**Alkaline fermentation of waste sludge causes a significant reduction of antibiotic
resistance genes in anaerobic reactors**

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ABSTRACT: Alkaline fermentation has been reported to be an effective method to recover valuable products from waste sludge. However, to date, the potential effect of alkaline pH on the fate of antibiotic resistance genes (ARGs) during anaerobic fermentation of sludge has never been documented. In this study, the target ARGs in sludge was observed to be removed effectively and stably when sludge was anaerobically fermented at pH 10. Compared with the control (without pH adjustment), the abundances of target ARGs at pH 10 were reduced by 0.87 (*sulII*), 1.36 (*sulIII*), 0.42 (*tet(O)*), 1.11 (*tet(Q)*), 0.79 (*tet(C)*) and 1.04 (*tet(X)*) log units. Further investigations revealed that alkaline fermentation shifted the community structures of potential ARGs hosts. Moreover, alkaline fermentation remarkably decreased the quantities and the ARGs-possessing ability of genetic vectors (plasmid DNA, extracellular DNA and phage DNA), which might limit the transfer of ARGs via conjugation, transformation and transduction. These results suggest that the shifted compositions of gene hosts and restricted gene transfer potential might be the critical reasons for the attenuation of ARGs at pH 10.

Keywords: Sludge alkaline fermentation; antibiotic resistance genes reduction; gene hosts; gene transfer potential

1. Introduction

In the last decade, the dissemination of antibiotic resistance genes (ARGs), which are considered as emerging contaminants, has become a global concern, posing risks to the environment and human health (Berendonk et al., 2015; Pruden et al., 2006). Once released from anthropogenic sources, some ARGs will eventually flow into wastewater treatment plants (WWTPs). It was reported that most (> 99%) of ARGs finally accumulated in waste sludge (Burch et al., 2013; Munir et al., 2011). Based on previous studies, 119 subtypes of ARGs were identified in sludge (Yang et al., 2014), and the concentration of some ARGs could be over 10^9 copies per gram of dry sludge (Ma et al., 2011; Munir et al., 2011). Given the

fact that the production of waste sludge increases dramatically every year (Feng et al., 2015), waste sludge would be a tremendous reservoir for ARGs.

Anaerobic treatment of sludge has been used widely to stabilize waste sludge, and at the same time some valuable products, such as short-chain fatty acids (SCFAs), can be recovered from sludge (Lee et al., 2014). The produced SCFAs could be supplied into WWTPs directly as complementary carbon sources for enhanced biological nutrient and phosphorus removal (Luo et al., 2015; Tong & Chen, 2007; Zhang & Chen, 2009). To improve the yield of SCFAs, alkaline fermentation of sludge, compared with traditional fermentation (i.e., without pH control), was reported to be beneficial to the hydrolysis of sludge protein and polysaccharide and inhibit the consumption of SCFAs by methanogens, resulting in a remarkably high SCFAs production (Chen et al., 2007; Zheng et al., 2013). However, to date, the potential effects of alkaline fermentation on the distribution of ARGs have never been reported.

It is well-known that the dissemination of ARGs mainly attributes to two processes: vertical gene transfer and horizontal gene transfer (HGT) (Gogarten & Townsend, 2005). The vertical transfer of ARGs mainly depends on the reproduction of antibiotic resistant bacteria (ARB) which has been suggested to be correlated with bacterial community compositions (Forsberg et al., 2014; Ma et al., 2011; Su et al., 2015). For example, the prevalence of *Pseudomonas* sp. in sludge was observed to account for the increase of the carried ARGs (Li et al., 2013). On the other hand, recent studies have confirmed that key genetic vectors, such as plasmids, extracellular DNA (eDNA) and phage DNA, which are extracted from sludge, can encode considerable copies of ARGs (Calero-Caceres et al., 2014; Zhang et al., 2011; Zhang et al., 2013). And, plasmids like pB10 were observed to transfer antibiotic resistance from *Escherichia coli* to sludge bacteria (Merlin et al., 2011). Thus, it is plausible that ARGs located in these vectors can be transferred from ARB to non-resistant bacteria by the pathways of HGT (i.e. conjugation, transformation and

transduction). Considering the shifted community structures during alkaline fermentation (Zheng et al., 2013), the dissemination of ARGs via vertical and horizontal transfer might also be disturbed. Unfortunately, to the best of our knowledge, no information is currently available on the fate of gene hosts as well as genetic vectors during sludge anaerobic treatment, especially alkaline fermentation.

The aim of this study, therefore, was to study the potential effect of long-term alkaline fermentation of sludge on the fate of ARGs, especially the sulfonamide and tetracycline resistance genes as they were the most widely detected ARGs in sludge (Ma et al., 2011; Munir et al., 2011). First, the quantities of target ARGs in the alkaline and conventional fermentation reactors were compared. Then, the underlying mechanisms for alkaline fermentation significantly reducing ARGs were explored from the following aspects: (1) the influences of alkaline fermentation on the community structures of potential ARGs hosts, (2) the fate of key mobile genetic vectors (plasmid, eDNA, and phage) under alkaline condition, and (3) the potential of HGT affected by alkaline pH value.

2. Materials and methods

2.1. Sludge preparation

Waste activated sludge was obtained from the secondary sedimentation tank of a municipal wastewater treatment plant, Shanghai, China. This wastewater treatment plant is operated with biological nutrient removal process. Before experiment, sludge was concentrated by settling at 4 °C for 24 h. The main characteristics of the concentrated sludge are as followings: pH 6.8 ± 0.1 , total suspended solids (TSS) 14960 ± 625 mg/L, volatile suspended solids (VSS) 10540 ± 582 mg/L, total chemical oxygen demand (TCOD) 14580 ± 592 mg/L, and soluble chemical oxygen demand (SCOD) 106 ± 8 mg/L.

2.2. Set-up and operation of the sludge fermentation reactors

In this study, six semi-continuous fermentation reactors were set up with 320 mL of sludge initially fed

in each one, and then all reactors were divided into two groups with three replicate in each set. During the entire fermentation process, the pH value in one group was adjusted to $\text{pH } 10.0 \pm 0.1$ by adding 4 M sodium hydroxide, but was not adjusted in other one which was set as the control. Every day, 40 mL of sludge mixture was wasted from each reactor, followed by adding the same amount of fresh sludge, which resulted in the sludge retention time (SRT) of 8 d ($\text{SRT} = 320/40 \text{ d}$) (Zheng et al., 2013). After the addition of sludge, reactors were flushed with nitrogen gas to remove oxygen, sealed, and placed in an air-bath shaker (120 rpm) at $35 \pm 1 \text{ }^{\circ}\text{C}$. During the operation period (100 d), the abundances of ARGs in the sludge were quantified frequently.

2.3. Extraction of various types of DNA

Sludge samples were firstly centrifuged at 4500 g and $4 \text{ }^{\circ}\text{C}$ for 10 min to obtain the liquid and solid phases, and then the various types of DNA were extracted (Figure S1, Supplementary data). To extract genomic DNA from the fermentation liquid (i.e. the liquid phase obtained by centrifugation), 10 mL of the supernatant was filtered with $0.45 \text{ }\mu\text{m}$ filters (Millipore, USA), and the filters were processed according to the reference (Munir et al., 2011). Also, 0.5 g (wet weight) of the pellet was used to extract the total genomic DNA of sludge following the Purkhold's protocol, which consists of lysozyme, proteinase K and sodium dodecyl sulfate (SDS) treatments (Purkhold et al., 2000). In addition, calf thymus DNA was used as the internal standard to measure the DNA extraction efficiencies from the two sets of examples (Chen et al., 2006). The data suggested that the discrepancy in extraction efficiencies between sludge samples could be ignored.

Considering the complexity of sludge, conventional method (alkaline lysis) seems to be non-ideal to extract plasmid DNA directly from sludge. Thus, a protocol using genomic DNA as the source material was applied (Zhang et al., 2011). Briefly, the extracted genomic DNA was further purified by using

QIAGEN Plasmid Mini Kit (Qiagen, USA) to concentrate plasmids and ATP-dependent Plasmid Safe DNase (Epicentre, USA) to remove remaining sheared genomic DNA.

For the extraction of extracellular DNA, a combined protocol was used according to the literatures (Corinaldesi et al., 2005; Zhang et al., 2013). Briefly, coupled with 0.2 g of acid-washed polyvinylpolypyrrolidone (PVPP), 1 g (wet weight) of the pellet was homogenized and washed 3 times using 0.1 M phosphate-buffered saline (PBS, pH 8.0). After the mixture was centrifuged at 500 g for 10 min, the supernatants were combined and again centrifuged at 10000 g and 4 °C for 20 min, and the re-obtained supernatants were filtered with a sterile 0.22 µm membrane filter (Millipore, USA). Finally, 1% cetyltrimethylammonium bromide (CTAB) was used to precipitate the extracellular DNA in the supernatant.

The phage DNA was extracted according to the reference (Calero-Caceres et al., 2014). Briefly, the pellet was homogenized 1:10 (w:v) with PBS (pH 7.4), suspended by magnetic stirring at room temperature for 30 min, and centrifuged at 10000 g and 4 °C for 20 min. The supernatants were purified using low protein-binding 0.22-µm-pore-size membrane filters (Millipore, USA), and then 100-fold concentrated by means of protein concentrators (100 kDa Amicon Ultra centrifugal filter units, Millipore, USA) according to the manufacturer's instructions. Finally, the concentrate was treated with chloroform and DNase (100 U/mL) to eliminate free DNA, and the phage DNA was isolated by the proteinase K digestion and SDS treatment (Colomer-Lluch et al., 2011). During this process, several controls were carried out to verify the specific treatment based on the published instructions (Calero-Caceres et al., 2014). All kinds of extracted DNA were further purified using the phenol-chloroform method (Zhang et al., 2013). Agarose gel electrophoresis was used to characterize the qualities of extracted DNA, and Nano Drop 2000 (Thermo Scientific, USA) was used to determine their extraction yields.

2.4. Quantification of target genes using real-time quantitative PCR

Sulfonamide resistance genes (*sul* I and *sul* II), tetracycline resistance genes (*tet*(O), *tet*(Q), *tet*(C) and *tet*(X)) and 16S rRNA genes were quantified using real-time quantitative PCR. The detailed information regarding the annealing temperatures and primers of target genes is recorded in Table S1 (Supplementary data). These assays were performed via a Step-One Real Time PCR System (Applied Biosystems, USA) in a total volume of 20 μ L containing 1 $\times$ SYBR Green PCR Master Mix (TaKaRa, Japan), 0.4 μ M each primer, and 4 ng of template DNA. The amplification program consisted of an initial denaturation at 95 $^{\circ}$ C for 10 min, 40 cycles of denaturing at 95 $^{\circ}$ C for 30 s, annealing at certain temperature (Table S1) for 30 s, and extension at 72 $^{\circ}$ C for 30 s, followed by a final extension at 72 $^{\circ}$ C for 30 s. Additionally, melting curves were also conducted with temperature lasting at certain annealing points for 30 s, ramping up to 95 $^{\circ}$ C in 20 min and then holding for 15 s to screen for nonspecific products,. The copy numbers of each target gene were determined via a standard curve generated by using 10-fold serial dilutions (Easy Dilution, Takara) of plasmids containing the target gene as a template. All PCR assays including the negative control were performed in triplicates. The efficiencies of the amplification of assessed genes were measured by diluting plasmid or sample DNA and calculated from the slopes of PCR reactions ($E=10^{(-1/\text{slope})}$). And the obtained data suggested that the amplification efficiencies of sample DNA and standard DNA have no significant differences. The quantities of ARGs in sludge were normalized to both grams of dry sludge and 16 rRNA genes. As for genes carried on genetic vectors, their amounts were assessed by the copies on per ng of DNA.

2.5. Identification of potential ARGs hosts in the fermented sludge

On day 100, sludge mixtures from all three reactors of the same set were mixed before being divided equally into three triplicates. As to these triplicates in each series, the random two reactors were spiked

with sulfamethoxazole (200 µg/mL) and tetracycline (20 µg/mL), respectively, to maintain the selective pressure based on previous studies (Gao et al., 2012; Li et al., 2013). The other one without any addition of antibiotics was operated as the control. During following 8 d incubation, all reactors were covered with aluminum foil, and sludge was centrifuged and re-suspended every 24 h. Also, the reactors exposed to antibiotics were re-spiked under initial concentration of specific antibiotic everyday (Li et al., 2013). After the incubation, DNA was extracted from sludge samples, and 16s rRNA genes were PCR-amplified with 338F and 533R primers. The PCR assays were conducted in a 25-µL reaction system containing 1 × Ex Taq reaction buffer (TaKaRa, Japan), 0.2 mM dNTPs, 0.4µM each primer, 2 U Ex Taq polymerase and 0.2 ng template DNA, and followed the temperature protocol described in a recent study (Li et al., 2013). After purification, PCR products were end-repaired, A-tailed and PE-adaptor ligated, and then sequenced on Illumina MiSeq 2000 according to the manufacturer's instructions. All raw reads which didn't match the primer exactly or contained ambiguous character (N) or had a shorter length (≤ 150 bp) were firstly abandoned. Then, chimeras were identified and removed for further analysis. A total of 307653 clean reads for 6 samples were generated. All clean reads have been submitted to the National Center for Biotechnology Information (NCBI) short-read archive (SRA) under accession numbers SRR2140592 to SRR2140597, and then subjected to the Silva Database for taxonomic assignment. The relative abundances (rb) of bacteria before and after the antibiotic incubation assays were compared, and bacteria whose initial relative abundance was $> 0.01\%$ and increased significantly ($\log_{1.5}(\text{rb-after}/\text{rb-before}) > 1$) were identified as the sulfonamide or tetracycline selected bacteria in the current study (Figures S2-S5, Supplementary data).

2.6. Analytic methods

The dry solid content of sludge was obtained by drying it at 105 °C overnight. The measurements of

SCFAs, TSS, VSS, TCOD, and SCOD were applied according to our previous publication (Luo et al., 2015). Zeta potential of the sludge mixture was measured by a streaming potential analyzer (EKA 1.00, Anton-Paar, Swiss) at temperature of 25 °C. Calcium and magnesium ions were measured by an atomic absorption spectrophotometer (AAnalyst 400, PerkinElmer, U.S.A). All tests in this study were performed in triplicate, and the one way analysis of variance (ANOVA) at a 0.05 level was used to analyze the data.

3. Results and discussion

3.1. Effects of alkaline fermentation on the distribution of ARGs in sludge

Figure 1 presents the absolute quantities of target genes in sludge sampled from two sets of reactors in the long-term operation. The abundances of all ARGs were relatively stable after day 60, which shared a similar trend with the accumulation of SCFAs (Figure S6, Supplementary data), suggesting that the distribution of ARGs might be associated with the performances of reactors. Additionally, it was significant that alkaline fermentation resulted in better removal of assessed ARGs than the conventional treatment ($p < 0.05$). In reactors without pH control, it was observed that 0.19 ~ 0.46 log unit of target genes were reduced on day 100, which was consistent with the previous observation (Ma et al., 2011). But the residual amounts of *sul* I, *sul* II, *tet*(O), *tet*(Q), *tet*(C) and *tet*(X) in sludge were still as high as 3.09×10^{12} , 7.33×10^{11} , 6.55×10^9 , 3.22×10^9 , 1.49×10^{11} and 9.92×10^{10} copies/g dry sludge, respectively. Nevertheless, when sludge was fermented at pH 10, the abundances of corresponding ARGs were significantly decreased to 4.16×10^{11} , 3.22×10^{10} , 2.50×10^9 , 2.51×10^8 , 2.39×10^{10} and 9.02×10^9 copies/g dry sludge. These data indicated that, compared with the conventional treatment, fermentation at pH 10 could further reduce 0.87, 1.36, 0.42, 1.11, 0.79 and 1.04 log unit of investigated genes, which implied that alkaline fermentation was more effective for the attenuation of ARGs.

Figure 1. Effects of alkaline fermentation on the distribution of sulfonamide (a and b) and tetracycline resistance genes (c-f) in the long-term operation. Error bars represent the standard deviations of triplicate measurements.

Considering the sludge samples might have different overall population sizes, in this study the measured ARGs were normalized to 16S rRNA genes (Figure S7, Supplementary data). It can be seen that the net reduction with the range from 0.17 to 1.17 log unit was induced by fermentation at pH 10, which shared a remarkable similar trend with that observed in Figure 1, confirming the high removal efficiencies of this process for all target genes. In addition, it was obvious from the data in Figure 1 and Figure S6 that all reduced ARGs remained relatively stable in the long-term pH 10 fermentation test. According to previous studies, treating sludge with the higher temperature could benefit the removal of some specific ARGs (Diehl & LaPara, 2010), but other genes such as *sul I* (Zhang et al., 2015), *tet(O)* (Wu et al., 2016), *tet(C)* and *tet(X)* (Ma et al., 2011) were somehow enriched. Also, pretreating sludge with thermal hydrolysis, ozone and microwave contributed to evident attenuation of ARGs, but some genes

tended to rebound during subsequent anaerobic digestion (Ma et al., 2011; Pei et al., 2016; Tong et al., 2016). Therefore, with respect to the target genes in the current study, alkaline fermentation seems to be a universal and stable method, benefiting their attenuation. Additionally, although the pH 10 condition was reported to promote the hydrolysis of waste sludge (Maspolim et al., 2015), our results in Table S2 (Supplementary data) indicated that alkaline fermentation actually didn't cause the significant release of ARGs into the fermentation liquid.

3.2. Effects of alkaline fermentation on the community structures of ARGs hosts in sludge

It has been reported that community structures were the primary determinants of ARGs content, contributing to the changes in ARGs patterns (Zhang et al., 2016). Thus, to explore the main reasons for alkaline fermentation remarkably reducing ARGs, the community structures of ARGs hosts involved in sludge fermentation systems were firstly investigated. The antibiotics selection experiments indicated that the compositions of tetracycline and sulfonamide selected bacteria at the genus level were significantly affected by alkaline fermentation (Figure 2). For example, the tetracycline selected bacteria in the conventional reactors were mainly affiliated to the genera of *Caldilinea* (31.1%), *Clostridium* (12.6%) and *Roseiflexus* (11.4%) (Figure 2a), while those in the alkaline fermentation reactors were closely related to *Clostridium sp.* (35.1%), *Corynebacterium sp.* (7.8%), and *Brevundimonas sp.* (7.8%) (Figure 2b). As to the sulfonamide selected bacteria, *Thauera* (25.3%), *Synergistes* (23.0%) and *Ilumatobacter* (9.5%) were the dominant genera during pH uncontrolled fermentation (Figure 2d), whereas *Bacillus sp.* (52.6%), *Corynebacterium sp.* (7.3%) and *Erysipelothrix sp.* (5.6%) were the major bacteria after alkaline fermentation (Figure 2e). Clearly, a number of genera have been selected as potential gene hosts in digested sludge. But it should be mentioned that the method applied in this study might not cover all possible hosts of target ARGs.

Figure 2. Effects of alkaline fermentation of sludge on the community structures of tetracycline (a and b) and sulfonamide selected bacteria (d and e) as well as the corresponding abundances (c and f) at the genus level.

At the phylum level (Table S3), on the other hand, the tetracycline and sulfonamide selected bacteria in the control reactors were mainly grouped into *Chloroflexi* (50.2%) and *Proteobacteria* (35.9%), respectively. However, those under alkaline condition were both affiliated to *Firmicutes* (48.6% & 68.3%). It had been observed that pH 10 condition was beneficial to the enrichment of *Firmicutes* (Maspolim et al., 2015). Also, *Firmicutes* were frequently associated with antibiotic resistance (Su et al., 2015). That might account for the observed shift of ARGs hosts in alkaline fermentation reactors. Additionally, the dominant genera, such as *Clostridium* and *Bacillus*, responsible for the hydrolysis and acidogenesis processes, were reported to be prevalent in alkaline fermentation systems (Liu et al., 2012). As *Clostridium sp.* had been reported to be the carrier of *tet*(O), *tet*(Q) or *tet*(C) (Chopra & Roberts, 2001), and *Bacillus sp.* was the shelter of *sul* I and *sul* II (Gao et al., 2012), it seems that the functional genera involved in SCFAs production could also serve as ARGs hosts.

It has been reported in the literature that the vertical transfer of ARGs was associated with the

reproduction of the bacterial hosts (Tian et al., 2016). In this study, for example, *Caldilinea* sp., the dominant genera of tetracycline selected bacteria in conventional reactors, might play an important role in the vertical transfer of corresponding genes. However, alkaline pH resulted in the apoptosis of these specific gene hosts (Figure 2b), which might contribute to the decline of the carried genes (Diehl & LaPara, 2010). As to the selected bacteria at pH 10, such as *Clostridium* sp. and *Bacillus* sp., although they were the hosts for ARGs, their total abundances went through a decrease (Figure 2), suggesting that the amounts of gene potential hosts were restricted. Thus, the shifted community structures caused by alkaline fermentation might disturb the vertical transfer of target genes in selected hosts, benefiting the reduction of corresponding ARGs. Similarly, it should be noted that there existed a risk of other non-investigated ARGs to be enriched at pH10 due to the community shifts.

3.3. Effects of alkaline fermentation on the potential of horizontal gene transfer

Horizontal gene transfer is a well-recognized process for the dissemination of ARGs, and it has been reported that anaerobic sludge microcosms could promote such process (Merlin et al., 2011). Due to the high densities of biomass (Ghosh et al., 2009), bacteria within sludge flocs could transfer genes via genetic vectors, i.e., plasmid, eDNA, and phage, by conjugation, transformation and transduction, respectively (Andersson & Hughes, 2010). Compared with the other two pathways, conjugation with plasmids was regarded as the most common one in the natural environment (Huddleston, 2014). Figure 3 showed that the concentration of plasmid DNA after alkaline fermentation was only 49.4% of that in the control, indicating that alkaline fermentation had significantly negative influences on the persistence of plasmid DNA in sludge ($p < 0.05$). As is well known, alkali can benefit the break-up of sludge flocs (Doğan & Sanin, 2009), and induce the solubilization of membrane proteins and saponification of the membrane lipids (Mendonca et al., 1994), which result in the release of plasmids. Also, plasmids were observed to

be degraded more quickly once they released from the host bacteria (Merlin et al., 2011). Thus, alkaline fermentation contributed to the fewer amount of plasmids transferable for conjugation. Meanwhile, results from Lin et al. (2016) suggested that more transferable plasmid could increase the transfer frequency, leading to higher numbers of transconjugants. It therefore seems that the conjugation process might be suppressed because of the fewer plasmids available at pH 10.

Figure 3. Effects of alkaline fermentation on the relative concentrations of genetic vectors (plasmid DNA, eDNA and phage DNA) in treated sludge. Error bars represent the standard deviations of triplicate measurements.

To further dig out the effect of pH 10 condition on the conjugation process, the carrying ability of plasmid DNA was investigated. Compared with the control the amount of *sul* I, *sul* II, *tet*(O), *tet*(Q), *tet*(C) and *tet*(X) on per ng of plasmid DNA was decreased by respectively 35.6%, 76.4%, 21.1%, 65.7%, 26.5% and 47.9% after fermentation at pH 10 (Figure 4a). It was reported that plasmids could possibly possess specific genetic locus for particular ARGs (Ma et al., 2014). The restricted carrying ability therefore implied that the locus tended to be inactivated by alkaline pH, contributing to lower transfer efficiency of assessed ARGs. On the other hand, it is well-known that bacteria conjugation requires cell-to-cell contact, and surface exclusion is considered as a barrier for this pathway (Thomas & Nielsen, 2005). However, the data in Figure 4b shows that alkaline pH remarkably decreased the zeta potential ($p < 0.05$), which is an

important parameter relevant with the surface charge of sludge mixture (Luo et al., 2015). With the electric charge dramatically dropped from -26.0 mV in the control to -36.6 mV under the alkaline condition, the interaction between anaerobic bacteria became harder due to the augmented mutual repulsion (Luo et al., 2015), suggesting that it became more difficult for the occurrence of bacteria conjugation at pH 10. Therefore, the genes transferred by conjugation via plasmids were restricted, which accounted for the reduced abundances of ARGs during sludge alkaline fermentation.

Figure 4. Effects of alkaline fermentation on the relative carrying ability of plasmids for target ARGs (a) and the zeta potential of sludge mixture (b). Error bars represent the standard deviations of triplicate measurements.

eDNA, which is released from the decomposed or disrupted cells or excreted from living cells (Thomas & Nielsen, 2005), is recognized as another important genetic vehicle and can transfer genetic traits once being integrated into recipient cells (Johnston et al., 2014). In the current study, the concentration of eDNA in the alkaline fermented sludge was only 15.1% of the control (Figure 3), suggesting that the gene vector for transformation was significantly limited ($p < 0.05$). Additionally, the data in Figure 5a confirm that eDNA is an reservoir for sulfonamide and tetracycline resistance genes, which was consistent with the observation by Mao et al. (2014). More importantly, it was observed that the carrying capabilities of eDNA for *sul I*, *sul II*, *tet(O)*, *tet(Q)*, *tet(C)*, and *tet(X)* were all reduced

remarkably in the pH 10 experiment ($p < 0.05$). The relative copy of *sul* II located on per ng of eDNA was decreased to 5.5% of the control. As for the other target ARGs, their relative copies were in the range of 40% ~ 70% of the control. Clearly, genes available for transformation via eDNA were significantly restricted by pH 10 fermentation.

Figure 5. Effects of alkaline fermentation on the relative carrying ability of eDNA for target ARGs (a) and the concentrations of measured Ca^{2+} and Mg^{2+} (b). Error bars represent the standard deviations of triplicate measurements.

The concentration of eDNA has been observed to be a key parameter affecting the transformation efficiency, and a proportional relationship between them was reported previously (Chung et al., 1989; Liu et al., 2014). It is reasonable to speculate that the limited eDNA caused by alkaline fermentation would negatively affect the transformation efficiency. Furthermore, the presence of a certain amount of bivalent ions, such as Ca^{2+} and Mg^{2+} , which are the essential components for the formation of sludge flocs and can be released during sludge anaerobic treatment (Novak et al., 2003), has been shown to promote the uptake of eDNA by indigenous bacteria (Chung et al., 1989; Liu et al., 2014). However, our observation shows that alkaline pH remarkably reduced the concentration of these two cations ($p < 0.05$) (Figure 5b), indicating the eDNA uptake might be negatively affected, which decreased the efficiency of transformation. Apparently, with restricted transferable genes and lower transformation efficiency, the dissemination of

ARGs via eDNA was blocked.

Figure 6. Effects of alkaline fermentation (pH 10) on the relative carrying ability of phage DNA for target ARGs. Error bars represent the standard deviations of triplicate measurements.

As to the dissemination of ARGs via transduction, phages have also been recognized as important mobile genetic elements and are distributed widely in sewage and sludge (Calero-Caceres et al., 2014; Uchiyama et al., 2014). The study of Calero-Caceres et al. (2014) indicated that sludge was a concentrated pool of phages, in which the number of somatic coliphages (an indicator of phages) was only 1.5 log unit lower than that of heterotrophic bacteria. Usually, phages were reported to have remarkable persistence and showed high survival ability after anaerobic treatment (Brown-Jaque et al., 2015). However, the current study indicated that alkaline fermentation caused a significant reduction (65%) of phage DNA ($p < 0.05$) (Figure 3). Also, the abundances of all target ARGs on per ng of phage DNA in the alkaline fermented sludge was much lower than those in the control (Figure 6). It is widely recognized that phages harboring ARGs could infect new hosts and introduce the ARGs into new cells, thereby conferring the given resistance (Calero-Caceres et al., 2014). Apparently, the lower amount and carrying ability of phage DNA may result in the declined efficiency of transduction, which also plays an important role in the spread of ARGs during sludge fermentation.

4. Conclusions

In summary, this study first confirmed that fermentation sludge at pH 10 could promote the removal of target ARGs in the range of 0.42 ~ 1.36 log unit. The reasons for the improved reduction were attributed to the shifted community structures and significantly decreased abundances of ARB, which disturbed and restricted the ARGs hosts in sludge. Meanwhile, with declined concentrations of genetic vectors (plasmid DNA, extracellular DNA and phage DNA) and the ARGs-possessing ability, as well as other factors such as lower zeta potential and $\text{Ca}^{2+}/\text{Mg}^{2+}$ concentration at high pH, the potential of HGT was inhibited, which promoted the reduction of assessed genes. Based on these results, alkaline fermentation turned out to be a promising way for the attenuation of ARGs in sludge.

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Appendix A. Supplementary data

This file contains additional Materials and Methods, Tables S1-S3 and Figures S1-S9. This information is available free of charge via the Internet at <http://www.journals.elsevier.com>.

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Graphical abstract