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Comparison of ozone and thermal hydrolysis combined with anaerobic digestion for municipal and pharmaceutical waste sludge with tetracycline resistance genes

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ABSTRACT

Biosolids from wastewater treatment plant (WWTP) are environmental reservoirs of antibiotic resistance genes, which attract great concerns on their efficient treatments. Anaerobic digestion (AD) is widely used for sewage sludge treatment but its effectiveness is limited due to the slow hydrolysis. Ozone and thermal hydrolysis pre-treatment were employed to improve AD efficiency and reduce antibiotic-resistant genes in municipal and pharmaceutical waste sludge (MWS and PWS, respectively) in this study. Sludge solubilization achieved 15.75–25.09% and 14.85–33.92% after ozone and thermal hydrolysis, respectively. Both pre-treatments improved cumulative methane production and the enhancements were greater on PWS than MWS. Five tetracycline-resistant genes (*tet*(A), *tet*(G), *tet*(Q), *tet*(W), *tet*(X)) and one mobile element (*int*I1) were qPCR to assess pre-treatments. AD of pre-treated sludge reduced more *tet* genes than raw sludge for both ozonation and thermal hydrolysis in PWS and MWS. Thermal hydrolysis pre-treatment was more efficient than ozone for reduction after AD. Results of this study help support management options for reducing the spread of antibiotic resistance from biosolids.

1. Introduction

Tetracycline antibiotics are commonly used in humans, livestock, and aquaculture (Martinez, 2009; Wang et al., 2016), and this has caused tetracycline resistant genes (*tet* genes) to emerge in bacteria which could be harmful to humans (Rizzo et al., 2013; Liu et al., 2014). Waste discharges, especially biosolids, from WWTP are major sources of diverse *tet* genes in the environment due to variety and density of microorganisms (Auerbach et al., 2007; Aydin et al., 2015a). *Tet* genes are reported at about 10^8 to 10^9 copies per gram TS of biosolids from full-scale municipal WWTP (Auerbach et al., 2007; Munir and Xagoraraki, 2011). Furthermore, biosolids from pharmaceutical (antibiotic production) WWTPs contain a higher concentration of *tet* genes (10^9 to 10^{13} copies per gram) (Aydin et al., 2015a; Liu et al., 2012). Thus, effective treatment of waste sludge may represent a strategy for reducing *tet* genes in the environment.

Anaerobic digestion (AD) is considered as an efficient, sustainable, and common way to treat waste sludge (Pei et al., 2015). AD offers the benefits of mass reduction, pathogen removal and the generation of methane gas (Pilli et al., 2011). However, AD is limited by the high retention time, restricted methanogenic production and low overall organic dry solid degradation efficiency due to slow hydrolysis (Abelleira-Pereira et al., 2015). AD has been expected to discourage selection of resistant bacteria, reduce horizontal transfer of antibiotic resistance genes (ARGs), and aid in removal of ARGs (Zhang et al., 2015; Ju et al., 2016; Ghosh et al., 2009; Ma et al., 2011). Ju et al. (2016) detected a wide spectrum of 323 ARGs during mesophilic AD and the results indicated that most ARGs could not be removed. Zhang et al. (2015) have proved that substantial reductions of 8 and 13 ARGs were achieved by thermophilic and mesophilic digestion among 35 major ARG subtypes detected, but the abundance of total ARGs and their diversity were not measureable changed. It has also been proved that conventional mesophilic AD process rarely decrease ARGs (Ghosh et al., 2009; Ma et al., 2011). Moreover, mesophilic digestion is more susceptible to ARG intrusion, which may be attributed to the high rate of ARB survival and/or horizontal gene transfer between raw sludge bacteria and the digester microbial community (Miller et al., 2016). Therefore, pre-treatments, such as ultrasonic, ozone, alkaline, and thermal processes (Pei et al., 2015; Braguglia et al., 2012; Chi et al.,

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2011; Cano et al., 2014) were combined with AD to enhance the efficiency, but the reduction of *tet* genes during the combined techniques has rarely been investigated.

Ozone oxidation is a commonly used oxidation technique for pre-treating sewage sludge (Bougrier et al., 2006; Pei et al., 2015). Studies revealed significant improvements in organic solid reduction and methane production (Braguglia et al., 2012; Erden et al., 2010; Silvestre et al., 2015), and positive effects on removal of Pharmaceutical and Personal Care Products (PPCPs) (Carballa et al., 2007), polycyclic aromatic hydrocarbons (PAHs) (Bernal-Martinez et al., 2009) when ozone treatment is combined with AD. Likewise, ozonation is one of the typical treatment methods in bacterial disinfection of wastewater and sludge (Oh et al., 2014; Macauley et al., 2006; Asfahl and Savin, 2012; Zhuang et al., 2015). Ozone could reduce more than 90% of antibiotic resistant bacteria (ARB) and ARGs in synthetic wastewater at 3 mg/L ozone concentration (Oh et al., 2014). Macauley et al. (2006) reported that the inactivation efficiency of ARB in swine lagoon could reach 3.3–3.9 log units at an ozone dose of 100 mg/L. Ozonation reduced 1.68–2.55 log units of *tet* genes in waste sludge from municipal WWTP with a dose of 177.6 mg/L (Zhuang et al., 2015). Thermal hydrolysis pre-treatment has been proven to enhance dewaterability (Donoso-Bravo et al., 2011), solubilization and biogas production (Abelleira-Pereira et al., 2015; Xue et al., 2015; Donoso-Bravo et al., 2011), lead to important economic savings (Cano et al., 2014), and overcome rate-limiting steps of hydrolysis of organic matter from AD (Ma et al., 2011). It is reported that thermal hydrolysis pre-treatment can reduce selective *tet* genes in municipal sludge from 1.59 to 2.60 log units (Ma et al., 2011).

In this study, waste sludges from municipal and pharmaceutical WWTPs were used during treatment. Ozone and thermal hydrolysis pre-treatments were applied and compared with respect to solubilization efficiency of organic components, enhancement of AD and reduction of *tet* genes. Biological methane potential (BMP) tests were performed for each pre-treatment to assess the efficiency and variation of *tet* genes during AD. Solubilization of organic matter and mass reduction of solids were measured, as were filterability characteristics, capillary suction time (CST), improvement in methane production and variations in five *tet* genes. *Tet* genes were selected according to their specific mechanisms: *tet*(A) and *tet*(G) for antibiotic efflux pumps, *tet*(Q) and *tet*(W) for target modification with ribosomal protection protein (RPP), and *tet*(X) for inactivating enzymes. The class 1 integron gene (*int*I1) was also measured as an indicator of the potential for horizontal gene transfer (HGT).

2. Materials and methods

2.1. Waste sludge

Pharmaceutical waste sludge (PWS) was obtained from the excess sludge tank of the WWTP of an antibiotic manufacturing plant that produces more than 1000 tons of oxytetracycline (OTC) every year in Hebei Province, China (Fig. S1a). The total concentrations of OTC were 1.14–12.36 mg/L in the SBR influent, 0.36–2.35 mg/L in the final effluent and 40.7–170.2 mg/kg dry TS in waste sludge. Municipal waste sludge (MWS) was obtained from a municipal WWTP (Fig. S1b) in the same city, which generates 500,000 m^3/day of treated wastewater. MWS was also obtained from the excess sludge tank. Physicochemical parameters of both waste sludges and the analytical methods were shown in Table S1.

2.2. Pre-treatment conditions and anaerobic biodegradability batch tests

2.2.1. Ozone oxidation

Ozone oxidation batch experiments were conducted in a bubble column with a sample volume of 2 L. Ozone was generated from pure oxygen gas by a CF-YG10 ozone generator (SMSM, Inc., China). The gas flow rate was 2 L/min with an ozone concentration of ~9 mg/L. Ozone was bubbled from the bottom of the reactor through a titanium alloy diffuser. Treatments of 0.1 g O_3/g TS were used as optimal concentration for biological sludge disintegration. The ozone transfer efficiency was over 90%. Experimental details were reported in Pei et al. (2015).

2.2.2. Thermal hydrolysis

The thermal hydrolysis reactor was made up of a 2 L reactor fed with the substrate and heated with steam until reaching the desired temperature, and a flash tank of 5 L where the steam explosion took place after the hydrolysis reaction was concluded. Operational conditions were constant: 170 °C, 8 bars and 30 min of hydrolysis time, which are optimized conditions as detailed by (Fdz-Polanco et al., 2008).

Disintegration degrees (DD_{COD}) were used to describe sludge solubilization efficiency (Abelleira-Pereira et al., 2015; Zhang et al., 2009) as follows:

$$DD_{COD} = \frac{SCOD_{pre-treated} - SCOD_0}{TCOD - SCOD_0} \quad (1)$$

$SCOD_{pre\text{-}treated}$ represents the supernatant COD of the pre-treated sludge (mg/L), $SCOD_0$ represents the supernatant COD of raw sludge (mg/L), and TCOD represents the total COD of raw sludge (mg/L).

2.2.3. Biological methane potential measurement

The effluent sludge from each pre-treatment (ozonation and thermal hydrolysis) and raw sludge were assessed for biochemical methane potential (BMP) assays which served to represent the AD treatment portion. BMP assays were conducted over 15 days using 500 mL serum bottles (effective volume 400 mL). Nitrogen was purged for 3 min to establish anaerobic conditions after 150 mL of inoculum and 250 mL of raw or pre-treated sludge were placed in the bottles. The inoculum was taken from a full-scale anaerobic digester in a municipal WWTP in Beijing, China. The pH, alkalinity, total solids (TS) and volatile solids (VS) of the sludge were 7.08, 2.71 g $CaCO_3$/L, 20.2 g/L and 8.8 g/L, respectively. BMP procedures are described in Pei et al. (2015). Methane was measured by displacement of 1 mol/L NaOH. All tests were carried out in triplicate and blank digestion tests (inoculum + water) were conducted in duplicate to correct for biogas produced from the inoculum.

2.3. DNA extraction and quantitative polymerase chain reaction (qPCR)

2.3.1. DNA extraction

Samples of PWS and MWS collected as follows: (1) raw sludge, (2) ozonation only, (3) thermal hydrolysis only, (4) AD only, (5) ozonation and AD, (6) thermal hydrolysis and AD. Each sludge sample of 2 mL was centrifuged at 10,000 rpm for 10 min at 4 °C to collect the pellet for DNA extraction. FastDNA SPIN kit for soil (MP Biomedicals) was used and DNA samples were stored at −20 °C for further analysis. The volume of DNA extraction solution for each sample was 80 μL. DNA in supernatant was also extracted to determine the release by pre-treatments. 20 mL of each supernatant sample was filtered through 0.22-μm polycarbonate

membranes (GTTP, Millipore, Ireland), and biomass on the membranes was collected in 2-mL sterilized tubes for DNA extraction using the Proteinase K method (Liu et al., 2012). Concentrations and quality of extracted DNA were confirmed with spectrophotometric analysis using a NanoDrop ND-1000 (Nanodrop, USA) and electrophoresis using 1% (weight/volume) agarose gel in 0.5 × TBE buffer.

2.3.2. PCR and qPCR of tet ARGs

Five *tet* genes (*tet*(A), *tet*(G), *tet*(Q), *tet*(W), *tet*(X)), class 1 integrons (*int*I1) and 16S rRNA genes were investigated by PCR and SYBR-Green real-time qPCR. Primers, annealing temperatures and the reaction matrix were described in Supporting Information (Tables S2–S4). In detail, the PCR product of each *tet* gene was purified using GeneJET Gel Extraction Kit (Thermo SCIENTIFIC, USA) and cloned using pMD18-T Vector (TaKaRa, Japan). Plasmids carrying each *tet* gene were extracted using TIANprep Mini Plasmid Kit (TIANGEN, China). Plasmid concentrations were determined by NanoDrop DN-1000.

qPCR was performed according to published methods (Liu et al., 2012). The melting process was automatically generated by ABI7300 software. Triplicate qPCR assays were performed for decimally diluted standard plasmids to obtain standard curves. Duplicate qPCR assays were performed for all samples and negative controls. To prevent inhibition of the sample matrix, 10–100 fold diluted samples were used for quantification. The following requirements were satisfied to obtain reliable quantification: R^2 higher than 0.99 for standard curves over 5 orders of magnitude and amplification efficiencies based on slopes between 90% and 110% (Table S5). All standard curves of qPCR were constructed from serial dilutions of cloned genes ranging from 10^9 (10^{11} for 16S rRNA) to 10^2 copies/μL, and the range of sample values were 10^8 (10^{11} for 16S rRNA) to 10^2 copies/μL.

Based on the calibration curves, the abundance of *tet* gene (copies/μL DNA) was calculated as below:

$$\text{abundance of } tet \text{ genes(copies/}\mu\text{L DNA)} = \frac{\text{DNA concentration(ng/}\mu\text{L)}}{\text{DNA molecular weight(g/mol)}} \times n \times 6.02 \times 10^{23} \times 10^{-9}$$

n represents the dilution multiple of DNA when prepared to qPCR and then the abundance was normalized to dry TS of the samples as below:

$$\text{abundance of } tet \text{ genes(copies/g dry TS)} = \frac{\text{abundance of tet genes(copies/}\mu\text{L DNA)} \times 80(\mu\text{L})}{2\text{mL} \times \text{TS(g/L)} \times 10^{-3}}$$

TS (g/L) was the total dry solid of each sludge sample.

2.4. Data analysis

To report an average performance of sludge treatment processes, multiple sampling events were measured with Microsoft Excel 2010. A paired sample Student's *t*-test was used to assess the significance of differences between different systems and samples based on P-values (p-value <0.05 was considered statistically significant), and log (gene concentrations) were used for t tests. Pearson's bivariate correlation analysis was performed to assess the relevance of *int*I1 and *tet* gene occurrences.

3. Results and discussion

3.1. Effects of treatments on AD efficiency

Effects of treatments on AD efficiency were shown in Table 1, including a) solubilization, b) mass reduction, c) filterability and d) methane production.

3.1.1. Solubilization

AD is limited by low efficiency of solids hydrolysis such as flocs, microflocs, aggregates of extracellular polymeric substances (EPS), recalcitrant compounds of proteins and lipids, and components of hard cell walls (Abelleira-Pereira et al., 2015; Carballa et al., 2011). Solubilization may positively influence subsequent anaerobic degradation (Xue et al., 2015). Thus, MWS and PWS were pretreated with ozone and thermal hydrolysis to increase solubilization and enhance AD efficiency.

As shown in Table 1a, both ozonation and thermal hydrolysis caused significantly increase of SCOD ($p < 0.05$), TN ($p < 0.05$), TP ($p < 0.05$) for PWS and MWS. Ozone generates highly oxidizing and non-selective radicals that can rupture cell membranes to release soluble organic matter and other compounds (Pei et al., 2015). Thermal hydrolysis can cause pressure differences in cells inducing burst (Bougrier et al., 2006). Both pre-treatments solubilized solid matter, reduced TS and VS and increased soluble organic matter in the supernatant.

The DD_{COD} for PWS after ozonation was lower than that after thermal hydrolysis ($p < 0.05$), but it was opposite for MWS. This might be explained by higher SCOD in raw PWS (Table S1) consumed more ozone at contact initiation between ozone and sludge (Zhang et al., 2009). Thermal hydrolysis released more TN and TP into the supernatant. Donoso-Bravo et al. (2011) reported that proteins chiefly contribute to increase of COD during pretreatment with thermal hydrolysis. Temperatures exceeding 170 °C promoted more protein solubilization (Bougrier et al., 2008; Wilson and Novak, 2009). That was similar with our results (Table 1a) and could explain why thermal hydrolysis released more TP and TN. Also, thermal hydrolysis released more DNA into supernatant than ozonation which would release TP and TN, too. This observation showed the potentially powerful advantage of thermal hydrolysis over ozonation for the destruction of cells, including influent ARBs.

3.1.2. Mass reduction

Table 1b showed the TS and VS percentage removal during processes. Both pre-treatments reduced TS and VS ($p < 0.05$), but the reduction for PWS was less than for MWS ($p < 0.05$). The lowest removal after AD was obtained by raw sludge without pretreatments for both sludges, but higher removal ($p < 0.05$) was observed for raw MWS than PWS. These may be explained by the presence of toxic and recalcitrant compounds in PWS from pharmaceutical wastewater such as drugs, pharmaceutical precursors, intermediates, and catalysts, which could not be degraded by microorganisms (Pei et al., 2015; Arslan-Alaton and Caglayan, 2006).

Pre-treatment with ozonation or thermal hydrolysis appeared to improve biodegradability and reduce toxicity (Pei et al., 2015), so pre-treatments prior to AD can enhance the efficiency all over the process. It is important to note that thermal hydrolysis coupled with AD had the highest percentage of mass reduction for both substrates compared to ozonation + AD. The difference could be related to the efficiency of the pretreatment alone in terms of property changes as presented in Table 1b. The removal efficiencies of TS and VS were higher ($p < 0.05$) for thermal hydrolysis and ozonation with PWS and very similar with MWS. This data is in agreement with previous publications studying on municipal waste

Table 1
Treatment effects on AD efficiency parameters including a) Solubilization, b) Mass reduction, c) Filterability and d) Methane production.

Treatment		Raw sludge		Ozonation		THydrolysis		AD only		Ozonation + AD		THydrolysis + AD	
Sludge sample		PWS	MWS	PWS	MWS	PWS	MWS	PWS	MWS	PWS	MWS	PWS	MWS
a) Solubilization	DD (%)	0.00 (0.00)[b]	0.00 (0.00)	25.09 (0.19)	15.75 (0.14)	33.92 (0.23)	14.85 (0.11)	-[c]	–	–	–	–	–
	TP[a] (mg/L)	35.44 (0.30)	7.04 (0.05)	43.95 (0.22)	8.08 (0.42)	46.89 (0.42)	8.74 (0.09)	–	–	–	–	–	–
	TN[a] (mg/L)	109.33 (1.83)	96.43 (0.88)	174.38 (2.14)	124.14 (2.08)	186.35 (1.98)	127.81 (0.83)	–	–	–	–	–	–
	DNA[a] (mg/L)	119.38 (2.34)	47.40 (1.67)	248.92 (3.28)	142.57 (0.95)	272.30 (4.22)	151.46 (3.58)	–	–	–	–	–	–
	Protein[a] (mg/L)	740.87 (6.93)	514.78 (3.45)	947.56 (5.33)	559.48 (1.47)	1106.99 (14.94)	572.59 (8.48)	–	–	–	–	–	–
b) Mass reduction	TS (%)	0.00 (0.00)	0.00 (0.00)	17.85 (0.14)	40.58 (2.01)	30.72 (0.23)	45.29 (0.38)	23.84 (0.23)	40.94 (4.32)	43.09 (2.33)	47.51 (3.85)	46.61 (1.84)	56.12 (4.29)
	VS (%)	0.00 (0.00)	0.00 (0.00)	20.77 (0.19)	42.41 (2.75)	29.02 (1.68)	33.99 (0.23)	33.27 (3.12)	42.08 (1.49)	56.43 (0.99)	58.65 (2.45)	61.75 (2.55)	61.46 (3.57)
c) Filterability	CST (s)	408.94 (5.22)	123.53 (2.89)	1948.32 (11.24)	150.13 (5.34)	72.85 (2.36)	41.66 (3.45)	148.64 (6.34)	113.13 (4.22)	42.52 (2.75)	12.24 (1.22)	38.72 (1.56)	13.15 (1.35)
d) Methane production	Cumulative methane (mL)	-[d]	–	–	–	–	–	77.52 (5.23)	137.24 (4.66)	173.33 (9.56)	180.56 (8.28)	440.02 (12.33)	356.34 (13.45)

[a] The concentrations of TP, TN, DNA and protein are that in supernatant.
[b] Standard deviations are shown in the parenthesis.
[c] Solubilization is shown after pre-treatments, so there are no solubilization parameters after AD.
[d] Biogas is cumulated during AD, so there are no production during pre-treatments.

sludge (Bougrier et al., 2006). In other words, thermal hydrolysis combined AD was more efficient than ozonation, and the efficiency was even more obvious when dealing with PWS.

3.1.3. Filterability

Ozonation and thermal hydrolysis alone or combined with AD can alter the physical characteristics of sludge (i.e., floc structure, bound water) affecting the general dewaterability or filterability (Braguglia et al., 2012). CST tests were carried out to evaluate filterability before and after AD of raw and pre-treated sludge. Table 1c showed higher CST value for raw PWS than MWS ($p < 0.05$), which indicates a higher dewaterability capacity from the MWS compared to PWS. After ozonation, a more significant increase in CST ($p = 0.0015$) value was observed in PWS than MWS ($p = 0.0203$). On the other hand, thermal hydrolysis significantly decreased the CST value for both MWS and PWS ($p < 0.05$). Similar results were reported in previous studies (Bougrier et al., 2006), which explained how thermal hydrolysis led to release of initial bound water from the sludge structure by breaking hydrogen bonds. Other study suggested that ozonation markedly increased specific colloidal charges (Braguglia et al., 2012), which means ozonation generated suspended fine particles that might decrease dewaterability and filtration.

The evaluation of each pretreatment combined with AD showed similar results despite the contrast effect when used alone. The filterability after AD is necessary because the dewatering process is normally carried out after AD, which may reach ~7% of the energy requirements in a conventional activated sludge WWTP (Donoso-Bravo et al., 2011). The parameter of dewaterability/filterability does not seem to be affected by the type of pre-treatment of the waste sludge when combined with AD. The organic matter in solution released by pre-treatments plays an important role in influencing the sludge dewaterability (Braguglia et al., 2009). However, the digestion of sludge, by consumption of the released organic matter, attenuated the negative/positive effects of pre-treatments and the filterability ameliorated during digestion process.

3.1.4. Methane production

Table 1d showed that both pretreatments improved methane production during AD ($p < 0.05$). Cumulative methane production after ozonation + AD increased 2.25 times in PWS and 1.31 times in MWS compared to raw sludge. Methane production after thermal hydrolysis + AD increased 5.71 times in PWS and 2.60 times in MWS compared to raw sludge. Pre-treatments enhanced methane production in PWS more than MWS ($p < 0.05$) due to the specific properties and composition of PWS. The total methane production for PWS appeared to be more ($p < 0.05$) after AD than that for MWS because of the abundant VS in PWS (Table S1). However, methane production per gram VS was greater in MWS ($p < 0.05$) (Table S6) and methane production was similar to that of TS and VS removal. Thermal hydrolysis improved AD in both PWS and MWS more so than ozonation. Bougrier et al. (2006) explained that ozone treatment might cause the formation of refractory compounds or intermediates that were not easily biodegradable. Other studies (Pei et al., 2015; Braguglia et al., 2012) suggested that ozonation did not improve anaerobic biodegradability of sludge.

3.2. Effects of treatments on removal of *tet* genes and *intI1*

Five tetracycline resistance genes (*tet*(A), *tet*(G), *tet*(Q), *tet*(W), *tet*(X)) and one mobile element (*intI1*) were quantified with qPCR to examine their response to various treatments: (1) raw sludge; (2) ozonation only; (3) thermal hydrolysis only; (4) AD only; (5) ozonation and AD; (6) thermal hydrolysis and AD. To compare absolute reductions of *tet* genes, gene quantities were normalized to grams of dry solids. Variation in 16S rRNA genes was also measured (Table S7).

3.2.1. Tet genes

Quantities of five *tet* genes in raw sludge ranged from 10^9–10^{13} copies/g dry TS in PWS and 10^9–10^{11} copies/g dry TS in MWS. Quantities of each *tet* gene during PWS processing were greater at least by one order of magnitude than that in MWS (Figs. 1 and 2, Table S8). The source of the PWS and MWS could explain these differences. Most of the tetracycline in WWTP was absorbed by the biomass (Aydin et al., 2015b). In this study, the total concentration of OTC-related compound (OTC and three hydrolysates) was 40.7–170.2 mg/kg dry solid in PWS. Aydin et al. (2015a) reported that antibiotic absorption by anaerobic sludge could stimulate

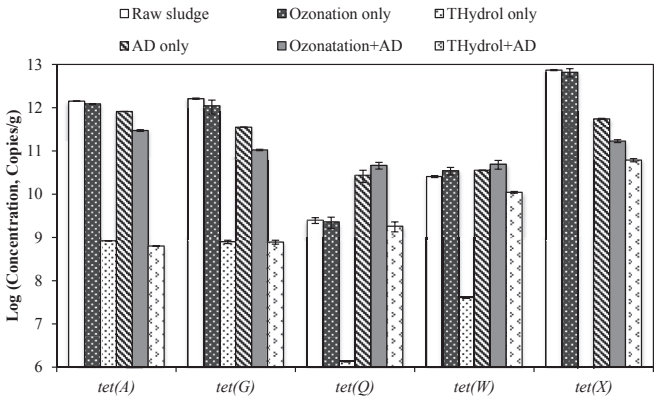

Fig. 1. Quantitative variation of *tet* genes during process in PWS (*tet*(X) for THydrol only was not detected).

acquisition of ARGs in the sludge. Thus, high OTC concentrations in PWS contributed to the increased *tet* genes due to antibiotic selective pressure.

3.2.1.1. Pre-treatments on reduction of tet genes. Pre-treatment with ozone slightly removed *tet* genes, reducing 0.04–0.17 log of five *tet* genes in PWS (Fig. 1 and Fig. S2) and 0.55–1.03 log in MWS (Fig. 2 and Fig. S3). This could be explained that the soluble organic matter in the PWS supernatant consumed part of ozone. Differences in *tet* gene removal between PWS and MWS were not observed after thermal hydrolysis which reduced all five *tet* genes ($P < 0.05$), ranging from 2.01 to 3.79 log units (except for *tet*(X) in PWS) in both PWS and MWS.

Each pre-treatment offered unique removal mechanism. During thermal hydrolysis, the high temperature and pressure sterilize the sludge, destroy cell walls, and release readily degradable components. Quantities of 16S rRNA in both PWS and MWS were reduced after thermal hydrolysis by 3 log units (Table S7). Ma et al. (2011) also confirmed that DNA was susceptible to hydrolytic destruction. Donoso-Bravo et al. (2011) reported that hydrolysis time had a small effect on sludge solubilization; thus, the reduction of DNA was a direct response to temperature and pressure changes in the thermal hydrolysis reactor. However, ozonation takes procedures to destroy *tet* genes because of non-selective oxidation of ozone, that is, ozone reacts with the soluble organic matters first and then the cell envelope before it touches *tet* genes (Zhuang et al., 2015). Hence, there is an inability of the dose for ozone to penetrate into the cytoplasm and achieve *tet* genes reduction, which means the dose 0.1 g O_3/g TS used in our study may not enough to gain more removal of *tet* genes although it has obtained the optimal solubilization. It suggested that more ozone was needed to achieve a desirable *tet* genes reduction which could then lead to a total *tet* genes removal regardless of the economy cost.

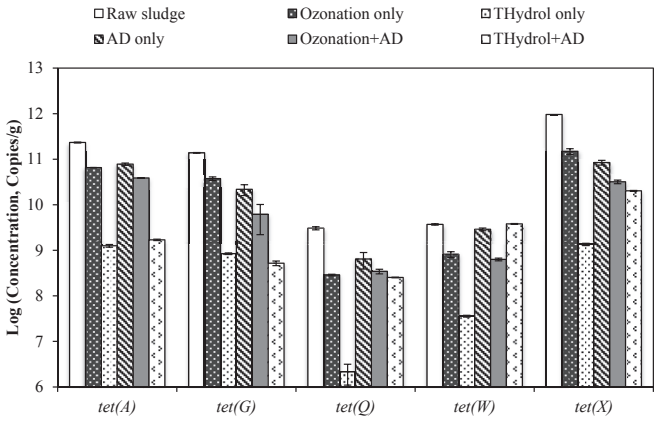

Fig. 2. Quantitative variation of *tet* genes during process in MWS.

Tet(X) was completely removed by thermal hydrolysis in PWS (Fig. 1) and this might be related to the physical characteristics of the microbe host. *Tet*(X) encodes an oxygenase enzyme which modifies and inactivates the tetracycline molecule. *Tet*(X) is found only in a strict anaerobe, *Bacteroides*, which is gram-negative bacteria (Roberts, 1994). Gram-negative bacteria with the lack of the peptidoglycan layer may be more susceptible to physical and oxidative attack (Kaneko et al., 2004), which could explain why *tet*(X) had a higher reduction.

When *tet* genes were normalized to 16S rRNA genes (relative abundance), rather than grams of solids (absolute abundance), the effect of the various treatment processes on *tet* genes maintained similar trend (Figs. S4 and S5), with two exceptions. First, since all DNA was similarly affected by thermal hydrolysis, normalization of *tet* genes to 16S rRNA genes masked the effect of the thermal hydrolytic pretreatment on *tet* genes. This result was similar with Ma et al. (2011)'s. Second, the absolute abundance of all *tet* genes (except *tet*(W) in PWS) was reduced by ozonation, but the relative abundance of some *tet* genes after ozonation was higher than raw sludge (*tet*(A), *tet*(Q) and *tet*(X) in PWS, *tet*(A), *tet*(G) and *tet*(W) in MWS).

3.2.1.2. AD for tet genes reduction. AD of raw sludge reduced all five *tet* genes in MWS ($p < 0.05$) (Fig. 2 and Fig. S3). In the case of PWS, *tet*(A), *tet*(G) and *tet*(X) were reduced ($p < 0.05$) while *tet*(Q), *tet*(W) increased ($p < 0.05$) (Fig. 1 and Fig. S2). PWS outcomes were consistent with the observations of Ma et al. (2011) with respect to reductions in *tet*(C), *tet*(G) and *tet*(X) genes and increases in *tet*(W) by mesophilic anaerobic digestion. Miller et al. (2012) reported that the mesophilic digester (37 °C, 20-day SRT) reduced levels of *int*I1 gene, but levels of *tet*(O) and *tet*(W) were the same or higher than in raw sludge. Results of Ghosh et al. (2009)'s study demonstrated that a conventional AD process rarely caused a significant decrease in the quantities of *tet* genes (*tet*(A), *tet*(O) and *tet*(X)), and even caused a rebound when subsequent after thermophilic digestion. Diehl and Lapara (2010) investigated effect of temperature on the fate of *tet* genes within AD treating municipal waste sludge, and they found that statistically significant reductions in the quantities of *tet*(A), *tet*(L), *tet*(O), *tet*(W) and *tet*(X) genes occurred in the anaerobic reactors at 37 °C, 46 °C and 55 °C, with the removal rates and removal efficiencies increasing as a function of temperature.

A sludge digester can physically destroy extracellular DNA through hydrolysis and biodegradation (Ma et al., 2011). *Tet* genes may be harbored by host bacterial cells and subject to amplification via cell growth or horizontal gene transfer or to reduction by differential survival during treatment processes (Ma et al., 2011). *Tet* genes were reduced by AD only except for *tet*(Q) and *tet*(W) in PWS. The mechanism of *tet*(Q) and *tet*(W) is target modification of ribosomal protection protein (RPP). A study (Aydin et al., 2015a) concluded that pharmaceutical wastewaters might promote mutations in ribosomal proteins among bacterial populations. High levels of antibiotics have been shown to increase and stimulate HGT and activate mobile genetic elements among the bacterial community (Beaber et al., 2004; Carles et al., 2005). Therefore, it could be assumed that PWS promoted ribosomal protein mutations by increasing HGT of target modification genes (*tet*(Q) and *tet*(W)) due to the high levels of OTC. That could explain the increase of *tet*(Q) and *tet*(W) in PWS after AD.

3.2.1.3. Combined pre-treatments and AD on reduction of tet genes. Compared to ozonation pre-treatment, ozonation combined AD removed more *tet* genes ($p < 0.05$) except for *tet*(Q) and *tet*(W), which rebounded in the anaerobic digester. For thermal hydrolysis, most *tet* genes rebounded after subsequent AD ($p < 0.05$) except *tet*(A) and *tet*(G) in PWS, which were not significantly different from quantities in thermal hydrolytically treated influent ($p = 0.051$ and 0.148, respectively). A similar rebound of ARGs in thermally hydrolyzed sludge after AD was reported by Ma et al. (2011).

Rebound of *tet*(Q), *tet*(W) and *tet*(X) might be related to the *tet* gene content in the inoculum which was mixed with the sludge samples before AD. Quantities of *tet* genes in inoculum (10^7–10^9) were almost the same before and after AD (Table S9). Although the quantities were lower than in raw PWS (10^9–10^{13}) and MWS (10^9–10^{11}), they became relatively high when compared to that in pre-treated sludge (10^6–10^9). Therefore, the *tet* genes in inoculum might be the source of the *tet*(Q), *tet*(W) and *tet*(X) rebound.

Anaerobic digestion after each pre-treatment was distinct from each other and from the digesters receiving raw sludge. The difference between the behavior found for each *tet* gene might be attributed to the resistance mechanism encoded by the *tet* gene. In 3.2.1.2, it has been concluded that the target modification genes may increase due to the high levels of OTC in PWS. Also, Zhang et al. (2015) reported that efflux pump remained to be the major antibiotic resistance mechanism in sludge samples, but the portion of ARGs encoding resistance via target modification increased in the anaerobically digested sludge. One possible explanation was that the microbial communities carrying ARGs of different resistance mechanisms reacted differently in different environment (Zhang et al., 2015). On the other hand, Ma et al. (2011) attributed the different behaviors of ARGs to variations of microbial community composition after pre-treatments. It was reported that the dominant bacterial species in the anaerobic digester after thermal hydrolysis was *Firmicutes*, indicating a favorable environment for fermentation after hydrolysis during pre-treatment. In order to understand variations in microbial community composition and confirm the proposed mechanism, further test is necessary.

Despite the rebound effect after thermal hydrolysis, AD of pre-treated sludge reduced more *tet* genes than that of raw sludge for both ozonation and thermal hydrolysis in PWS and MWS, which demonstrated that pre-treatments, especially thermal hydrolysis, reduced *tet* genes in the effluent of pre-treatment combined AD. Even rebounded, *tet* genes did not reach the extent of AD only, which was shown in Figs. S2 and S3.

3.2.2. Class 1 integron (intI1)

The class 1 integron is important for gene transfer among bacteria, and it is frequently reported to carry one or more gene cassettes that encode antibiotic resistance (Henriques et al., 2006). Quantitative changes in the *intI*1 gene were depicted in Fig. 3. Significant reduction ($p < 0.05$) of *intI*1 was observed during the treatment process, and *intI*1 rebounded with *tet* genes during AD after thermal hydrolysis pre-treatment. Almost all *intI*1 in MWS was reduced by ozone pre-treatment, and it did not rebound during the subsequent AD. That may attribute to the different characters between MWS and PWS. There were higher levels of antibiotics in PWS than MWS, and high levels of antibiotics might increase and stimulate HGT and activate mobile genetic elements (Beaber et al., 2004; Carles et al., 2005). Therefore, *intI*1 in PWS may be difficult to reduce due to its activity and rapid proliferation. Further study is necessary to confirm why the different removal efficiencies were only observed from ozonation rather than thermal hydrolysis. Pearson's bivariate correlation analysis were performed to correlate *intI*1 and *tet* gene occurrence. Table S10 and S11 showed that *tet*(A) and *tet*(X) during ozonation-AD in PWS and *tet*(G) and *tet*(Q) during ozonation-AD in MWS were positively correlated with intI1 ($R^2 = 0.999$, 0.999, 0.818 and 0.892, respectively; $P < 0.05$). This suggests that horizontal gene transfer may be critical for sludge digestion. Significant correlation was not found between *intI*1 and other *tet* genes during treatments.

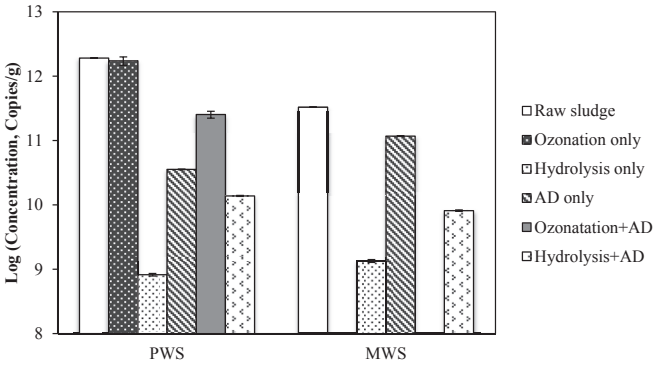

Fig. 3. Quantitative variation of *intI*1 during process (*intI*1 in MWS for Ozonation only and Ozonation + AD were not detected).

4. Conclusions

Ozone and thermal hydrolysis were used as pre-treatments before anaerobic digestion of municipal and pharmaceutical waste sludge. Both pre-treatments improved solid solubilization and filterability of sludge when combined with AD. Pre-treatments improved removal of TS, VS and production of methane during AD, especially for PWS. Reductions of five tetracycline resistance genes and *intI*1 were enhanced during AD after pre-treatments. Thermal hydrolysis was more efficient for anaerobic biodegradability and reduction of *tet* genes compared to ozonation. Additional studies are needed to understand how pre-treatments affect *tet* genes encoding different resistance mechanisms.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.04.058.

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