies/g TS, which was removed by 80.0 % in mesophilic ADS (Figs. 2 & 4–5).

Compared to the thermophilic ADS, mesophilic AD further relatively reduced the total ARGs abundance by 76.9 % - 82.3 % in the sludge across three seasons. The average total ARG removal efficiency during mesophilic AD was 79.7 % (from 1.32 × 10^{12} to 2.69 × 10^{11} gene copies/g TS) (Fig. 5). For *intI1*, mesophilic AD reduced the average absolute abundance of *intI1* by 6.9 % to 2.37 × 10^{10} gene copies/g TS in the mesophilic ADS (Fig. 2).

Overall, TPAD can remove >90 % of the total targeted ARGs and *intI1* in MS. TPAD reduced the *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* by 82.2 % - 99.8 % and entirely removed the *blaTEM*, *drfA1*, and *tetB* in sludge (Figs. 5 & 6). Among the TPAD process, thermophilic AD mainly contributed to 60.6 %–66.8 %, 77.6 %–82.0 %, 56.3 %–84.9 %, 62.9 %–79.0 % and 84.1 %–90.1 % removals of *sul1*, *sul2*, *mefA*, *tetA* and *tetX*, respectively, while mesophilic AD only contributed to the further 8.0 %–36.6 % removals of these ARGs (Fig. 6). The sole thermophilic AD process completely removed the ARGs of *blaTEM*, *drfA1*, and *tetB*. For *aac(6′)-Ib-cr* and *ermb* removal, mesophilic AD contributed to higher removal efficiencies of 51.9 %–56.1 % and 48.7 %–59.1 %, respectively, while thermophilic AD process contributed to slightly lower removal efficiencies of 42.6 %–46.7 % and 24.2 %–38.0 %, respectively (Fig. 6). Overall, TPAD achieved an average total ARG removal efficiency of 92.4 % (from 3.53 × 10^{12} to 2.69 × 10^{11} gene copies/g TS) (Figs. 5 & 6), where thermophilic AD contributed to 62.6 % of the total removal efficiency (92.4 %) and mesophilic AD contributed to the further 29.8 % of the total removal efficiency (92.4 %). For *intI1*, TPAD reduced the average absolute abundance of *intI1* by 92.5 % in the sludge (Fig. 6), where thermophilic AD contributed to 91.9 % of the total removal efficiency (92.5 %).

3.3. Correlation between ARGs and intI1/microbial community and effect of TPAD on microbial community in the sludge

The correlation between the ten detected ARGs and the *intI1*/microbial community at the genus level is shown in Fig. 7. It should be noted that bacteria exhibited positive correlations with ARGs, whereas archaea did not display any correlations with ARGs. Therefore, results related to archaea were omitted in this study. The results suggested that nine bacteria (among the top 30 in the absolute abundance) had significant positive correlations with nine ARGs ($R > 0.8$, $p < 0.05$) (Fig. 7), where *tetB* did not significantly correlate to the bacteria. The nine bacteria are all mesophilic bacteria, including *Comamonadaceae*, *p-251-o5*, *Lachnospiraceae*, *Arcobacter*, *Flavobacterium*, *Prevotella*, *Pseudarcobacter*, *Aeromonas*, and *Butyrivibrio*. These bacteria are regarded as the ARB, which are potential hosts for ARGs due to the strong positive correlations between these bacteria and ARGs. The *intI1* is considered as an indicator of HGT potential, which can facilitate ARGs transfer (Wu et al., 2016). The results revealed that *intI1* had a significant correlation (R > 0.8, p < 0.05) with seven ARGs including *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* in this study (Fig. 7).

Fig. 8 further shows the absolute abundance of the top 30 bacteria (at the genus level) in MS, thermophilic ADS, and mesophilic ADS. TPAD reduced the abundance of the 9 ARB by 1–3 magnitudes, i.e., *Comamonadaceae*, *p-251-o5*, *Lachnospiraceae*, *Arcobacter*, *Flavobacterium*, *Prevotella*, *Pseudarcobacter*, *Aeromonas*, and *Butyrivibrio*, through thermophilic AD and mesophilic AD (Fig. 8 and Figs. S1–3). For instance, the average absolute abundances of *Flavobacterium* and *Butyrivibrio* decreased from 5.7 × 10^{10} and 3.6 × 10^{10} gene copies/g TS in MS to 2.3 × 10^{10} and 8.9 × 10^{9} gene copies/g TS in the thermophilic ADS, respectively, which were then decreased to 6.6 × 10^{7} and 6.5 × 10^{7} gene copies/g TS in the mesophilic ADS, respectively. The decreased absolute abundance of these ARB contributed to the removal of ARGs.

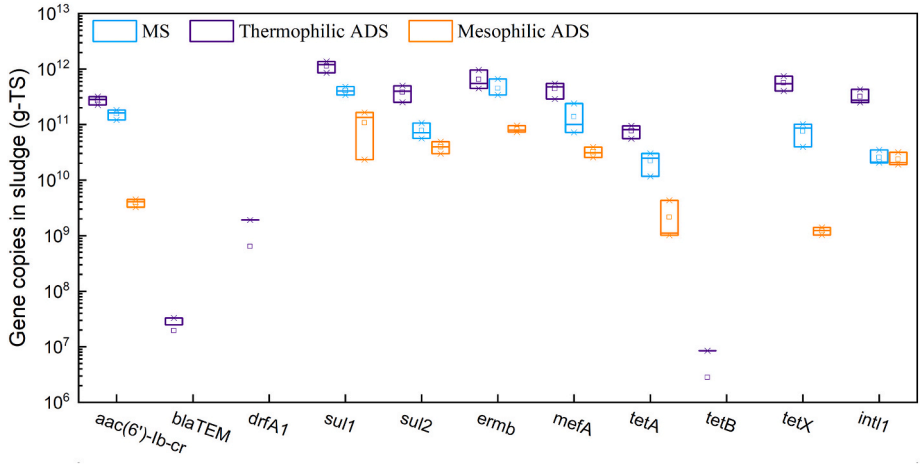

Fig. 5. Absolute abundances of ARGs and *intI1* in sludge. The unshown items indicate the ARGs were not detected in the sample.

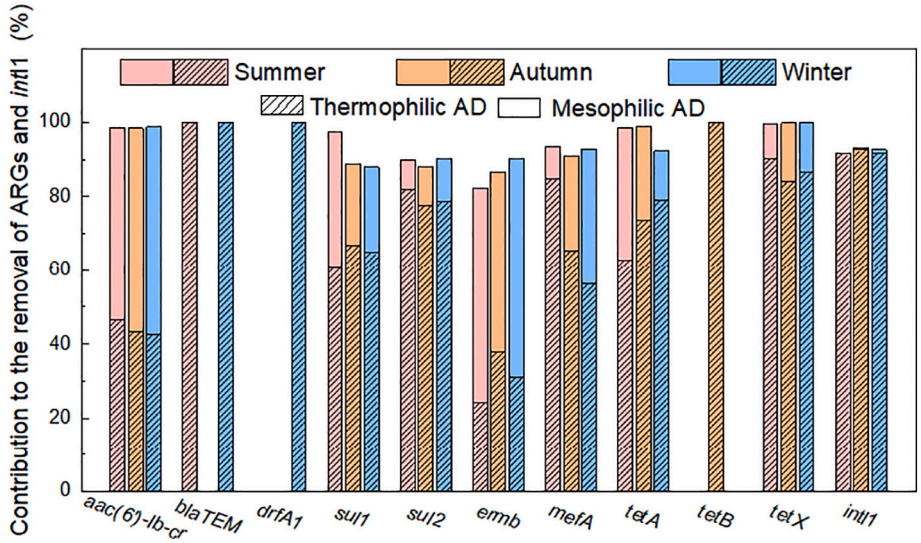

Fig. 6. Contribution to the removal of ARGs and *intI1* from thermophilic and mesophilic AD. The unshown bars indicate the ARGs were not detected in the season.

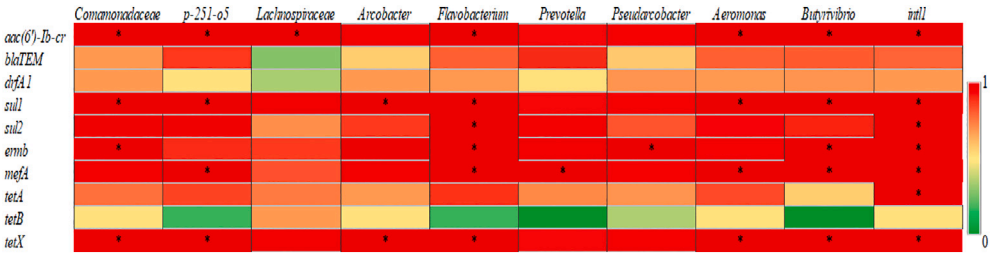

Fig. 7. Correlation between ARGs and *intI1*/microbial community at the genus level. An asterisk (*) indicates a significant positive correlation ($R > 0.8$, $p < 0.05$).

4. Discussion

This study investigated the fate of ARGs in the sludge during the TPAD in a full-scale WWTP. The results indicated that TPAD reduced 10 common ARGs by 82.2 % - 100.0 % and reduced the average total absolute abundance of ARGs by 92.4 % throughout 3 seasons summer, autumn, and winter. Therefore, TPAD can act as a solution that minimizes the risks associated with ARG dissemination considering the spread of ARGs from ADS to the environment during sludge disposal (Ju et al., 2016; Zhang et al., 2021; Zou et al., 2020), The great effectiveness of TPAD on ARGs removal in this study supports that the continued and future implementation of TPAD in WWTPs will promote the safe reuse of sludge. Therefore, TPAD can contribute to human health and environmental safety when it is applied to sludge disposal and reuse.

TPAD has comparable or better performance in ARGs removal compared to other biological sludge treatment methods, such as mesophilic AD, thermophilic AD, and aerobic digestion, while it is less powerful than thermal sludge treatment methods such as incineration and pyrolysis. Sole mesophilic AD was reported to reduce 20 %–45.8 % of ARGs, which exhibited a milder removal effect on ARGs than TPAD (Zhang et al., 2022; Mortezaei et al., 2023). In some cases, ARGs regrew and new ARGs appeared in mesophilic AD (Guo et al., 2017; Jang et al., 2017). Thermophilic AD was reported to have higher ARGs removal efficiencies of 65.0 % - 75.8 % compared to mesophilic AD (Tian et al., 2016; Mortezaei et al., 2023), while it was also reported unable to reduce ARGs and even lead to the regrowth of ARGs in some studies (Zhang et al., 2015). Similarly, aerobic digestion sometimes causes ARGs regrowth, while studies also reported up to three orders of ARGs removal were achieved by aerobic digestion (Zhang et al., 2022). On the contrary, thermal sludge treatment, i.e. pyrolysis and incineration, can eliminate ARGs to undetectable levels due to their high temperatures ranging from 300 °C - 700 °C and 200 °C -600 °C, respectively (Chu et al., 2020; Costa et al., 2023).

The total absolute abundance of targeted ARGs in sludge seasonally varied before and after TPAD in the WWTP. Generally, the total absolute abundance of ARGs in winter MS is higher than those in summer and autumn. The total absolute abundance of ARGs in winter MS was 4.49×10^{12} gene copies/g TS, which was 1.55 and 1.40 times of those in summer and autumn (2.88×10^{12} and 3.21×10^{12} gene copies/g TS), respectively. Correspondingly, the total absolute abundance of ARGs after TPAD was 3.57×10^{11} gene copies/g TS in winter, which was 1.99 and 1.32 times higher than in summer and autumn (1.79×10^{11} and 2.70×10^{11} gene copies/g TS). More ARGs in winter sludge may be related to the amount of medicine (including antibiotics) used in different seasons. Typically, winter is a high-incidence season for respiratory diseases such as influenza and pneumonia, when more medicine like antibiotics is used and then discharged to WWTPs (Walker, 2012; Wang et al., 2019). The use of large amounts of antibiotics leads to the accumulation and proliferation of ARGs in WWTPs. The higher abundance of ARGs in winter sludge tends to lead to a greater spread of ARGs than in summer and autumn sludge (Birošová et al., 2014; Shen et al., 2022). Therefore, surpassing the abundance of ARGs in sludge in winter is a potential problem for sustaining a lower ARGs level in discharged sludge (mesophilic ADS). Strategies such as SRT changing, pretreatment, and post-AD treatment have been reported to improve the removal of ARGs in anaerobic digestion (Gurmessa et al., 2020). Extending SRTs promoted ARGs removal in mesophilic AD, while shortening SRTs facilitated ARGs removal in thermophilic AD (Mortezaei et al., 2023). Post-AD treatments, such as improving sludge storage, composting of ADS, and converting ADS into biochar, facilitate the removal of ARGs in ADS (Li et al., 2018; Jang et al., 2019; Gurmessa et al., 2020). These strategies desire future research to improve the ARGs

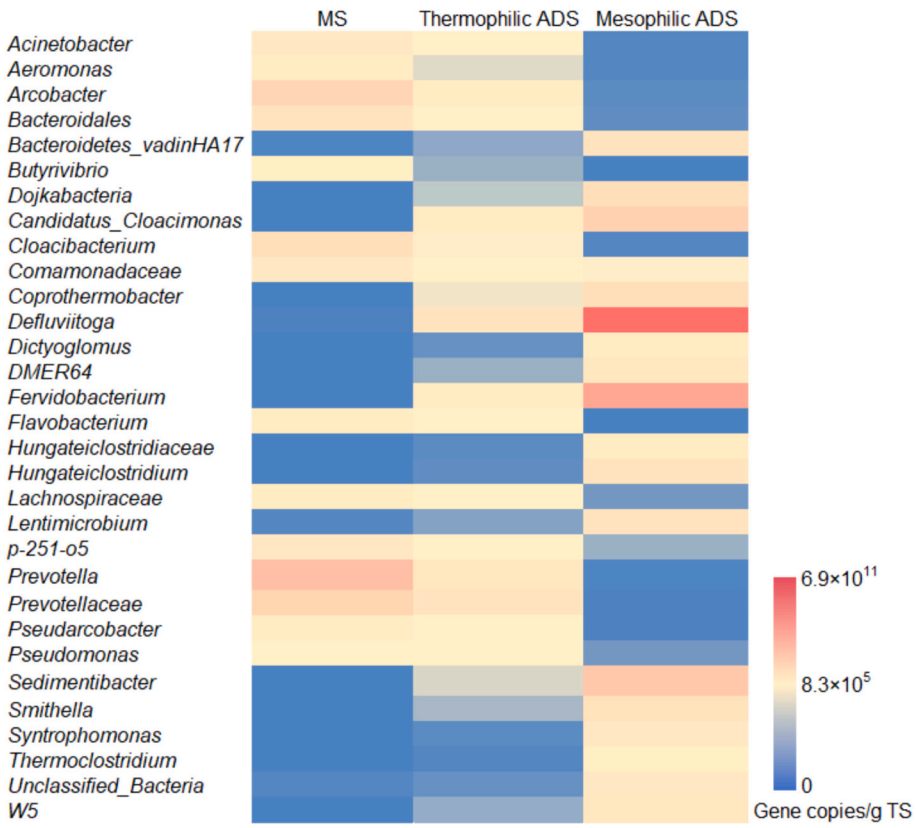

Fig. 8. Heatmap of the top 30 most abundant microorganisms (at the genus level) in MS, thermophilic ADS, and mesophilic ADS.

removal of winter sludge in TPAD.

Among TPAD processes, thermophilic AD played a key role for an average ARGs removal efficiency of 62.6 %, and mesophilic AD led to a further ARGs removal efficiency of 29.8 %, resulting in a total average ARGs removal efficiency of 92.4 % during TPAD. The higher removal efficiency of ARGs during thermophilic AD indicates that thermophilic AD mainly contributed to the removal of ARGs in the TPAD process. This is closely related to the higher temperature used in thermophilic AD compared to normal mesophilic AD. Previous research has demonstrated that temperature is one of the most important parameters affecting the removal of ARGs in an anaerobic environment (Tian et al., 2016; Zhang et al., 2022). Sun et al. (2016) reported that thermophilic AD (55 °C) contributed to a 60 % higher reduction of detected ARGs removal than mesophilic AD (35 °C). Mortezaei et al. (2023) indicated less abundant ARGs in the thermophilic AD than in the mesophilic AD, where the removal rates of ARGs were 75.8 % vs. 45.8 %, respectively. This study conducted the thermophilic AD under 55 °C temperature with a SRT of 11 days, which facilitated the removal of ARGs.

High temperature during thermophilic AD enhanced the elimination of mesophilic ARB and *intI1*, which consequently promoted the removal of ARGs. It has been reported that a lot of mesophilic bacteria, including the ARBs, cannot survive for long at 55 °C (Miller et al., 2016; Wu et al., 2016). This is consistent with the shift of microbial community during thermophilic AD in this study. The absolute abundance of mesophilic bacteria including ARB decreased, while the absolute abundance of thermophilic bacteria (i.e., *Defluviitoga*, *Fervidobacterium*, *Thermoclostridium*, *and Dictyoglomus*) increased in the community (Fig. 8) (Baudrexl et al., 2019; Maus et al., 2016; Saiki et al., 1985). Furthermore, high temperature during the thermophilic AD contributed to the removal of *intI1*, suppressing the HGT potential. This is also in accordance with the previous studies that *intI1* was attenuated in an anaerobic environment under high temperature (37–55 °C) (Miller et al., 2016; Xu et al., 2020; Zhang et al., 2015). It has been reported that thermophilic AD (50–60 °C) removed >75 % fractions of *intI1* (Ghosh et al., 2009). Wu et al. (2016) reported a two-phase thermophilic digestion (thermophilic acidogenic phase reactor + mesophilic methanogenic phase reactor) achieved ~70 % removal of *intI1*. In this study, the absolute abundance of *intI1* was reduced by 91.7 % – 93.1 % through the thermophilic AD, while it reduced by <1 % in the subsequent mesophilic AD compared to its abundance in MS (Sections 3.1–3.2). Simultaneously, *intI1* was positively correlated with seven ARGs including *aac(6′)-Ib-cr*, *sul1*, *sul2*, *ermb*, *mefA*, *tetA*, and *tetX* (Section 3.3), suggesting that these seven ARGs were heavily harbored on *intI1*. The reduced abundance of *intI1* in the thermophilic AD thereby contributed to the removal of ARGs because of the less HGT among the microbial community.

Although thermophilic AD was the major contributor to ARGs removal, the subsequent mesophilic AD also contributed to a further 29.8 % average total ARG removal. This can be attributed to alter in the microbial community because of the temperature variation from thermophilic AD to mesophilic AD. It has been reported that microbial communities are the main determinants of ARG abundance in ADS (Aziz et al., 2022). Additionally, sole mesophilic AD was also extensively reported to remove ARGs (Guo et al., 2017). For example, Zhang et al. (2021) reported that mesophilic AD achieved a 40 % removal of ARG. Furthermore, the absolute abundance of *intI1* did not regrow and kept a consistent level in mesophilic AD after it was reduced by 92.5 % through thermophilic AD. This suggests mesophilic AD process combined with thermophilic AD (i.e., TPAD) provides an effective environment for the ARGs removal in WWTPs.

5. Conclusions

The effect of TPAD on the fate of ARGs in the sludge was evaluated in a full-scale WWTP. The following conclusions can be drawn from this study:

- TPAD reduced the average absolute abundance of targeted ARGs by 92.4 % in the sludge in a WWTP across summer, autumn, and winter. TPAD also effectively removed the *intI1* by 92.5 %. This indicates TPAD can reduce the spread of ARGs from the sludge to the natural environment through sludge reuse or disposal.
- Thermophilic AD plays a major role in the ARGs and *intI1* removals. The sole thermophilic AD process completely removed the ARGs of *blaTEM*, *drfA1*, and *tetB*. Thermophilic AD contributed to the 62.6 % and 91.9 % of the average total ARGs removal (92.4 %) and *intI1* removal (92.5 %), respectively. Mesophilic AD contributed a further 29.8 % and 0.6 % of the average total ARGs removal (92.4 %) and *intI1* removal (92.5 %), respectively.
- The removal of ARGs through TPAD is related to the decreased abundance of ARB (potential microbial hosts for ARGs) and the decreased abundance of *intI1*. It is attributed to the high temperature and the temperature variation during the TPAD process.
- The total absolute abundance of targeted ARGs in sludge seasonally varied before and after TPAD in the WWTPs, which was higher in winter than in summer and autumn. This is related to the abundant use of antibiotics against the high-incidence respiratory infection.

CRediT authorship contribution statement

Huan Liu: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Zehao Zhang:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Xuan Li:** Writing – review & editing. **Ting Zhou:** Writing – review & editing. **Zhenyao Wang:** Visualization. **Jibin Li:** Methodology. **Yi Li:** Visualization. **Qilin Wang:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2024.171555.

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