Distribution of tetracycline resistance genes in anaerobic treatment of waste sludge: The role of pH in regulating tetracycline resistant bacteria and horizontal gene transfer

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ABSTRACT: Although pH value has been widely regarded as an important factor that affects resource recovery of waste sludge, the potential influence of diverse pHs on the distribution of tetracycline resistance genes (TRGs) during sludge anaerobic treatment is largely unknown. Here we reported that in the range of pH 4-10, 0.58-1.18 log unit increase of target TRGs was observed at pH 4, compared with that at pH 7, while 0.70-1.31 log unit further removal were obtained at pH 10. Mechanism study revealed that varied pHs not only altered the community structures of tetracycline resistant bacteria (TRB), but also changed their relative abundances, benefitting the propagation (acidic pHs) or attenuation (alkaline pHs) of TRB. Further investigation indicated that the amount and gene-possessing abilities of key genetic vectors for horizontal TRGs transfer were greatly promoted at acidic pHs but restricted under alkaline conditions.

Keywords: diverse pHs; sludge anaerobic treatment; tetracycline resistance genes; tetracycline resistant bacteria; horizontal transfer

1. Introduction

With the growing application of biological wastewater treatment, a huge amount of waste sludge is generated. In China, for example, 34 million metric tonnes of waste sludge were produced in 2015, posing a great threat to the environment (Feng et al., 2015). Nowadays, anaerobic treatment has been widely used to solve the sludge problem, because it can recover energy and some valuable products from waste sludge (Zhang et al., 2011a). The literature reported that the pH value is a critical parameter for anaerobic sludge treatment, affecting the solubilization of organic matters (Maspolim et al., 2015), which subsequently determines the yields of short-chain fatty acids or methane (Chen et al., 2007; Zhang et al., 2011a). For instance, the previous study found that alkaline fermentation was able to enhance the hydrolysis of waste sludge, and thus improved the efficiency of anaerobic treatment (Chen et al., 2007). However, the potential impacts of diverse pHs on the fate of sludge-accumulated contaminants,

especially antibiotic resistance genes (ARGs), remain unclear.

With the extensive applications of antibiotics in medical and veterinary fields, the dissemination of ARGs has posed potential risks to the environment and human health (Berendonk et al., 2015; Zhang et al., 2015). ARGs could be released from anthropogenic sources, and then entered wastewater treatment plants. It was reported that more than 99% of these ARGs were eventually accumulated in waste sludge (Burch et al., 2013), which led to sludge becoming tremendous and potential reservoirs of antibiotic resistance. As one of the extensively studied ARGs, tetracycline resistance genes (TRGs) were frequently detected in waste sludge, and their concentration was reported at about 10^8 to 10^{13} copies per gram (Pei et al., 2016). During last decades, a few studies observed that sludge treatments were functional for the removal of some TRGs, but the concentrations of residual TRGs in treated sludge were still considerable (Burch et al., 2013; Ma et al., 2011). To improve the removal efficiency, attentions were paid to parameters such as temperature (Diehl & LaPara, 2010; Ma et al., 2011) and pretreatment (Pei et al., 2016). Nevertheless, to date, it is unknown whether the diverse pHs of anaerobic treatment would affect the fate and removal of TRGs in waste sludge.

It is well-known that antibiotic resistant bacteria (ARB) are the hosts and sources for the propagation of ARGs (Berendonk et al., 2015; Gao et al., 2012). In sludge, tetracycline resistant bacteria (TRB) could account for up to 3.97% of the total bacterial community, and the abundances of specific TRGs were observed to positively associate with the related genus (Li et al., 2013). Also, some researchers found that the dynamic of ARGs was strongly affected by the bacteria community structures (Ma et al., 2011; Su et al., 2015). Meanwhile, because sludge has the high dense and diversity of microbial communities, it is easy for the dissemination of ARGs from ARB to non-resistant bacteria by horizontal gene transfer (HGT) (Su et al., 2015). Previous studies have indicated that the genetic vectors, such as plasmids, extracellular DNA

(eDNA) and phages, are able to encode considerable concentrations of TRGs (Calero-Caceres et al., 2014; Mao et al., 2014; Zhang et al., 2011b), suggesting that these key vectors play vital roles in the propagation of tetracycline resistance. Although the changes in pHs were reported to shift the bacterial community structures (Zheng et al., 2013), their potential effects on the patterns of TRB and the fate of genetic vectors have seldom been considered.

The aim of this study was to investigate the effects of different pHs on the distribution of TRGs during sludge anaerobic treatment. Firstly, the abundances of target TRGs (*tet*(O), *tet*(Q), *tet*(C) and *tet*(X)) were examined during sludge treatment at pH 4-10. Then, the underline mechanisms for pH significantly affecting the fate of TRGs were explored by analyzing the shift in the community structure and the changes in the relative abundance of TRB. Also, the key genetic vectors (plasmid, extracellular DNA, and phage) involved in horizontal gene transfer were characterized to dig out their potential roles in the distribution of TRGs when sludge was treated under the diverse pH conditions.

2. Materials and methods

2.1 Set-up and operation of sludge anaerobic reactors. To investigate the effects of diverse pHs on the distribution of TRGs during anaerobic treatment, sludge was fermented at pH 4-10 in order to transform particulate organic matters into soluble substances (i.e., Phase I) (Figure S1), and then digested for methane production under the neutral condition (i.e., Phase II) according to the literature (Zhang et al., 2011a). Briefly, in Phase I, reactors were adjusted to 4, 5, 7, 9 and 10, respectively, and the sludge retention time (SRT) was maintained at 8 d. Meanwhile, the pH value of all reactors in phase II was controlled at pH 7, and the SRT was maintained at 9 d. To maintain the concentration of anaerobic granular sludge, wasted granular sludge was put back to digesters by sifting wasted sludge through an aperture 0.2 mm sifter. All reactors were sealed and mixed by stirrers at a speed of 100 rpm in a 35 ± 1 °C temperature-controlled

room. On day 100, sludge was sampled from all reactors for the extraction of various DNA, i.e. genomic DNA, plasmid DNA, eDNA and phage DNA (see Supplementary data).

2.2 Identification of tetracycline resistant bacteria in sludge. To identify the potential TRB in treated sludge, stress incubation assays were adopted according to the literature (Li et al., 2013). Briefly, on Day 100, sludge mixtures obtained from the same sets of reactors in phase I were firstly mixed, and then re-divided into two duplicates. As to these duplicates, one was spiked with tetracycline (20 µg/mL) to maintain the selective pressure (Li et al., 2013). The other one was conducted without the addition of tetracycline and set as the control. During following incubation of 8 days, all reactors were covered by aluminum foil, and sludge was centrifuged, re-suspended and re-spiked under initial concentration of tetracycline every day (Li et al., 2013). Then, sludge was sampled and used for Illumina MiSeq sequencing. The library preparation, sequencing and data analysis were detailed in Supplementary data. The relative abundances (rb) of bacteria before and after the stress experiment were compared, and bacteria whose initial relative abundances were > 0.01% and increased significantly ($\log_{1.5}(\text{rb-after}/\text{rb-before}) > 1$) were considered as the TRB in this study.

2.3 Analytical methods. *Tet(O)*, *tet(Q)*, *tet(C)*, *tet(X)* and 16S rRNA genes were quantified using real-time quantitative PCR (Q-PCR), and the detailed procedure is presented in the Supplementary data.

The dry solid content of sludge was obtained by drying it at 105 °C overnight. The measurements of TSS, VSS, TCOD, and SCOD were in accordance with our previous publication (Chen et al., 2007). The conjugation experiments were detailed in the Supplementary data.

3. Results and discussion

3.1 Effects of the pHs on the distribution of TRGs during sludge anaerobic treatment. Figure 1 reveals that all target TRGs showed highly similar responses to diverse pHs, although they represented for

different kinds of resistance mechanisms (*tet*(O) and *tet*(Q) for ribosomal protection, *tet*(C) for efflux pumps and *tet*(X) for tetracycline transformation) (Diehl & LaPara, 2010; Ma et al., 2011). In terms of acidic pHs, the absolute quantities of assessed TRGs were significantly increased compared with the control (pH 7) ($p < 0.05$). The maximum abundances could reach up to 5.83×10^{10} , 4.58×10^{10} , 6.99×10^{11} and 6.53×10^{11} copies / g dry sludge for *tet*(O), *tet*(Q), *tet*(C) and *tet*(X), respectively, at pH 4, which were 0.58, 1.18, 0.90 and 0.82 log unit higher than those in the control. In addition, it should be noted that the increased abundances of TRGs in low pH-treated sludge were even higher than those in feed sludge (2.45×10^{10} for *tet*(O), 8.56×10^9 for *tet*(Q), 1.01×10^{11} for *tet*(C) and 1.99×10^{11} for *tet*(X)), suggesting that the acid conditions could benefit the propagation of TRGs. In contrast, when sludge was treated in alkaline conditions, the corresponding abundances were significantly declined as the pH value increased ($p < 0.05$). The net reduction for *tet*(O), *tet*(Q), *tet*(C) and *tet*(X) was 0.45, 0.94, 0.47, and 0.41 log unit at pH 9, and 0.70, 1.31, 0.92 and 1.03 log unit at pH 10, respectively. **The data in Table S2 indicated that the reduced TRGs in sludge were not transferred to the fermentation liquid at alkaline pHs.** Apparently, alkaline conditions were capable of facilitating the attenuation of TRGs.

To compare target TRGs proportionally between samples which might have different overall population sizes, gene quantities were also normalized to 16S rRNA genes (Figure S2). It was obvious that the relative abundances of assessed genes were dramatically increased at pH 4 and 5, but declined at pH 9 and 10, which shared a remarkably similar trend with that of the absolute abundances in Figure 1. For instance, the relative abundance of *tet*(Q) was only 8.50×10^{-4} in the control (pH 7), however, the corresponding abundances were 3.01×10^{-2} at pH 4 and 1.43×10^{-4} at pH 10. Therefore, the obtained results suggested that pH values were critical in the distribution of TRGs. Besides, Table S3 shows that the concentrations of TRGs were mainly altered in the phase I, thus, the investigation of the underlying

mechanism would focus on the tetracycline resistant bacteria and horizontal gene transfer in this stage.

3.2 Effects of the pHs on the community structures and abundances of TRB in sludge. As the hosts of ARGs, antibiotic resistant bacteria play vital roles in the persistence and proliferation of ARGs (Gao et al., 2012). Figures 2a-2e summarize the community profiles of TRB in each set of reactors at genus level. The majority of genera were *Prevotella* (pH 4), *Caldisericum* (pH 5), *Pelobacter sp.* (pH 7), *Pseudomonas* (pH 9) and *Clostridium* (pH 10), which accounted for 50.3%, 55.5%, 26.3%, 37.5% and 26.2% of the total TRB, respectively. At the phylum level, *Proteobacteria*, *Caldiserica* and *Firmicutes* were the most dominant bacteria responsible for tetracycline resistance, and *Proteobacteria* seemed to be more prevalent at lower pHs, while *Firmicutes* tended to be enriched at higher pHs (Figure S3). Clearly, the community compositions of TRB were affected by the value of pHs.

Figures 2f further shows that the relative abundances of TRB in the control (pH 7) accounted for 4.2% of the total bacteria, similar to the data reported in the literature (4.0%) (Li et al., 2013). Whereas, the relative abundance was increased dramatically at the acidic pHs, reaching up to 9.9% at pH 5 and 10.7% at pH 4, respectively. In contrast, under the alkaline conditions, the abundances were significantly declined to 2.6% (pH 9) and 1.4% (pH 10). Furthermore, our data also confirmed that the total quantities of bacteria were not significantly affected by the pHs in the long-term treatments ($p > 0.05$) (Figure S4). Therefore, these results suggested that the acidic pHs promoted the propagation of TRB, whereas the alkaline pHs resulted in their attenuation, which shared a highly similar trend with that of TRGs.

Previous study had reported that ARGs concentrations were positively correlated with the abundances of corresponding bacteria hosts (Li et al., 2013). Among the identified TRB in Figure 2a-2e, a number of dominant genera have already been demonstrated as the vital hosts for specific ARGs, according to the

Antibiotic Resistance Genes Database (ARDB) and limited publications. For instance, *Clostridium* sp. was reported as the carrier of *tet*(O) and *tet*(Q), *Acetobacter* sp. was found to harbor the plasmids carrying *tet*(C), and *Pseudomonas* sp. was the shelter of both *tet*(C) and *tet*(X) (ARDB). In addition, *Prevotella* sp. was resistant to tetracycline on account of *tet*(Q) (Chopra & Roberts, 2001). While, *Caldisericum* was observed to positively correlated with *tet*(O) (Peng et al., 2016). Thus, under acidic conditions, for example, the enrichment as well as the elevated abundances of *Prevotella* and *Acetobacter* might be the critical reasons for the propagation of *tet*(Q) and *tet*(C) at pH 4. Whereas, under alkaline conditions, although dominant genera, such as *Clostridium* and *Pseudomonas*, were associated with target genes, the significantly decreased abundances could still result in the attenuation of target TRGs. As to the other genera identified in Figure 2, although they were lack of clear gene hosting information, they might still play important roles in the distribution of target genes. Overall, above results indicated that sludge anaerobic treatment at diverse pHs altered the community structures and abundances of TRB, and thus led to the changed distribution of target TRGs.

3.3 Effects of the pHs on the potential of horizontal gene transfer. Horizontal gene transfer has been regarded as an important mechanism for the widespread distribution of ARGs. Figure 3a shows three possible pathways mainly responsible for this process, i.e., conjugation with plasmids replicated from cell to cell, transformation by uptake of eDNA, and transduction through infection of phages (Huddleston, 2014). Among these pathways, conjugation with plasmids has been reported to play a major role in HGT (Huddleston, 2014), and the contribution of plasmids such as pB10 in the dissemination of ARGs was also observed in anaerobic digesters (Merlin et al., 2011). Therefore, the impacts of diverse pHs on the potential of plasmids-mediated transfer of tetracycline resistance were investigated.

Figure 3b presents the yields of plasmids in sludge treated by varied pHs. It was observed that the

amounts of plasmids DNA in per g dry sludge were respectively increased by 27.8 % and 18.9% at pH 4 and 5. Whereas, corresponding amounts in the conditions of pH 9 and 10 were decreased by 18.1 % and 42.2%. The similar trends were observed when comparing the carrying abilities of plasmids in the range of pH 4-10 (Figure S5a). At pH 4, the numbers of plasmid-borne *tet*(O), *tet*(Q), *tet*(C) and *tet*(X) could reach up to 265.2%, 332.4%, 184.8% and 168.9% of the control. Whereas, at pH 10, it was found that the corresponding abilities were only 76.8%, 17.7%, 79.8% and 45.9% of the control, respectively. Apparently, diverse pHs affected the amounts of plasmids and carried TRGs available for conjugation.

It is well-known that conjugation relies on the replication of plasmids from the donor to the recipient (Huddleston, 2014). Thus, the pH-altered amount of plasmids DNA as well as their gene carrying abilities might affect the transfer of target TRGs. Additionally, some publications have documented that the process of conjugation could be affected by parameters such as temperature (Inoue et al., 2005) and agitation (Merlin et al., 2011). However, in the current study, pHs were found to have no significant impacts on the transfer frequency of plasmids, which was consistent with the results in the literature (Qiu et al., 2012). It is therefore reasonable to conclude that treating sludge under acidic conditions benefits the transfer of plasmids-borne TRGs, but reduces the potential of conjugation at alkaline pHs.

eDNA is another critical genetic vector by which the harbored ARGs can be taken up and integrated by indigenous bacterial via transformation (Mao et al., 2014). Figure 3c demonstrates that the eDNA concentrations at pH 9 and 10 were 31.9% and 14.4% of the control, respectively, whereas those at pH 4 and pH 5 dramatically increased to 416% and 256% of the control, correspondingly. It seems that alkaline pHs were harsh for the persistence of eDNA, while acid pHs were advantageous to their excretion. Additionally, results in Figure S5b reveals that the amounts of target TRGs located on per ng eDNA were decreased under alkaline conditions, but elevated at acidic pHs. The variation trends were similar to those

of plasmids DNA shown in Figure S5a.

It is well-known that eDNA is a vital component of extracellular polymeric substances (EPS) in sludge. Previous studies suggested that alkaline conditions were more beneficial for the solubilization of sludge EPS than acidic or neutral conditions (Maspolim et al., 2015), and thus promoted the release of eDNA into the fermentation liquid, in which eDNA exhibited worse persistence (Mao et al., 2014). This might be the critical reason for the significant reduction of eDNA at pH 9 and 10 (Figure 3c). In addition to originating from the lysis of decomposing or disrupted cells, eDNA could be derived from the excretion of living cells (Zhang et al., 2013). For example, the dominant genus of *Acetobacter* (Figure 2a) under acidic conditions was reported to benefit the production of eDNA by the biosynthesis of EPS (Czaczyk & Myszka, 2007), and DNA was not easily degraded under such condition (Palmgren et al., 1996). That might account for the dramatically increased yields of eDNA at pH 4. It was reported that the activity of bacterial transformation was proportional to the remaining concentration of eDNA (Pote et al., 2003). Thus, the altered amounts of eDNA would result in the differential potential of transformation. Taking the changed carrying abilities of eDNA into consideration, it can therefore speculate that acidic treatments of sludge promote the eDNA transformation, whereas parallel treatments under alkaline conditions inhibit the transfer of eDNA-borne TRGs.

Due to the considerable persistence in sludge, phages are also regarded as important sources for the propagation of ARGs (Calero-Caceres et al., 2014). It can be seen from Figure 3d that compared with the control, alkaline pHs caused a significant reduction of phage DNA in sludge (75.3% at pH 9 and 85.6% at pH 10), but acidic pHs, on the contrary, resulted in the remarkable increase (114.4% at pH 4 and 41.5% at pH 5) ($p < 0.05$). Figure S5c further demonstrates that the TRGs-possessing performances of phage DNA were also decreased under acidic conditions and increased at alkaline pHs. The most significant increase

could be 204.4%, 264.5%, 155.5% and 238.9% for *tet*(O), *tet*(Q), *tet*(C) and *tet*(X), respectively, at pH 4. Whereas, the dramatic decrease even resulted in the absence of *tet*(O) and *tet*(Q) in phage DNA (Figure S5c). 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). It was reported that the percentage of bacteria infected by phages was positively related to the amount of phages (Kasman et al., 2002). Thus, the increased amounts of phage DNA as well as their gene carrying abilities could contribute to the promoted dissemination of target genes at acidic pHs. On the other hand, corresponding decrease at alkaline pHs might lead to the inhibition of gene propagation through transduction.

4. Conclusions

This study demonstrated that the abundances of target TRGs were significantly increased under acidic conditions, but decreased at alkaline pHs. One of the critical reasons was attributed to the regulated community structures and abundances of TRB. Furthermore, the potential of gene transfer via genetic vectors was promoted at pH 4 and 5, but inhibited at pH 9 and 10, which also account for the propagation or attenuation of target TRGs. Clearly, these results indicated that more attentions should be paid when sludge were treated at acidic pHs, whereas alkaline conditions seemed to be more promising for the removal of ARGs.

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Supplementary data

This file contains additional Materials and Methods, Tables S1-S3, and Figures S1-S5. This materials is available free of charge via the Internet at <http://www.journals.elsevier.com>.

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Figure 1. Effects of diverse pHs on the distribution of TRGs in treated sludge. Error bars represent the standard deviations of triplicate measurements.

Figure 2. Effects of the pHs on the community structures (a-e) and the abundances (f) of tetracycline resistant bacteria at the genus level.

Figure 3. HGT pathways depending on genetic vectors (a). Relative yields of plasmids DNA

(b), extracellular DNA (c), and phage DNA (d) extracted from sludge treated by diverse pHs.

Error bars represent the standard deviations of triplicate measurements.

Figure captions

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Graphical abstract