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Free ammonia pretreatment enhances the removal of antibiotic resistance genes in anaerobic sludge digestion

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ABSTRACT: Sludge has been recognized as a reservoir of antibiotic resistance genes (ARGs) in the wastewater treatment plants. Our previous study has demonstrated that free ammonia (FA, i.e., NH_3-N) pretreatment is an effective method for enhancing anaerobic digestion of sludge. However, the effect of FA pretreatment on the removal of ARGs in the anaerobic sludge digestion is still unknown. In this study, several ARGs representing various antibiotic classes and integrase gene (*intI1*) which is crucial for horizontal transfer of ARGs were chosen. This study demonstrated for the first time that combined FA pretreatment (420 mg NH_3-N/L for 24 h) and anaerobic digestion could enhance the removal of *aac(6')-Ib-cr*, *blaTEM, sul2, tetA*, *tetB* and *tetX* from sludge by 17~74% compared with anaerobic digestion without FA pretreatment, resulting in a lower ARGs abundance in the anaerobically digested sludge. This is caused by the removal of tested ARGs during FA pretreatment and the reduced abundance of potential bacterial hosts of ARGs due to FA pretreatment during anaerobic digestion. The removal of *IntI1* was not significantly affected by FA pretreatment and *intI1* only had a significant correlation with one ARG *sul1* in this study, indicating that *intI1* did not play a large role in the fate of the tested ARGs in this study. This study indicated that FA pretreatment for anaerobic digestion could potentially reduce the spread of ARGs from the sludge to the natural environment during sludge disposal or reuse.

Keywords

Antibiotic resistance genes; Free ammonia; Anaerobic digestion; Wastewater treatment plants

1. Introduction

Antibiotics have been widely used for human and livestock to treat infectious diseases and promote the growth of livestock (Zhuang et al., 2015). Though the effectiveness of antibiotics has significantly benefited mankind, the intensive use of antibiotics has led to the spread of antibiotic resistance among microorganisms. Antibiotic resistance genes (ARGs), as the main reason for microorganisms to be able to withstand the bacteriostatic or bactericidal effects of antibiotics (Martínez et al., 2014), have been widely found in soil, surface water, groundwater, PM 2.5, and even deep ocean sediments (Allen et al., 2010; Brown and Balkwill, 2009; Ouyang et al., 2020). The spread of ARGs not only posed a global threat to the public well-being, but also affected the development of industries such as veterinary medicine and agriculture (Teuber, 2001).

Generally, the spread of ARGs is considered a result of natural selection when microorganisms are exposed to antibiotic compounds (Negreanu et al., 2012). In unaltered states, up to 95% of antibiotics can be exerted from humans or animals, which enter into the sewers and are eventually collected by wastewater treatment plants (WWTPs) (Negreanu et al., 2012). This alters the selective pressure and leads to the occurrence of ARGs in the WWTPs microbial community. Furthermore, the ARGs can disseminate through vertical gene transfer by cell reproduction, and/or horizontal gene transfer by mobile genetic elements in the WWTPs microbial community (Shao et al., 2018; Xue et al., 2019).

Recent studies suggested that sludge from WWTPs might be a crucial source of ARGs to the environment because most of the ARGs in a WWTP eventually end up in the sludge (Xue et al., 2019; Yang et al., 2014). In particular, ARGs in sludge can reach a very high concentration. For instance, the abundances of tetracycline resistance genes *tetA* and *tetX* in sludge reached

1×10^{12} and 1×10^{13} gene copies/g-TS (total solids), respectively (Auerbach et al., 2007), which is about eight orders of magnitude higher than that of soil samples (1×10^{4} - 1×10^{5} gene copies/g-TS) (Duan et al., 2017; Nõlvak et al., 2016). Therefore, the existence of ARGs in sludge may lead to the spread of the ARGs from sludge to soil, considering a large amount of sludge is reused for agriculture in many regions such as Australia (>65%) and the United States (>50%) (Australian and New Zealand Biosolids Partnership, 2019; the United States Environmental Protection Agency, 2019). The potential spread of ARGs may have an adverse effect on the environment (e.g., soil) and increase the risk of sludge reuse to human health (Chen et al., 2016; Ross and Topp, 2015).

Anaerobic digestion is a typical sludge treatment method, which achieves sludge reduction and energy recovery (Batstone et al., 2002). Recently, additional benefits of anaerobic digestion have been observed with ARGs removal, where the total abundances of *tetA, tetB, tetC, tetW, tetX, sul1* and *sul2* were reduced in anaerobic digestion (Ma et al., 2011; Pei et al., 2016). However, the efficiency of anaerobic digestion is often limited by the poor biodegradability of the sludge (Carrère et al., 2010). Thus, various pretreatment methods such as ultrasonic and thermal pretreatment have been applied to enhance sludge biodegradability and sludge reduction (Bougrier et al., 2006). Additional benefits of pretreatment have been observed recently on the removal of ARGs. For instance, ultrasonic pretreatment enhanced the removal of the targeted ARGs by 50% during anaerobic digestion and further reduced the abundance of the bacterial hosts of ARGs (Wang et al., 2019).

Free ammonia (FA., i.e., NH_3) pretreatment was found as an economically attractive and environmentally friendly approach to enhance the anaerobic digestion efficiency, as it only relies on a by-product (i.e., FA) of wastewater treatment (Wang, 2017; Wei et al., 2017a). Our

previous study demonstrated that FA pretreatment at 420 mg N/L for 24 h enhanced sludge biodegradability by 20% (Wei et al., 2017a). However, the effect of FA pretreatment on the removal of ARGs in anaerobic sludge digestion is still largely unknown. Understanding this effect will be beneficial and essential for the practical application of the FA pretreatment strategy.

This study aimed to assess the effect of FA pretreatment on the abundances of ARGs in anaerobic sludge digestion. The full-scale secondary sludges with and without FA pretreatment were subjected to anaerobic digestion tests. Nine ARGs representing various antibiotic classes were quantified by real-time quantitative polymerase chain reaction (RT-qPCR). The integrase gene (*intI1*) of Class 1 integrons was also quantified as a representative of mobile genetic elements which could affect the horizontal transfer of ARGs. The abundances of ARGs and *intI1* in FA-pretreated sludge were quantified to evaluate the fate of ARGs and *intI1* during the FA pretreatment. Microbial community analysis and the correlation between ARGs and *intI1*/microbial community were also determined to reveal the abundance change of the potential bacterial hosts of ARGs, which aimed to further understand the potential mechanisms underpinning the effect of FA pretreatment on the removal of ARGs in anaerobic sludge digestion.

2. Materials and methods

2.1 Sludge sources

Both secondary sludge and inoculum were used to conduct the experiments. The secondary sludge was collected from the thickener of a WWTP conducting biological nitrogen and phosphorus removal. The WWTP has a sludge retention time (SRT) of 12 - 16 d. The inoculum was collected from a lab-scale mesophilic anaerobic digester receiving real secondary sludge

from the same WWTP. The mesophilic anaerobic digester has an SRT of 15 d. The secondary sludge and inoculum were used for the anaerobic digestion tests to be described below.

2.2 Pretreatment of secondary sludge with FA

Batch experiments were conducted to evaluate the impact of FA pretreatment on the abundances of ARGs and *intI1*. One liter of secondary sludge was evenly distributed into two batch reactors as control and experimental reactors, respectively. For the experimental reactor with FA pretreatment, a certain amount of ammonium stock solution (3.0 M) was added to obtain an ammonia concentration of 500 mg N/L. pH was adjusted and maintained at 10.0 ± 0.1 using NaOH solution. The pretreatment was conducted in a temperature-controlled room (25 °C) with a stirring speed of 500 rpm using a magnetic stirrer for 24 h. The total ammonia nitrogen, pH and the temperature collectively resulted in an FA concentration of 420 mg NH_3-N/L, which was determined by the formula $S_{(NH_4^+-N+NH_3-N)} \times 10^{pH}/(K_b/K_w+10^{pH})$ (Anthonisen et al., 1976). The $S_{(NH_4^+-N+NH_3-N)}$ is the total ammonia nitrogen concentration. The K_b/K_w is equal to $e^{6,344/(273+T)}$. This ammonia concentration (i.e., 420 mg NH_3-N/L) was selected based on our previous study, which demonstrated that FA pretreatment at 420 mg NH_3-N/L for 24 h led to the highest methane production potentially with a large economic advantage (Wei et al., 2017a). The control reactor was set up without ammonium addition or pH control. For both reactors, sludge samples were taken both before and after pretreatment for the determination of ARGs and *intI1* using RT-qPCR, as described below.

2.3 Anaerobic digestion tests

Anaerobic digestion tests were performed to evaluate the effect of FA pretreatment on the removal of ARGs during anaerobic sludge digestion. The serum vials (160 mL) with a working volume of 100 mL were used for the anaerobic digestion tests. The inoculum and the secondary

sludges with and without FA pretreatment were added into two separate serum vials, resulting in a VS (volatile solids) based inoculum to sludge ratio of approximately 2.0. The vials were flushed with helium gas for 2 min (1 L/min) to ensure an anaerobic condition. After that, a rubber stopper with an aluminum crimp cap was used to seal the vials. The sealed vials were then put into an incubator operated at 37 °C. Blank was also set up, which only contained inoculum and MilliQ water (i.e., without secondary sludge). The anaerobic digestion tests lasted for 45 days. At the end of the anaerobic digestion tests, the anaerobically digested sludges with and without FA pretreatment as well as blank were sampled to analyze ARGs, *intI1,* and microbial community as described in the following sections.

2.4. Quantification of ARGs and *intI1*

Sludge samples were collected into centrifuge tubes and then centrifuged at 10,000 rpm and 4 °C for 10 min to collect the pellet for DNA extraction. 0.25 g pellet of each sample was used for DNA extraction using the Fast DNA Spin Kit for Soil (MP Biomedicals, USA) according to the manufacture's instruction. The integrity of extracted DNA was measured by gel electrophoresis (1% agarose). The concentration and purity of extracted DNA were confirmed by a NanoDrop ND-1000 (NanoDrop, USA).

RT-qPCR was used to quantify the abundance of the target genes in this study. One aminoglycoside and fluoroquinolone resistance gene (*aac(6')-Ib-cr*), one beta-lactamase resistance gene (*blaTEM*), two sulfonamide resistance genes (*sul1 and sul2*), and five tetracycline resistance genes (*tetA, tetB, tetG, tetM* and *tetX*) were chosen to represent ARGs in this study. The selected ARGs represent various antibiotic classes. The *intI1* was also included in the quantification to represent the change of mobile genetic elements. The *intI1* is prevalent in the bacterial community and plays a key role in the spread of ARGs (Amos et al.,

2018). The 16s rRNA gene was also quantified to represent the total bacterial biomass in the sludge (Burch et al., 2013). Each DNA sample was amplified in triplicate. The detailed information of annealing temperatures, primers and q-PCR reaction matrix of target genes is listed in the supporting information (Tables S1 and Text S1, Supporting Information).

In this study, the absolute abundances of the target genes were normalized to the gram of TS (i.e., gene copies/g-TS). The relative abundances of the target genes were normalized to 16S rRNA genes (i.e., gene copies/16s rRNA) as an indicator of the proportion of bacteria carrying ARGs and *intI1*.

2.5 Microbial community analysis

The microbial community was analyzed by a high-throughput sequencing method. PCR primers 341F (5'-CCTACGGGAGGCAGCAG-3') and 806R (5'-ATTACCGCGGCTGCTGG-3') were used to amplify the V3–V4 regions of 16S rRNA genes. The extracted DNA in each sample was amplified in triplicate in PCR to acquire amplicons (see Tables S1 for the details of PCR). The amplicons were further purified with GeneJET^{TM} Gel Extraction Kit (Thermo Scientific, USA). A fluorometer of Qubit 2.0 (Thermo Scientific, USA) was used to determine the concentrations of the amplicons. Amplicons were then subjected to small fragment library construction and pair-end sequencing using the Illumina NovaSeq PE250 platform (Illumina, USA).

The sequencing reads of each sample was performed under specific filtering conditions to obtain the high-quality reads according to the Cutadapt (v1.9.1) quality-controlled process (Martin, 2011). Subsequently, the PCR chimeras were filtered using UCHIME (v4.2) algorithm

(Edgar et al., 2011) by comparing it with Silva (v132) database (Quast et al., 2012). Then, the clean reads of each sample were finally obtained.

Sequences analysis was performed by UPARSE software (v7.0.1001) (Edgar, 2013). Sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs). The representative sequence for each OTU was screened for further annotation. For each representative sequence, the Silva (v132) database was used based on the Mothur algorithm to annotate taxonomic information (Quast et al., 2012).

2.6 Data analysis

The correlation between ARGs and *IntI1*/microbial community was determined by spearman's rank correlation coefficient (R) using SPSS 25.0 (IBM, USA), which is a powerful tool for providing new insights into ARGs and their potential bacterial hosts in complex environmental examples (Song et al., 2017). A correlation between bacterial genus and an ARG with a $R > 0.8$ and $p < 0.05$ was considered as a potential bacterial host for the selected ARGs (Li et al., 2015). Visualization of the correlation between ARGs and *IntI1*/microbial community was produced by Matlab R2020a (MathWorks, USA) platform. The abundance of bacteria was normalized to gram of TS to obtain its absolute abundance and was also visualized using heatmap by Heml (v1.0).

3 Results

3.1 Effects of FA pretreatment on the fate of ARGs and *intI1* in the sludge prior to anaerobic digestion

The abundances of ARGs and *intI1* in the sludge with and without FA pretreatment were quantified to evaluate the effect of FA pretreatment on the fate of ARGs and *intI1*. The absolute

abundances of *aac(6')-Ib-cr, blaTEM, sul1*, *sul2*, *tetA*, *tetB*, *tetG, tetM, tetX and intI1* were ranged from 7.8×10^6 to 3.4×10^9 gene copies/g-TS (Fig. 1). FA pretreatment decreased the absolute abundances of *blaTEM, sul1, sul2, tetA*, *tetB* and *tetX* by 21%, 8%, 16%, 73%, 38% and 76%, respectively (Fig. 2). Although *tetA*, *tetB, tetX, tetM* and *tetG* all belong to the tetracycline resistance genes, different impacts of FA pretreatment were observed on *tetM* and *tetG* (Figs. 1 and 2). FA pretreatment increased the absolute abundance of *tetM* by more than three times, while an insignificant change was observed in the absolute abundance of *tetG*. The insignificant abundance change of *tetG* could be attributed to the fact that *tetG* fragment was more conservative to FA pretreatment in comparison to *tetA, tetB* and *tetX* (Zhang and Zhang, 2011). The reason for the increased absolute abundance of *tetM* during FA pretreatment required further study. In addition, FA pretreatment did not significantly (p>0.05) affect the absolute abundances of *aac(6')-Ib-cr* and *intI1* (Figs. 1 and 2).

(Position for Fig. 1)

(Position for Fig. 2)

Overall, FA pretreatment reduced the total absolute abundance of the tested ARGs by ~10% in the sludge, indicating that some ARGs could be removed by FA directly in the sludge pretreatment. In contrast, the absolute abundance of *intI1* in the sludge was not significantly affected by FA pretreatment.

To determine the proportion of bacteria carrying ARGs and *intI1*, ARGs were normalized to 16S rRNA gene abundance to get the relative abundance (Fig. 3). Similar trends to the absolute abundances were found in the relative abundances of ARGs and *intI1* (Fig. 3). FA pretreatment decreased the relative abundances of *blaTEM, sul1, sul2, tetA*, *tetB* and *tetX* by 15~76%. In

contrast, FA pretreatment increased the relative abundance of *tetM* by more than three times (Fig. 3). In terms of the relative abundances of *aac(6')-Ib-cr*, *tetG and intI1*, FA pretreatment showed insignificant impact (p>0.05). In total, FA pretreatment slightly decreased the relative abundance of the total tested ARGs by ~10%. These results indicate that FA pretreatment could reduce the proportion of the targeted ARGs in the biomass but had a negligible effect on *intI1*.

(Position for Fig. 3)

3.2 FA pretreatment enhanced the removal of ARGs in anaerobic sludge digestion

The absolute abundances of ARGs and *intI1* in untreated sludge and anaerobically digested sludge without FA pretreatment were compared in Fig. 1. Compared with untreated sludge, anaerobic digestion reduced the absolute abundance of *aac(6')-Ib-cr, sul1, sul2*, *tetA, tetB, tetG*, *tetX* and *intI1* by 22~89% (Fig. 4), resulting in an overall reduction of about 30% in the total absolute abundance of the tested ARGs. It is evident that FA pretreatment enhanced the removal of ARGs in anaerobic sludge digestion. Compared with untreated sludge, the absolute abundance of *aac(6')-Ib-cr, sul1, sul2, tetA, tetB, tetG, tetX* and *intI1* was removed by 25~95% in the anaerobically digested sludge with FA pretreatment (Fig. 4). Overall, combined FA pretreatment and anaerobic digestion could reduce the total absolute abundance of the tested ARGs by 40%, from 6.0×10^9 gene copies/g-TS in the untreated sludge to 3.6×10^9 gene copies/g-TS in the anaerobically digested sludge with FA pretreatment.

(Position for Fig. 4)

In particular, the removal of *aac(6')-Ib-cr*, *blaTEM*, *sul2*, *tetA, tetB* and *tetX* was enhanced by 17%, 58%, 19%, 52%, 42% and 74%, respectively, in the anaerobically digested sludge with

FA pretreatment, in comparision to anaerobically digested sludge without FA pretreatment (Fig. 5). In addition. FA pretreatment did not (p>0.05) significantly affect the abundances of *sul1*, *tetM* and *intI1*, and slightly increased the abundance of *tetG* by 14% in the anaerobically digested sludge (Fig. 5). Overall, FA pretreatment enhanced the removal of the total absolute abundance of the tested ARGs by ~15% in the anaerobic digestion.

(Position for Fig. 5)

The effects of FA pretreatment on the relative abundances of the tested ARGs and *intI1* in the anaerobic digestion were shown in Fig. 3. The results were consistent with the trend of absolute abundance. Compared with the anaerobically digested sludge without FA pretreatment, FA pretreatment reduced the relative abundances of *aac(6')-Ib-cr, blaTEM, sul2, tetA, tetB* and *tetX* by 20~75% in the anaerobically digested sludge. On the contrary, FA pretreatment increased the relative abundances of *tetG* by 14% in the anaerobically digested sludge. The relative abundance of *tetM*, *sul* and *intI1* remained unchanged (p>0.05) at around 2.5×10^{-4}, 9.2×10^{-3} and 7.7×10^{-4} gene copies/16s rRNA. Overall, FA pretreatment decreased the relative abundance of the total tested ARGs in the digested sludge by 15%. These indicate that FA pretreatment could decrease the proportion of the targeted ARGs in the anaerobically digested biomass, but does not significantly affect *intI1*.

3.3. Correlation between ARGs and *intI1*/microbial community and effect of FA pretreatment on microbial community in anaerobic sludge digestion

The correlation between the nine tested ARGs and *intI1*/microbial community (at the genus level) was shown in Fig. 6. The results suggested that fourteen bacteria (among the top 30 in the absolute abundance) had a significant positive correlation with nine ARGs selected in this

study (R>0.8, p<0.05) (Fig. 6). *TetB and tetX* shared seven potential bacterial hosts, i.e., *Thauera*, *unidentified_Nitrosomonadaceae*, *Halomonas, Acidaminobacter, Sulfuritalea, Aquabacterium* and *Microbacterium.* In addition, *tetA* and *sul2* shared most of their potential bacterial hosts with *tetB and tetX*, except *Anaeromyxobacter* and *Sulfuritalea*. Four bacterial hosts, i.e., *Thauera*, *Aquabacterium*, *Microbacterium* and *Sulfuritalea* were identified for s*ul1. TetM* had three potential bacterial hosts, which were *Methanoculleus*, *Smithella* and *Methanosarcina.* In addition, *aac(6')-ib-cr*, *blaTEM* and *tetG* each had one sole potential bacterial host, which were *unidentified_Archaea, Lactobacillus* and *Dechloromonas*, respectively. The results also revealed that *intI1* only had a significant correlation (p < 0.05) with one ARG *sul1* in this study (Fig. 6). This demonstrated that *intI1* may not be the main factor affecting the fate of the tested ARGs.

(Position for Fig. 6)

The discrepant changes of the bacterial abundances may explain the different ARGs abundances in the anaerobically digested sludges with and without FA pretreatment. In our study, most of the bacterial hosts of the ARGs were affiliated into three phyla, which were *Proteobacteria*, *Bacteroidetes* and *Euryarchaeota. Proteobacteria* was the predominant bacterial phylum, which included eight bacterial hosts (genus level) in this study. As shown in Fig. S1, FA pretreatment significantly decreased the absolute abundance of the *Proteobacteria* from 2.0×10^{10} to 1.1×10^{10} gene copies/g-TS during anaerobic sludge digestion. Fig. 7 further showed the difference in the abundance of the bacteria (at the genus level) in the anaerobically digested sludges with and without FA pretreatment. Compared to the digested sludge without FA pretreatment, FA pretreatment reduced the abundance of most potential bacterial hosts in the digested sludge. For instance, the abundances of *Thauera* and *Lactobacillus* decreased from

325 1.8×10^8 and 5.0×10^8 gene copies/g-TS in the digested sludge without FA pretreatment to
326 1.4×10^8 and 7.4×10^6 gene copies/g-TS in the digested sludge with FA pretreatment,
327 respectively. Furthermore, compared to anaerobically digested sludge without FA pretreatment,
328 FA pretreatment reduced the total absolute abundances of the bacteria genera (i.e.,
329 *unidentified_Archaea, Lactobacillus, unidentified_Nitrosomonadaceae, Thauera, Halomonas,*
330 *Acidaminobacter, Anaeromyxobacter, Aquabacterium, Sulfuritalea*), that were associated with
331 the decreased ARGs in the digested sludge from 1.8×10^9 to 1.0×10^9 gene copies/g-TS. These
332 may explain the lower abundance of ARGs in the anaerobically digested sludge with FA
333 pretreatment. In contrast, the total abundances of potential bacterial hosts of *sul1* and *tetM* were
334 relatively stable in digested sludges with and without FA pretreatment, which was consistent
335 with the negligible changes of their abundances in anaerobically digested sludge due to FA
336 pretreatment. In addition, the abundance of the potential bacterial host of *tetG* (i.e.,
337 *Dechloromonas*) increased from 1.7×10^8 gene copies/g-TS in the digested sludge without FA
338 pretreatment to 6.3×10^8 gene copies/g-TS in the digested sludge with FA pretreatment. This
339 may contribute to the increased abundance of *tetG* in the digested sludge with FA pretreatment
340 compared to the digested sludge without FA pretreatment.
341
342 (Position for Fig. 7)
343
344 **4. Discussion**
345 This study reveals for the first time that FA pretreatment could enhance ARGs removal in
346 anaerobic sludge digestion. This was experimentally demonstrated through the anaerobic
347 digestion tests with or without FA pretreatment. Nine widely detected ARGs were tested in the
348 experiments. The combined use of FA pretreatment and anaerobic digestion reduced the total
349 absolute abundance of the tested ARGs by ~15% in comparison to anaerobic digestion without

FA pretreatment. This is caused by the removal of tested ARGs during FA pretreatment and the reduced abundance of potential bacterial hosts of ARGs due to FA pretreatment during the anaerobic digestion process. The removal of *IntI1* was not significantly affected by FA pretreatment, indicating that *intI1* was not the main factor affecting the fate of the tested ARGs in this study.

4.1 Potential mechanisms of enhanced removal of ARGs in combined use of FA pretreatment and anaerobic digestion

Our results indicated that some ARGs were removed during FA pretreatment. FA is the unionized form of ammonium (NH_4^+). Previous studies have shown that FA could inactivate a variety of microorganisms, as FA could easily diffuse through the cell membrane to reach the cytoplasm, resulting in proton imbalance or potassium deficiency (Martinelle and Häggström, 1993). Therefore, FA could potentially inactivate or kill antibiotic resistant bacteria during the pretreatment. Additionally, a recent study has indicated that FA could induce DNA damage (Zhang et al., 2020). As a consequence, the DNA that carries ARGs might be damaged, which leads to a decrease in the absolute abundances of ARGs including *blaTEM*, *sul1*, *sul2*, *tetA*, *tetB* and *tetX* in this study.

The relative abundances of ARGs also decreased after FA pretreatment, which may be because 16S rRNA genes were less affected by FA treatment than ARGs (Fig. 1). This might be related to different positions of ARGs and 16s rRNA genes in the microorganisms. 16S rRNA genes are typically located at bacterial genomic DNA. They are tightly coiled and are around by the nucleotide-related proteins (Olins and Olins, 2003). These proteins may protect the 16S rRNA gene from being damaged by FA to some extent. In terms of ARGs, many of them are located at plasmids that are widely distributed in the cytoplasm (Pogliano, 2002). Therefore, ARGs are

more vulnerable to FA attack than 16S rRNA genes. In terms of *intI1*, though some of them could also be attacked by FA, DNA damage may result in SOS response (a stress-response system in bacteria) of cell, increase the conjugative transfer frequency and activate *intI1* expression (Erill et al., 2007; Hocquet et al., 2012). As a consequence, FA pretreatment did not significantly affect the abundance of *intI1* in sludge.

It has been reported that the microbial community is the primary determinant of ARGs abundance in the anaerobically digested sludge (Guo et al., 2017; Ma et al., 2011). In this study, FA pretreatment changed the microbial community in the anaerobically digested sludge, which could be caused by the different selective pressure due to the effect of FA on microbes (Wang., 2017; Wang et al., 2017). For example, our study found that FA reduced the abundance of *Thauera* in the anaerobically digested sludge (Fig. 7). As *Thauera* was the potential bacteria host of *sul2*, *tetA*, *tetB* and *tetX* (Fig. 6), the enhanced removal of these ARGs was clearly linked with the FA pretreatment. Moreover, the total abundance of the potential bacterial hosts of the decreased ARGs was lower in the digested sludge with FA pretreatment than that in the digested sludge without FA pretreatment, contributing to the reduced abundance of the ARGs (Figs. 6 and 7). As described in Section 3.3, the relative stable total abundances of potential bacterial hosts may result in negligible changes in the abundances of *sul1* and *tetM* in the anaerobically digested sludges with and without FA pretreatment. In terms of *tetG*, the abundance of its potential bacterial host (i.e. *Dechloromonas*) was higher in the digested sludge without FA pretreatment than that in the digested sludge with FA pretreatment, which could potentially lead to an increased *tetG* abundance after implementing FA pretreatment (Figs. 6 and 7).

399 *IntI1* plays an important role in the horizontal gene transfer among bacteria which could also
400 be a mechanism affecting the fate of ARGs in the anaerobic sludge digestion under certain
401 conditions (Zhang et al., 2019). For instance, Zhang et al. (2019) concluded that the changes
402 of *intI1* could be related to the decrease of *sul1*, *tetG*, *ereA* and *sul2* in the anaerobically
403 digested sludge with microwave pretreatment. In our study, the abundance of *intI1* remained
404 similar in the anaerobically digested sludges both with and without FA pretreatment and was
405 only found to be positively correlated with *sul1*. This suggested that some *sul1* genes may be
406 harbored in *intI1* and *intI1* may not affect the removal of most of ARGs in anaerobic sludge
407 digestion in this study.
408
409 **4.2 FA pretreatment as a potential strategy for enhancing ARGs removal in the**
410 **anaerobically digested sludge**
411 Our previous study demonstrated that FA pretreatment could enhance methane production
412 during anaerobic digestion of primary and secondary sludges (Wang, 2017; Wei et al., 2017a,
413 2017b). This study showed for the first time that, in addition to the enhanced methane
414 production, FA pretreatment could also enhance the ARGs removal in anaerobic sludge
415 digestion.
416
417 Sludge from WWTPs has been regarded as an essential resource for agriculture (Sharma et al.,
418 2017). For example, more than 65% and 50% of the sludge are been reused for agriculture in
419 Australia and the United States, respectively (Australian and New Zealand Biosolids
420 Partnership, 2019; the United States Environmental Protection Agency, 2019). Sludge is the
421 major by-product of the wastewater treatment process. It has a nutrient value that can condition
422 soils and improve their structure and water retention. These benefits create strong motivations
423 for the reuse of sludge. However, such an opportunity may disappear if the risk to human health

due to ARGs spread through sludge outweighs the fertilizing benefits. This study indicates that the combined use of FA pretreatment and anaerobic digestion could enhance the removal of ARGs, thereby mitigating the risk of sludge reuse to human health. This provided promising support for the safe application of reused sludge as an organic fertiliser globally to support the transition to a circular economy.

Importantly, FA is a by-product of wastewater treatment and can be attained directly from the anaerobic digestion liquor of the WWTPs, which contain an FA concentration of 30-560 mg NH_3-N/L (Wang et al., 2017). Therefore, the FA pretreatment strategy requires negligible external chemical input. The FA pretreatment strategy is also sophisticated in its simplicity, easing its uptake. Its implementation only requires the installation of a small, simple mixing tank and minor retrofitting of existing WWTPs. Therefore, this FA pretreatment strategy is potentially economically attractive and environmentally friendly.

This study tested nine representatives of ARGs that are widely detected in the wastewater environment. The results indicated that FA pretreatment may potentially be able to enhance the removal of the other un-tested ARGs in anaerobic sludge digestion. It has been reported that the potential bacterial hosts of certain ARGs could be commonly shared with a variety of other ARGs (Tian et al., 2019). For example, *Thauera* was the host of *strB* (a streptomycin resistance gene), *aadA2* (an aminoglycoside resistance gene) and *qacH* (a fluoroquinolone resistance gene) (Tian et al., 2019). Therefore, the decrease in the abundance of *Thauera* in the anaerobically digested sludge with FA pretreatment (Fig. 7) observed in this study may also result in an enhanced removal of *strB, aadA2* and *qacH.*

It should be highlighted that this is only a proof-of-concept study that demonstrated the

feasibility of the FA pretreatment strategy in enhancing ARGs removal in anaerobic digestion. Therefore, the optimization of the FA pretreatment strategy (e.g., FA concentration) was not conducted in this study. A higher ARGs or *intI1* removal may be achievable after optimizing the FA pretreatment strategy. Furthermore, though promising results were achieved in our laboratory studies, full-scale trials are required to fully reveal the potential of this FA pretreatment strategy. In addition, this study only investigated a selective of the known ARGs using RT-qPCR to represent various antibiotic classes. Future studies should focus on conducting metagenomic sequencing technique to reveal the broad spectrum profile of ARGs.

5. Conclusions

In this study, the effect of FA pretreatment on the removal ARGs in the anaerobic sludge digestion was evaluated by anaerobic digestion tests. The following conclusions can be drawn from this study:

- FA pretreatment at 420 mg NH_3-N/L for 24h enhanced the removal of the total tested ARGs by ~15% in the anaerobically digested sludge. This revealed that FA pretreatment could potentially reduce the spread of ARGs from the sludge to the natural environment through sludge reuse.
- FA pretreatment had a negligible effect on the abundance of *intI1*.
- The enhanced removal of ARGs was likely attributed to the removal of tested ARGs during FA pretreatment and the reduced abundance of potential bacterial hosts of ARGs due to FA pretreatment during the anaerobic digestion process.

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606 **List of figures**

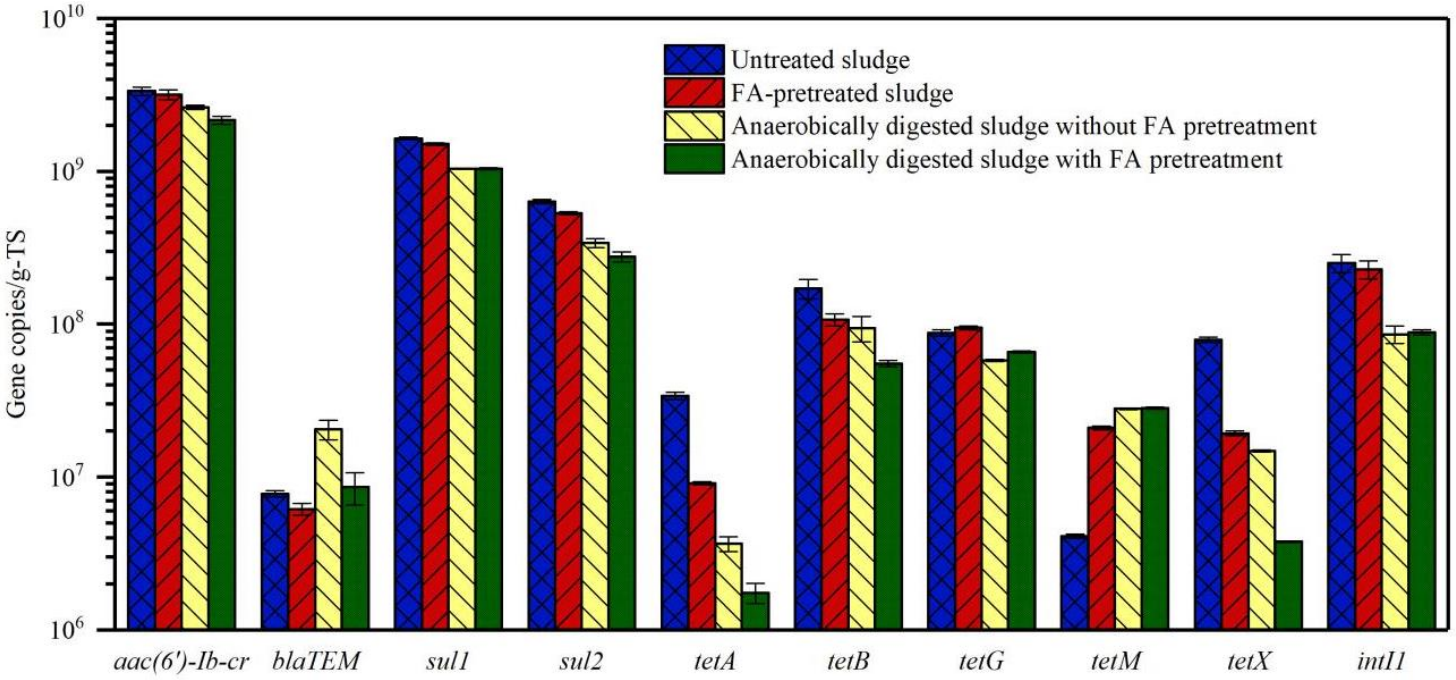

Figure 1. Absolute abundances of ARGs and *intI1* in different sludges

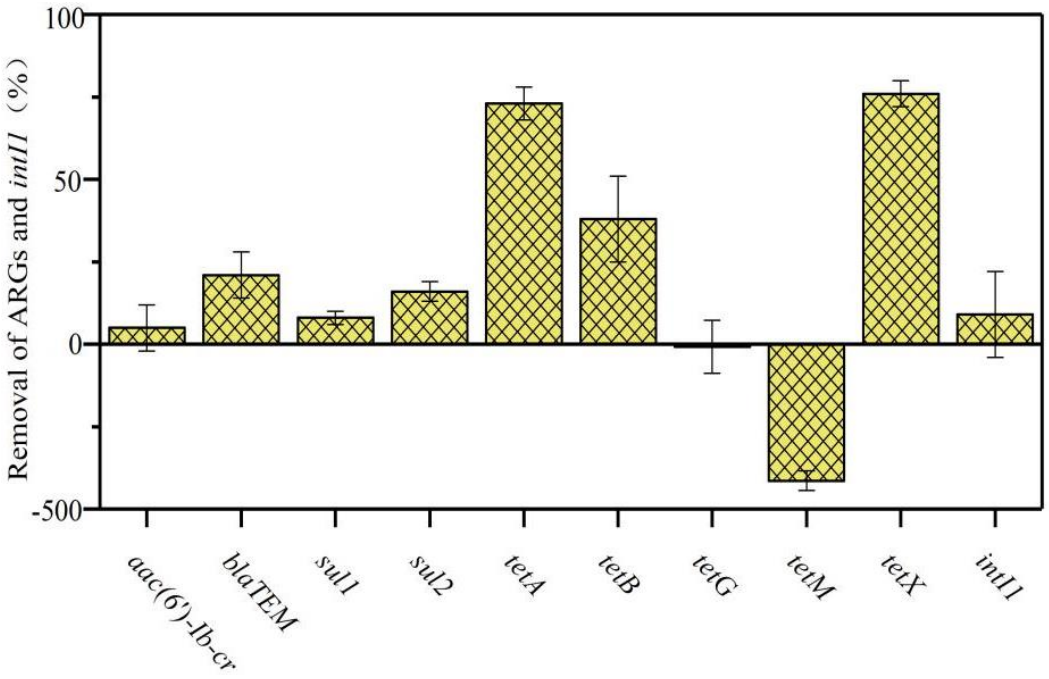

Figure 2. Removal ratios (%) of ARGs and *intI1* in sludge during FA pretreatment. Negative means the target genes increased after FA pretreatment.

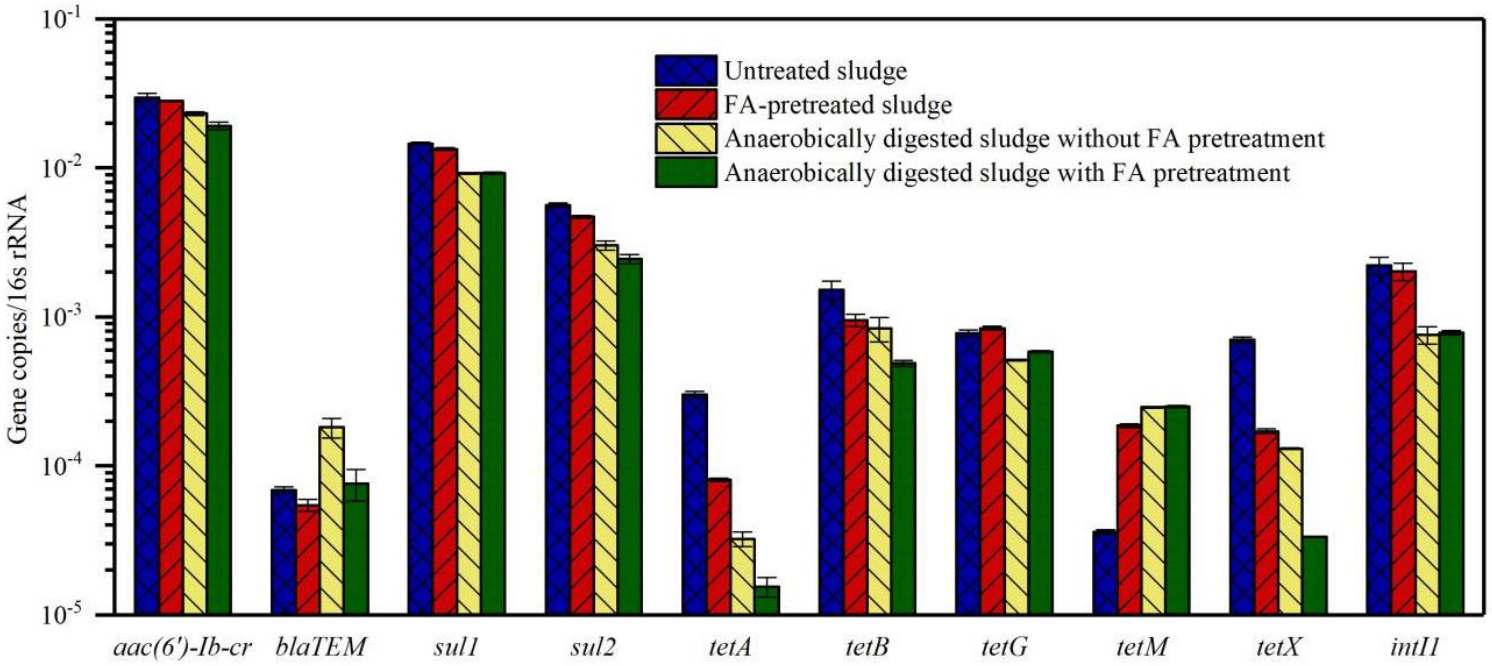

Figure 3. Relative abundances of ARGs and *intI1* in different sludges

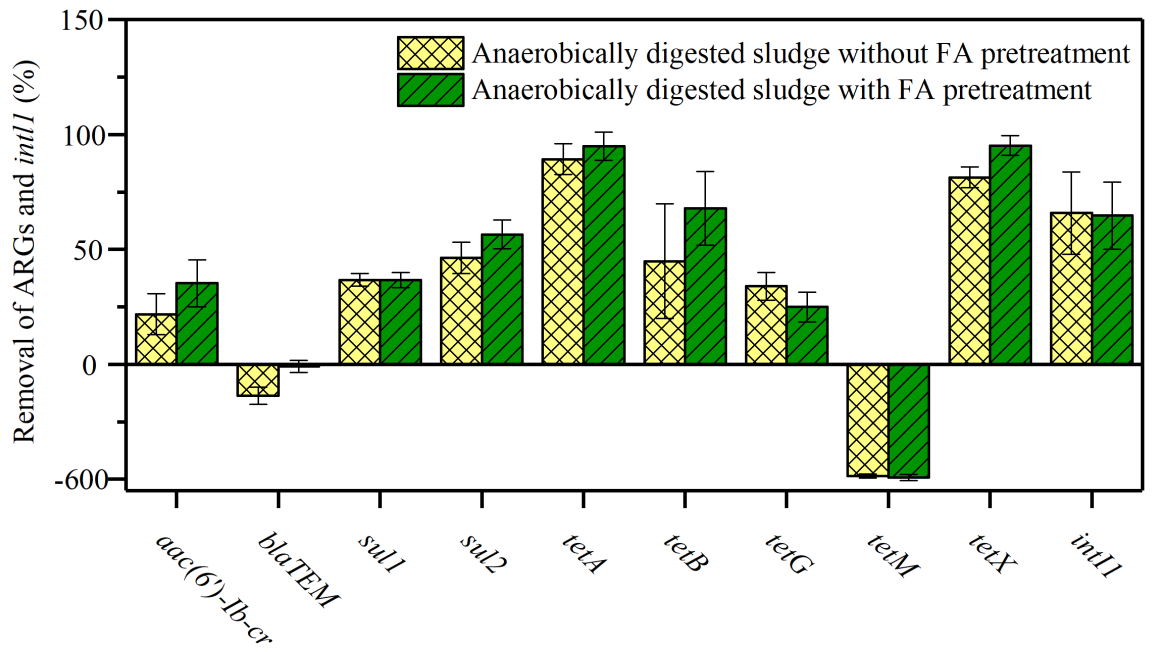

Figure 4. Removal ratios (%) of ARGs and *intI1* in anaerobically digested sludge with and without FA pretreatment compared to untreated sludge. Negative means the target genes increased.

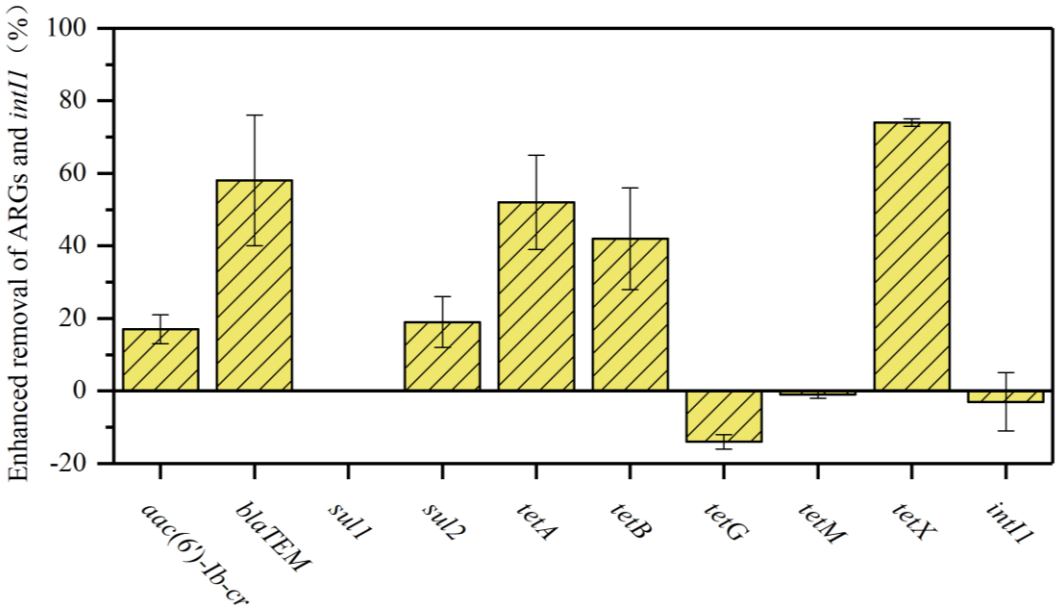

Figure 5. Enhanced removal (%) of ARGs and *intI1* in the anaerobically digested sludge with FA pretreatment compared with the anaerobically digested sludge without FA pretreatment. Negative means the target genes increased in the anaerobically digested sludge with FA pretreatment.

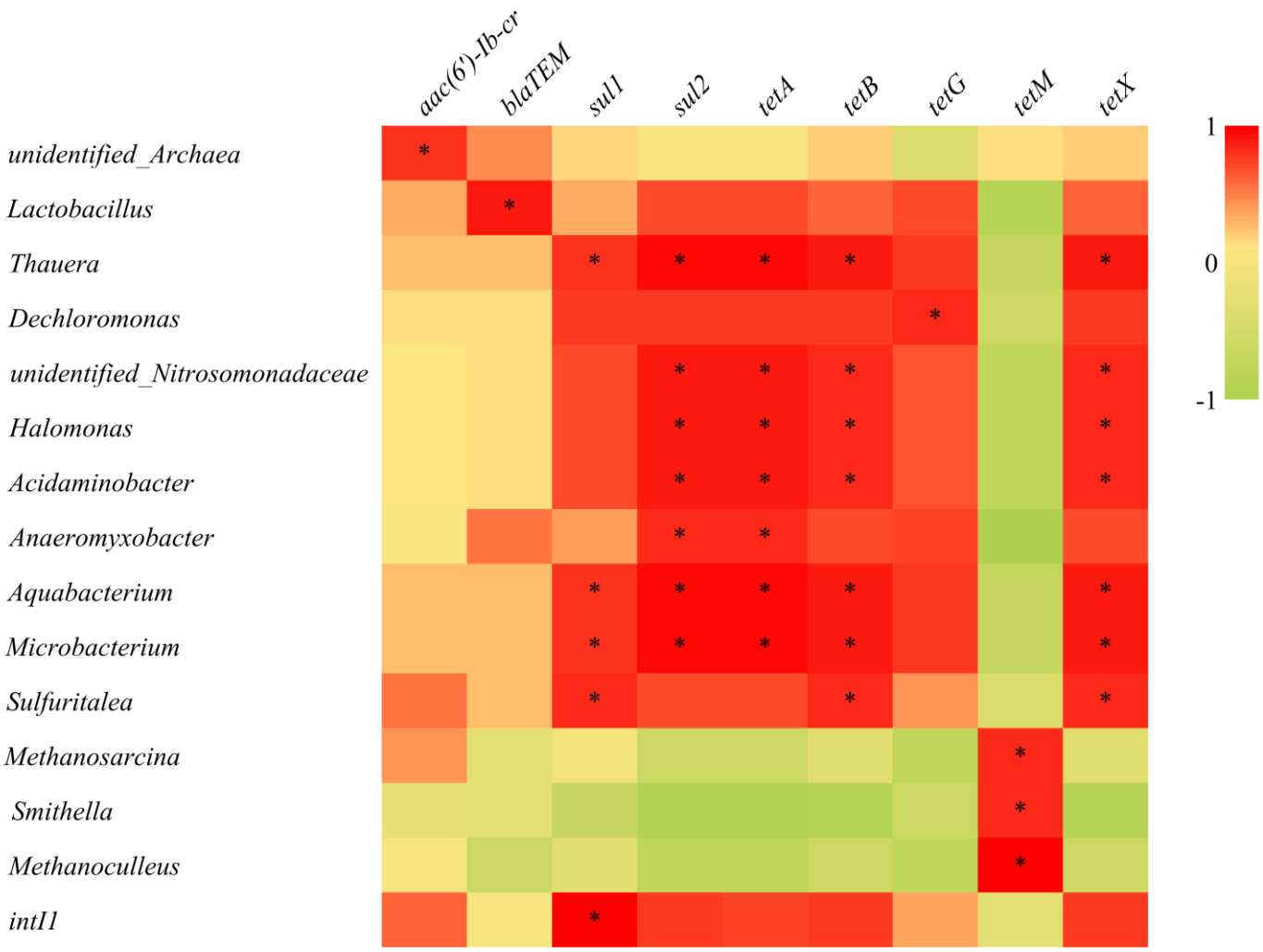

Figure 6. Correlation between ARGs and *intl1*/microbial community at the genus level. An asterisk (*) indicates a significant positive correlation (R>0.8, P < 0.05). The scale bar showed the R value between ARGs and *intl1*/microbial community.

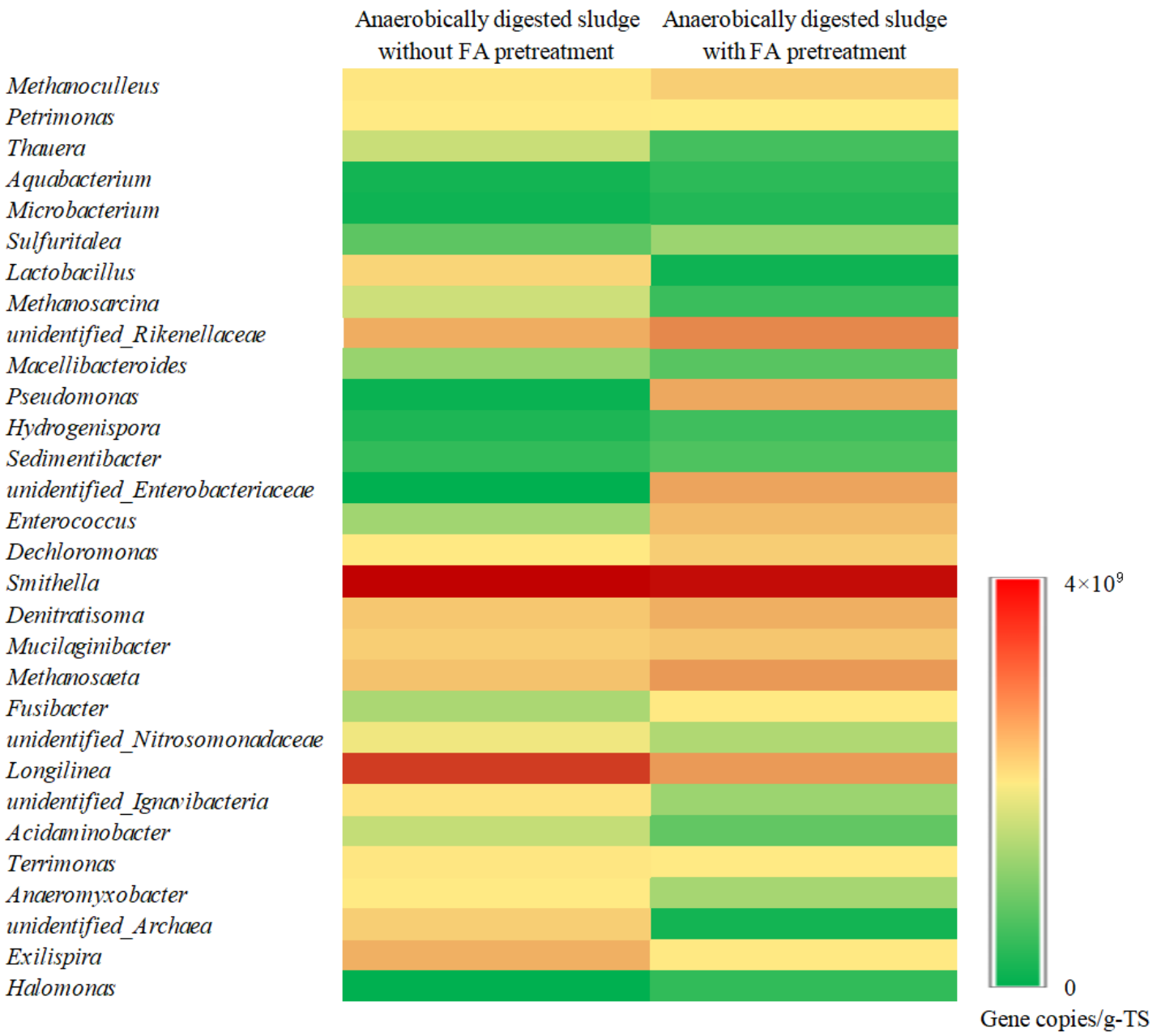

Figure 7. Heatmap of the top 30 most abundant bacteria (at the genus level) in the anaerobically digested sludge with and without FA pretreatment.