Microplastics deteriorate the removal efficiency of antibiotic resistance genes during aerobic sludge digestion

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ABSTRACT: Sludge from wastewater treatment plants (WWTPs) is considered to be reservoirs of antibiotic resistance genes (ARGs), which can be efficiently removed by sludge treatment processes, e.g., aerobic sludge digestion. However, recent studies report microplastics, which also accumulate in sludge, may serve as carriers for ARGs. In the presence of microplastics, whether ARGs can still be efficiently destroyed by aerobic sludge digestion remains to be urgently investigated. In this study, the fate of ARGs during aerobic digestion was investigated with and without the addition of three prevalent categories of (i.e., polyvinyl chloride (PVC), polyethylene (PE), and polyethylene terephthalate (PET)). Nine ARGs and class 1 integron-integrase gene (*intI1*) that represents the horizontal transfer potential of ARGs were tested in this study. Compared with the control, the ARGs removal efficiency decreased by 129.6%, 137.0%, and 227.6% with the presence of PVC, PE, and PET, respectively, although a negligible difference was observed with their solids reduction efficiencies. The abundance of potential bacterial hosts of ARGs and *intI1* increased in the reactors with the addition of microplastics, suggesting that microplastics potentially selectively enriched bacterial hosts and promoted the horizontal transfer of ARGs during aerobic sludge digestion. These may have contributed to the deteriorated ARGs removal efficiency. This study demonstrated that microplastics in sludge would decrease the ARGs removal efficiency in aerobic digestion process, potentially leading to more ARGs entering the local environment during sludge disposal or utilization.

Keywords: *Antibiotic resistance genes; Microplastics; Aerobic digestion; Wastewater treatment plants*

1. Introduction

In the last few decades, the diffusion of antibiotic resistance has become one of the greatest concerns to public health globally (Laxminarayan et al., 2013). The massive use of antibiotics in the household has resulted in the widespread presence of antibiotic resistance genes (ARGs) in wastewater treatment plants (WWTPs) (Karkman et al., 2018). ARGs can be broadly transmitted among the microbial community structure in WWTPs through cell reproduction of antibiotic resistance bacteria, and/or horizontal transfer mediated by mobile genetic elements (MGEs) (Karkman et al., 2018; Li et al., 2019). In particular, only a small proportion of ARGs in WWTPs is discharged into the environment with the effluent, and most (>99%) ARGs will be ultimately stored up by sludge (Yang et al. 2014).

As an essential part of WWTPs, sludge digestion is commonly applied to stabilize biological sludge (Wang et al., 2017). Aerobic digestion is a widely used sludge treatment technology, especially in small-scale WWTPs. Aerobically digested sludge is often used to improve soil condition and water retention due to its nutrient value (Navas et al., 1998). For example, in the United States, 51% of the sludge is utilized for land application (U. S. Environmental Protection Agency, 2019). However, ARGs in digested sludge may eventually enter the local environment (Bondarczuk et al., 2016; Chen et al., 2016).

As such, considerable researches have been conducted to investigate the fate of ARGs during the aerobic sludge process (Jang et al. 2019). A recent study revealed that superior ARGs removal performance was achieved with aerobic digestion due to the rapid volatile solids (VS) removal as well as narrow ranges of potential ARGs hosts (Jang et al. 2015).

Despite the high ARG removal efficiency as demonstrated in previous studies, the

presence of microplastics adds uncertainty to the aerobic sludge digestion process. Increasing evidence reveals that microplastics would enter WWTPs due to human activities, and eventually accumulate in sludge (Carr et al., 2016; Liu et al., 2021). So far, up to 170 particles/g-TS (TS: total solids) of microplastics have been observed in sewage sludge (Lares et al., 2018; Talvitie et al., 2015; Sun et al., 2019; Liu et al., 2021). To date, the impact of microplastics on the fate of ARGs during aerobic digestion remains unclear. A recent study showed that exposure to environmentally relevant concentrations of microplastics could increase oxidative stress and altered the microbial community structure during aerobic sludge digestion (Wu et al., 2019a; Seeley et al., 2020). The increased oxidative stress could potentially accelerate the generation of reactive oxygen species (ROS), which accelerates horizontal gene transfer (HGT) and leads to the proliferation of ARGs (Dwyer et al., 2009; Chen et al., 2019). Besides, changes in the microbial community structure could also affect the abundance of ARGs (Zarei-Baygi et al., 2020). Therefore, we raise the hypothesis that the presence of microplastics in the sludge may alter the fate of ARGs during aerobic digestion and thereby the level of ARGs in digested sludge.

The aim of this study is to evaluate the impacts of microplastics on the fate of ARGs during aerobic sludge digestion. The secondary sludge was harvested from a municipal WWTP and subjected to aerobic digestion tests without and with the addition of three prevalent categories of microplastics (i.e., polyvinyl chloride (PVC), polyethylene (PE), polyethylene terephthalate (PET)). Nine ARGs and class 1 integron-integrase gene (*intI1*) in the sludge were quantified before and after aerobic digestion to evaluate the ARGs removal efficiency in aerobic digestion. Through microbial community structure analysis

and the correlations between ARGs and *intI1*/microbial community structure, the variation of the potential microbial hosts of ARGs were revealed, and the underlying mechanisms of how microplastics affect the fate of ARGs during aerobic sludge digestion were further understood. To the best of our knowledge, this is the first study to investigate the impacts of microplastics on the fate of ARGs in aerobic sludge digestion. The results further improved the understanding of the roles of microplastics on sludge treatment in WWTPs in regards to ARGs.

2. Materials and methods

2.1 Sources of secondary sludge and microplastics

The secondary sludge was harvested from a secondary sedimentation tank of a municipal WWTP in Australia (sludge retention time (SRT) = 12-16 days). The main properties of the sludge were as follow: TS 7.6 ± 0.2 g/L, VS 5.4 ± 0.2 g/L, pH 7.3 ± 0.1, total chemical oxygen demand (TCOD) 8.7 ± 0.2 g/L and soluble chemical oxygen demand (SCOD) 0.2 ± 0.1 g/L.

In this study, aerobic digestion tests were applied to assess the impacts of three categories of microplastics (i.e., PVC, PE, and PET) on the fate of ARGs in aerobic sludge digestion. The PVC, PE and PET are three types of prevalent microplastics in the sludge. (Liu et al., 2019, 2021; Sun et al., 2019). The spherical PVC, PE, and PET microplastics were purchased at Zhongcheng Plastic Products Co. Ltd. Located in Guangzhou, China, with an average diameter of 600 μm. The morphologies and size of microplastics were observed and confirmed using scanning electron microscopy (Zeiss Supra 55VP, German) with a lithography system (Raith Elphy Plus E-beam, German). In addition, the background

116 concentration and characteristics of microplastics in the secondary sludge were determined
117 as described in supporting information (SI, Text S1). The background concentration of
118 microplastics in the secondary sludge were enumerated at around 15 ± 3 particles/g-TS
119 with an average size of 600 ± 50 μm.
120
121 *2.2 Aerobic digestion experiments design*
122 Four aerobic sludge digesters (R1 - R4) were set up with four 2.5 L Erlenmeyer flasks,
123 as shown in Fig. 1. A total of 6000 mL of secondary sludge was poured into R1 to R4
124 equally. Afterwards, PVC, PE and PET microplastics were added into R2, R3 and R4,
125 respectively, reaching 170 particles/g-TS of microplastics. To our best knowledge, 170
126 particles/ g-TS is the highest concentration of microplastics observed in sludge (Sun et al.,
127 2019; Liu et al., 2021). R1 was carried out as a control group without exogenous
128 microplastics (Figure 1). During this experiment, dissolved oxygen (DO) was maintained
129 at about 5.0 mg/L, four pH automatic controllers were applied to control the pH in the
130 reactor at 7.3 ± 0.1 by adding 0.1 M NaOH solution. The aerobic digestion tests lasted for
131 25 days, aligning with the SRT of the full-scale aerobic digestion in Australia. It is worth
132 noting that all the equipment was plastic-free to avoid the introduction of additional
133 microplastics.
134 Herein, the effects of PVC, PE and PET microplastics at 170 particles/g-TS on the
135 degradability of aerobic sludge digestion were appraised by measuring VS degradation of
136 sludge and inorganic nitrogen production. Sludge was sampled every 2-3 days from each
137 digester for analyzing TS, VS, and inorganic nitrogen (i.e., NH_4^+–N, NO_2^-–N and NO_3^-–N)
138 during the experiment. Triplicate tests were conducted to ensure accuracy. The VS

degradation of sludge in the *i* day (day$_i$) was calculated by (VS_{day0}- VS_{day_i})/VS_{day0} × 100%.

The sludge of each digester before and after aerobic digestion was also sampled into sterile glass bottles for the quantification of target genes and microbial community structure analysis. The variations in the abundance of ARGs in different digesters were compared to determine the effects of microplastics on the removal efficiency of ARGs in aerobic sludge digestion. The change of MGEs abundance and microbial community structure was also measured before and after aerobic digestion in each reactor to uncover the underlying mechanisms.

(Position for Fig.1)

2.3 Analytical methods, DNA preparation and gene quantification

The inorganic nitrogen concentrations of the samples were determined using an NH_4^+ photometric test kit (Merck Millipore, USA), NO_3^- and NO_2^- vial test kit (Hack, Australia), respectively. The concentrations of TS and VS were analyzed based on the standard methods (WE Federation and APH Association, 2005).

DNA extraction was performed for each sludge sample using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) following the instructions. The integrity, purity and concentration of the extracted DNA were detected by electrophoresis on a 1% agarose gel and Nanodrop ND-1000 (Nanodrop, USA), respectively. The extracted DNA was firstly stored at -20 ℃ and then transport to Sangon Biotech Co. Ltd. (Shanghai, China) for analysis on dry ice within 48 hours.

In this study, real-time quantitative PCR (RT-qPCR) was applied to quantify the target

genes. Nine common ARGs types were selected as representatives of the various classes of ARGs. One aminoglycoside and fluoroquinolone resistance gene (*aac(6')-Ib-cr*), one trimethoprim resistance gene (*drfA1*), three macrolide resistance genes (*ermB, ermF* and *mefA*), two sulfonamide resistance genes (*sul1* and *sul2*), and two tetracycline resistance genes (*tetA* and *tetX*) were chosen in this study. The gene of *intI1* was quantified to represent the frequency of HGT which involves the dissemination of ARGs (Stokes and Gillings, 2011). The 16S rRNA gene in samples was quantified as the total bacterial biomass in the sludge (Low et al., 2000). Triplicate amplification of each gene was performed to ensure accuracy. Details about the target gene such as annealing temperature and primer are listed in SI, Table1 S1. For better quantification and comparison, both absolute abundances (i.e., gene copies/g-TS) and relative abundances (i.e., gene copies/16S rRNA) of target genes were calculated in this study. The relative abundances are the reflection of the percentage of microbes carrying ARGs and *intI1* (Ma et al., 2011).

2.4 Microbial community structure analysis

The high-throughput sequencing of 16S rRNA gene was performed by Illumina HiSeq 2500 platform (Illumina, USA). The V3–V4 regions of 16S rRNA genes were amplified with PCR primers as showed in supporting information (SI, Table S1). The raw data of each sample was analyzed following Cutadapt (v1.9.1) quality-controlled process and was compared with Silva (v138.1) database using USEARCH (v11) algorithm to obtain the clean reads. The rest of the bioinformatics analysis was conducted as described in our previous study (Zhang et al., 2021). A heatmap that uncovered the changes of microbial community structure was prepared by HemI (http://hemi.biocuckoo.org/).

185

186 *2.5 Network analysis*

187 The spearman's rank correlation coefficient (R) was employed to identify the
188 relationship between ARGs and *intI1*/microbial community structure using SPSS software
189 (Version 25.0, IBM, USA) in this study. The value of R could provide a cogent tool for the
190 statistical identification of potential microbial hosts of ARGs in complex environments
191 (Chen et al., 2016). Statistically, a potential microbial host for the tested ARGs refers to a
192 microbial genus with a correlation of $R > 0.8$ and $p < 0.05$ with an ARG (Chen et al., 2016).
193 The correlation between ARGs and *intI1*/microbial community structure was visualized by
194 MATLAB R2020b (MathWorks, USA).

195

196 **3. Results**

197 *3.1 Effects of microplastics on aerobic sludge digestion*

198 Fig. 2(A) shows the VS degradation of secondary sludge with and without microplastics
199 during aerobic digestion. In the 25 day period, the secondary sludge without microplastics
200 was degraded by 46.4% ± 3.9% in aerobic digestion. During the same period, the secondary
201 sludge with PVC, PE and PET was degraded by 43.8% ± 4.2%, 43.6% ± 3.3% and 44.3%
202 ± 4.1% in aerobic digestion, respectively, representing no significant ($p > 0.05$) difference
203 in comparison to the control reactor. In addition, aerobic sludge digestion would result in
204 the release of inorganic nitrogen (Metcalf and Eddy, 1991). The production of inorganic
205 nitrogen production in aerobic sludge digestion during the 25 days was shown in Fig 2(B).
206 The total inorganic nitrogen production of aerobically digested sludge with PVC, PE and
207 PET in the same period was 26.9 ± 2.2, 25.0 ± 2.9 and 26.0 ± 3.2 mg-N/g-VS, respectively,

approximating to that of the control reactor (i.e. 26.1 ± 3.1 mg-N/g-VS). Aligning with the data of the degradability and inorganic nitrogen production, PVC, PE and PET microplastics at 170 particles/g-TS showed no significant effect on the aerobic digestion of secondary sludge.

(Position for Fig. 2(A) & 2(B))

3.2 Effect of microplastics on the fate of ARGs and intI1 in the aerobic sludge digestion

The absolute abundance of tested ARGs and *intI1* in sludge before and after aerobic digestion were compared to evaluate the effect of microplastics on the fate of ARGs and *intI1* in aerobic sludge digestion. The absolute abundances of *aac(6')-Ib-cr, ermB, ermF, dfrA1, sul1, sul2, tetA, tetX* and *mefA* were ranged from 1.7×10^{9} to 6.4×10^{12} gene copies/g-TS in secondary sludge before aerobic digestion (Fig. 3). Aerobic digestion reduced absolute abundances of various tested ARGs by 59.6 - 96.7% to 9.4×10^{7} - 4.9×10^{11} gene copies/g-TS in the aerobically digested sludge from control reactor (Figs. 3 and 4). This is equivalent to a total ARGs removal efficiency of 85.3% (from 1.0×10^{13} to 1.5×10^{12} gene copies/g-TS) in aerobic digestion. For *intI1*, aerobic digestion decreased the absolute abundance of *intI1* by 86.1% (Fig. 4), from 6.4×10^{11} in secondary sludge to 8.9×10^{10} gene copies/g-TS in aerobically digested sludge (Fig. 3).

(Position for Fig. 3)

(Position for Fig. 4)

231 In comparison to the control reactor, it is manifested that PVC, PE, PET microplastics
232 deteriorate the removal efficiencies of ARGs and *intI1* during aerobic digestion, resulting
233 in higher levels of ARGs in the digested sludge from the reactors with microplastics. The
234 absolute abundance of tested ARGs was ranged from 1.5×10^{8} to 2.0×10^{12} gene copies/g-
235 TS in aerobically digested sludge with PVC microplastics (Fig. 3), equivalent to an ARGs
236 removal efficiency of 32.1 - 91.3% in aerobic digestion (Fig. 4). Compared with that of the
237 control reactor, PVC microplastics decreased the removal efficiencies of absolute
238 abundance of *aac(6')-Ib-cr*, *ermB*, *ermF*, *dfrA1*, *sul1*, *tetA*, *tetX* and *mefA* by 57.0% to 905.3%
239 in the digested sludge (Fig. 5). The *sul2* was the only exception that remained at almost the
240 same level as that of the control reactor. Overall, the exposure of PVC microplastics in
241 aerobic digestion deteriorated the removal efficiency of the total absolute abundance of
242 tested ARGs by 129.6% compared with the control reactor (Fig. 5). In addition, PVC
243 microplastics also increased the abundance of *intI1* to 2.0×10^{11} gene copies/g-TS in the
244 aerobically digested sludge with PVC microplastics (Fig. 3). This revealed that PVC
245 microplastics in aerobic digestion also deteriorated the removal efficiency of *intI1* by 120.5%
246 compared with the control reactor (Fig. 5).
247
248 (Position for Fig. 5)
249
250 Similar to PVC microplastics, PE microplastics also deteriorated the removal efficiency
251 of ARGs and *intI1* during aerobic digestion (Figs. 3 and 5). The absolute abundance of
252 tested ARGs ranged from 1.1×10^{8} to 1.9×10^{12} gene copies/g-TS in the aerobically digested
253 sludge with PE microplastics (Fig. 3), with the removal efficiencies of *aac(6')-Ib-cr*, *ermB*,

254 *ermF*, *dfrA1*, *sul1*, *sul2, tetA and tetX* ranged from 28.7% to 93.8% (Fig. 4). The *mefA* was
255 the only exception, which was increased by 193.8% (Fig. 4). Compared with aerobically
256 digested sludge from the control reactor, PE microplastics decreased the removal efficiency
257 of absolute abundance of various tested ARGs by 11.5% to 997.8%, and reduced the
258 removal efficiency of the total absolute abundance of tested ARGs by 137.0% (Fig. 5). The
259 exposure of PE microplastics also increased the abundance of *intI1* by 85.0% compared
260 with aerobically digested sludge from control reactor (Fig. 5), from 8.9×10^{10} in the
261 aerobically digested sludge to 1.7×10^{11} gene copies/g-TS in the aerobically digested sludge
262 with PE microplastics (Fig. 3).
263 The deterioration of ARGs removal efficiency in aerobic digestion also occurred with
264 PET microplastics exposure. In the aerobically digested sludge with PET microplastics, the
265 absolute abundance of tested ARGs ranged from 1.3×10^{8} to 2.4×10^{12} gene copies/g-TS (Fig.
266 3), with the removal efficiencies of *aac(6')-Ib-cr*, *ermB*, *ermF*, *dfrA1*, *sul1*, *tetA* and *tetX*
267 ranged from 46.0% to 92.5% (Fig. 4). On the contrary, the absolute abundance of *mefA*
268 increased by 59.3% and the absolute abundance of *sul2* remained at almost the same level
269 as that of the secondary sludge (Fig. 4). Compared with aerobically digested sludge from
270 control reactor, PET microplastics reduced the removal efficiency of various tested ARGs
271 by 33.7% to 984.1% (Fig. 5), resulting in a decreased removal efficiency of the total
272 absolute abundance of the tested ARGs by 227.6%, from 1.5×10^{12} gene copies/g-TS in the
273 aerobically digested sludge from control reactor to 4.8×10^{12} gene copies/g-TS in the
274 aerobically digested sludge with PET microplastics. Likewise, the exposure of PET
275 microplastics also decreased the removal efficiency of *intI1* by 107.4% compared with
276 aerobically digested sludge from control reactor (Fig. 5), from 8.9×10^{10} in the aerobically

277 digested sludge from control reactor to 1.9×10^{11} gene copies/g-TS in the aerobically
278 digested sludge with PET microplastics (Fig. 3).
279 When it comes to the relative abundance of ARGs, it is clear that these three categories
280 of microplastics (i.e., PVC, PE and PET) exert similar impacts on the fate of ARGs in
281 aerobic digestion. In comparison to aerobically digested sludge from control reactor, PVC,
282 PE and PET microplastics increased the total relative abundance of ARGs by 23.5%, 43.5%
283 and 48.1%, respectively (Fig. 6). This suggests that the percentage of microbial carrying
284 ARGs and *intI1* was increased due to the presence of microplastics, which could be caused
285 by 1) changes in microbial community structure; 2) elevated HGT.
286
287 (Position for Fig. 6)
288
289 *3.3 Correlation between ARGs and intI1/microbial community structure*
290 To fully understand the contributions of the microbial community structure changes and
291 HGT to the fate of ARGs, the correlation between the tested ARGs and microbial
292 community structure*/intI1* was explored in this study. Twenty-one bacteria genera (among
293 the top 30 dominant bacterial genera) showed a significant positive correlation (i.e., $R>0.8$,
294 $p<0.05$) with tested nine ARGs (Fig. 7), indicating that they could be the potential hosts of
295 the nine ARGs. The gene of *mefA* had the largest number (eight) of potential microbial
296 hosts among the tested ARGs, including *unclassified_Planctomycetaceae,*
297 *unclassified_Anaerolineaceae, Plasticicumulans, unclassified_Betaproteobacteria,*
298 *Piscinibacter, Armatimonadetes_gp5, Thauera and unclassified_Verrucomicrobia*. The
299 genes of *ermB*, *ermF*, *sul2* and *tetX* each had only two potential microbial hosts, which

were the least among the detected ARGs. The genes of *ermB, ermF* and *tetX* shared one potential microbial host, i.e., *unclassified_polyangiaceae*. Their other microbial hosts are *Cetobacterium*, *Azospira* and *unclassified_Rhodocyclaceae*, respectively. The potential microbial hosts of *sul2* were *unclassified_Anaerolineaceae* and *Plasticicumulans*. In addition, *intI1* was found to be significantly correlated ($p < 0.05$) with five ARGs (i.e., *aac(6')-Ib-cr*, *ermB*, *ermF*, *dfrA1* and *tetX*) in this study (Fig. 7). This suggested that these five ARGs may mainly be harbored in *intI1* and increased with HGT within bacteria. Therefore, *intI1* likely contributed to the fate of tested ARGs.

(Position for Fig. 7)

3.4 Effect of microplastics on microbial community structure in aerobic sludge digestion

Fig. 8 exhibited the abundance of bacteria genera in the aerobically digested sludges with and without microplastics. Obviously, the exposure of different microplastics altered the microbial community structure in the aerobically digested sludge, in particular the abundance of potential microbial hosts of ARGs. Compared with the aerobically digested sludge from control reactor, PVC, PE and PET microplastics increased the abundance of potential microbial hosts in the aerobically digested sludge. Take *Plasticicumulans,* the potential microbial hosts of *mefA* and *sul2* (Fig. 8), as an example. The abundance of *Plasticicumulans* increased from 2.8×10^{11} gene copies/g-TS in the aerobically digested sludge from control reactor to 3.2×10^{11}, 4.0×10^{11} and 3.1×10^{11} gene copies/g-TS due to PVC, PE and PET microplastics, respectively. Overall, PVC, PE and PET microplastics increased the total absolute abundances of the twenty-one bacteria genera that were

associated with the tested ARGs from 2.4×10^{12} in the aerobically digested sludge from the control reactor to 3.5×10^{12}, 3.4×10^{12} and 3.2×10^{12} gene copies/g-TS in the aerobically digested sludge with PVC, PE and PET microplastics, respectively. This might be one of the reasons for the higher abundance of ARGs in the aerobically digested sludge of microplastics.

(Position for Fig. 8)

4. Discussion

Herein, our study uncovered that microplastics could decrease ARGs removal efficiency in aerobic sludge digestion. This was verified by four aerobic sludge digestion tests with or without three prevalent categories of microplastics (i.e., PVC, PE and PET). Overall, the total absolute abundance of all nine tested ARGs reduced by approximately 85.3% in aerobic digestion without exogenous microplastics. However, with the presence of PVC, PE and PET microplastics, the removal efficiencies of ARGs during aerobic digestion were seriously hindered. Compared with aerobically digested sludge from control reactor, PVC, PE and PET microplastics rose the total absolute abundance of ARGs by 129.6%, 137.0% and 227.6%, respectively. Our study revealed that microplastics altered the microbial community structure in digested sludge, and increased the abundance of potential microbial hosts of tested ARGs. This is likely the major reason for the increased ARGs abundance in the aerobically digested sludge with microplastics. Besides, microplastics also increased the abundance of *intI1* in sludge after aerobic digestion, suggesting a potential enhancement of horizontal transfer of ARGs among bacteria due to microplastics.

346

347 *4.1 Potential mechanisms of deteriorated ARGs removal during aerobic digestion in the*
348 *presence of microplastics on the removal efficiency*

349 Conventionally aerobic digestion is regarded as an efficient technique for ARGs
350 reduction, as it provides rapid VS removal as well as narrow ranges of potential ARGs'
351 hosts (Jang et al. 2015), which is consistent with the decreased abundance of potential
352 microbial hosts of ARGs owing to aerobic digestion in the control reactor. Although in this
353 study, the VS removal was not significantly impacted by microplastics, the abundance of
354 potential microbial hosts of ARGs was increased due to the presence of microplastics in
355 digested sludge in comparison to that of the control reactor. Take *ermF* for instance, the
356 microbial hosts of *ermF* (i.e., *Azospira* and *unclassified_Polyangiaceae*, Fig. 6) rose from
357 1.0×10^{10} gene copies/g-TS in aerobically digested sludge of control to 1.5×10^{10}, 1.2×10^{10},
358 2.0×10^{10} gene copies/g-TS in aerobically digested sludge with PVC, PE and PET
359 microplastics, respectively. This is likely the major reason for the deteriorated ARGs
360 removal efficiency in reactors with exogenous microplastics during aerobic digestions. The
361 change of microbial community structure during aerobic sludge digestion due to
362 microplastics is likely caused by two reasons: 1) toxicity of microplastics; 2) biofilm
363 formation on the surface of microplastics.

364 The toxicity of microplastics was observed in previous studies (Jeong and Choi, 2019).
365 For instance, PVC microplastics inactivated algae in freshwater by inducing ROS (Wu et
366 al., 2019a); PE microplastics inhibited the reproduction of springtail, *Folsomia candid*a in
367 soil by changing the gut microbial community structure (Ju et al., 2019). Thus, PVC, PE
368 and PET microplastics exposure may produce different selective pressure to the control

reactor (aerobic digestion without microplastics) on bacteria in the sludge and thus altered the microbial community structure. Moreover, microplastics have relatively high surface areas, which allows the development of biofilms (Rummel et al., 2017; Ogonowski et al., 2018; Wu et al., 2019b). Recent studies found that the bacteria attached to microplastics were largely different from the aqueous environment as well as the biofilms on leaf and stone surfaces under the same condition (Wu et al., 2019b). Further, Pham et al. (2021) indicated that the biofilms on microplastic surfaces in sludge were significantly different from those attached to sand in sludge. Therefore, it is likely that biofilms formed on the surface of microplastics in the sludge altered the variation of microbial communities during aerobic sludge digestion. The microplastic in sludge potentially provided selection pressure on the bacteria where the preferable bacteria of the microplastics particles may multiply more, leading to the alteration of the microbial community structure during aerobic sludge digestion.

Additionally, in the control reactor, aerobic digestion decreased the abundance of *intI1* in aerobically digested sludge (Fig. 3 & 5), suggesting that aerobic digestion likely provided an environment that could reduce the chance or frequency of bacteria interaction and thus reduce the frequency of HGT. On the contrary, compared with that of the control reactor, the abundance of *intI1* in the digested sludge was elevated by the exposure of microplastics. This could be another nonnegligible mechanism for the increased abundance of ARGs observed in digested sludge in comparison to the control group. The results of correlations between ARGs and *intI1* in this study showed that *aac(6')-Ib-cr*, *ermB*, *ermF*, *dfrA1* and *tetX* were correlated ($R>0.8$, $p<0.05$) with *intI1*. This indicates that a large number of the above five ARGs may be harboured in *intI1* and horizontally diffused among

392 bacteria along with *intI1* (De la Cruz and Davies., 2000). This allows more non-antibiotic
393 resistance bacteria to acquire the ability to resist antibiotics. The increased abundance of
394 *intI1* is likely caused by biofilm formed on the surface of microplastics (Arias-Andres et
395 al., 2018; Wang et al., 2020; Zhang et al., 2020). The biofilm developed on the surface of
396 the microplastics normally has a higher density of bacteria than the inherent aqueous
397 environment, leading to an increased gene exchange frequency among bacteria
398 (Huddleston, 2014). Moreover, biofilms can promote the stability of the plasmid (one of
399 the MGEs) and expand the host range of HGT (Madsen et al., 2012). Thus, the
400 microplastics in the sludge likely increased the frequency and probability of horizontal
401 ARGs transfer during aerobic digestion in this study. Meanwhile, there is growing evidence
402 that microplastics are positively correlated with ROS production in bacteria (Lenz et al.,
403 2016). ROS could activate *intI1* expression in cells and increase the HGT frequency
404 (Dwyer et al., 2009; Han et al., 2019). Hence, microplastic-induced ROS production in
405 cells may also be one of the reasons for the decreased ARGs removal efficiency in aerobic
406 sludge digestion due to microplastics.
407
408 *4.2 Potential adverse environmental effects caused by the presence of microplastics in*
409 *aerobic sludge digestion*
410 Our study proved that microplastics could deteriorate the ARGs removal efficiency in
411 aerobic sludge digestion. Sludge is considered to be a considerable resource for land
412 application especially in agriculture, which can be used either for land improvement or
413 compost (Fytili and Zabaniotou, 2008; Smith, 2009). The nutritional value of sludge creates
414 a powerful incentive for the reuse of sludge in agriculture. However, this study implies that

415 due to the presence of microplastics in the sludge, the insufficient removal of ARGs during
416 aerobic digestion may lead to more diffusion and transfer of ARGs from the sludge into the
417 local environment during the sludge utilization. In addition, it has been observed that
418 microplastics can become the vectors of the ARGs to enter different new environments
419 (Caruso, 2019; Dong et al., 2020; Su et al., 2021). Thus, the ARGs accumulated in reused
420 sludges may transfer to other environments under the influence of external factors such as
421 wind and earthworm movement, posing a greater great threat to the environment
422 (Evangeliou et al., 2020; Rillig et al., 2017). Nevertheless, it might become a Pyrrhic
423 victory if the risk to public health caused by the diffusion of ARGs and microplastics
424 outweighs the sake of fertilization via sludge utilization.

425 Furthermore, this study applied newly manufactured microplastics of the same size. In
426 real-world scenarios, most of the microplastics present in the sludge are so-called 'aged'
427 microplastics, which may have a larger surface area and tend to form more biofilms, due
428 to historical physical damage, chemical oxidation or biodegradation (Bandow et al., 2017;
429 Hüffer et al., 2018; Su et al., 2021). Thus, a greater deterioration in the removal of ARGs
430 during aerobic digestion is expected due to the presence of aged microplastics. In addition
431 to the aging factor, the surface of microplastics can adsorb different chemical substances
432 (e.g. antibiotics and bactericides) (Li et al., 2018; Ma et al., 2019) and heavy metals (e.g.
433 copper and silver) that present in wastewater or sludge (Brennecke et al., 2016). A large
434 number of those substances have been proved to be associated with the proliferation of
435 ARGs (Alonso et al., 2001). Accordingly, in aerobic sludge digestion, a higher
436 accumulation of ARGs in aerobic sludge digestion due to microplastics may occur under
437 real conditions than in the present experiment.

It is worth emphasized that this is a prototype experiment demonstrating that the exposure of microplastics could decrease the removal efficiency of ARGs in aerobic sludge digestion. Only three different categories (PVC, PE and PET, 600 μm) of microplastics in the same concentration (170 particles/g-TS) were conducted in this study. Despite informative results in our studies, more comprehensive trials (e.g., the size and concentration of microplastics) are recommended for further investigations. Besides, only a few known ARGs were selected as representatives to be investigated using RT-qPCR in our study. In future research, metagenomic sequencing and high-throughput qPCR could be the feasible methods to uncover the fate of more ARGs.

5. Conclusions

Our study investigated the impact of PVC, PE and PET microplastics on the fate of ARGs in aerobic sludge digestion. The main conclusions are:

- PVC, PE and PET microplastics at 170 particles/g-TS showed no significant effect on the sludge degradation during aerobic digestion;

- PVC, PE and PET microplastics at 170 particles/g-TS decreased the removal efficiency of the total abundance of tested ARGs by 129.6%, 137.0%, and 227.6% in aerobically digested sludge, respectively. This suggests that microplastics could accelerate the transfer of ARGs from the sludge to the local environment during sludge disposal or utilization;

- PVC, PE and PET microplastics increased the abundance of potential microbial hosts of ARGs, and also the abundance of *intI1* during aerobic sludge digestion. These could be the major reasons for the increased ARGs abundance in aerobically digested sludge.

CRediT authorship contribution statement

Zehao Zhang: Conceptualisation, Investigation, Methodology, Data curation, Formal analysis, Writing - original draft. **Huan Liu**: Methodology, Establishing experiment, Sampling, Visualization, Writing - review & editing. **Haiting Wen**: Visualization, Writing - review & editing. **Li Gao:** Sampling, Writing - review & editing. **Yanyan Gong**: Writing - review & editing. **Wenshan Guo**: Writing - review & editing. **Zhiyao Wang**: Writing - review & editing. **Xuan Li**: Writing - review & editing. **Qilin Wang**: Supervision, Conceptualisation, Project administration, Funding acquisition, Writing - review & editing.

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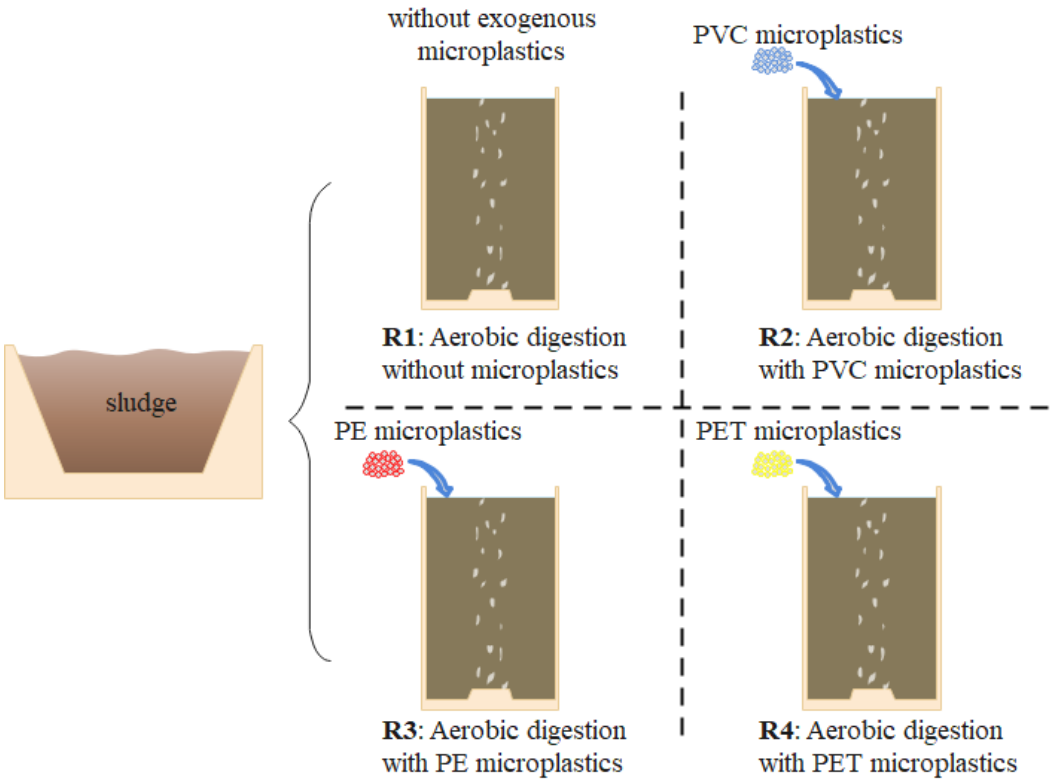
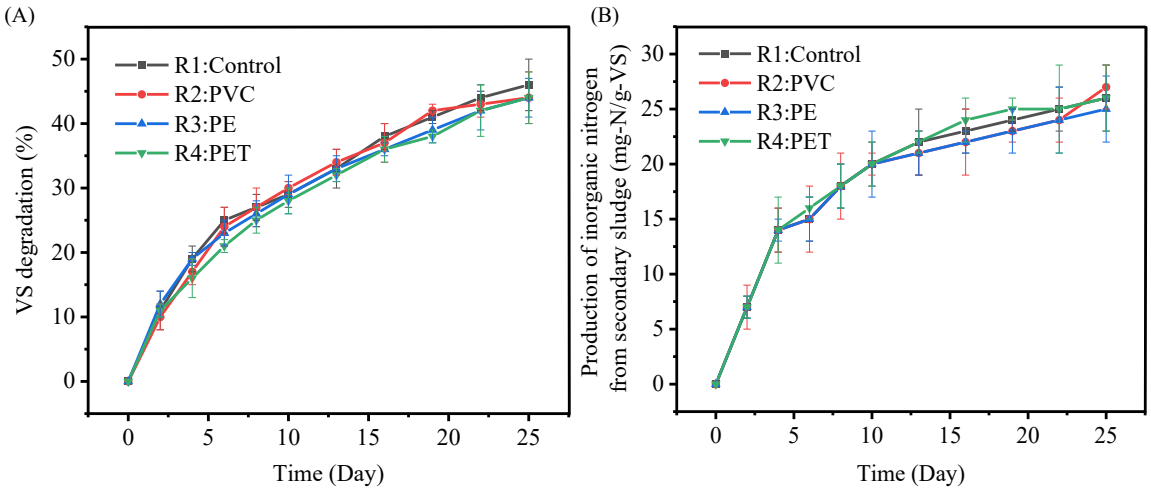


Figure 1. Aerobic sludge digestion experiments setup.



633

634 **Figure 2**. A) VS degradation (%) of secondary sludge with and without microplastics

635 during aerobic digestion; B) Inorganic nitrogen production from secondary sludge with and

636 without microplastics during aerobic sludge digestion. Error bars represent standard

637 deviations of triplicate tests.

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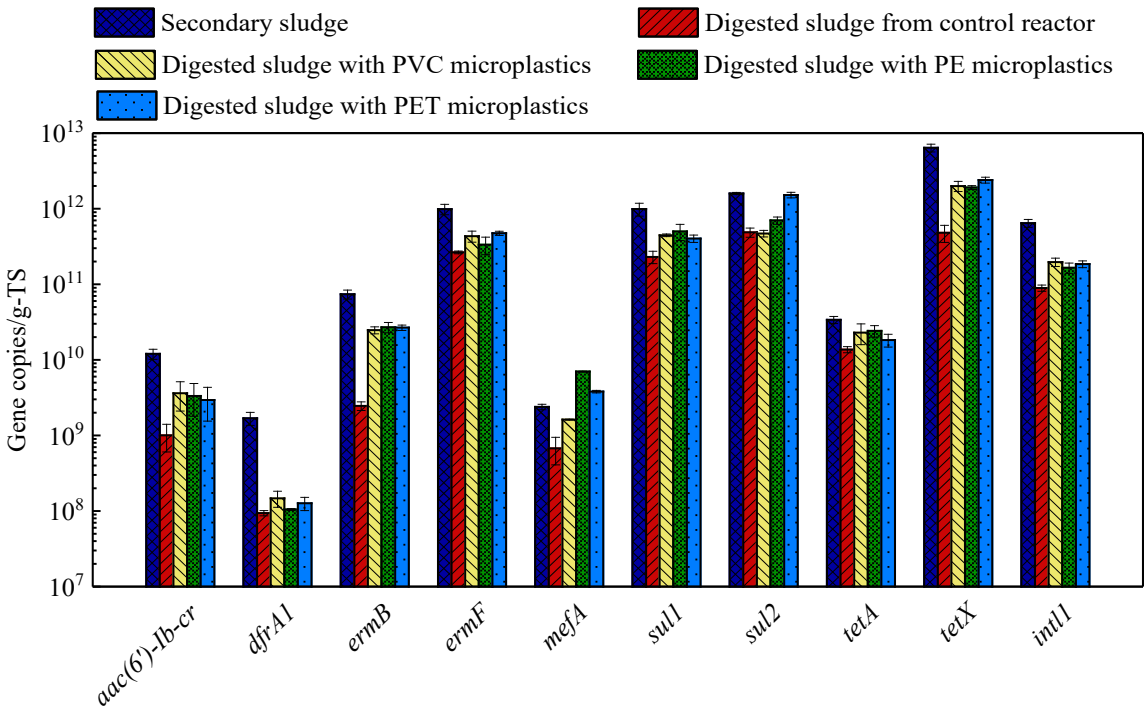

642

643 **Figure 3**. Absolute abundances of targeted ARGs and *intI1* in different sludges. Error bars

644 represent standard deviations of triplicate tests.

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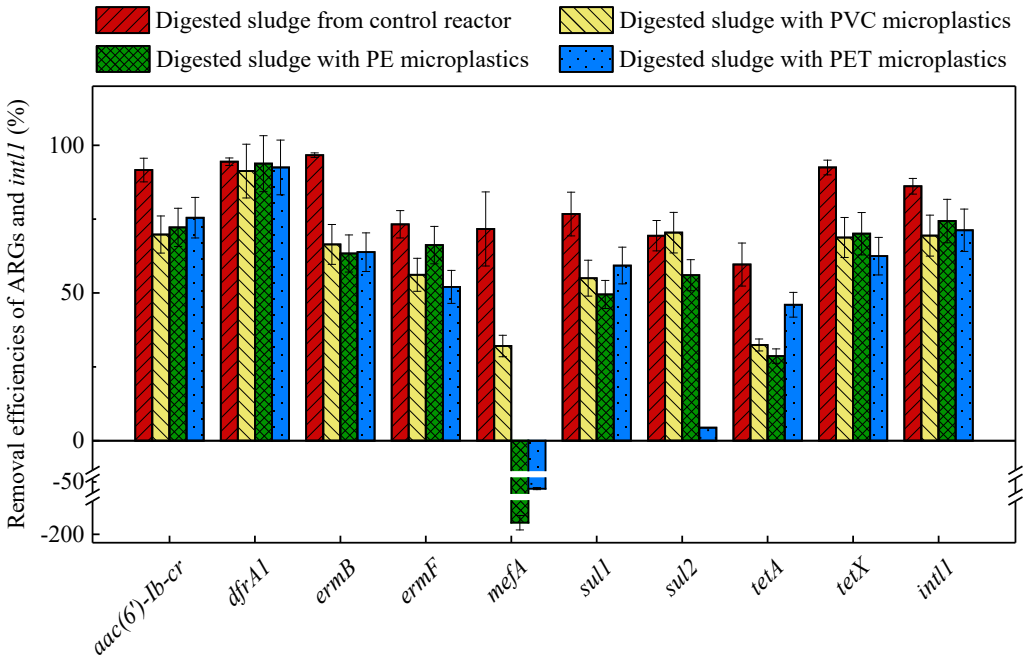

649

650 **Figure 4**. Removal efficiencies of targeted ARGs and *intI1* (%) due to aerobic sludge

651 digestion in the four reactors. Removal efficiencies (%) = ($N_{secondary}$ -

652 $N_{digested}$)/$N_{secondary}$×100% . $N_{secondary}$: ARGs or *intI1* in secondary sludge; $N_{digested}$: ARGs

653 or *intI1* in aerobically digested sludge from four reactors. Error bars represent standard

654 deviations.

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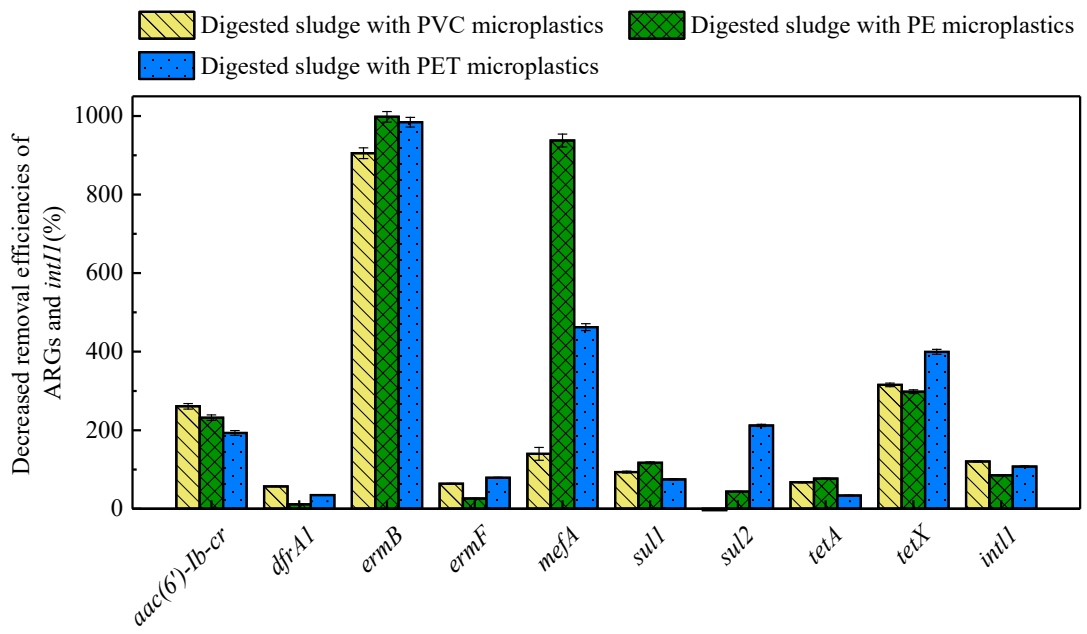

658

659 **Figure 5**. Decreased removal efficiencies of targeted ARGs and *intI1* (%) in aerobically

660 digested sludges with PVC, PE and PET microplastics compared to aerobically digested

661 sludges. Decreased removal efficiencies (%) = $(N_{microplastics} - N_{control})/N_{control} \times 100\%$.

662 $N_{microplastics}$: ARGs or *intI1* in aerobically digested sludge with microplastics; $N_{control}$: ARGs

663 or *intI1* in aerobically digested sludge from control reactor. Error bars represent standard

664 deviations.

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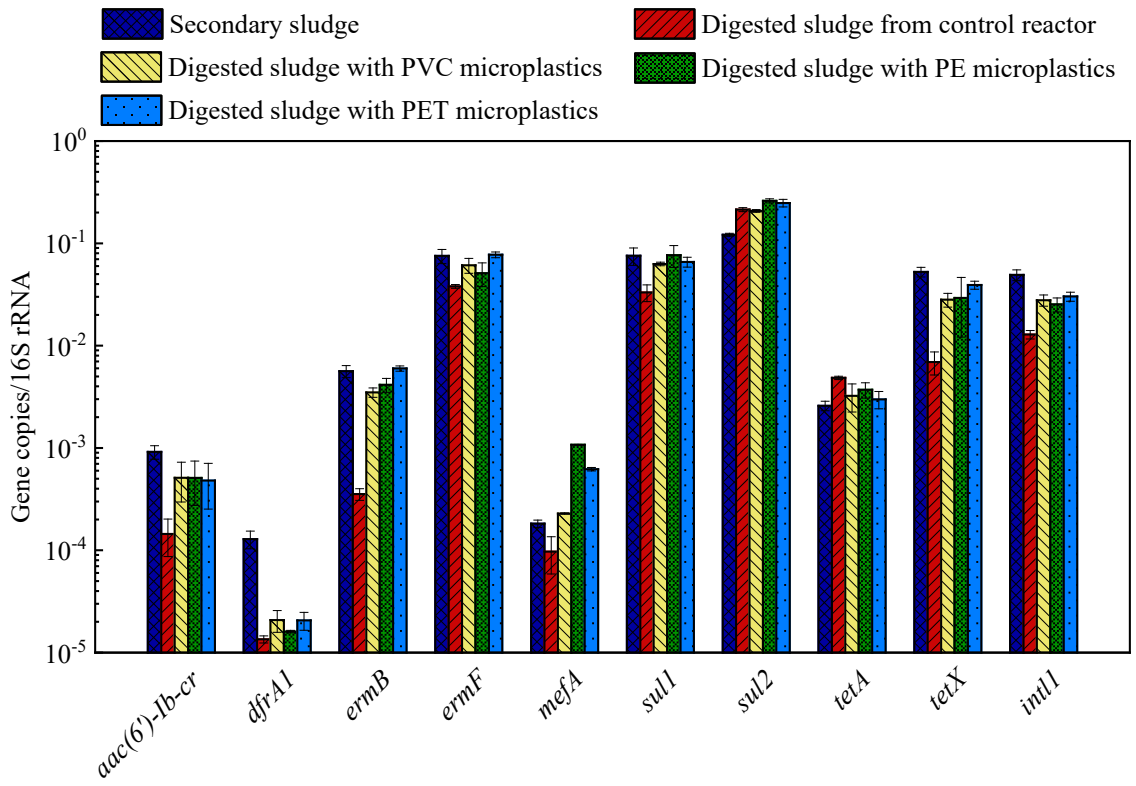

668

669 **Figure 6**. Relative abundances of ARGs and *intI1* in different sludges. Error bars represent

670 standard deviations of triplicate tests.

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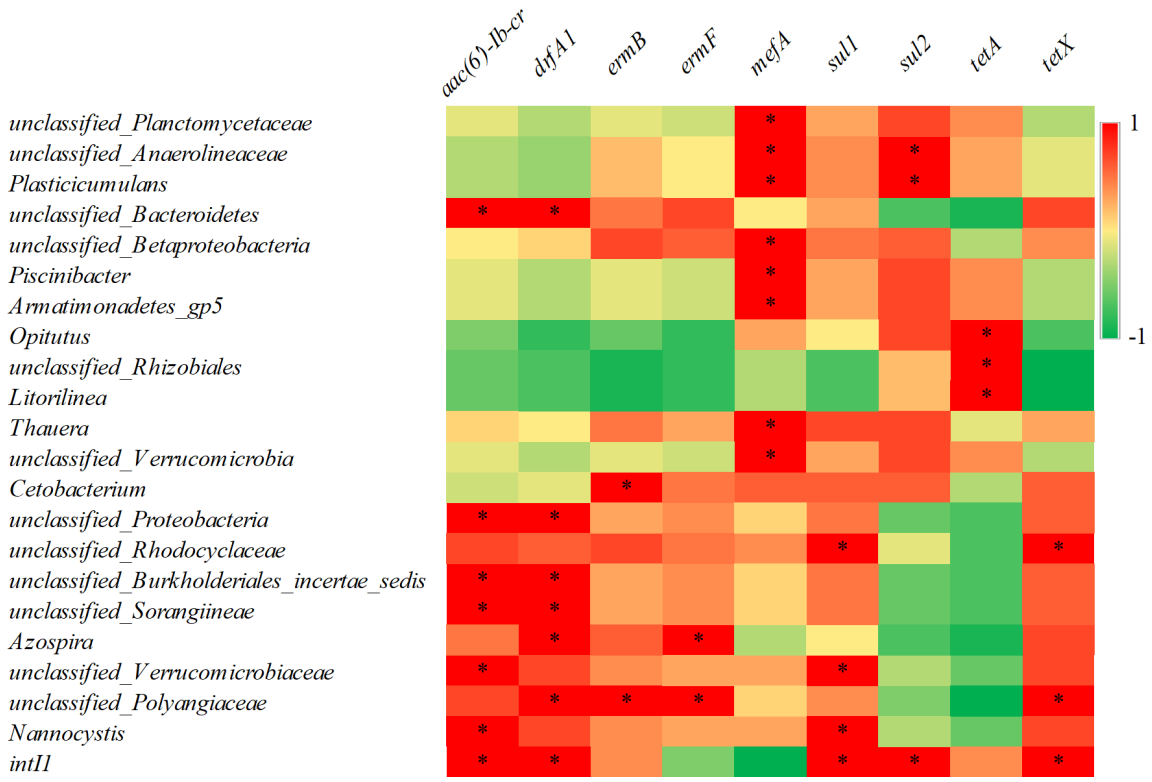

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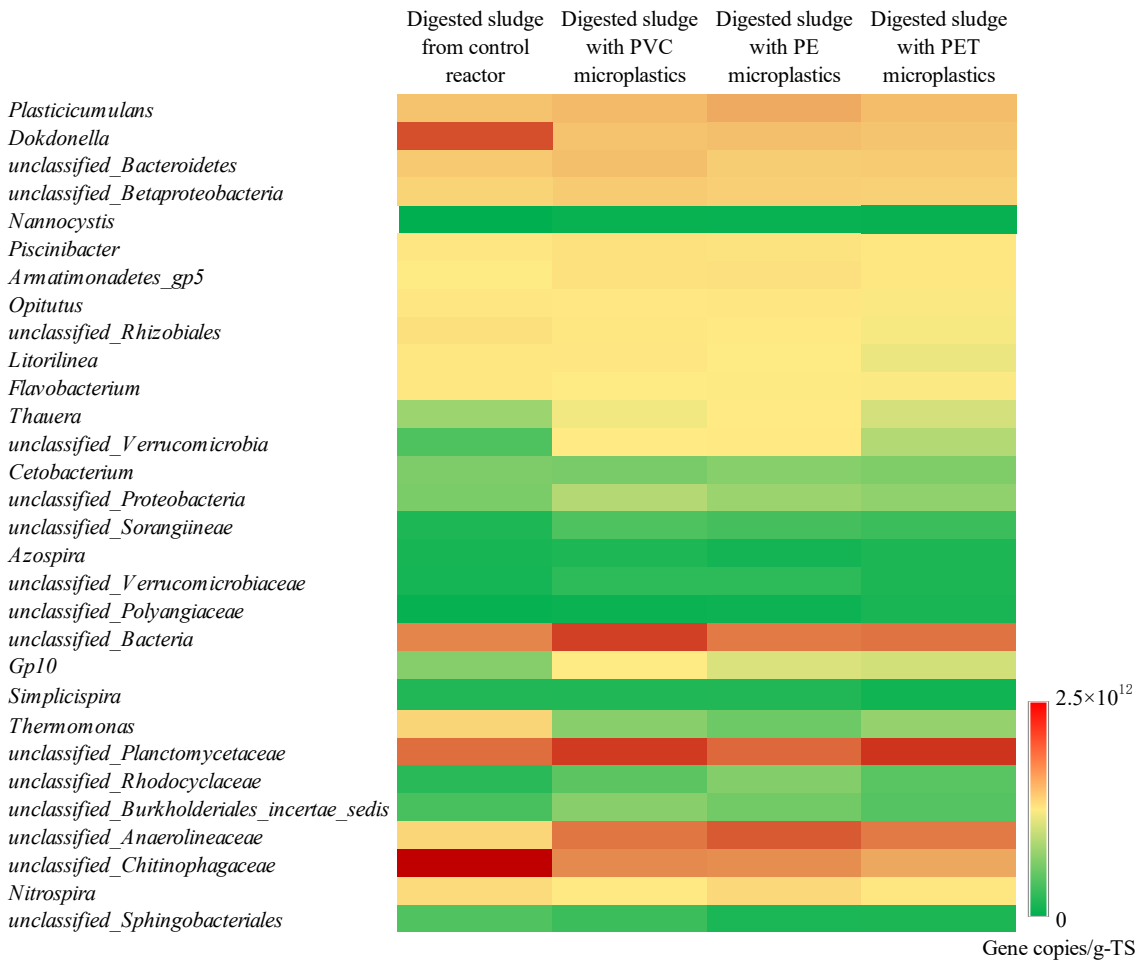
674 **Figure 7**. Correlation between ARGs and *intI1*/microbial community structure at the genus

675 level. An asterisk (*) indicates a significant positive correlation (R>0.8, p<0.05). The scale

676 bar showed the R value between ARGs and *intI1*/microbial community structure.



677

678 **Figure 8**. Heatmap of the top 30 most abundant bacterial genera in the aerobically digested

679 sludges with and without PVC, PE and PET microplastics.