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Seasonal monitoring of bacteria and archaea in a full-scale thermophilic anaerobic digester treating food waste-recycling wastewater: Correlations between microbial community characteristics and process variables

Joonyeob Lee [a,1], Gyuseong Han [a,1], Seung Gu Shin [a,*], Taewoan Koo [a], Kyungjin Cho [b], Woong Kim [c], Seokhwan Hwang [a,*]

[a] School of Environmental Science and Engineering, Pohang University of Science and Technology, Pohang, Gyeongbuk 790-784, Republic of Korea
[b] Center for Water Resource Cycle Research, Korea Institute of Science and Technology, 39-1 Hawolgok-Dong, Seongbuk-Gu, Seoul 136-791, Republic of Korea
[c] Department of Environmental Engineering, Kyungpook National University, Daegu, Republic of Korea

HIGHLIGHTS

- Na^+ and lipid could affect COD removal and bacteria community.
- Na^+ and lipid could affect archaeal quantity but not its community structure.
- *Gelria* and *Cardiocoprobacter* were negatively correlated with Na^+ and lipid.
- NH_3 had no correlation with COD removal or with total microbial populations.
- *Methanoculleus*, *Methanobacterium*, *Tepidanaerobacter* responded differently to NH_3.

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ABSTRACT

Microbial population size, community structure, and diversity, and the correlations of these characteristics with process variables were investigated in samples taken seasonally over two years from a full-scale thermophilic anaerobic digester treating food waste-recycling wastewater (FRW). The organic component of the FRW consisted of carbohydrate (35% of volatile solids), protein (34%) and lipid (30%). The chemical oxygen demand (COD) removal efficiency of the anaerobic digestion (AD) system negatively correlated with Na^+ (2.9–7.7 g/L) and lipid (3.3–22.8 g/L) concentrations, which varied significantly over the two years. *Tepidanaerobacter*, *Anaerobaculum*, *Defluviitoga*, *Keratinibaculum*, *Gelria*, *Tepidimicrobium*, *Caldicoprobacter*, *Bacillus*, and *Syntrophaceticus* were the major bacterial genera, and *Methanoculleus* and *Methanobacterium* were the major archaeal genera. Concentrations of Na^+ and lipid in the digester were negatively correlated with total bacterial and archaeal populations determined by real-time quantitative PCR. These concentrations could also significantly affect the bacterial community structure (e.g., negative correlations with *Gelria*), but not archaeal community structure. Lipid concentration was negatively correlated with bacterial diversity, but was not correlated with archaeal diversity. Ammonia concentration in the digester (2.0–4.3 g N/L) had no significant correlation with COD removal or total bacterial/archaeal populations, but could significantly affect both bacterial and archaeal community structures, including syntrophic acetate-oxidizing bacteria and hydrogenotrophic methanogens. These results indicate that Na^+, lipid and ammonia are among the key parameters that affect the process performance of a thermophilic AD system treating FRW and/or the microbial communities in it.

* Corresponding authors.
E-mail addresses: candlit@postech.ac.kr (S.G. Shin), shwang@postech.ac.kr (S. Hwang).
[1] Joonyeob Lee and Gyuseong Han contributed equally to this work.

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1. Introduction

Food waste is one of the three largest components by weight of the organic waste stream in Korea; 8.4 million tons of food waste and wastewater (14.3% of total organic waste generation) was generated in 2011 [1]. In Korea, food waste has been selectively collected among household wastes by law since 2003, and > 95% is recycled as animal feed or compost. During the recycling process, 3.4 million tons of food waste-recycling wastewater (FRW, also referred to as food waste leachate or food wastewater) have been generated annually, and constituted 41% of the total annual food waste and wastewater generation in 2011 [1]. FRW is a high-strength organic wastewater, that has 48–200 g chemical oxygen demand (COD)/L [2]. Due to the large quantity and the high organic material content, significant environmental effects are anticipated if FRW is released into the ecosystem without adequate treatment.

Anaerobic digestion (AD) has been considered as a treatment option that can reduce the quantity of organic waste, and simultaneously generate CH_4, which can be used as a fuel. The Korean government has invested considerable money to install full-scale anaerobic digesters in major cities in Korea to treat food waste and/or FRW; at present, twenty full-scale AD plants are being operated.

AD consists of four sequential biochemical processes: hydrolysis, acidogenesis, acetogenesis, and methanogenesis. Process imbalance between acidogenesis and methanogenesis may occur (i.e., the total concentration of volatile fatty acids (VFAs) [TVFA] produced by acidogens exceeds the amount of VFAs consumed by methanogens) as a result of hydraulic or organic overloading, the presence of compounds that inhibit anaerobes, or changes in process conditions and influent substrate [3]. The content of major organic components (i.e., carbohydrate, protein, and lipid) would also affect the stability and efficiency of AD because they are degraded into various metabolites via different biochemical pathways [4]. FRW is reported to contain high contents of lipid (30.5 g/L; 37% of volatile solids (VS)), protein (24.6 g/L; 30% of VS), and Na^+ (2.1 g/L for FRW, 6.9 g/L for food waste), so potentially-inhibitory compounds such as long chain fatty acids (specified as lipid), ammonia, and Na^+ could be primary concerns in AD of FRW [2,5,6]. Furthermore, in a full-scale AD system, the characteristics of the feedstock can vary unpredictably; such fluctuations may cause imbalance between acidogenesis and methanogenesis, and can change the microbial community structure as well as process stability and performance [7,8]. In fact, substrate utilizations and inhibitory effects are correlated with growth of specific microorganisms and to specific shifts in the microbial communities during variations of process performance, but the natures and degrees of these correlations remain unknown. In general, process functionality (and metabolic rate) is higher but microbial diversity is lower in thermophilic AD than in mesophilic AD, so stable operation may be difficult to attain in thermophilic AD systems [9,10]. Thus, understanding of correlations within and among process variables and microorganisms in a thermophilic AD system would provide new insight into the interactions that affect the stability of the process.

Many studies have used high-throughput sequencing methods to quantify microbial community structures in AD systems, but reference data for profiling of anaerobic microbial communities on various types of organic waste are limited [11]. Moreover, variations of microbial communities in a full-scale thermophilic AD system treating FRW in the presence of variations in inhibitory effects (e.g., by Na^+ and lipid) have not been reported to the best of our knowledge. Therefore, in this study, two-year variations (covering four seasons) of microbial communities of a full-scale thermophilic anaerobic digester treating FRW were analyzed using 454 pyrosequencing. Non-metric multidimensional scaling was conducted to investigate temporal variation in microbial community structure over this period. Correlations within and among process variables and microbial communities in the AD system were determined using correlation tests and visualized using redundancy analysis.

2. Materials and methods

2.1. Full-scale anaerobic digester

A full-scale anaerobic digester (working volume 2200 m^3) fed with FRW in city of Gwangju, Korea was chosen for investigation of microbial community structures. The digester is a thermophilic (58.5 °C) continuously-stirred tank reactor (CSTR), and so it is abbreviated as TC. Hydraulic retention time of the digester varied from 15.5 d to 17.5 d.

2.2. Sampling and DNA extraction

The organic and inorganic composition of FRW varies widely over seasons [2], so the effects of this variation may affect process variables and microbial communities in the thermophilic AD system treating FRW. To consider its temporal variations, eight seasonal samples were collected from October, 2010 to July, 2012. The samples were numbered chronologically: TC1 = Oct 2010; TC2 = Jan 2011; TC3 = Apr 2011; TC4 = Jul 2011; TC5 = Oct 2011; TC6 = Jan 2011; TC7 = Apr 2012; TC8 = Jul 2012). Samples were collected from the influent pipe and directly from the digester, and stored in an ice box as soon as possible.

Total genomic DNA was extracted from the TC samples by using an automated nucleic acid extractor (Magtration System 6GC, PSS, Chiba, Japan). Before extraction of genomic DNA, 200 μl of sample was centrifuged at 16 000 *g* for 10 min, and 100 μl of the supernatant was decanted. Then the pellet was washed twice in three steps: (1) the pellet was added with 100 μl of deionized distilled water and resuspended; (2) the suspension was centrifuged; (3) 100 μl of supernatant was decanted. Finally, the pellet was gently suspended and applied to an automated nucleic acid extractor (Magtration System 6GC, PSS Co., Japan). The pellets including the extracted DNA were eluted with 100 μl of Tris–HCl buffer (pH 8.0) and stored at −20 °C until further analyzed.

2.3. Physicochemical analysis

A gas chromatograph (6890 Plus, Agilent, Palo Alto, CA) with an HP Innowax capillary column and flame ionization detector was used to measure VFAs and ethanol. The pH, chemical oxygen demand (COD) and VS concentrations were determined according to the procedures in Standard Methods [12]. Carbohydrate concentration was measured using the phenol–sulfuric acid method [13]. Protein concentration and total ammonia nitrogen [ammonia] were determined using the Kjeldahl method [12]. Lipid concentrations [lipid] were measured by gravimetric analysis after extraction of lipid using chloroform: methanol (1:2 v/v) [14]. Sodium ion concentration [Na^+] and chloride ion concentration were measured using ion chromatography (790 Personal IC, Metrohm, Switzerland). All analyses were performed in duplicate.

2.3.1. Pyrosequencing analysis

Several fusion primers were designed under the guidance of Macrogen (Seoul, Korea) and synthesized by Bioneer (Daejeon, Korea). The fusion primers consisted of adapter A or B and the oligonucleotide for amplifying the target sequence. The combined primers A-787f (5′-ATTAG ATACC CNGGT AG-3′) and B-1492r

(5′-GNTAC CTTGT TACGA CTT-3′) were used to amplify the V5–V9 of 16S rRNA genes of extracted genomic DNA [15]. After an initial denaturation at 95 °C for 5 min, 30 cycles of polymerase chain reaction (PCR) were performed (denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 1 min), followed by a final extension step at 72 °C for 7 min. The PCR products were purified on 1% agarose gel by using a genomic DNA purification kit (GeneAll Biotechnology, Seoul, Korea). All of the 454 pyrosequencing analyses were performed at Macrogen (Seoul, Korea) following the manufacturer's protocol (454 Life Science, Branford, USA). An "in-house" program (Macrogen, Seoul, Korea) was used to remove the adapter sequences from all amplified reads before further analysis. Reads with low nucleotide-quality scores (< 20), short sequences (< 270-bp), and potentially-chimeric sequences were discarded and MOTHUR with CD-HIT-OTU was used to cluster the remaining sequences that had ⩾ 97% sequence similarity as operational taxonomic units (OTUs). Taxonomic classification of the OTUs was performed using the SILVA database. The nucleotide sequences obtained from the 454 pyrosequencing were deposited in NCBI's sequence read archive (PRJNA315957).

2.4. Real-time quantitative polymerase chain reaction analysis

Real-time quantitative polymerase chain reaction (QPCR) (LightCycler 480, Roche) was used to quantify the populations (16S rRNA gene copies) of total bacteria and total archaea in the digester. TaqMan probe-primer sets were used: for bacteria, BAC338F (5′-ACTCC TACGG GAGGC AG-3′), BAC516F (5′-TGCCA GCAGC CGCGG TAATA C-3′), and BAC805R (5′-GACTA CCAGG GTATC TAATC C-3′); for archaea, ARC787F (5′-ATTAG ATACC CSBGT AGTCC-3′), ARC915F (5′-AGGAA TTGGC GGGGG AGCAC-3′), and ARC1059R (5′-GCCAT GCACC WCCTC T-3′) [16].

2.5. Statistical analysis

Non-metric multidimensional scaling (NMS) using Sorensen (Bray–Curtis) distance was conducted to visualize how the similarity of microbial community structures in the digester changed over time (PC-ORD 5.0, MjM software, OR, USA). Sorensen (Bray–Curtis) distance is dominantly used for NMS of ecological community data because it provides better sensitivity in heterogeneous data sets and gives less weight to outliers than does Euclidean distance [17]. Relative abundances of bacterial OTUs or archaeal OTUs obtained by 454 pyrosequencing analysis were used as the data sets. Process variables with $p < 0.1$ were selected and used as the second matrix to construct a Joint NMS ordination plot. Each main matrix was processed for ordination such that the stress (< 10) and the instability (< 10^{-4}) criteria were met [17].

Redundancy analysis (RDA) to visualize the correlations among environmental variables and microbial community structures in two-dimensional space was performed using CANOCO 4.5 (Plant Research International, The Netherlands). In this study, the relative abundances of bacterial genera from the 454 pyrosequencing analysis were used as dependent variables, and process variables selected by forward selection (e.g., [lipid], [Na^+], [TVFA], and [ammonia] in the digester; lipid removal, COD removal) were used as independent variables. The statistical significance of the model was tested by Monte Carlo permutation against 999 random datasets. Pearson correlation tests were conducted using R software packages with optparse and gclus libraries to quantify correlation coefficients within and among process variables and microbial communities.

3. Results and discussion

3.1. Variations in process performance along with changes in process variables in a full-scale thermophilic AD system treating FRW

Temporal variation and effect of process variables on process performance of the full-scale thermophilic AD system fed with FRW were investigated using the eight seasonal samples. The two-year averages of the FRW feedstock were 161.5 ± 27.3 g COD/L and 71.4 ± 14.7 g VS/L. Carbohydrate (24.7 ± 14.8 g/L; 35% of VS), protein (24.3 ± 9.1 g/L; 34%) and lipid (21.3 ± 4.0 g/L; 30%) contributed evenly to the organic contents of the FRW (Fig. 1a). These values confirm that the characteristics of the FRW used in this study agreed with the year-round observations as previously reported [2]. The coefficient of variation (CV) was 60% for carbohydrate, 32% for lipid and 32% for protein. These variations were higher than those of VS (21%) and COD (17%); i.e., temporal variations in composition exceeded those in total organic concentration. The high CV of carbohydrate content resulted mainly from relatively lower values of carbohydrate contents in summer samples (17% of VS), TC4 and TC8, compared to spring (29%), fall (34%), and winter (54%) samples. [TVFA] was higher in the summer (19.0 g/L) than in other seasons (7.7–10.9 g/L) (Fig. 1a). One possible explanation for these opposing trend is that high temperature during summer may have increased microbial acidification of carbohydrate during transport and storage processes.

In this study, COD removal efficiency was considered a key parameter in assessment of the process performance of the AD system. COD removal varied from 26% to 80% over time (Fig. 1b). More carbohydrate (22.3 ± 15.3 g/L; 90% of influent) was degraded than lipid (12.7 ± 5.1 g/L; 60% of influent) or protein (9.4 ± 5.6 g/L; 38% of influent) in the system. Poor degradation of protein compared to carbohydrate and lipid is often observed in full-scale anaerobic digesters fed with FRW [18], and suggests that FRW may contain high contents of refractory protein. Lipid removal increased as COD removal increased ($r = 0.907$, $p < 0.05$), and was more similar to the trend in variation with COD than were carbohydrate and protein removal (Fig. 1); these results suggest that lipid removal could be the main factor that determines COD removal in the TC digester.

[Lipid], [Na^+], and [ammonia] in the digester can directly influence process performance and microbial communities in an AD system [19]. [Lipid] varied more among samples (CV = 89%), than did [Na^+] (36%), or [ammonia] (23%) (Fig. 1c). High [lipid] in the digester can inhibit microorganisms (1) by limiting substrate transfer between the cell and the aqueous phase, or (2) by disrupting cellular homeostasis [20,21]. Moreover, compared to mesophilic conditions, inhibition of lipids (including long chain fatty acids) is more problematic under thermophilic conditions due to the presence of higher portion of their unionized forms [22]. In this study, [Lipid] in the digester increased as [lipid] in the influent FRW increased ($r = 0.789$, $p < 0.05$); i.e., high [lipid] in the digester may be closely related to high [lipid] in the influent FRW (Fig. 1c). COD removal decreased as [lipid] in the digester increased ($r = -0.889$, $p < 0.05$); this trend suggests that lipids may inhibit digestion in the digester. For instance, COD removal remained above the average (61%) when [lipid] in the digester was < 5.0 g/L, but decreased drastically to 26–44% when it was > 5.0 g/L (Fig. 1b and c). This trend is consistent with a previous study that reported 5 g lipid/L as an inhibition threshold in AD of protein- and lipid-rich organic wastes [23].

[Na^+] can inhibit growth of microorganisms if it is high enough to cause dehydration of cells by elevated osmotic pressure [19,24,25]. The IC_{50} of sodium for methanogenic activity, assessed

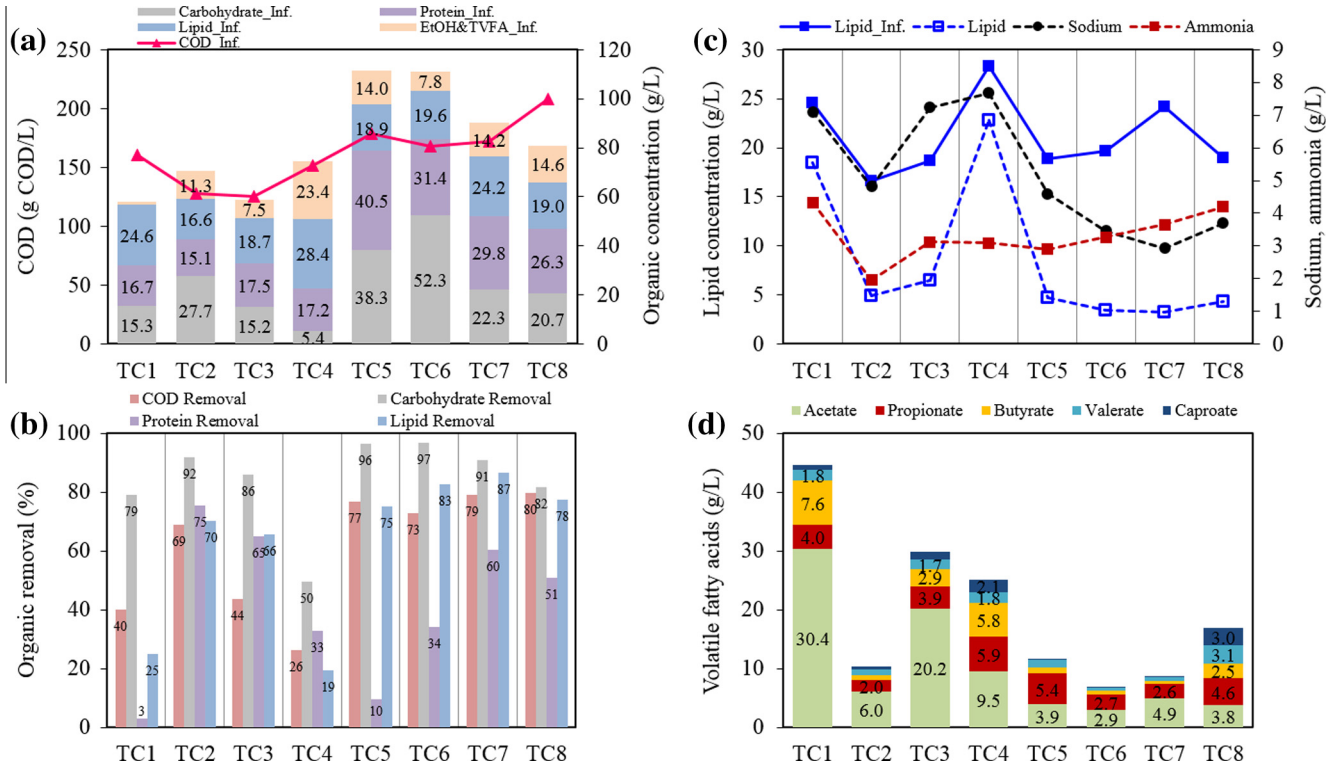

Fig. 1. (a) Temporal variations in organic profiles of food waste-recycling wastewater fed to the full-scale thermophilic CSTR digester. (b) Changes in organic removals. (c) Changes in concentrations of possible inhibitors in the digester. (d) Changes in concentrations of volatile fatty acids in the digester. Inf. = influent; removal = portion (%) of organics removed from the influent. TC1 = Oct, 2010; TC2 = Jan, 2011; TC3 = Apr, 2011; TC4 = Jul, 2011; TC5 = Oct, 2011; TC6 = Jan, 2011; TC7 = Apr, 2012; TC8 = Jul, 2012.

on methanogens alone or together with syntrophic bacteria, varies widely from 3 g/L to 16 g/L, mainly due to the differences in the degree of acclimation of microbial communities and in the environmental conditions in the AD systems [19,25]. In this study, [Na^+] in the digester varied from 2.9 g/L to 7.7 g/L; [Na^+] in the digester was negatively correlated with COD removal ($r = -0.908$, $p < 0.05$) (Fig. 1c). COD removal decreased significantly when [Na^+] in the digester reached 7.1–7.7 g/L (e.g., in TC1, TC3, and TC4); this response is consistent with a previous report that thermophilic AD was severely inhibited by the presence of 7.8 g Na^+/L [26].

High [ammonia] can also inhibit methane production by inhibiting methanogens and bacteria; reported IC_{50} of ammonia for methane production is $1.7 \leqslant$ [ammonia] $\leqslant 14$ g/L, depending on inoculum and the environmental conditions in the AD system [19,27]. Neither [ammonia] nor [free ammonia] showed significant correlation with any organic removals in this study; i.e., the ranges of [ammonia] and [free ammonia] in the digester (2.0–4.3 g N/L and 0.1–2.0 g N/L, respectively) might not have significantly affected the entire process performance during the two-year operation (Fig. 1c).

VFAs can accumulate when: (1) the influent loading or characteristics are high or vary widely, so that methanogens cannot completely process the VFAs generated by bacteria, (2) compounds (including Na^+, lipid, ammonia and increased VFAs themselves) that inhibit methanogens occur in the digester [3,19]. COD removal decreased as [TVFA] and concentrations of acetate and butyrate increased ($r = -0.776$, -0.690, -0.833, respectively, all $p < 0.1$) (Fig. 1d); these results are consistent with previous reports that suggest that the concentrations of VFAs in the digester can be used as an indicator of process instability [3].

3.2. QPCR analysis of total microbial populations

To investigate the relationship between variations in microbial population and the perturbations in [Na^+] and [lipid], total bacterial and archaeal populations of the eight seasonal samples were quantified using QPCR. The total population of bacteria was $1.4 \times 10^{10} \pm 1.5 \times 10^{10}$ rRNA gene copies/mL, and the total population of archaea was $9.1 \times 10^{7} \pm 1.1 \times 10^{8}$ 16S rRNA gene copies/mL (Supplementary Fig. 1), and both were positively correlated with variation in COD removal ($r = 0.782$, $p < 0.05$ and $r = 0.664$, $p < 0.1$, respectively) but negatively correlated with [Na^+] and [lipid] in the digester (Supplementary Fig. 1); the second of these results suggests that high [Na^+] and [lipid] inhibited the growth of both bacteria and archaea, and reduced overall process performance.

3.2.1. Pyrosequencing analysis of microbial population profile in total

454 pyrosequencing of bacterial 16S rRNA gene amplicons from eight samples resulted in 58 342 non-chimeric sequence reads. Reads that shared $\geqslant$ 97% similarity in sequences were grouped as OTUs; after this preprocess, 17 073 reads (2 134 ± 783 reads for each sample) remained as rigid reads and were grouped into 116 OTUs for further analysis. In this study, the ratio R of classified reads to total bacterial reads decreased as taxonomic levels of methanogens moved from the phylum to the species. R was 1.00 at the phylum level, 0.83 at the class level, 0.82 at the order level, 0.80 at the family level, and 0.77 at the genus level, but only 0.20 at the species level. Because R decreased significantly between phylum and class, and between genus and species, further analysis focused on the phylum and the genus.

All of the bacterial reads (17 073 reads) were classified into one of nine phyla: Firmicutes (66.6 ± 20.1%), Synergistetes

(19.1 ± 18.6%), Thermotogae (10.4 ± 8.9%), Bacteroidetes (2.2 ± 3.5%), Atribacteria (1.1 ± 2.0%), Proteobacteria (0.4 ± 0.6%), Tenericutes (0.2 ± 0.5%), Actinobacteria (0.006 ± 0.018%), Spirochaetes (0.004 ± 0.011%) (Supplementary Fig. 2). The overwhelming dominance of Firmicutes has been commonly reported in lab-scale [28] and full-scale thermophilic anaerobic digesters [29]. In this study, of 116 OTUs (i.e., total number of bacterial OTUs found in this study), 90 (78%) were classified into Firmicutes and a large portion of unclassified reads at the class level (92%) belonged to Firmicutes. This result indicates that Firmicutes was highly diverse and contributed significantly to the decrease in *R* between the phylum and class levels. Phylum Atribacteria (8% of total unclassified reads at the class level) has no cultivated representatives, and is only found in cultivation-independent genomic analyses such as metagenomic analyses [30]. Reconstruction of genomic information of Atribacteria genotypes suggests that these bacteria can ferment carbohydrate and organic acids to ethanol, acetate, and hydrogen.

Results of various previous metagenomic studies of anaerobes have suggested correlations between process variables (i.e., environmental variables) or process performance and bacteria at the phylum level [8,29,31]. One report demonstrated that the types and compositions of substrates fed to the AD systems determined the ratio R_{FB} of Firmicutes to Bacteroidetes (e.g., high R_{FB} for easily-degradable substrates, low R_{FB} for fibrous substrate) [31]. In this study, R_{FB} showed no such a relationship with composition of the substrate fed to the TC digester. In fact, investigation of the correlation between process variables and bacteria communities of AD systems may be inadequate if it is based solely on bacterial data at the phylum level. This is because bacteria vary greatly in both phenotype and genotype even if they belong to the same phylum; thus, these correlations should be conducted at the finest taxonomic level possible (i.e., genus or species).

At the genus level, 13218 reads (77.4% of the total bacterial reads) were classified into 45 genera. Sixteen bacterial genera that showed relative abundance > 0.5% in one of the eight samples were considered high-rank groups: *Tepidanaerobacter* (20.6 ± 15.1%), *Anaerobaculum* (19.1 ± 18.6%), *Defluviitoga* (10.4 ± 8.9%), *Keratinibaculum* (8.1 ± 8.3%), *Gelria* (7.9 ± 8.2%), *Tepidimicrobium* (3.4 ± 5.0%), *Caldicoprobacter* (2.5 ± 1.2%), *Bacillus* (1.8 ± 4.4%), *Syntrophaceticus* (1.7 ± 2.8%), *Ruminiclostridium* (0.6 ± 1.1%), *Lactobacillus* (0.4 ± 0.6%), *Halocella* (0.3 ± 0.3%), *Proteiniphilum* (0.3 ± 0.6%), *Methyloversatilis* (0.2 ± 0.3%), *Syntrophomonas* (0.2 ± 0.2%), *Haloplasma* (0.1 ± 0.3%) (Fig 2a). Among these sixteen genera, *Tepidanaerobacter*, *Anaerobaculum*, *Defluviitoga*, *Keratinibaculum*, *Gelria*, *Tepidimicrobium*, *Caldicoprobacter*, *Bacillus*, and *Syntrophaceticus* were dominant (96% of the average abundance of classified bacterial genera and each contributing ⩾ 1.0% on average) (Fig. 2a).

454 pyrosequencing of the eight samples yielded 8130 non-chimeric archaeal 16S rRNA gene sequence reads; 3822 (478 ± 642 reads per sample) remained as rigid reads and were grouped into nine OTUs for further analysis. All (3822) of the archaeal reads were classified into seven methanogen genera: *Methanoculleus* (63.7 ± 44.4%), *Methanobacterium* (30.7 ± 40.2%), *Methanolinea* (2.2 ± 4.2%), *Methanothermobacter* (1.6 ± 4.4%), *Methanospirillum* (0.9 ± 2.2%), *Methanogenium* (0.9 ± 2.2%), and *Methanosaeta* (0.1 ± 0.2%) (Fig. 2b). Hydrogenotrophic methano-

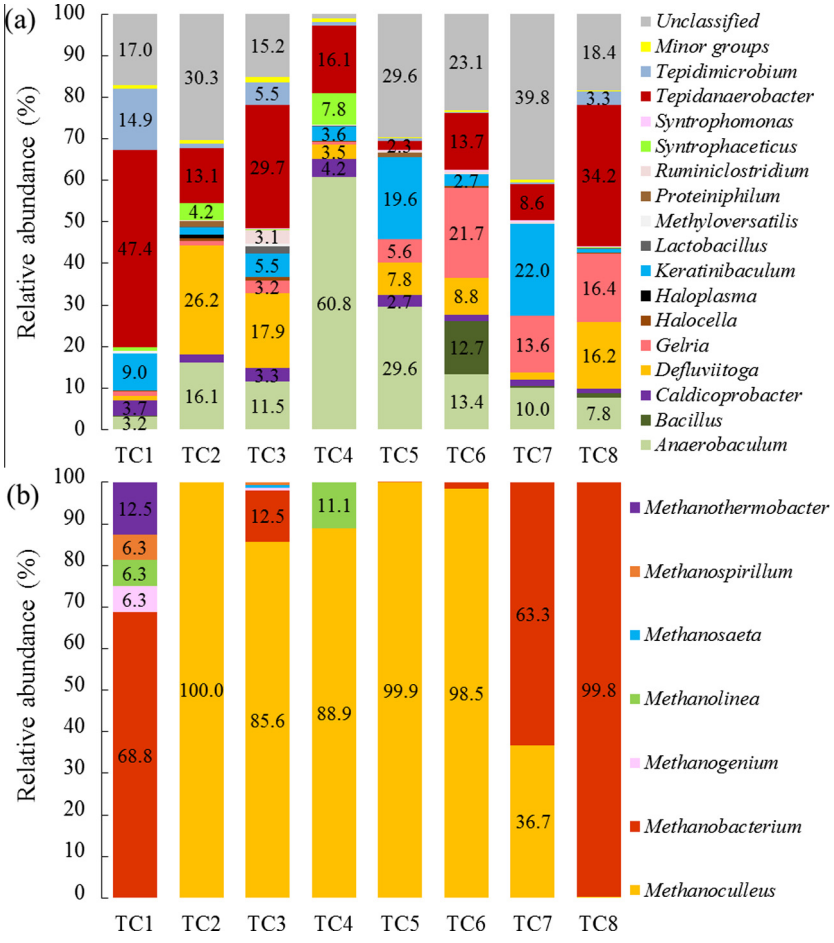

Fig. 2. Temporal variations of genera in (a) bacterial communities, and (b) archaeal communities in the thermophilic CSTR digester fed with food waste-recycling wastewater. Bacteria communities with relative abundance < 0.5% categorized as *Minor groups*.

gens (HMs) constituted 99.9% of total archaeal reads. The dominance of HMs is generally observed in AD systems that are ammonia-stressed (> 3 g N/L) due to presence of selective inhibition on aceticlastic methanogens [27]. High [ammonia] in the TC digester (3.3 ± 0.8 g N/L on average) supports this trend. At the genus level, taxonomic resolution was much lower for bacteria (19.6%) than for archaea (100%); the difference may occur because bacteria are more diverse than archaea, so that 16S rRNA based analysis of bacteria is likely to be more vulnerable to limitations in short-read length of 16S rRNA amplicons in 454 pyrosequencing and incomplete 16S rRNA gene databases [32].

3.3. Variations in microbial community structures

NMS was conducted to visualize the similarity of microbial community structure based on the relative abundances of OTUs across the sample set in the full-scale thermophilic AD system treating FRW (Fig. 3). The Joint NMS result for bacteria indicated that bacterial community structure dynamically shifted over time, and that all temporal shifts were in different directions; the lack of consistent change suggests that bacterial community structure was influenced more by environmental variations in the digester than by temporal variations (Fig. 3a). [Lipid], [Na^+], [TVFA], and [ammonia] in the digester and COD removal had significant effects ($p < 0.1$) on in bacterial community structures among the samples. The samples located in the upper half of the NMS (TC1, TC3, TC4) had high [lipid], [Na^+], and [TVFA] in the digester and low COD removals compared to the other samples; this result indicates that community structure varied in response to [lipid], [Na^+], and [TVFA] in the digester and to COD removal. The vector to [ammonia] in the digester was at 90° to the vectors to [lipid] and [Na^+] in the digester; this means that the effects of [ammonia] are independent of those of [lipid], [Na^+]. [TVFA] in the digester showed positive correlation with [lipid] and [Na^+], and negative correlation with COD removal; this result agrees well with previous study that suggested that high VFA concentrations in the digester may indicate process instability and inhibition of the AD system [3].

In the Joint NMS ordination plot based on archaeal OTUs, significant shifts of methanogen community structure occurred three times: TC1 to TC2, TC6 to TC7, and TC7 to TC8 (Fig. 3b). The shifts are mainly explained by shifts of dominant methanogen from *Methanoculleus* to *Methanobacterium* (i.e., a negative correlation between these most-abundant genera, $r = -0.971$, $p < 0.05$) (Fig. 2b). [Ammonia] in the digester was a significant process variable ($p < 0.1$) among process variables to explain variations in archaeal community structures among the samples (Fig. 3b). The vector to [ammonia] in the digester points up axis 2; this orientation suggests that [ammonia] could have a significant effect on the significant shifts of archaeal community structures. The opposite correlations of *Methanobacterium and Methanoculleus* with [ammonia] ($r = 0.829$, $p < 0.05$ and $r = -0.874$, $p < 0.05$, respectively) supported this hypothesis.

In this study, [lipid], [Na^+], and [TVFA] in the digester (3.3–22.8 g lipid/L, 2.9–7.7 g Na^+/L, 6.7–44.6 g TVFA/L) were important process variables that could affect both total bacterial population (i.e., bacterial 16S rRNA gene copies/L) and bacterial community structure (i.e., relative compositions of bacteria OTUs). They seemed also to influence variations in total archaeal population, but not archaeal community structure. In contrast, [ammonia] in the digester (2.0–4.3 g N/L; 0.1–2.0 g free ammonia N/L) was the only factor that affected the shifts of archaeal community structures, but it had no significant correlation with COD removal and total bacterial population.

These observations suggest that if specific microorganisms are suppressed by increasing [ammonia], their functions might be replaced by other microorganisms with similar roles without major reduction in overall process performance in this thermophilic AD system, at least within the given ranges of [ammonia]. Furthermore, the fact that [ammonia] had similar effects on bacterial and archaeal community structures implies that perturbing [ammonia] could cause critical changes in syntrophic consortia (of bacteria and archaea) that perform methanogenesis in the TC digester. This speculation is supported by the results that [ammonia] in the digester was positively correlated with relative abundances of *Tepidanaerobacter* (a genus that includes syntrophic acetate-oxidizing bacteria, and that comprised 20.6 ± 15.1% of total bacteria; $r = 0.657$, $p < 0.1$) and of *Methanobacterium* (an HM genus that comprised 30.7 ± 40.2% of total archaea; $r = 0.829$, $p < 0.05$).

3.4. Variations of dominant microbial genera along with process variables

RDA was performed to visualize the correlations and the trends among process variables and microbial communities through eight

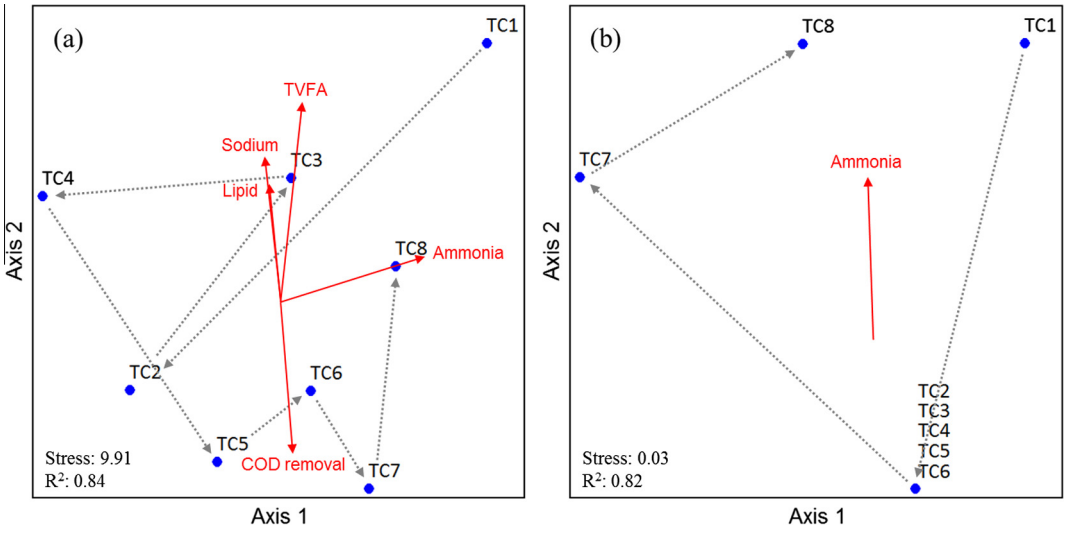

Fig. 3. Joint NMS ordination plots for TC samples based on Sorensen (Bray–Curtis) distance with relative abundance of (a) bacterial OTUs, and (b) archaeal OTUs. Grey arrows (dotted lines): the community structure shifts over time. Red arrows (solid lines): correlation vectors of the process variables with significant factors $p < 0.1$. Significance was tested by Monte Carlo permutation against 999 random datasets. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

seasonal variations in the thermophilic AD system (Fig. 4). The correlations were double-checked using Pearson's correlation coefficient at 90% and 95% confidence intervals (Supplementary Table 1). The RDA ordination plot (Fig. 4) had model $p < 0.05$, which means that the model was statistically significant at the 95% confidence interval, and eigenvalue = 0.55, which means that the independent variables explained 55% of the variance in the response variables.

The genera *Tepidanaerobacter* and *Syntrophaceticus* include syntrophic acetate-oxidizing bacteria (SAOB) such as *Tepidanaerobacter acetatoxydans* and *Syntrophaceticus schinkii*, which establish syntrophic relationships with HMs [33,34]. *T. acetatoxydans* was detected in this study; this species can grow in co-culture with HMs including *Methanoculleus* sp. and *Methanobacterium* sp., which were dominant methanogens in the TC digester [33,35]. In this study, the facts that (1) HMs constitute 99.9% in total methanogens, (2) *Tepidanaerobacter* was the most abundant bacteria genus, and (3) the average abundance of *Tepidanaerobacter* was twelve times higher than that of *Syntrophaceticus*, suggest that hydrogenotrophic methanogenesis coupled with *T. acetatoxydans* could be the main metabolic pathway to generate methane by acetate degradation in the TC digester. Furthermore, the relative abundance of *Tepidanaerobacter* increased as [ammonia] increased ($r = 0.657$, $p < 0.1$) (Fig. 4; Supplementary Table 1); i.e., *Tepidanaerobacter* was not inhibited by [ammonia] within the range of 2.0–4.3 g N/L (0.1–2.0 g free ammonia N/L) in the TC digester. This conclusion is consistent with the previous report that *T. acetatoxydans* could grow at 0–7 g N/L; whereas *Thermacetogenium phaeum*, another common thermophilic SAOB, could grow at 0–3 g N/L [35]. Although the genus *Syntrophaceticus* can also grow at high [ammonia] = 6.4 g N/L, it grows optimally in mesophilic conditions (25–45 °C), so it was uncommon in the TC digester [34]. The relative abundance of *Tepidanaerobacter* was correlated positively with the relative abundance of *Methanobacterium* ($r = 0.598$) and negatively with the relative abundance of *Methanoculleus* ($r = 0.713$, $p < 0.05$); these opposite relationships suggest that *Methanobacterium* might be a preferred syntrophic partner in methanogenesis under the ammonia-stressed condition in the TC digester.

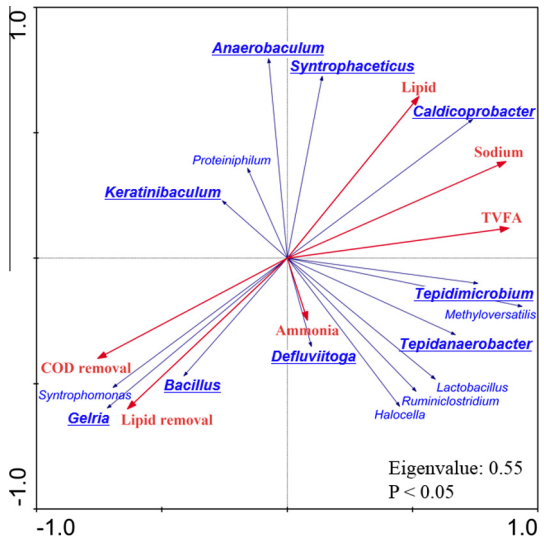

Fig. 4. Redundancy analysis showing correlations between process variables (red arrows) and bacteria genera (blue arrows). All process variables applied in this analysis were significant ($p < 0.1$) except ammonia ($p < 0.2$). Significance was tested by Monte Carlo permutation against 999 random datasets. Dominant bacteria genera (⩾ 1.0% in relative abundance on average) are expressed in bold and underlined. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Anaerobaculum (19.1%) was the second most-dominant genus, and could significantly contribute to carbohydrate removal ($r = 0.692$, $p < 0.1$) in the TC digester (Supplementary Table 1). This conclusion is consistent with literature reports that *Anaerobaculum hydrogeniformans* ferments mainly sugars to acetate, hydrogen and carbon dioxide, possibly in syntrophy with HMs [36]. *Anaerobaculum* was abnormally dominant at TC4 (60.8%) when NaCl concentration was the highest (10.7 g/L) among the samples (Fig. 2a), this response is consistent with the observation that *A. hydrogeniformans* requires high NaCl concentration (10 g/L) for optimum growth.

Defluviitoga (10.4%) was the third dominant genus. It consists of one known species, *Defluviitoga tunisiensis*, a thermophilic (55 °C as the optimum), slightly halophilic (5 g NaCl/L as the optimum) sulfur-reducing bacterium isolated from a whey digester [37]. This bacterium can degrade a variety of sugars to acetate, H_2, and CO_2. Previous studies reported that the genus *Defluviitoga* was the most dominant bacterium in thermophilic AD systems fed with protein-rich wastes such as stillage [38] or food waste [9], but the exact roles of this bacteria in those systems were not determined. Both wastes included high protein content, so the positive correlation between protein removal and the relative abundance of *Defluviitoga* ($r = 0.685$, $p < 0.1$) in the TC digester (Supplementary Table 1) may not be coincidental.

Keratinibaculum (8.1%) was the fourth dominant genus; it includes one known species, *Keratinibaculum paraultunense*, a thermophilic (55 °C as the optimum), slightly halophilic (2 g NaCl/L as the optimum) bacterium that uses keratins and other proteins [39]. To our knowledge, this is the first detection of *Keratinibaculum* as a dominant bacterium in an AD system; however, the relative abundance of this genus was not significantly correlated with any of the process variables considered in this study.

Gelria (7.9%) was the fifth dominant genus. It includes one known species, *Gelria glutamica*, which was isolated from a propionate-oxidizing methanogenic enrichment culture [40]. *G. glutamica* is reported to ferment glutamate in syntrophic relationship with *Methanobacterium* sp., and can solely oxidize sugars to acetate, propionate, H_2 and CO_2 under thermophilic conditions. The relative abundances of *Gelria* and *Syntrophomonas* were correlated negatively with [Na^+] and [lipid], but positively with COD removal and lipid removal (Fig. 4). This suggested that these genera may make critical contribution to lipid or COD removals, but are drastically inhibited by the [Na^+] and [lipid] ranges in the TC digester (Fig. 1). Inhibition of *Gelria* and *Syntrophomonas* by [Na^+] and [lipid] has not been reported previously, so the contributions of these genera to COD removal or lipid removal were investigated. *Gelria* could contribute to COD removal by degrading glutamate in syntrophic relationship with HMs. Previous research also supports the significance contribution of *Gelria* to production of H_2, which may then be utilized by HMs, in a thermophilic AD system to degrade cellulose and microbial protein [41]. Thus, *Gelria* sp. may be an indicator of process performance in thermophilic AD systems that treat food waste or food wastewater. *Syntrophomonas* consists of species known to oxidize long chain fatty acids (i.e., lipid) in syntrophic relationship with methanogens [42]; this relationship is consistent with the positive correlation between lipid removal and the relative abundance of this genus.

Caldicoprobacter (2.5%) was the only genus in which the relative abundance was positively correlated with [Na^+] in the digester ($r = 0.939$, $p < 0.05$) (Fig. 4; Supplementary Table 1); i.e., *Caldicoprobacter* may be halophilic or halotolerant at least within the ranges of 2.9–7.7 Na^+ g/L, and this is consistent with the characteristics of *C. faecale* (recently reclassified from *Acetomicrobium faecale*), which is halotolerant (< 50 g NaCl/L) [43]. Moreover, the relative abundance of *Caldicoprobacter* was negatively correlated with COD removal ($r = 0.918$, $p < 0.05$), so they may indicate abnor-

Table 1
The correlations between three types of diversity (of bacteria and archaea) and process variables in the TC samples. Numbers: Pearson correlation coefficients; yellow: $p < 0.1$; pink: $p < 0.05$; empty box: $p \geqslant 0.1$.

		Concentrations in the digester (g/L)				Organic removal efficiencies (%)			
Domain	Measure	Na^+	NH_4^+	Lipid	TVFA	COD	Carbohydrate	Protein	Lipid
Bacteria	Richness	.	.	-0.716	.	.	0.765	.	0.627
	Evenness	.	.	-0.811	-0.645	0.640	0.789	.	0.749
	Shannon index	.	.	-0.848	.	0.640	0.860	.	0.768
Archaea	Richness	.	.	.	0.663	.	.	.	.
	Evenness	.	.	.	.	.	.	.	.
	Shannon index	.	.	.	0.683	.	.	.	.

mality of process performance in thermophilic AD systems treating food waste or food wastewater.

Tepidimicrobium (3.4%) showed the highest positive correlation with [TVFA] in the digester ($r = 0.901$, $p < 0.05$) (Fig. 4; Supplementary Table 1). This relationship is consistent with reports that all species in this genus are acidogens that produce VFAs under thermophilic conditions [44]. A positive correlation of the relative abundance of *Tepidimicrobium* with [TVFA] in the digester has been noted previously, and the presence of this genus is considered an indicator of process acidification [45].

Among the dominant genera, *Tepidanaerobacter*, *Anaerobaculum*, *Defluviitoga*, *Ketarinibaculum*, and *Tepidimicrobium* had no significant correlation with [Na^+] or [lipid] in the digester (Fig. 4; Supplementary Table 1); therefore variations of [Na^+] or [lipid] within the given ranges might do not seem to affect the relative abundances of those genera in bacterial community structures in the TC digester. However, [ammonia] was a possible factor to explain variations in these genera: as [ammonia] increased, relative abundances of *Tepidanaerobacter*, *Tepidimicrobium*, and *Defluviitoga* increased, whereas relative abundances of *Anaerobaculum* and *Keratinibaculum* decreased (Fig. 4).

Bacterial and archaeal diversity indices were separately quantified based on rigid OTUs ($\geqslant$ 0.5% relative abundance in one of eight samples). In an ecosystem, species richness *S* represents the number of species present; and evenness *E* represents their relative abundances. The Shannon diversity index *H*, which considers both *S* and *E*, is generally used to quantify species diversity of various ecosystems [9,46,47].

For bacteria, [lipid] in the digester correlated negatively with *S*, *E*, and *H*; i.e., inhibition due to high [lipid] decreased overall bacterial diversity (Table 1). Removals of COD, VS, carbohydrate, and lipid positively correlated with *H*; these trends indicate that increasing the diversity of the bacterial community could stabilize and increase process performance of the AD system. All of these results are consistent with previous studies and the general concept in ecology that a high diversity of microorganisms increases the functionality of an ecosystem [9,48].

For archaea, [TVFA] in the digester was positively correlated with H; this relationship indicates that process instability could occur as archaeal diversity increased. This conclusion concurs with a previous report that methanogen diversity increases in the presence of inhibitory effects [49]. It also indicates that bacteria and archaea could have different diversity responses, but not different population size responses, to inhibitory effects in AD systems.

4. Conclusions

In this study, microbial communities were investigated in a full-scale thermophilic anaerobic digester treating FRW, a high-strength and complex organic wastewater. Moreover, variations in microbial community characteristics (i.e., population size, community structure, diversity) and their correlations with process variables were elucidated using biomolecular tools (i.e., QPCR and 454 pyrosequencing) and statistical computations (i.e., NMS, RDA and Pearson correlation test). Eight seasonal observations of two-year operation of the digester gave statistically meaningful results as follows.

(1) Temporal variation of organic composition in the FRW was high; high [lipid] in the digester may result from high [lipid] in the influent FRW. [Na^+] and [lipid] in the digester was negatively correlated with COD removal of the AD system, but [ammonia] was not.
(2) *Tepidanaerobacter*, *Anaerobaculum*, and *Defluviitoga*, *Keratinibaculum*, *Gelria*, *Tepidimicrobium*, *Caldicoprobacter*, *Bacillus*, and *Syntrophaceticus* were dominant bacterial genera, and *Methanoculleus* and *Methanobacterium* were dominant archaeal genera.
(3) Both total bacterial and archaeal populations were positively correlated with COD removal but negatively correlated with [Na^+] and [lipid] in the digester. This result suggests that high [Na^+] and [lipid] within the ranges might inhibit the growth of both bacteria and archaea, and reduce COD removal of the AD system.
(4) The bacterial community structure could be significantly affected by [Na^+], [lipid] and [ammonia] in the digester. *Gelria* and *Cardiocoprobacter* showed opposite responses to [Na^+] and [lipid] in the digester, and may be microbial indicators of process abnormality in thermophilic CSTR AD systems that treat FRW. [Ammonia] in the digester could solely affect both bacterial and archaeal community structures, especially in the dominance and the variations in SAOB (i.e., *Tepidanaerobacter*) and HMs (i.e., *Methanoculleus*, *Methanobacterium*) genera, which were explained by variation in [ammonia].
(5) Bacterial diversity was negatively correlated with [lipid]. Archaeal diversity was positively correlated with [TVFA].

To summarize, high [Na^+] > 7 g Na^+/L and high [lipid] > 5 g lipid/L might result in decrease of overall bacteria and archaeal functionalities in the thermophilic AD system; in turn, this repression would reduce COD removal. Moreover, [Na^+], [lipid], and [ammonia] were among the key parameters that determine process performance and/or the underpinning microbial communities for thermophilic AD system treating high-strength and complex organic wastewater.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2016.04.097.

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