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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