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The role of zero valent iron on the fate of tetracycline resistance genes and class 1 integrons during thermophilic anaerobic co-digestion of waste sludge and kitchen waste

Pin Gao [a, *], Chaochao Gu [a], Xin Wei [a], Xiang Li [a], Hong Chen [a], Hanzhong Jia [b], Zhenhong Liu [a], Gang Xue [a], Chunyan Ma [a, **]

[a] College of Environmental Science and Engineering, State Environmental Protection Engineering Center for Pollution Treatment and Control in Textile Industry, Donghua University, Shanghai 201620, China
[b] Laboratory of Environmental Sciences and Technology, Xinjiang Technical Institute of Physics & Chemistry, Key Laboratory of Functional Materials and Devices for Special Environments, Chinese Academy of Sciences, Urumqi 830011, China

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ABSTRACT

Activated sludge has been identified as a potential significant source of antibiotic resistance genes (ARGs) to the environment. Anaerobic digestion is extensively used for sludge stabilization and resource recovery, and represents a crucial process for controlling the dissemination of ARGs prior to land application of digested sludge. The objective of this study is to investigate the effect of zero valent iron (Fe^0) on the attenuation of seven representative tetracycline resistance genes (*tet*, *tet*(A), *tet*(C), *tet*(G), *tet*(M), *tet*(O), *tet*(W), and *tet*(X)), and the integrase gene *intI1* during thermophilic anaerobic co-digestion of waste sludge and kitchen waste. Significant decrease ($P < 0.05$) in the quantities of *tet* (except *tet*(W)) and *intI1* genes was observed at Fe^0 dosage of 5 g/L, whereas no significant differences ($P > 0.05$) were found for all gene targets between digesters with Fe^0 dosages of 5 and 60 g/L. A first-order kinetic model favorably described the trends in concentrations of *tet* and *intI1* gene targets during thermophilic anaerobic digestion with or without Fe^0. Notably, *tet* genes encoding different resistance mechanisms behaved distinctly in anaerobic digesters, although addition of Fe^0 could enhance their reduction. The overall results of this research suggest that thermophilic anaerobic digestion with Fe^0 can be a potential alternative technology for the attenuation of *tet* and *intI1* genes in waste sludge.

1. Introduction

Antibiotic resistance genes (ARGs) have been regarded as emerging contaminants in recent years (Pruden et al., 2006), and were frequently found in different environmental compartments (Allen et al., 2010; Gao et al., 2012; Munir et al., 2011). It is commonly considered that municipal wastewater treatment plants (WWTPs) are one of the most important sources of ARGs into the environment (Gao et al., 2015; Rizzo et al., 2013). Activated sludge, which is produced from WWTPs, has been identified as a significant reservoir of ARGs (Calero-Cáceres et al., 2014), and the potential land application of WWTP sludge may result in the dissemination of ARGs (Chen et al., 2016).

Numerous technologies have been used for reducing ARG quantities in activated sludge (Burch et al., 2013; Ma et al., 2011; Zhang et al., 2016), and anaerobic digestion was demonstrated as an efficient way. In China, approximately 34 million tons of waste sludge was produced in 2015 (Feng et al., 2015), most of which was treated by anaerobic digestion. Great efforts have been undertaken to enhance the sludge reduction and to improve transformation of organic matters into sustainable resources and energy (Cao and Pawłowski, 2012). However, researches on the evolution of ARGs during sludge anaerobic digestion are limited, and the effect of alternative technologies may have on ARG attenuation in this process is still poorly understood.

A few studies have investigated the removal of ARGs from waste sludge during anaerobic digestion, and effect of different operational parameters was studied, such as temperature (Diehl and

* Corresponding author. 2999 North Renmin Road, Songjiang District, Shanghai 201620, China.
** Corresponding author. 2999 North Renmin Road, Songjiang District, Shanghai 201620, China.
E-mail addresses: pingao@dhu.edu.cn (P. Gao), machunyan@dhu.edu.cn (C. Ma).

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LaPara, 2010; Zhang et al., 2015), retention time (Ma et al., 2011), pH (Huang et al., 2016), and various pretreatment options (Pei et al., 2016; Zhang et al., 2016). As an example, temperature was demonstrated as a crucial variable for ARG removal, because high temperature could efficiently inactivate and destroy bacterial cells carrying ARGs during sludge anaerobic digestion. Then thermophilic digestion process was found to outperform mesophilic digestion in reducing a portion of ARGs (Diehl and LaPara, 2010). However, similar or poorer reduction performance of several ARGs was observed in thermophilic digestion as compared with mesophilic process (Ma et al., 2011). This fact suggested that different ARGs probably responded distinctly to the operational conditions during sludge anaerobic digestion. Also, the evolution of bacterial communities, especially ARG carriers under different digestion conditions, might contribute to the different fate of ARGs (Zhang et al., 2016).

Previous studies have demonstrated that zero valent iron (Fe^0) could enhance anaerobic biological processes and alter the phylogenetic distribution of dominant bacteria in anaerobic digesters (Feng et al., 2014; Liu et al., 2012; Yang et al., 2013). This is not only that Fe^0 can rapidly decline the oxidation-reduction potential (ORP) in anaerobic systems, but also can be an electron-donor for participating microbial metabolism (Karri et al., 2005; Newman and Kolter, 2000). Thus, the microbial communities carrying ARGs probably behave differently in the presence of Fe^0 during anaerobic digestion. Guo et al. (2014) reported that exposure to iron in gut microbiota of mouse might result in changes in ARGs (i.e., *tet*(M), *tet*(O), and *tet*(Q)) owing to the alteration of gut microbial diversity. Nevertheless, the effect of Fe^0 on the fate of ARGs within anaerobic digesters treating waste sludge has seldom been investigated.

The aim of this work is to assess the potential of Fe^0 for the attenuation of ARGs during thermophilic anaerobic co-digestion of waste sludge and kitchen waste. The purpose of adding kitchen waste was to adjust C/N ratio of the anaerobic digestion system, which has been demonstrated as a feasible and beneficial option for improving the digestion performance (Jang et al., 2016; Zhang et al., 2016). Seven frequently detected genes encoding tetracycline resistance (*tet* genes) including *tet*(A), *tet*(C), *tet*(G), *tet*(M), *tet*(O), *tet*(W), and *tet*(X), were selected. Also, the integrase gene *intI1* of class 1 integrons was quantified for exploring the potential of horizontal gene transfer. Finally, a first-order kinetic model was used to describe the evolution of the above gene targets in the presence or absence of Fe^0 during anaerobic digestion.

2. Material and methods

2.1. Experimental set-up

Three series of lab-scale anaerobic digesters were initiated using 1 L conical bottles with a working volume of 0.8 L. The feed sludge was the concentrated activated sludge collected from the secondary sedimentation tank in a municipal WWTP in Songjiang district in Shanghai, which has a total treatment capacity of 300,000 equivalent inhabitants. Wastewater treated is a mixture of municipal and industrial wastewater (~30%). The main biological treatment consists of anoxic, anaerobic, and aerobic (A/A/O) units. The collected sludge was concentrated by gravity settling to a volatile suspended solids (VSS) of about 12.20 g/L. Kitchen waste was collected from the dining hall of Donghua University in Songjiang campus, which was homogenized using a food crusher (Philips) before use, achieving an average VSS level of 55.60 g/L. The loaded co-substrate of waste sludge and kitchen waste was mixed thoroughly at a mass ratio of 1:3 (VSS), resulting in a final C/N mass ratio of about 26:1. All digesters were placed in a water bath to control temperature at 50±1 °C and mixed by mechanical stirrers with a speed of 120 rpm. To achieve a mean sludge retention time (SRT) of 24 days, about 0.27 L of mixture from each digester was replaced every 8 days with the feed co-substrate. Digester pH was measured using a pH meter (S220-K, Mettler Toledo, Switzerland). Biogas was collected into Tedlar bags (E-Switch) for analysis, and the volume was measured by a glass syringe.

All digesters were operated until steady-state performance achieved with respect to biogas production and volatile solids removal. Afterwards, 0, 5 and 60 g/L Fe^0 powder (40 μm in diameter, purity>98%, Shanghai Chemical Reagent Co., China) was added into these three anaerobic digesters, respectively. To investigate the effect of Fe^0 on the fate of gene targets, the experiments were performed at a discrete time interval of 8 days.

2.2. Sample collection and DNA extraction

Samples were collected from each digester every 2 days over four sampling events at a discrete time interval. Each sample was centrifuged at 10,000 rpm for 10 min, and about 0.5 g of the pellet was used for DNA extraction in triplicate using a FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA). Triplicate total genomic DNA was homogenized together to average out bias sampling and extraction. DNA concentration and purity were determined with a Qubit 2.0 DNA quantification system (Invitrogen, Carlsbad, CA, USA).

2.3. Real-time quantitative PCR (qPCR)

Seven *tet* genes including three efflux pump genes of *tet*(A), *tet*(C), and *tet*(G); three ribosomal protection genes of *tet*(M), *tet*(O), and *tet*(W); and one enzymatic modification gene of *tet*(X) (Chopra and Roberts, 2001), as well as *intI1* and 16S rRNA genes were quantified by qPCR (LightCycler® 96, Roche Diagnostics GmbH, Mannheim, Germany). The primer sequences, amplicon sizes, and annealing temperatures used for all gene targets were listed in Table 1. Each qPCR run began with 10 min of initial denaturation at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing for 20 s at the primer-specific annealing temperature, and extending for 30 s at 72 °C. A 20 μL mixture was used for each reaction, containing 10 μL of FastStart Essential DNA Green Master (Roche Diagnostics GmbH, Mannheim, Germany), 1.5 μL each of forward and reverse primers (4 μmol/L), 1 μL of template genomic DNA, and 6 μL of DNA-free water.

Standards were prepared by initial PCR amplification of genes from concentrated sludge, and synthesized by Sangon Biotech (Shanghai) Co., Ltd. Ten-fold serial dilutions of plasmid DNA were prepared and run on the thermal cycler for constructing standard curves (R^2>99%). The amplification efficiencies were examined in the range of 88%–101% for a minimum of seven points on each standard curves. Melting curves were conducted for all assays to verify that nonspecific amplification did not occur. Each reaction was run in triplicate with a standard curve and a negative control using DNA-free water instead of template genomic DNA.

2.4. Data analysis

The quantities of gene targets were presented as the arithmetic means of triplicate qPCR assays, and data were calculated using Microsoft Excel 2010. All plots for gene targets were generated with OriginPro 8.5 software (OriginLab Corporation, USA). Pearson bivariate correlation analysis was performed using SPSS 19.0 (IBM, USA) to reveal relevance between *tet* genes and *intI1*, and *t* tests were also conducted to assess the significance of differences between samples. A *P*-value of <0.05 was considered statistically significant. A first-order kinetic model was used to fit the quantities