Water Research 106 (2016) 71–78

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Impact of fulvic acids on bio-methanogenic treatment of municipal solid waste incineration leachate

Yan Dang [a, c, 1], Yuqing Lei [a, 1], Zhao Liu [a], Yiting Xue [a, 2], Dezhi Sun [a, *], Li-Ying Wang [c, d], Dawn E. Holmes [b, c]

[a] Beijing Key Laboratory for Source Control Technology of Water Pollution, Engineering Research Center for Water Pollution Source Control and Eco-remediation, College of Environmental Science and Engineering, Beijing Forestry University, 35 Tsinghua East Road, Beijing 100083, China
[b] Department of Physical and Biological Sciences, Western New England University, Springfield, MA 01119, USA
[c] Department of Microbiology, University of Massachusetts Amherst, Morrill IV N Science Center, Amherst, MA 01003, USA
[d] State Key Laboratory of Bioreactor Engineering and Institute of Applied Chemistry, East China University of Science and Technology, Shanghai 200237, China

ARTICLE INFO

Article history:
Received 7 February 2016
Received in revised form
21 September 2016
Accepted 22 September 2016
Available online 23 September 2016

Keywords:
Fulvic acids
Incineration leachate
Methanogenesis
Electron shuttle
EGSB reactor

ABSTRACT

A considerable amount of leachate with high fulvic acid (FA) content is generated during the municipal solid waste (MSW) incineration process. This incineration leachate is usually processed by downstream bio-methanogenic treatment. However, few studies have examined the impact that these compounds have on methanogenesis and how they are degraded and transformed during the treatment process. In this study, a laboratory-scale expanded granular sludge bed (EGSB) reactor was operated with MSW incineration leachate containing various concentrations of FA (1500 mg/L to 8000 mg/L) provided as the influent. We found that FA degradation rates decreased from 86% to 72% when FA concentrations in the reactor were increased, and that molecular size, level of humification and aromatization of the residual FA macromolecules all increased after bio-methanogenic treatment. Increasing FA influent concentrations also inhibited growth of hydrogenotrophic methanogens from the genus *Methanobacterium* and syntrophic bacteria from the genus *Syntrophomonas*, which resulted in a decrease in methane production and a concomitant increase in CO_2 content in the biogas. Sequences most similar to species from the genus *Anaerolinea* went up as FA concentrations increased. Bacteria from this genus are capable of extracellular electron transfer and may be using FA as an electron acceptor for growth or as a shuttle for syntrophic exchange with other microorganisms in the reactor. In order to determine whether FA could serve as an electron shuttle to promote syntrophy in an anaerobic digester, co-cultures of *Geobacter metallireducens* and *G. sulfurreducens* were grown in the presence of FA from raw leachate or from residual bioreactor effluent. While raw FA stimulated electron transfer between these two bacteria, residual FA did not have any electron shuttling abilities, indicating that FA underwent a significant transformation during the bio-methanogenic treatment process. These results are significant and should be taken into consideration when optimizing anaerobic bioreactors used to treat MSW incineration leachate high in FA content.

1. Introduction

With economic development and significant increases in population densities worldwide, the amount of municipal solid waste (MSW) being generated around the world has reached epic proportions. In fact, it has been estimated that about 1.3 billion tons of MSW are currently being generated per year, and that by 2025 this will likely increase to 2.2 billion tons per year (Hoornweg and Bhada-Tata, 2012). Therefore, it does not seem too surprising that methods designed to reduce the volume of MSW are in high demand. Incineration followed by bio-methanogenic treatment is one method that shows promise, with the dual advantage of reducing volume while producing energy at the same time (Chou et al.,

* Corresponding author.
E-mail address: sdzlab@126.com (D. Sun).
[1] Both authors contributed equally.
[2] Present address: Environmental Monitoring Station of Ningdong Energy and Chemical Industry Base, Ningxia 750411, China.

http://dx.doi.org/10.1016/j.watres.2016.09.044
0043-1354/

2009).

Prior to incineration, fresh MSW is stored in bunkers for several days, where a considerable amount of leachate can be generated. In fact, it is estimated that one ton of MSW will produce 0.2 m^3 of leachate which means that 1.3 billion tons of MSW will produce 260 million m^3 of leachate (Ozkaya, 2005). The leachate contains humic substances which are complex heterogeneous organic compounds formed during the decay and transformation of organic matter (Schmidt et al., 2011). Humic substances are resistant to biodegradation and can react physically and chemically with many pollutants and increase their negative impact on the environment. For example, they can form complexes with metals that can influence their speciation and toxicity (Christensen et al., 1996), they can enhance the solubility of hydrophobic pollutants such as phthalates and polycyclic aromatic hydrocarbons (Chiou et al., 1986; Marttinen et al., 2003), and they can react with chlorine to form harmful by-products like trihalomethanes and haloacetic acids (Singer, 1999).

In addition to their ability to interact with pollutants, humic substances can also shuttle electrons between bacteria and extracellular electron acceptors (Scott et al., 1998) or between syntrophic bacteria in a process referred to as direct interspecies electron transfer (DIET) (Liu et al., 2012; Lovley et al., 1999; Smith et al., 2015b). This shuttling ability can also significantly influence carbon and electron flow within microbial ecosystems, particularly in environments where methanogenesis is the terminal electron accepting process. While studies have shown that humic substances can enhance syntrophy between certain bacteria where metals or nitrate serve as the terminal electron acceptor, the high mid-point potential of humic substances' redox couple does not allow reduction of carbon dioxide to methane (Liu et al., 2012). Studies have also shown that humic substances can inhibit hydrolytic enzymes involved in cellulose degradation, which is an important rate-limiting step during anaerobic digestion of biomass (Azman et al., 2015; Fernandes et al., 2015). Methanogens utilize substrates (i.e. acetate, H_2, CO_2, formate) generated by cellulose hydrolysis and the degradation of other complex organics to drive methanogenesis. If these substrates are no longer available, methanogenesis cannot occur and biogas production will drop significantly (Gunaseelan, 1997).

Aquatic humic substances in leachate can be divided into two groups (fulvic acids (FA) and humic acids) determined by their molecular size, redox abilities, and solubility in water (Christensen et al., 1998). The majority (>95% w/w) of humic substances found in incineration leachate are classified as FA, which tend to have smaller molecular weights (MW) and higher redox abilities than humic acids (Christensen et al., 1998; Kang et al., 2002; Ritchie and Perdue, 2003). Although FAs are significantly more abundant in incineration leachate, most studies done thus far have focused on the impact that humic acids can have on methanogenic systems (Liu et al., 2015a).

Therefore, the purpose of this study was to investigate how FA compounds can impact bio-methanogenic treatment of MSW incineration leachate, and how these compounds are degraded and transformed during the treatment process. We exposed a laboratory expanded granular sludge bed (EGSB) reactor treating incineration leachate to increasing concentrations of FA. We found that elevated FA content in the leachate decreased biogas production rates and organics degradation efficiencies, and changed the microbial community in the bioreactor. We also found that the structure of FA changed significantly during bio-methanation and that while FA molecules extracted from raw leachate could serve as electron shuttles between bacteria, macromolecules extracted from digester effluent lacked all shuttling abilities. These results should help with future attempts to optimize anaerobic digesters treating MSW incineration leachate.

2. Materials and methods

2.1. Bioreactor influent composition

The fresh leachate used as the feedstock in this study was obtained from an MSW-to-energy incineration plant in Beijing, China. The characteristics of the leachate are shown in Supplementary Table S1. Raw leachate diluted with tap water to desired concentrations (COD values ranging from 4000 mg/L to 17,000 mg/L) was provided as influent for the bioreactors.

2.2. Laboratory-scale EGSB reactor treatment of fresh leachate

A laboratory-scale EGSB reactor was operated to treat incineration leachate amended with FA. Anaerobic granular sludge with a volatile suspended solids (VSS) to total suspended solids (TSS) ratio of 0.72 was obtained from a full-scale up-flow anaerobic sludge bed (UASB) reactor treating wastewater collected from a brewery in Henan, China and provided as an inoculum at a concentration of 6.6 g VSS/L. The reactor, schematically shown in our previous study (Dang et al., 2013) had a working volume of 2.0 L and was stably operated at 33 ± 1 °C throughout the study. Internal recirculation was applied and the liquid up-flow velocity was maintained at 2.0 m/h. Hydraulic retention time (HRT) was kept at 2.0 d, and the sludge retention time (SRT) was between 28 d and 39 d.

During the start-up period (first 118 days), leachate provided as the influent was stepwise increased from a COD concentration of ~4000 mg/L to ~17,000 mg/L. Once the bioreactor had acclimated to a COD concentration of 17,000 mg/L, the experimental stage (days 119–222) was started and various concentrations of FA (stepwise from 1500 mg/L to 8000 mg/L) extracted from raw leachate was added to the influent. FA concentrations in the influent were monitored at each step until the end of the experiment to ensure that influent FA was stable and that desired concentrations were added to the bioreactors. Throughout the experiment, COD (~17,000 mg/L), free ammonia (23–32 mg/L) and calcium (800–1500 mg/L) concentrations were far below loads that inhibited activity in previous studies (Dang et al., 2014; Liu et al., 2015b; Ye et al., 2011).

2.3. Analysis of fulvic acids

FA were extracted from raw and treated leachate with methods previously described (He et al., 2006). The molecular weight (MW) distribution of FA was analyzed by high performance liquid chromatography (HPLC, Lumtech, Germany) with a refractive index detector (RID), and a PL aquagel-OH column (300 mm × 7.5 mm, Waters, USA) at 30 °C. De-ionized water was used as the mobile phase with a flow rate of 1.0 mL/min, and polyethylene glycols with MW of 2,000, 4,000, 10,000 and 20,000 Da (PEG, Merck, Germany) were used as standards.

FA elemental composition was determined with an Element Analyzer (EA1110-FISONS). UV–visible absorption spectra were recorded on a Thermo Evolution300 spectrophotometer (Thermo, USA), and infrared spectra were recorded with KBr pellets (100 mg KBr + 1 mg samples) on a Bruker Vertex 70/80 Fourier transform infrared spectroscopy (FTIR) spectrometer (Bruker, Germany). For nuclear magnetic resonance (NMR) spectra, solutions were prepared by dissolving 10 mg of each FA sample in 0.5 mL of 1.0 N NaOD (in D_2O). ^1H NMR spectra were recorded at 400 MHz on a Bruker AV III NMR spectrometer (Bruker, Germany). ^{13}C NMR spectra were also attempted, but failed due to the complex structure of FA analyzed in this study.

2.4. High-throughput sequencing

Sludge samples were collected from the EGSB reactor at the end of each step; day 118 (1500 mg/L FA), day 162 (4000 mg/L FA) and day 218 (8000 mg/L FA). A sludge sample collected during the recovery period of the EGSB reactor was also analyzed. The E.Z.N.A. Soil DNA Kit (OMEGA, USA) was used to extract total DNA.

The V4 region of the 16S rRNA gene was amplified by the polymerase chain reaction (PCR) with the primer set (515F/806R) (Peiffer et al., 2013). PCR reactions (20 μL final volume) were carried out in triplicate and consisted of ~0.5 ng template DNA, 200 nM of each primer, 250 nM dNTP, and 1 × fastPfu buffer (Takara, Dalian, China). High-throughput sequencing was done on an Illumina Hiseq 2000 platform (Illumia, San Diego, USA) by Novogene Biotechnology Co., Ltd. (Beijing, China), and yielded more than 11,300 sequences for each sample with an average read length of 250.4 base pairs. Sequences were placed into various operational taxonomic units (OTUs) with Pyrosequencing Pipeline software (https://pyro.cme.msu.edu). Raw sequence files have been submitted to the NCBI Sequence Read Archive (SRA) database under accession no. SRR2880885–2880889.

2.5. Co-cultures of G. metallireducens and G. sulfurreducens

Co-cultures of *Geobacter metallireducens* (strain GS-15) and *G. sulfurreducens* (strain PCA) were obtained from Derek Lovley's laboratory at the University of Massachusetts Amherst. Cultures were grown under an N_2:CO_2 atmosphere (80:20, v/v) at 30 °C, as previously described (Coppi et al., 2001; Smith et al., 2015b; Summers et al., 2010) in freshwater medium with 13 mM ethanol provided as the electron donor and 40 mM fumarate provided as the electron acceptor. Cysteine and DL vitamins were omitted from the medium to rule out the possibility that these compounds might act as electron shuttles (Smith et al., 2015b). FA extracted from the influent or effluent were added to defined co-cultures at a final concentration of 0.2 g/L. Control co-cultures were also grown in the presence of 0.2 g/L anthraquinone-2,6-disulfonate (AQDS).

2.6. Analytical methods

COD, biochemical oxygen demand (BOD), alkalinity and NH_4^+-N were determined by standard methods (Apha, 2005), pH was measured with a Hach sensION+pH3® pH meter (Hach, USA), and a TOC/TN analyzer (multi N/C 3000, Analytik Jena AG, Germany) was used to measure the TOC of leachate samples. Biogas volume was recorded with a gas container connected to a gas meter (FMA4000; Omega; USA), and methane and carbon dioxide concentrations were determined by gas chromatography (thermal conductivity detector, TCD) (Agilent, USA). Organic acids were monitored by HPLC analysis (Nevin et al., 2008), and ethanol concentrations were monitored with gas chromatography as previously described (Morita et al., 2011).

3. Results and discussion

3.1. Impact of fulvic acids on treatment efficiency of the EGSB reactor

The performance of the EGSB reactor during the entire period of operation is shown in Fig. 1. During the start-up period (Stage I: Acclimation), the volume of leachate in the influent was gradually increased until day 51 when the COD was ~17,000 mg/L (BOD_5 ~11,000 mg/L) and concentrations of NH_4^+-N and FA were ~300 mg/L and 1500 mg/L, respectively. The COD removal efficiency was largely maintained at >96%, total volatile fatty acids in the effluent were <13 mmol/L, and alkalinity in the reactor gradually increased to 8000 mg/L ($CaCO_3$ calculated), suggesting that the acclimation period was successful (Ahring et al., 1995). Biogas production gradually increased from 0.33 L (standard temperature and atmospheric pressure, STP)/d to 5.69 L (STP)/d and methane content increased from 50.10% to 78.52%.

During Stage II (FA degradation; days 119–222), COD influent concentrations were maintained at 16,000–19,000 mg/L, and concentrations of FA extracted from raw leachate were gradually increased from 1500 mg/L to 8000 mg/L. When FA concentrations in the influent were below 3000 mg/L, COD removal efficiencies were >94%, however, COD removal efficiencies gradually decreased to 81% when FA concentrations were increased to 4000 mg/L and 8000 mg/L. Total biogas and methane production were similar to what was observed in the start-up period during the first 25 days of Stage II when FA concentrations were ~3000 mg/L; total biogas was ca. 5.31 L (STP)/d, methane content was ~72%. When FA concentrations were increased from 4000 mg/L to 8000 mg/L, total biogas production increased slightly to 6.40 L (STP)/d, however, methane content went down to ~60% while CO_2 content increased from ca. 24%–35%. It is likely that the slight increase in CO_2 production resulted from higher COD concentrations associated with increasing influent FA (Sobotka et al., 1983). However, it is also possible that some of the increased CO_2 resulted from inhibition of CO_2 reduction to methane by FA. Acetate concentrations increased from ~11 mM to ~15 mM and the removal efficiencies of complex organic matter other than FA decreased from 97% to 92% during this period. This may be explained by increased acetogenic activity and decreased activity by cellulolytic bacteria.

Although conversion of organic compounds to methane seemed to be slightly inhibited during this period, total volatile fatty acids in the effluent stayed lower than 22 mM and bioreactor alkalinity remained >11,000 mg/L ($CaCO_3$ calculated), indicating that the EGSB reactor was operating favorably without any risk of acidification according to guidelines set by Leitão et al. (2006). In addition, even when COD concentration in the influent was increased to 3820 mg/L, BOD_5 concentrations in the effluent were maintained at 100–300 mg/L.

For stage III of the experiment (days 223–261, recovery phase), FA concentrations in the influent were dropped back down to initial levels (1500 mg/L), and the ability of EGSB reactors to recover from exposure to high FA concentrations was investigated. After 25 days of operation, the COD removal efficiency went up to >95%, CO_2 content dropped to 21.80%, and methane content increased to 71.93%. These results demonstrate that the bio-methanogenic treatment process could recover completely once FA content in the bioreactor was reduced.

3.2. Degradation and transformation of fulvic acids

FA degradation efficiencies were calculated during Stage II (days 119–222) (Supplementary Fig. S1). Efficiencies dropped slightly when FA influent concentrations were increased; ~86% at FA influent concentrations up to 3000 mg/L compared to 72% when influent concentrations were increased to 8000 mg/L.

The MW distribution of residual FA in the effluent also changed when the reactor was exposed to high FA concentrations (Fig. 2). When influent FA was increased from 1500 mg/L to 8000 mg/L, the proportion of large molecules (MW > 20 kDa) in the effluent increased from 54% to 68% while small molecules decreased from 31% to 14%.

These results demonstrate that the removal efficiency of large molecules (>20 kDa) was reduced when FA concentrations in the influent were high. It is possible that increased concentrations of FA in the influent (a) inhibited growth of microorganisms capable of

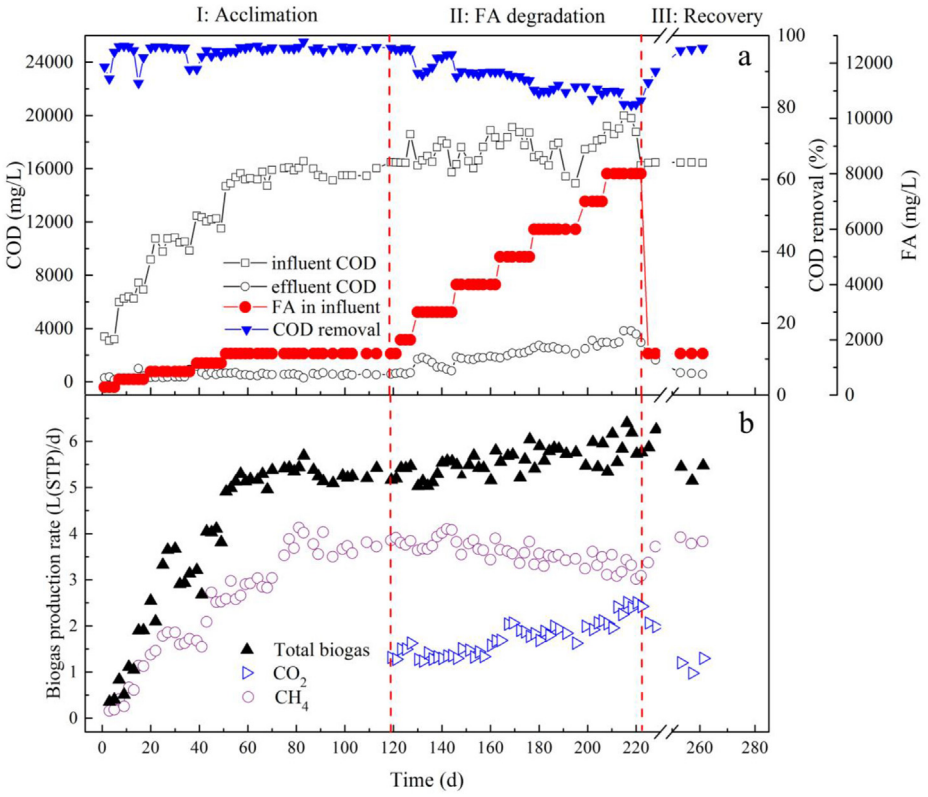

Fig. 1. Measurements taken during operation of the EGSB bioreactor of (a) FA, influent COD, effluent COD, COD removal efficiencies and (b) total biogas, CO_2 and CH_4 production. CO_2 production was only monitored during Stages II and III.

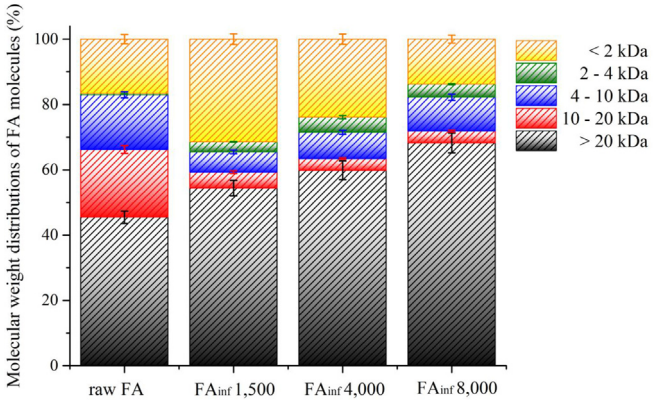

Fig. 2. Molecular weight (kDa) distributions of FA molecules present in raw leachate and various effluents. Error bars represent standard deviations from triplicate measurements. FA_{inf} represents FA concentrations (mg/L) in bioreactor influents.

macromolecular FA degradation, and/or (b) enhanced humification of small and medium sized molecules. It is important to understand this phenomenon as formation of these larger macromolecules makes further treatment of leachate more difficult.

The elemental composition of FA molecules detected in raw leachate also differed significantly from the composition of molecules found in effluents exposed to all but the highest concentration of FA (Table 1). As concentrations of FA in the influent were increased from 1500 to 4000 mg/L, the proportion of carbon increased ~9%, hydrogen decreased ~2-fold, oxygen decreased ~8%, and nitrogen increased 1.6 fold. The proportions of all four of these elements in effluents from the bioreactor exposed to 8000 mg/L FA, on the other hand, were almost the same as those detected in raw leachate indicating that the elemental composition of these FA molecules did not change much after methanogenic treatment in the presence of high FA concentrations.

In order to further evaluate the chemical structure of FA collected from various treatments, the atomic ratio of H/C was calculated. Samples with H/C ratios <1 tend to have a primarily aromatic framework (Aiken et al., 1985), while those with ratios greater than one are likely to contain structures with functional aliphatic groups. Similar to results from elemental analyses, H/C ratios of raw leachate and effluent from bioreactors fed 8000 mg/L FA were both high (1.95 and 1.93 respectively). Ratios from 1500 mg/L and 4000 mg/L FA effluents, on the other hand, were about 2.6 fold lower and appeared to be severely aromatized.

Spectroscopic measurements also indicated that the degree of aromatization and humification increased after bio-methanogenic treatment. The specific UV absorbance at 254 nm ($SUVA_{254}$) increased from 0.99 L/(mg·m) in raw leachate FA to 2.4, 3.7, and 3.3 L/(mg·m) in FA1500, FA4000, and FA8000 effluent samples. These results differed from those determined with H:C calculations; aromaticity increased in all effluent samples, even those exposed to 8000 mg/L FA. This can be explained by the fact that an abundance of conjugated aromatic compounds in FA8000 effluent can result in an underestimation of aromaticity determined by H:C calculations. The UV-Vis spectral ratio E_2/E_3 (UVA_{250}/UVA_{365}) decreased from 3.9 in raw leachate to 3.6, 3.4, and 2.95 in FA1500, FA4000, and FA8000 effluent, which was consistent with MW distribution results.

FTIR spectroscopic and ^1H NMR studies also showed apparent differences between FA extracted from raw leachate and effluent samples (Supplementary Fig. S2). Similar to UV-visible spectral and elemental analyses, FTIR spectral analysis showed that

Table 1
Proportion of carbon, hydrogen, oxygen and nitrogen, and the atomic ratio of hydrogen to carbon in FA molecules collected from various samples.

	FA in raw leachate	FA in eff. (FA_{inf} = 1500 mg/L)	FA in eff. (FA_{inf} = 4000 mg/L)	FA in eff. (FA_{inf} = 8000 mg/L)
C	44.70%	54.64%	52.94%	45.13%
H	7.26%	3.37%	3.42%	7.27%
O	43.32%	34.97%	36.00%	42.72%
N	4.72%	7.01%	7.64%	4.89%
H/C	1.95	0.74	0.78	1.93

bio-methanogenic treatment increased aromatization of FA compounds. According to ^{1}H NMR, the number of protons on methylene chains (region I) was greater in all effluent samples, however, the increase was not as significant in the FA8000 sample as it was in the FA1500 and FA4000 samples (Table 2 and Supplementary Fig. S3). The number of protons in region II only increased in the FA8000 effluent sample, and protons in region III decreased in all three effluent samples indicating that oxygen containing functional groups (i.e. carboxyl and hydroxyl groups) were being degraded even in the presence of high FA concentrations. The number of protons from region IV was lower than raw leachate in both the FA1500 and FA4000 samples, but was higher in the FA8000 sample. These results indicate that aromatic side groups were only degraded at low FA concentrations and that additional aromatic side groups were formed during the bio-methanogenic treatment process when FA concentrations in the influent were 8000 mg/L.

3.3. Effect of fulvic acids on microbial communities associated with bioreactor granules

Analysis of 16S rRNA gene sequences showed that as FA concentrations were increased in the influent, the proportion of bacteria from the phylum *Chloroflexi* (primarily species from the family *Anaerolinaceae*) increased significantly (Fig. 3A), but dropped back down during the recovery period. The majority of archaeal sequences from all four conditions were methanogenic (82–99% of the sequences).

Firmicutes and methanogenic archaeal sequences decreased with increasing FA concentrations, however, while *Firmicutes* sequences stayed proportionately low during recovery, methanogens rebounded. These results are consistent with the finding that *Firmicutes* are key players in degradation of complex organic matter (Dang et al., 2013; Schwarz, 2001), which is inhibited by humic substances. This drop in methanogenic archaea is also consistent with a decrease in methane production by the digester.

The majority of methanogenic sequences clustered with the hydrogenotrophic genus *Methanobacterium* and the acetoclastic genus *Methanosaeta*. Proportions of these genera changed with exposure to increasing concentrations of FA; *Methanobacterium* accounted for 38% of the sequences at low concentrations of FA compared to 17% in FA8000 effluents (Fig. 3B). These results suggest that FA inhibited growth of methanogenic archaea in the bioreactors, particularly those using hydrogen as an electron donor for growth. This might result from inhibition of microbial enzymes involved in hydrogen production by FA, or from abiotic reaction of hydrogen with FA, making H_2 inaccessible to hydrogenotrophic methanogens for respiration. It has already been shown that humic-like substances can inactivate microbial extracellular activity by binding to proteins resulting in aggregation, complexation and precipitation of humic substance-protein complexes (Coates et al., 2002; Wetzel, 1993).

More detailed analysis of the bacterial community structure showed that a genus of bacteria from the phylum *Firmicutes*, *Syntrophomonas*, appeared to be adversely impacted by high concentrations of FA. The proportion of *Sytrophomonas* from FA1500 and FA4000 samples was ca. 13% but dropped down to only 4% in the FA8000 sample and never recovered. *Sytrophomonas* species are known to be important members of the microbial community in anaerobic digesters (Lin and Lu, 2015; Smith et al., 2015a) and have been shown to form syntrophic partnerships with methanogenic archaea that promote bio-methanation (Li et al., 2015; Zhao et al., 2016).

16S rRNA gene sequences from the family *Anaerolinaceae* (phylum *Chloroflexi*), on the other hand, increased significantly in the presence of FA and accounted for 40% of the bacterial sequences in the FA8000 sample. These sequences dropped back down to 18% of the bacterial community during the recovery period, suggesting that growth of these organisms was stimulated by FA compounds. *Anaerolinaceae* are frequently associated with anaerobic digestion processes (Narihiro et al., 2015), and can transfer electrons to 2,4,6-iodobenzene derivatives and respire Fe(III) (Kawaichi et al., 2013), suggesting that they would be capable of electron transfer to humic substances such as FA. *Chloroflexi* have also been shown to oxidize aromatic compounds during anaerobic respiration (Fennell et al., 2004), and a significant portion of the carbon in FA is aromatized (Khan and Schnitzer, 1972). Therefore, it is also possible that these organisms are using FA as electron donors for growth.

3.4. Electron shuttling capabilities of FA tested in co-cultures of G. metallireducens and G. sulfurreducens

Syntrophic partnerships found in co-cultures or anaerobic digesters can be stimulated by addition of conductive materials and/or electron shuttles via DIET (Chen et al., 2014; Dang et al., 2016; Liu et al., 2012; Smith et al., 2015b; Zhao et al., 2015). The concept of DIET referred to a syntrophic partnership between two bacteria

Table 2
Relative contributions of ^{1}H chemical shift regions according to ^{1}H NMR spectral analysis of FA molecules extracted from raw leachate and various effluent samples.

Chemical shift region δ (ppm)	Assignment	Relative contribution (%)			
		FA in raw leachate	FA in eff. (FA_{inf} = 1500 mg/L)	FA in eff. (FA_{inf} = 4000 mg/L)	FA in eff. (FA_{inf} = 8000 mg/L)
I (0.4–1.7)	Terminal CH_3, and CH_2, CH of methylene chains, etc.	20.67%	37.99%	43.68%	30.84%
II (1.7–3.0)	CH_3, and CH_2, CH proton α to aromatic or carboxyl groups, etc.	21.89%	19.71%	20.77%	31.59%
III (3.0–4.5)	Protons on carbon α to oxygen, carbohydrates, etc.	51.61%	38.19%	30.89%	30.77%
IV (6.0–8.0)	Aromatic protons (including quinone, phenol, etc.)	5.84%	4.11%	4.67%	6.81%

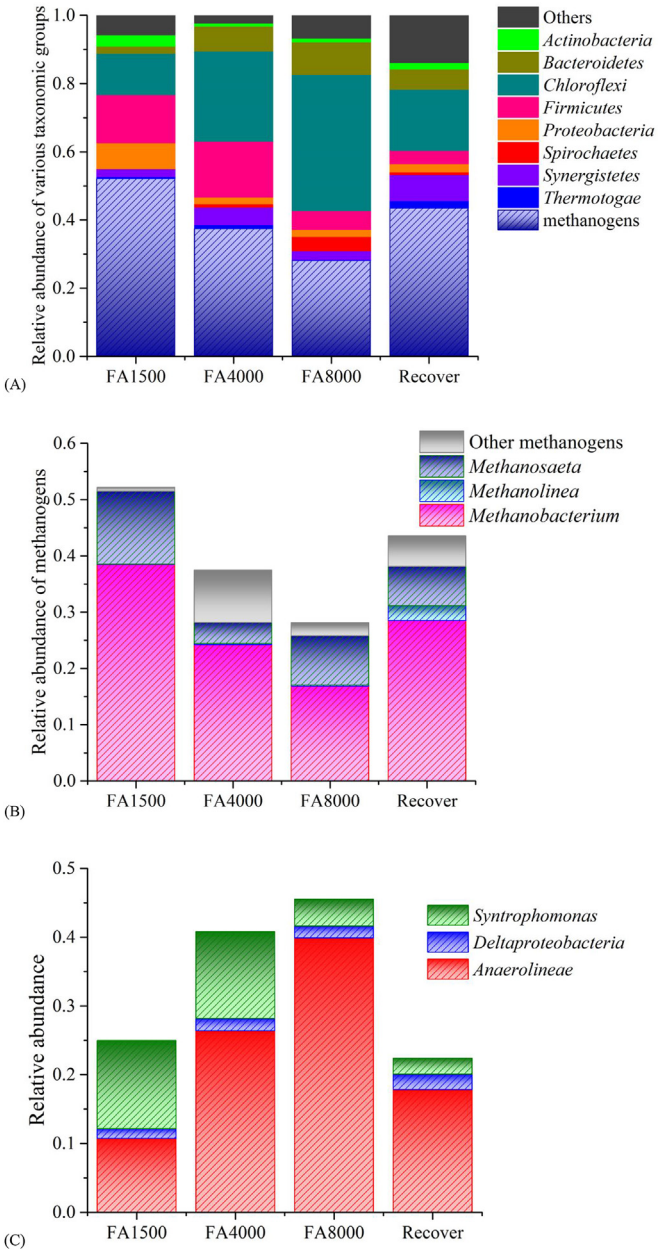

Fig. 3. Analysis of 16S rRNA gene sequences in DNA extracted from granules collected from bioreactors exposed to 3 different FA concentrations (1500 mg/L, 4000 mg/L, or 8000 mg/L) or from a recovery bioreactor. (A) Relative abundance of sequences from various taxonomic groups; (B) Relative abundance of sequences from methanogenic archaea; (C) Relative abundance of sequences most similar to organisms that are capable of extracellular electron transfer. Sequences that accounted for less than 1% of the population were grouped into the category "Others".

that transfer electrons directly from electron-donating cells to electron-accepting cells, which is considered as an alternative pathway in syntrophic metabolism. It has already been shown that the humic substance analogue, AQDS, can serve as an electron shuttle and promote DIET between two anaerobic metal respiring *Geobacter* species, a genus frequently associated with anaerobic digesters (*G. metallireducens* and *G. sulfurreducens*). However, attempts made to stimulate DIET between a co-culture of *G. metallireducens* and *Methanosaeta/Methanosarcina* with AQDS were unsuccessful because the high mid-point potential of the AQDS/AHQDS redox couple does not enable reduction of carbon dioxide to methane (Liu et al., 2012; Smith et al., 2015b).

In order to evaluate whether untreated or treated FA could serve as an electron shuttle and stimulate syntrophy, ethanol metabolism by co-cultures of *G. metallireducens* and *G. sulfurreducens* in the presence of FA was examined. Results showed that addition of untreated FA from raw leachate to the co-cultures promoted ethanol metabolism and succinate production (Fig. 4). In contrast, addition of treated FA extracted from all three EGSB reactor effluents (FA1500, FA4000, FA8000) inhibited growth of the co-cultures, indicating that FA is significantly transformed during the methanogenic bio-treatment process and can no longer serve as an electron shuttle to stimulate syntrophic interactions.

4. Conclusions

These results show that bio-methanogenic treatment transformed FA from leachate into molecules that were much more difficult to degrade and had reduced electron shuttling capabilities. In addition, addition of FA to anaerobic digesters inhibited growth of methanogens and syntrophic bacteria. This, in turn, caused an overall reduction in methane production and bioreactor efficiencies.

This study also found that the larger FA molecules formed during bio-methanogenic treatment of influent with high FA concentrations were resistant to biodegradation. These compounds pose a major threat to the environment if released without further

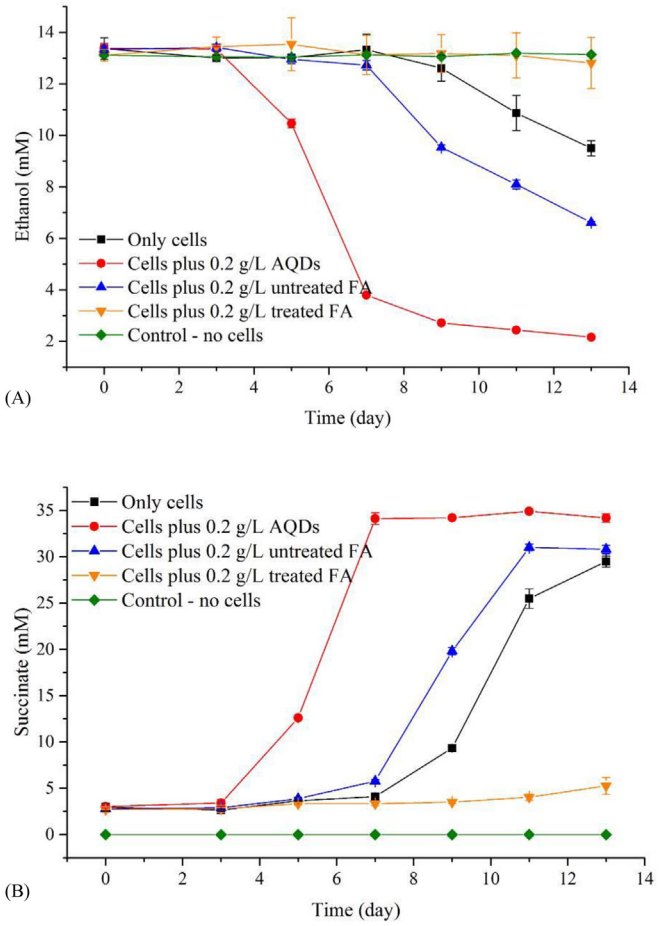

Fig. 4. (A)Ethanol consumption and (B) succinate produced from fumarate reduction by a syntrophic co-culture of *G. metallireducens* and *G. sulfurreducens* with amendments of treated/untreated FA or AQDS. The error bars represent standard deviations of the mean for triplicate cultures.

treatment. Therefore, we suggest that these residual FA macromolecules should be further processed by chemical or physical methods such as advanced oxidation, coagulating or sedimentation prior to release. Results from this study should be taken into consideration in future attempts to optimize anaerobic digesters treating MSW incineration leachate.

Notes

The authors declare no competing financial interest.

Contribution declarations

Yan Dang, Yuqing Lei, Dezhi Sun and Dawn E Holmes conceived the experiments. Yan Dang and Yuqing Lei operated the EGSB reactor. Yuqing Lei, Zhao Liu and Yiting Xue help to performed spectroscopic experiments. Yan Dang, Dawn E Holmes and Li-Ying Wang performed *Geobacter* co-culture experiments. Yan Dang and Dawn E Holmes analyzed the data and wrote the manuscript.

All authors have seen the manuscript at all stages, discussed the data and agreed to the content.

Acknowledgment

We thank Professor Runcang Sun and Dr. Jialong Wen from Beijing Forestry University for the NMR tests. This research was financially supported by the Beijing Science and Technology Plan Projects (Beijing Science and Technology Commission), and the Fundamental Research Funds for the Central Universities (BLYJ201401), as well as the National Natural Science Foundation of China (51678051).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.09.044.

References

Ahring, B.K., Sandberg, M., Angelidaki, I., 1995. Volatile fatty acids as indicators of process imbalance in anaerobic digestors. Appl. Microbiol. Biotechnol. 43 (3), 559–565.

Aiken, G.R., McKnight, D.M., Wershaw, R.L., MacCarthy, P., 1985. Humic Substances in Soil, Sediment, and Water: Geochemistry, Isolation and Characterization. John Wiley & Sons.

Apha, A., 2005. WEF (2005) Standard Methods for the Examination of Water and Wastewater. American Public Health Association, American Water Works Association, and Water Environment Federation.

Azman, S., Khadem, A.F., Zeeman, G., van Lier, J.B., Plugge, C.M., 2015. Mitigation of humic acid inhibition in anaerobic digestion of cellulose by addition of various salts. Bioengineering 2 (2), 54–65.

Chen, S., Rotaru, A.-E., Liu, F., Philips, J., Woodard, T.L., Nevin, K.P., Lovley, D.R., 2014. Carbon cloth stimulates direct interspecies electron transfer in syntrophic co-cultures. Bioresour. Technol. 173, 82–86.

Chiou, C.T., Malcolm, R.L., Brinton, T.I., Kile, D.E., 1986. Water solubility enhancement of some organic pollutants and pesticides by dissolved humic and fulvic acids. Environ. Sci. Technol. 20 (5), 502–508.

Chou, J.-d., Wey, M.-Y., Liang, H.-H., Chang, S.-H., 2009. Biotoxicity evaluation of fly ash and bottom ash from different municipal solid waste incinerators. J. Hazard. Mater. 168 (1), 197–202.

Christensen, J.B., Jensen, D.L., Christensen, T.H., 1996. Effect of dissolved organic carbon on the mobility of cadmium, nickel and zinc in leachate polluted groundwater. Water Res. 30 (12), 3037–3049.

Christensen, J.B., Jensen, D.L., Grøn, C., Filip, Z., Christensen, T.H., 1998. Characterization of the dissolved organic carbon in landfill leachate-polluted groundwater. Water Res. 32 (1), 125–135.

Coates, J.D., Cole, K.A., Chakraborty, R., O'Connor, S.M., Achenbach, L.A., 2002. Diversity and ubiquity of bacteria capable of utilizing humic substances as electron donors for anaerobic respiration. Appl. Environ. Microbiol. 68 (5), 2445–2452.

Coppi, M.V., Leang, C., Sandler, S.J., Lovley, D.R., 2001. Development of a genetic system for *Geobacter sulfurreducens*. Appl. Environ. Microbiol. 67 (7), 3180–3187.

Dang, Y., Holmes, D.E., Zhao, Z., Woodard, T.L., Zhang, Y., Sun, D., Wang, L.-Y., Nevin, K.P., Lovley, D.R., 2016. Enhancing anaerobic digestion of complex organic waste with carbon-based conductive materials. Bioresour. Technol. 220, 516–522.

Dang, Y., Ye, J., Mu, Y., Qiu, B., Sun, D., 2013. Effective anaerobic treatment of fresh leachate from MSW incineration plant and dynamic characteristics of microbial community in granular sludge. Appl. Microbiol. Biotechnol. 97 (24), 10563–10574.

Dang, Y., Zhang, R., Wu, S., Liu, Z., Qiu, B., Fang, Y., Sun, D., 2014. Calcium effect on anaerobic biological treatment of fresh leachate with extreme high calcium concentration. Int. Biodeterior. Biodegrad. 95, 76–83.

Fennell, D.E., Nijenhuis, I., Wilson, S.F., Zinder, S.H., Häggblom, M.M., 2004. Dehalococcoides ethenogenes strain 195 reductively dechlorinates diverse chlorinated aromatic pollutants. Environ. Sci. Technol. 38 (7), 2075–2081.

Fernandes, T.V., van Lier, J.B., Zeeman, G., 2015. Humic acid-like and fulvic acid-like inhibition on the hydrolysis of cellulose and tributyrin. BioEnergy Res. 8 (2), 821–831.

Gunaseelan, V.N., 1997. Anaerobic digestion of biomass for methane production: a review. Biomass bioenergy 13 (1), 83–114.

He, P.-j., Xue, J.-f., Shao, L.-m., Li, G.-j., Lee, D.-J., 2006. Dissolved organic matter (DOM) in recycled leachate of bioreactor landfill. Water Res. 40 (7), 1465–1473.

Hoornweg, D., Bhada-Tata, P., 2012. What a waste: a global review of solid waste management.

Kang, K.-H., Shin, H.S., Park, H., 2002. Characterization of humic substances present in landfill leachates with different landfill ages and its implications. Water Res. 36 (16), 4023–4032.

Kawaichi, S., Ito, N., Kamikawa, R., Sugawara, T., Yoshida, T., Sako, Y., 2013. Ardenticatena maritima gen. nov., sp. nov., a ferric iron-and nitrate-reducing bacterium of the phylum 'Chloroflexi'isolated from an iron-rich coastal hydrothermal field, and description of Ardenticatenia classis nov. Int. J. Syst. Evol. Microbiol. 63 (8), 2992–3002.

Khan, S., Schnitzer, M., 1972. The retention of hydrophobic organic compounds by humic acid. Geochim. Cosmochim. Acta 36 (7), 745–754.

Leitão, R.C., Van Haandel, A.C., Zeeman, G., Lettinga, G., 2006. The effects of operational and environmental variations on anaerobic wastewater treatment systems: a review. Bioresour. Technol. 97 (9), 1105–1118.

Li, H., Chang, J., Liu, P., Fu, L., Ding, D., Lu, Y., 2015. Direct interspecies electron transfer accelerates syntrophic oxidation of butyrate in paddy soil enrichments. Environ. Microbiol. 17 (5), 1533–1547.

Lin, B., Lu, Y., 2015. Bacterial and archaeal guilds associated with electrogenesis and methanogenesis in paddy field soil. Geoderma 259–260, 362–369.

Liu, F., Rotaru, A.-E., Shrestha, P.M., Malvankar, N.S., Nevin, K.P., Lovley, D.R., 2012. Promoting direct interspecies electron transfer with activated carbon. Energy Environ. Sci. 5 (10), 8982–8989.

Liu, K., Chen, Y., Xiao, N., Zheng, X., Li, M., 2015a. Effect of humic acids with different characteristics on fermentative short-chain fatty acids production from waste activated sludge. Environ. Sci. Technol. 49 (8), 4929–4936.

Liu, Z., Dang, Y., Li, C., Sun, D., 2015b. Inhibitory effect of high NH_4^+–N concentration on anaerobic biotreatment of fresh leachate from a municipal solid waste incineration plant. Waste Manag. 43, 188–195.

Lovley, D.R., Fraga, J.L., Coates, J.D., Blunt-Harris, E.L., 1999. Humics as an electron donor for anaerobic respiration. Environ. Microbiol. 1 (1), 89–98.

Marttinen, S.K., Kettunen, R.H., Rintala, J.A., 2003. Occurrence and removal of organic pollutants in sewages and landfill leachates. Sci. Total Environ. 301 (1), 1–12.

Morita, M., Malvankar, N.S., Franks, A.E., Summers, Z.M., Giloteaux, L., Rotaru, A.E., Rotaru, C., Lovley, D.R., 2011. Potential for direct interspecies electron transfer in methanogenic wastewater digester aggregates. MBio 2 (4) e00159–00111.

Narihiro, T., Nobu, M.K., Kim, N.K., Kamagata, Y., Liu, W.T., 2015. The nexus of syntrophy-associated microbiota in anaerobic digestion revealed by long-term enrichment and community survey. Environ. Microbiol. 17 (5), 1707–1720.

Nevin, K.P., Richter, H., Covalla, S., Johnson, J., Woodard, T., Orloff, A., Jia, H., Zhang, M., Lovley, D., 2008. Power output and columbic efficiencies from biofilms of *Geobacter sulfurreducens* comparable to mixed community microbial fuel cells. Environ. Microbiol. 10 (10), 2505–2514.

Ozkaya, B., 2005. Chlorophenols in leachates originating from different landfills and aerobic composting plants. J. Hazard. Mater. 124 (1), 107–112.

Peiffer, J.A., Spor, A., Koren, O., Jin, Z., Tringe, S.G., Dangl, J.L., Buckler, E.S., Ley, R.E., 2013. Diversity and heritability of the maize rhizosphere microbiome under field conditions. Proc. Natl. Acad. Sci. 110 (16), 6548–6553.

Ritchie, J.D., Perdue, E.M., 2003. Proton-binding study of standard and reference fulvic acids, humic acids, and natural organic matter. Geochim. Cosmochim. Acta 67 (1), 85–96.

Schmidt, M.W., Torn, M.S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I.A., Kleber, M., Kögel-Knabner, I., Lehmann, J., Manning, D.A., 2011. Persistence of soil organic matter as an ecosystem property. Nature 478 (7367), 49–56.

Schwarz, W., 2001. The cellulosome and cellulose degradation by anaerobic bacteria. Appl. Microbiol. Biotechnol. 56 (5–6), 634–649.

Scott, D.T., McKnight, D.M., Blunt-Harris, E.L., Kolesar, S.E., Lovley, D.R., 1998. Quinone moieties act as electron acceptors in the reduction of humic substances by humics-reducing microorganisms. Environ. Sci. Technol. 32 (19), 2984–2989.

Singer, P., 1999. Humic substances as precursors for potentially harmful disinfection by-products. Water Sci. Technol. 40 (9), 25–30.

Smith, A.L., Skerlos, S.J., Raskin, L., 2015a. Membrane biofilm development improves

COD removal in anaerobic membrane bioreactor wastewater treatment. Microb. Biotechnol. 8 (5), 883–894.
Smith, J.A., Nevin, K.P., Lovley, D.R., 2015b. Syntrophic growth via quinone-mediated interspecies electron transfer. Front. Microbiol. 6.
Sobotka, M., Votruba, J., Havlik, I., Minkevich, I., 1983. The mass—energy balance of anaerobic methane production. Folia Microbiol. 28 (3), 195–204.
Summers, Z.M., Fogarty, H.E., Leang, C., Franks, A.E., Malvankar, N.S., Lovley, D.R., 2010. Direct exchange of electrons within aggregates of an evolved syntrophic coculture of anaerobic bacteria. Science 330 (6009), 1413–1415.
Wetzel, R.G., 1993. Humic compounds from wetlands: complexation, inactivation, and reactivation of surface-bound and extracellular enzymes. Proc. Int. Assoc. Theor. Appl. Limnol. 25, 122–122.
Ye, J., Mu, Y., Cheng, X., Sun, D., 2011. Treatment of fresh leachate with high-strength organics and calcium from municipal solid waste incineration plant using UASB reactor. Bioresour. Technol. 102 (9), 5498–5503.
Zhao, Z., Zhang, Y., Quan, X., Zhao, H., 2016. Evaluation on direct interspecies electron transfer in anaerobic sludge digestion of microbial electrolysis cell. Bioresour. Technol. 200, 235–244.
Zhao, Z., Zhang, Y., Woodard, T., Nevin, K., Lovley, D., 2015. Enhancing syntrophic metabolism in up-flow anaerobic sludge blanket reactors with conductive carbon materials. Bioresour. Technol. 191, 140–145.