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Case Study

Feeding frequency influences process performance and microbial community composition in anaerobic digesters treating steam exploded food waste

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ABSTRACT

In anaerobic digestion, studies of feeding frequency have produced conflicting results. Hence, the effect of feeding frequency on process variables and microbial community structure was investigated by comparing a laboratory-scale digester fed steam exploded food waste 10 times daily vs. one fed an equivalent amount once daily. The Frequently Fed Digester (FFD) produced on average 20% more methane and had lower effluent concentrations of long-chain fatty acids. Greater daily fluctuations in acetate, pH and biogas production rate could explain the lower specific methane yield and β-oxidation. Feeding frequency also influenced the microbial community whereby *Tenericutes* (42%) dominated in FFD but *Firmicutes* (31%) was most abundant in the Daily Fed Digester (DFD). Feeding frequency effects are therefore postulated to occur more often in digesters fed labile feedstocks at high organic loading rates.

1. Introduction

Increasing interest in food waste anaerobic digestion (AD) has resulted in a number of laboratory-scale studies (as reviewed in Braguglia et al., 2018; Ren et al., 2018; Wang et al., 2018). However, laboratory-scale and full-scale anaerobic digesters often differ in physical characteristics such as size, stirring speed and feeding frequency. For example, laboratory continuously stirred tank reactors (CSTRs) are commonly fed once daily although full-scale AD plants are fed continuously throughout the day. Furthermore, AD systems often operate at high organic loading rates (OLRs) as a strategy to increase methane yields from food waste. Hence, when translating results from semi-continuous laboratory studies to continuously fed full-scale AD-systems, the feeding frequency's influence on digester performance is often critically overlooked.

Recently, some researchers suggested that feeding frequency influences methane yields in anaerobic digesters, but results are conflicting (Conklin et al., 2006; De Vrieze et al., 2013; Lv et al., 2014; Mulat et al., 2016; Ziels et al., 2017). Most tests omitted continuous feeding, which best represents operating practices for full-scale food waste plants. Only two studies investigated approximately continuous feeding frequencies (Mulat et al., 2016; Ziels et al., 2017), but both used fiber based substrates. Hence, research on how feeding frequency influences food waste AD is lacking.

Food waste AD is a complex process whereby multiple sub-processes influence the outcome and require active monitoring to ensure stable and efficient digester performance. Four trophic levels are defined in the anaerobic decomposition of organic matter by microbes. First, particulate material is solubilized, followed by production of organic acids. Next, acetate is generated and oxidized, and finally methane is produced. When feeding digesters once daily, researchers report that the aforementioned product concentrations fluctuate (De Vrieze et al., 2013; Lv et al., 2014; Mauky et al., 2015; Mulat et al., 2016). Moreover, changes in product concentrations influence process thermodynamics (Fukuzaki et al., 1990) and modify the environment to select new microbial taxa (Conklin et al., 2006). Therefore, it was expected that feeding frequency will affect process performance and microbial community composition in anaerobic digesters receiving food waste.

In this study, feeding once-per-day vs. 10-times-per-day was compared at a high organic loading rate (21 gCOD/L/d) during the AD of steam exploded food waste. Process performance was monitored at two time interval resolutions; that is, observations were made every 24 h immediately before feeding, herein referred to as "daily process performance", and observations were also made at multiple time-points within a 24-h period, herein referred to as "within-day process performance". Finally, microbial community diversity was characterized

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and taxa whose abundance changed due to the different feeding frequencies were identified.

2. Materials and methods

The effect of feeding frequencies was compared by using laboratory CSTRs fed steam exploded food waste. Biogas production was measured with an automated system for continuous sampling, and effluent samples were taken for analysis of process parameters and microbial community structure.

2.1. Digester configuration

Two laboratory digesters (BELACH BIOTEKNIK AB, Stockholm, Sweden), a Daily-Fed Digester (DFD) and a Frequently-Fed Digester (FFD), were operated with a maximum working volume of 6 L at 37 °C and a stirrer speed of 100 rpm. Gas volume was measured with water displacement, and gas composition was measured every hour using gas chromatography as previously described (Zamanzadeh et al., 2016). To begin, the digesters were filled with 3 L of identical inoculum from a laboratory CSTR, D0, which had been operating for 8 months digesting hygienized (pretreated at 70 °C for 1 h) food waste. The inoculum digester was fed once daily, and was operated with an organic loading rate (OLR) of 6 gCOD/L/d and a hydraulic retention time (HRT) of 60 days. Digester operation of DFD and FFD began by feeding steam exploded food waste at the target OLR of 21 gCOD/L/d, and they were operated as batch digesters until the full digester volume of 6 L was reached after 7 days. The average HRT of both digesters was 10 days in continuous mode. A high OLR and low HRT was used because these reflect the operating conditions at food waste AD plants (Nagao et al., 2012).

Digester feeding and discharge occurred daily. To investigate the effect of feeding frequencies, one digester (DFD) was fed semi-continuously, i.e. once daily, similar to the feeding pattern in most food waste AD studies, whereas the other (FFD) was fed by a continuous feeding system operated at maximum frequency. The continuous feeding system automatically delivered feed to the digester at 2.4 h intervals and was refilled daily. When the full digester volume was reached, discharge was taken from both digesters immediately before feeding or refilling of the continuous feeding system.

Digester C1 operated in parallel with the experimental reactors, and served as a control to DFD in that C1 received the same food waste, but without the steam explosion pre-treatment, and with the same OLR. The inoculum source for C1 also differed from the experimental digesters, and came from a digester operated at high organic loading rates.

2.2. Food waste characteristics

The substrate in DFD and FFD was food waste that originated from health facilities and restaurants and was obtained from Norwegian Food Recycling (Norsk Matretur AS, Lørenskog, Norway) after hygienization at 70 °C for 1 h. The food waste was steam exploded at the Biogas Laboratory at the Norwegian University of Life Sciences (Ås, Norway) at 135 °C for 20 min in order to make it more easily degradable. Both the untreated and steam exploded food waste were stored at 5 °C until it was fed to the digesters.

The characteristics of the hygienized versus the steam exploded food waste differed (Table 1). Mainly, the steam exploded food waste was more dilute due to water from the steam that is added to the substrate when it is steam exploded. The steam exploded food waste also contained less concentrated acetic acid and lactic acid compared to the hygienized food waste. The lactic acid concentration was the highest of the solubles measured and the four compounds lactic acid, acetic acid, propionic acid and glucose that together summed to 56.7 gCOD/L corresponding to 83% of the soluble COD in the feed substrate.

Table 1
Food waste (FW) characteristics (average ± standard deviation). Concentrations of acids and glucose on the basis of gCOD/L are given in parenthesis.

	Unit	Steam Exploded FW	Hygienized FW
TCOD	g/L	208.3 ± 4.7	292 ± 9.7
SCOD	g/L	68.7 ± 5.3	97.1 ± 0.9
TS	%	11.8 ± 0.2	17.6 ± 0.7
VS/TS	%	89.5	89.6 ± 0.5
TCOD/VS		1.97 ± 0.05	1.85 ± 0.03
pH		4.3 ± 0.1	3.9 ± 0.08
TAN	mg/L	283 ± 21	378 ± 23
Lactic acid	g/L (COD)	31.3 ± 0.4 (33.4 ± 0.4)	61.3 ± 0.2 (65.3 ± 0.2)
Acetic acid	g/L (COD)	2.20 ± 0.04 (2.34 ± 0.04)	3.646 ± 0.005 (3.886 ± 0.005)
Propionic acid	g/L (COD)	2.71 ± 0.05 (4.10 ± 0.08)	4.40 ± 0.01 (6.65 ± 0.02)
Glucose	g/L (COD)	0.18 ± 0.02 (0.19 ± 0.02)	0.15 ± 0.06 (0.15 ± 0.06)

2.3. Sampling and chemical analysis

Total solids (TS), volatile solids (VS), ash, pH, chemical oxygen demand (COD), total ammonia nitrogen (TAN), VFAs, and long-chain fatty acids (LCFAs) were measured in samples taken after the daily discharge on days 38, 44 and 46, and in additional samples for VFA on days 18, 19, 25 and 29.

For within-day process performance, the digesters were sampled for analysis of acetic acid at nine different time points: 1, 2, 3, 5, 7, 11, 14, 17 and 24 h after feeding. To minimize process disturbance caused by sampling, sampling was done at different time points on different days. For example, the 5th hour sample was collected on day 27 while the 7th hour sample was collected on day 28. Because the acetic acid concentration in the 24th hour sample (immediately before feeding) remained low (< 300 mg/L) in the time period for the within-day process performance sampling, the use of different sampling days for within-day acetic acid variation was considered justified. Samples from time points 1, 2 and 24 h after feeding were sampled on several days, and average values were used for further analysis.

TS, VS and ash were determined gravimetrically by drying at 105 °C and subsequent burning at 550 °C. The pH was measured using a pH electrode (Orion GD9156BNWP, Thermo Scientific, MA, USA) and pH/ISE meter (Orion Dual Star, Thermo Scientific). Chemical oxygen demand (COD) and total ammonia nitrogen (TAN) were determined using Merck Spectroquant® commercial kits (Merck KGaA, Darmstadt, Germany). Soluble COD (SCOD) was analyzed after filtering the sample through a 0.45 μm cellulose acetate syringe filter. Volatile fatty acids (VFAs) in the effluent were analyzed with a Dionex 3000 HPLC as previously described by Estevez et al. (2014). Substrate VFAs were analyzed using the same instrument equipped with an Aminex® HPX-87H column, 300 × 7.8 mm and a Micro-guard cation H+ guard column (Cat.No.: 125-0129, Bio-Rad Laboratories Inc., Hercules, CA, USA). The analysis was done isocratic with 0.4 mM aqueous H_2SO_4 with a flow of 6 mL/min at 50 °C. VFAs were detected with a UV detector at 210 nm, while glucose was detected with refractor index (RI) (RefractoMax521, ERC Inc., Saitama, Japan).

The samples taken at the daily discharge were also analyzed for long-chain fatty acids (LCFA) with gas chromatography mass spectrometry (GC–MS). The GC used was an Agilent 6890 GC equipped with a Gerstel PTV injector with solvent evaporation and the MS was an Agilent 5973 MSD in Selected Ion Monitoring (SIM) mode. A CP-SIL 8 CB column (Varian) with inner diameter 250 μm, length 50 m and 0.25 μm film thickness was used for separation of the fatty acid esters. The carrier gas was Helium with a flow of 29 cm/L. The injector temperature was initially 50 °C and the solvent was evaporated with a flow of 50 mL/min for 1.89 min. The injector temperature then increased by

270 °C/min until 320 °C was reached and held for 1.2 min. The column oven was first held at 40 °C for 1.89 min, followed by a 20 °C/min ramp to 160 °C and a hold for 2 min, followed by an 80 °C/min ramp to 270 °C, followed by a 50 °C/min ramp to 325 °C, which was held for 2 min. Prior to injection, an internal standard of 50 μg of nonadecanic acid-ester (C19:0) was added to the samples, and then the samples were extracted and methanolyzed. 0.1 g digestate was added to a 20 mL centrifuge tube, followed by 4 mL DCM/MeOH (2:1), 0.1 mL 1 M HCl and 5 mL 0.9% aqueous NaCl. The centrifuge tube was then mixed by hand for 1 min and then centrifuged at 2500 rpm for 5 min. After centrifugation, the water phase was removed and the DCM phase dried under N_2 at 60 °C. 200 μL BCl_3-methanol 14% w/w (Aldrich B1252 -100 mL) was added to the dried extract, sealed and heated at 60 °C for 20 min for the methanolysis to occur. The derivatized extract was cooled to room temperature, and 1 mL milliQ water was added with 1 mL hexan and vortexed for 20 s. The hexan phase was then transferred to a GC-vial for analysis.

2.4. DNA extraction and purification

Samples for microbial community analysis were collected by withdrawing effluent at the start of the experiment and after 38, 44 and 46 days of digester operation. The samples were collected in 15 mL centrifuge tubes and immediately stored at −20 °C until DNA extraction. Genomic DNA from each sample was extracted in triplicate using the Power-Soil DNA Isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). The entire process was carried out according to the manufacturer's protocol, with minor modification of the bead beating whereby a Precellys®24 homogenizer was used at 5000 rpm for 20 s. The extracted and purified DNA was pooled for each individual sample and measured fluorometrically on a Qubit™ fluorometer (Life Technologies, Eugene, OR, USA) using the Quant-IT™ dsDNA HSAssay Kit (Invitrogen, Carlsbad, CA, USA). On average, the obtained concentration was 45 ± 15 ng/μL DNA from each digester sample.

2.4.1. Sequence library preparation

We selected the V3-V4 hypervariable region of the 16S rRNA gene to produce amplicon libraries for Illumina MiSeq sequencing. Indexed amplicons were prepared in two sequential PCR steps whereby in the first we amplified using the 16S-specific primers from Takahashi et al. (2014), Pro341F (5′-CCTACGGGNBGCASCAG-3′) and Pro805R (5′GACTACNVGGGTATCTAATCC-3′), onto which the Illumina adapter sequence was included. In the second PCR, a unique 12 bp index was integrated into the reverse site of each sample library using NEXTflex™ 16S V4 Amplicon-Seq Kit 2.0 (Bioo Scientific Corporation, Austin, TX, USA).

The first PCR was carried out in a 50 μL reaction volume consisting of 46 ng digester DNA, 1.0 unit of Platinum® *Pfx* DNA polymerase (Invitrogen), 1 mM $MgSO_4$ and 0.3 μM of each primer. The PCR reaction began with an initial denaturation at 94 °C for 5 min, followed by 25 cycles of denaturation at 94 °C for 15 sec, annealing at 55 °C for 30 s, extension at 68 °C for 45 s, and a final elongation at 68 °C for 10 min. The first PCR products were purified using Agencourt AMPure XP beads (Agencourt Bioscience Corporation, Beverly, MA, USA) following the protocol of the NEXTflex™ kit. In the second PCR, twelve amplification cycles were used, following the NEXTflex™ protocol. The PCR products were purified with AMPure beads, and DNA concentrations were measured on Qubit. Amplicon libraries were normalized and pooled in equimolar concentrations to create the multiplexed library pool. This resulting library pool was further purified by gel extraction (E-Gel 1% agarose, Invitrogen, and MinElute Gel extraction Kit, Qiagen, Hilden, Germany). The final quality of the library was verified by agarose gel electrophoresis in addition to spectrophotometry with Nanodrop 1000 (Thermo Scientific, Waltham, MA, USA). The multiplexed library pool was sequenced on an Illumina MiSeq with paired-end 300 bp cycle run using MiSeq reagent kit V3 (Illumina Inc., San Diego, CA, USA) at the Norwegian Sequencing Center in Oslo, Norway.

2.4.2. Sequence analysis

The sequence analysis was performed with Quantitative Insights Into Microbial Ecology (QIIME) version 1.9.1 (Caporaso et al., 2010) and other stand-alone programs. The paired-end reads were merged using PEAR (Zhang et al., 2014) before quality filtering with PRINSEQ (Schmieder and Edwards, 2011) using a minimum quality score 20, average quality score 30, minimum length 300 and maximum length 500, before downstream analysis. VSEARCH (Rognes et al., 2016) was used for detection of chimeric sequences (uchime_denovo with default parameters), followed by open reference clustering with USEARCH61 (at 97% sequence identity) of non-chimera sequences and de novo picking of operational taxonomic units (OTUs). The OTUs were assigned to taxonomy with QIIME's uclust-based taxonomy assigner with the Greengenes database, and singletons were removed as they tend to contain disproportionate errors. The arithmetic mean of values from the PCR triplicates was used in the data analysis.

After quality filtering with PRINSEQ, 822 859 sequences remained for downstream analysis. 135 376 of these sequences were identified as chimeras (among which 70 070 were singletons) and removed resulting in a final 687 483 sequences used in the analysis of the microbial community. The lowest number of sequences in any sample was 20 175. A total of 179 genera and 9382 OTUs were identified.

2.4.3. Data accessibility

Sequence data are available at NCBI Sequence Read Archive under accessions SRR6484246 to SRR6484281 as part of BioProject PRJNA430711.

2.5. Data exploration and statistical analysis

Statistical testing of the process parameters was done using the two-sided paired student *t*-test at 0.05 significance.

The β-diversity was examined by calculating the Bray-Curtis, Jaccard, and UniFrac distance metrics (Lozupone and Knight, 2005) at a cutoff at 15 000 sequences per sample. ANalysis Of SIMilarity (ANOSIM) was conducted via the compare_categories.py script in QIIME with 1000 permutations.

Differential abundance analysis was performed with the LEfSe algorithm (Segata et al., 2011). In LEfSe, pairwise comparisons were done only between samples from the same day, and the threshold logarithmic LDA score was set to 4.5 to limit complexity. The alpha value was set to 0.05 for all statistical tests.

3. Results and discussion

3.1. Daily process performance

Significantly higher biogas and methane yields were observed in FFD, which produced on average 20% more methane (Table 2). The daily volumetric methane yield of this digester was 6.3 ± 0.6 L/L/d. This contrasts previous findings. For example, Mulat et al. (2016) found lower methane yields with frequent feeding, while Ziels et al. (2017) found no difference. However, their substrates and organic loading rates differed from this study. For instance, Mulat et al. (2016) used a lignocellulosic substrate and Ziels et al. (2017) co-digested cow manure and oleate. Furthermore, hydrolysis limits AD of both cow manure and lignocellulosic materials (Shrestha et al., 2017), whereas methanogenesis limits food waste AD (Braguglia et al., 2018). Since hydrolysis and methanogenesis have different optimal conditions (Kumanowska et al., 2017), the substrate could explain why improved methane yields were observed with higher feeding frequency in this study contrary to previous findings.

Lower methane yields accompanied 14 g/L higher LCFA concentration in the daily-fed digester when compared to the frequently

Table 2
Process parameters (average ± standard deviation) measured on samples from day 38, 44 and 46. Acid concentrations on the basis of mgCOD/L is given in parenthesis.

	unit	DFD	FFD	p-value
pH		7.80 ± 0.17	7.80 ± 0.09	1
TS	% of ww[1]	5.5 ± 0.6	5.4 ± 0.4	0.4
VS/TS	%	77 ± 2	76 ± 2	0.1
Ash	% of ww[1]	1.29 ± 0.08	1.29 ± 0.01	0.9
TCOD	g/kg	72 ± 10	61 ± 4	0.1
TCOD/VS		1.69 ± 0.05	1.51 ± 0.01	0.1
SCOD	g/L	13.3 ± 1	12.7 ± 0.8	0.5
PCOD	g/kg	58 ± 9	48 ± 4	0.2
TAN	g/L	1.8 ± 0.3	1.52 ± 0.03	0.2
FAN	mg/L	140 ± 44	116 ± 19	0.3
Acetic acid	mg/L (COD)	95 ± 98 (101 ± 104)	33 ± 58 (35 ± 62)	0.2
Propionic acid	mg/L (COD)	636 ± 813 (962 ± 1229)	56 ± 67 (85 ± 101)	0.3
$COD_{reduction}$	%	65 ± 6	71 ± 2	0.2
Volumetric biogas yield	L/L/d	8 ± 2	10 ± 1	< 0.05
Specific CH_4 yield	mL/$gCOD_{added}$	236 ± 49	305 ± 35	< 0.05
Specific CH_4 yield	mL/gVS_{added}	465 ± 86	601 ± 56	< 0.05
COD_{CH4}/ $COD_{reduced}$	%	109 ± 20	121 ± 14	0.1
Volumetric methane yield	L/L/d	4.9 ± 0.9	6.3 ± 0.6	< 0.05

[1] ww = wet weight.

fed digester (Table 3). This equaled a stoichiometric methane potential of 490 mL/L/d. Hence, it explained one third of the difference in methane yields and accounted for more than the difference in TCOD between the two digesters (Table 2).

Propionic acid was the dominant VFA in the digester effluent and displayed the greatest difference between the two digesters (Fig. 1A). Specifically, DFD propionate concentrations peaked at 2500 mg/L while the FFD propionate concentrations never exceeded 40 mg/L. This indicates that propionic acid oxidation was inhibited in DFD but not in FFD. Because propionate and LCFA accumulation follow LCFA inhibition (Labatut et al., 2014; Ma et al., 2015), it can be inferred that lower feeding frequency caused fluctuations in metabolites that synergized with the inhibition mechanism and lowered methane yields as well as increased concentrations of particulate COD, LCFA, and propionic acid in DFD. Further, although hydrogen partial pressure was not measured, propionate oxidation demands a lower hydrogen partial pressure compared to the other VFAs (van Lier et al., 1994), making this the first VFA to accumulate if hydrogen partial pressures rise.

3.2. Within-day process performance

Biogas production rate, biogas methane concentration, pH, temperature and acetic acid concentrations fluctuated within the 24-h feeding intervals (Table 4, Figs. 1B and 2). In DFD, the apparent gas production rate spiked immediately after feeding, before it rapidly decreased (Fig. 2). When the gas production rate increased, the biogas methane concentration dropped. It was also observed that gas production rate and methane concentration in FFD varied, but significantly less. The pH and temperature also varied significantly more in DFD (Table 4), suggesting that the buffering capacity in the digesters could withstand smaller amounts of feed with a pH of 4.3, but at higher loadings the pH dropped. Further, DFD's acetic acid concentration increased immediately after feeding, to 1500 mg/L before declining below 300 mg/L, which was the concentration immediately before feeding (Fig. 1B). Observing these fluctuations are paramount because acetate inhibits β-oxidation and propionate oxidation (Beaty and Mcinerney, 1989; Fukuzaki et al., 1990; Lier et al., 1993), and pH fluctuations impair methanogens (Sowers et al., 1984). Hence, they could explain why LCFA accumulated in the daily-fed digester and not in the frequently fed one.

Studies usually disregard within-day process parameter fluctuations because they collect samples immediately before each feeding event. Prior studies of feeding frequency corroborate the fluctuations observed here, and there are studies that demonstrate even larger fluctuations in the less frequently fed digesters (Mauky et al., 2015; Mountfort and Asher, 1978; Mulat et al., 2016). Such fluctuations result from changes in the physical, chemical and biological conditions of the reactor. For example, a low substrate pH reduces digester pH immediately after feeding and the labile substrate influx results in increased microbial acidogenesis. Furthermore, acetic acid's within-day variation indicates that acetogenesis proceeded at a higher rate than methanogenesis in DFD. Moreover, the decrease in biogas methane concentration immediately after feeding can be explained by: 1) low feed temperature causing digester heating after feeding (Table 4), releasing CO_2 more rapidly than CH_4, 2) feed acidity (Table 1) reducing digester pH (Table 3) and leading to more rapid release of CO_2 to the gas phase and inhibition of methanogens or 3) increased microbial acidogenesis resulting in pH reduction combined with CO_2 production.

3.3. Microbial community diversity and structure

DFD and FFD developed microbial communities of divergent composition, and this result is consistent for three common distance metrics (Fig. 3). Both FFD and DFD started from the same inoculum, D0, and thus began with the same microbial community composition, but then diverged during operation of the reactors under different feeding regimes as is consistent with previous results (Ferguson et al., 2016). This divergence was evident along the main ordination axis of the β-diversity plots (Fig. 3), which explains 45–50% of the variation for either the weighted UNIFRAC, Bray-Curtis, or binary Jaccard distances. The

Table 3
Concentration of long-chain fatty acids in reactor effluent.

	DFD		FFD		p-value
	(g/kg)	(gCOD/kg)	(g/kg)	(gCOD/kg)	
Lauric acid	0.026 ± 0.005	0.07 ± 0.01	0.009 ± 0.005	0.03 ± 0.02	< 0.05
Myristic acid	0.29 ± 0.04	0.8 ± 0.1	0.09 ± 0.03	0.3 ± 0.1	< 0.01
Pentadecanoic acid	0.030 ± 0.004	0.09 ± 0.01	0.012 ± 0.005	0.04 ± 0.1	< 0.005
Palmitoleic acid	0.032 ± 0.004	0.09 ± 0.01	0.017 ± 0.004	0.05 ± 0.01	< 0.05
Palmitic acid	3.3 ± 0.5	9.4 ± 1	0.8 ± 0.3	2.2 ± 0.9	< 0.01
Linoleic acid	0.30 ± 0.03	0.86 ± 0.07	0.23 ± 0.03	0.66 ± 0.09	< 0.05
Oleic acid	1.5 ± 0.2	4.4 ± 0.5	0.7 ± 0.2	1.9 ± 0.7	< 0.01
Stearic acid	1.7 ± 0.2	4.9 ± 0.5	0.7 ± 0.3	2.1 ± 1	< 0.05
Sum LCFAs	7.1 ± 0.9	21 ± 3	3 ± 1	7 ± 3	< 0.01

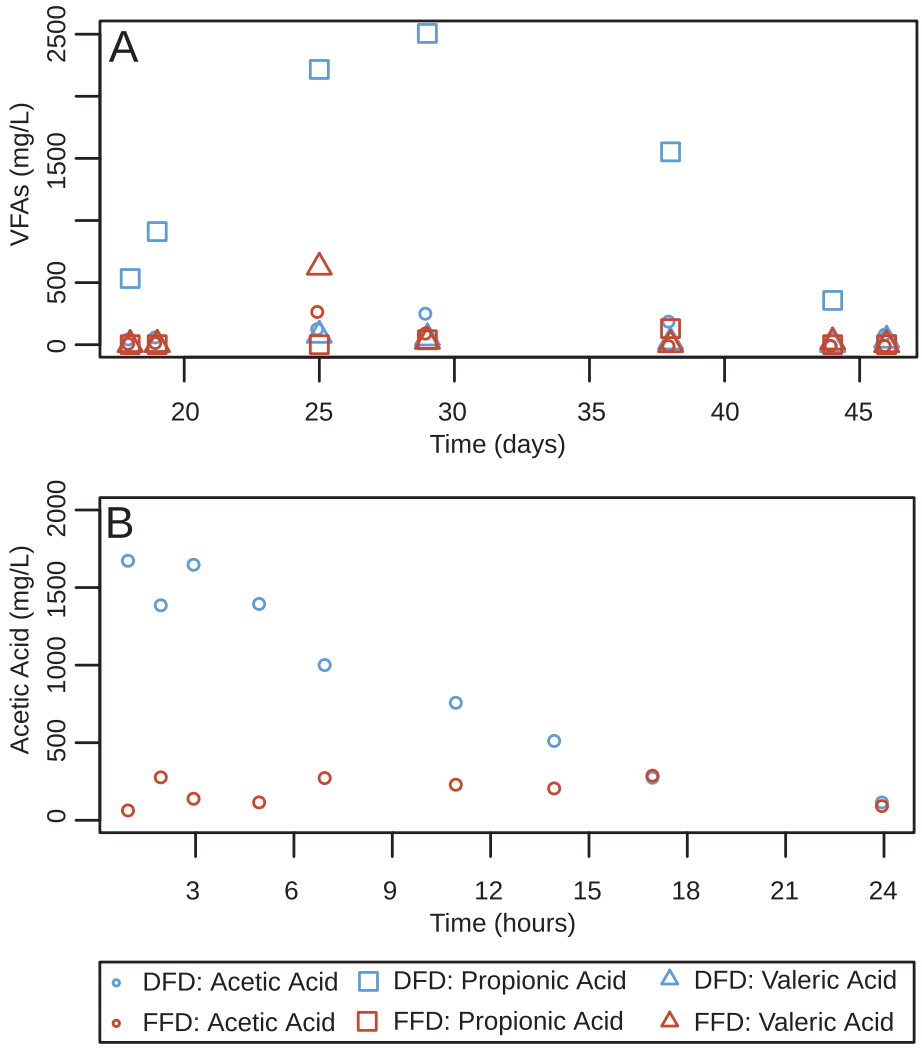

Fig. 1. VFA concentrations in digester effluent. A) Concentration of major VFAs in digester effluent immediately before feeding and refilling of automatic feeding device. B) Within-day concentration of acetic acid. Time-points are hours post-feeding of DFD.

Table 4
Mean ± standard deviation of within-day ranges for select process parameter values, which indicates greater stability in FFD.

Variation over 24 h	unit	DFD	FFD	p-value
CH_4	%-points	28 ± 2	6 ± 2	< 0.001
Gas flow	mL/min	91 ± 16	64 ± 12	< 0.001
pH		0.50 ± 0.09	0.04 ± 0.01	< 0.001
Temp	°C	2.2 ± 0.9	0.11 ± 0.04	< 0.001

microbial community in the semi-control, C1, whose feeding regime and operation most closely resembles DFD, overlapped with DFD in ordination plots for the Bray-Curtis and the binary Jaccard distances; however, for the Weighted UNIFRAC metric, reactor C1 separated to a minor extent along the second ordination axis explaining 30% of the variation (Fig. 3). Weighted UNIFRAC considers the phylogenetic tree of taxa present in two samples and weights branch lengths according to the relative abundance of those taxa present in one sample vs. the other (Lozupone et al., 2007). Two dominant taxa, *Acholeplasma* and *Candidatus* Cloacamonas, differed in abundance between DFD and C1 (Fig. 4), and differences in how weighted UNIFRAC and Bray-Curtis metrics weight dominant taxa could account for the separation of C1 and DFD in the weighted UNIFRAC ordination. Regardless, clustering of C1 and DFD demonstrates that feeding frequency was a stronger influence on microbial community composition than inoculum source or pre-treatment method (the food waste input to DFD was steam-exploded whereas that to C1 was not).

Alpha-diversity, evenness and richness were significantly different in the two digesters (Table 5), with a richer, more diverse and even community in DFD, which is consistent with the findings of De Vrieze et al. (2013). The main taxon whose abundance differed between FFD and DFD was an uncharacterized genus in the *Acholeplasmataceae* (Fig. 4). This genus had a relative abundance of 38% in FFD and consisted of a single OTU. Moreover, its abundance was a mere 0.003% in DFD (Table 6).

Tenericutes' dominance in FFD and relatively high abundance in DFD (16%) contrasts previous findings that report it as a minor phylum in anaerobic digesters (Nelson et al., 2011). The *Tenericutes* observed in this study belong to the *Acholeplasmataceae* and comprise two OTUs, one associated with the genus *Acholeplasma*, and another that is an uncharacterized lineage within the *Acholeplasmataceae*. *Acholeplasma* are predominantly associated with animals and have been isolated from mammalian fluids. The phytoplasmas, a candidatus genus of uncultured, plant-associated bacteria, group phylogenetically into the *Acholeplasmataceae*. Hence, one can reason that bacteria within the *Acholeplasmataceae* would grow on food waste comprised of plant and animal matter in a 37 °C digester. Furthermore, an isolate from a laboratory biogas reactor was shown to have a 16S sequence 92% similar to *A. morum* and to produce acetic acid, suggesting that this organism may play a role as an acetogen fermenting amino acids (Cibis et al.,

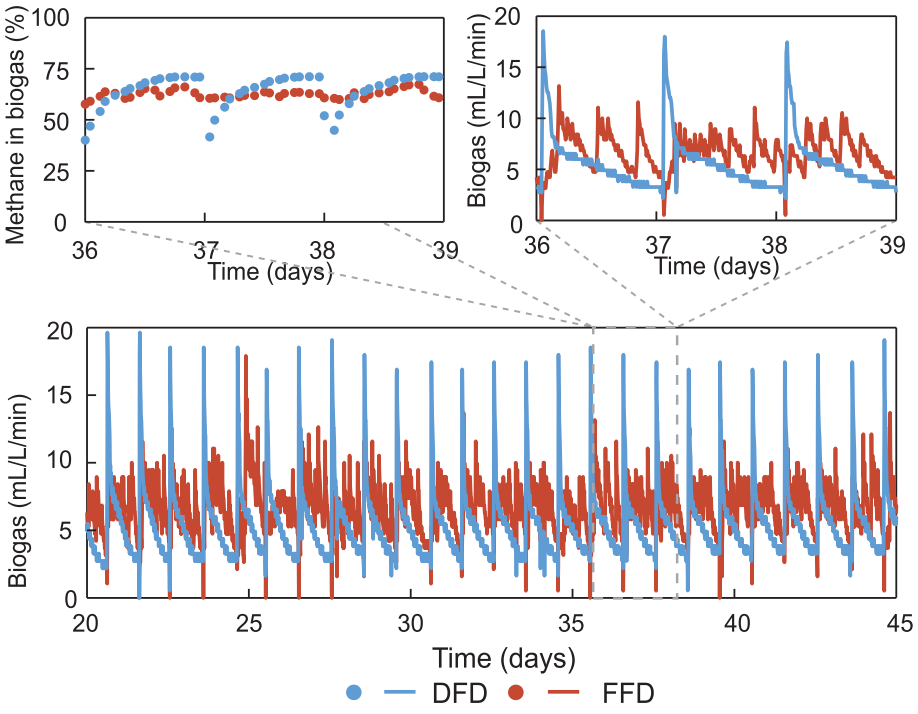

Fig. 2. Variation in biogas production rate and methane concentration in biogas.

2016). Previously, increase in *Tenericutes* abundance has been observed after increasing the OLR of food waste and chicken waste digesters (Fitzgerald et al., 2015; Guo et al., 2014; Li et al., 2016; Yi et al., 2014; Ziganshina et al., 2015). However, the functionality of these species in anaerobic digesters is largely unknown. Nevertheless, it appears from the results in this study that in addition to high OLR, high feeding frequency stimulates *Tenericutes*' abundance and selects for specific *Tenericutes* species.

In addition to *Tenericutes*, LEfSe analysis showed that taxa belonging to four other phyla were differentially abundant in DFD and FFD (Table 6). For example, *Bacteriodales* and *Clostridiales* were more abundant in DFD. For *Bacteriodales,* an unknown genus belonging to an unknown family was differentially abundant, hence the functioning of these organisms are unknown. However, the *Bacteriodales* includes proteolytic bacteria (Rivière et al., 2009), and the higher TAN concentration in DFD indicates higher proteolytic activity in this digester. Moreover, *Clostridiales* abundance has been shown to positively correlate with several parameters related to lower methane production values, including VFAs (Vrieze et al., 2015). Another differentially abundant taxa within the phylum *Firmicutes* was the RFN20 genus of the family *Erysipelotrichaceae,* which was more abundant in DFD (6.3% vs. 0.08%). *Erisypelotrichaceae* have previously been observed in anaerobic digesters, for example, *Erisypelotrichaceae* comprised 12.8% of the microbial community in rice straw fermentation liquor (Zhao et al., 2012). In other studies of microbial communities in anaerobic digesters, the genus RFN20 comprised between 0.2 and 2.1% of microbes digesting petrochemical oil refinery waste activated sludge (Wang et al., 2016) and 0.04–5.49% of those digesting marine macroalgae (Zhang et al., 2017). However, since little is known of the ecophysiology of these taxa in AD, it was not possible to infer why feeding frequency influenced their abundance.

Furthermore, *Candidatus* Cloacamonas, which fall within the candidate division WWE1, was also differentially abundant in DFD. However, W22 belonging to WWE1 was almost as abundant as *Candidatus* Cloacamonas in DFD (6.6% vs. 4.7%). Organisms within the WWE1 phylum are commonly found in AD systems and may contribute to the breakdown of cellulose and the fermentation of sugars and proteins found in food waste (Ju and Zhang, 2014; Pelletier et al., 2008). Moreover, experiments have suggested that WWE1 organisms can ferment cellulose hydrolysis intermediates (Limam et al., 2014). Metagenomic recovery of the genome sequence of *Candidatus* Cloacamonas acidaminovorans, suggests it is a hydrogen producing syntroph (Pelletier et al., 2008). On the other hand, the genus W22 (more abundant in FFD) has been observed at 25% abundance in a 37 °C

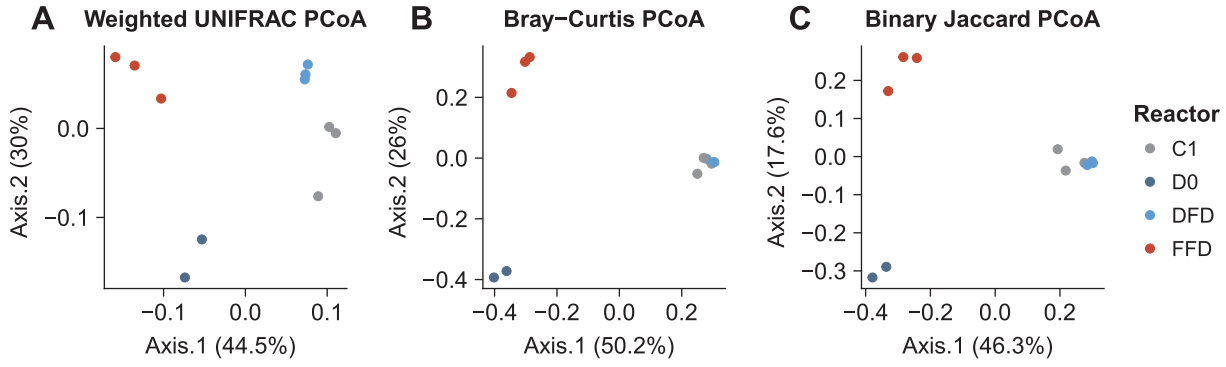

Fig. 3. PCoA of A) the weighted UNIFRAC distance metric. B) The Bray-Curtis dissimilarity. C) The Binary Jaccard index.

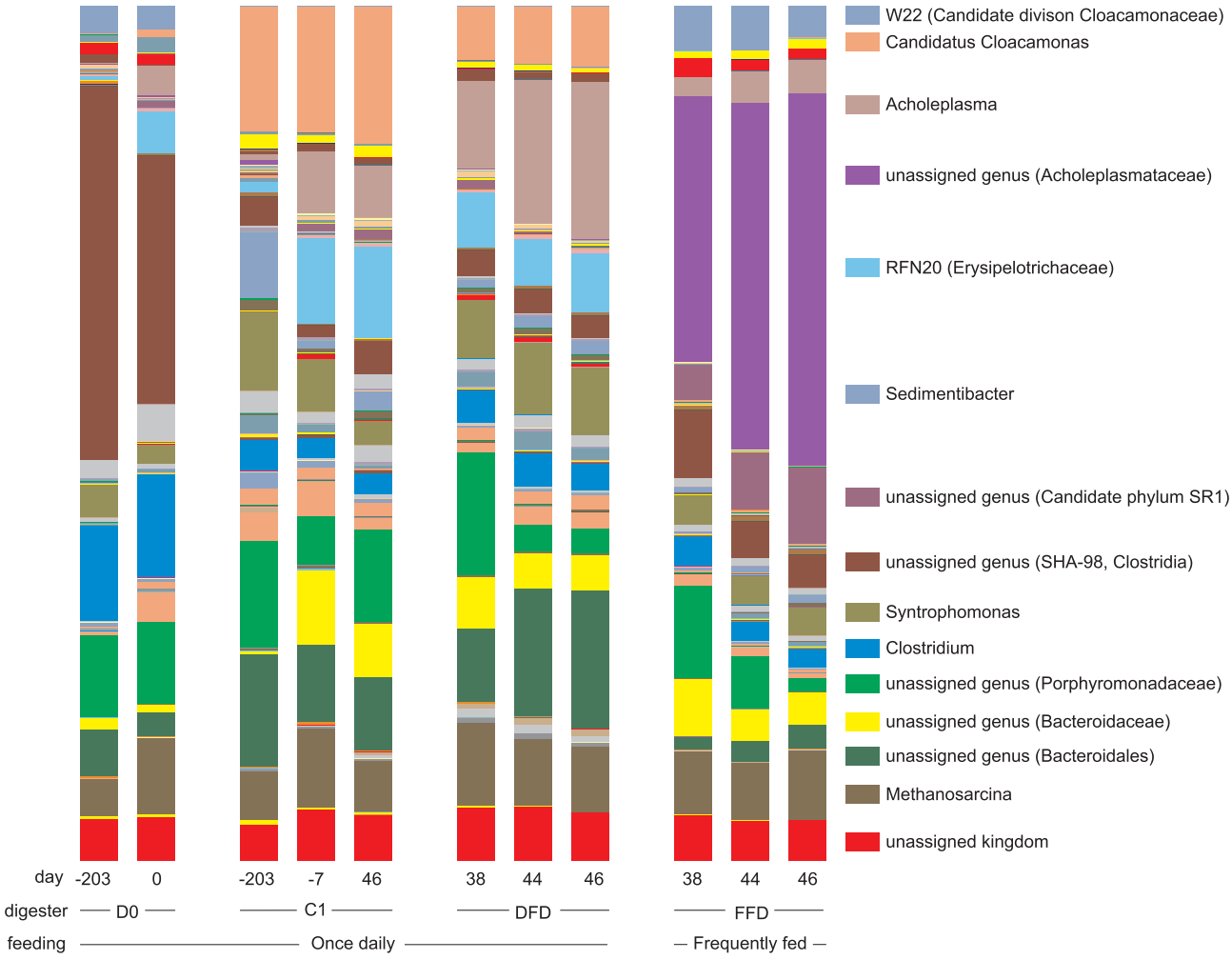

Fig. 4. Stacked bar plot of relative abundance of taxa in each sample. Each sample is the mean of three PCR replicates. Colors indicate taxa as displayed in the figure legend. Only taxa with a relative abundance above 2% are labeled. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
Mean ± standard deviation of alpha diversity indices in DFD and FFD.

	DFD	FFD	p-value
Simpson	0.94 ± 0.01	0.83 ± 0.05	< 0.05
Shannon	5.9 ± 0.2	4.5 ± 0.4	< 0.01
Gini	0.971 ± 0.005	0.985 ± 0.003	< 0.05
Richness (observed OTUs)	1009 ± 108	716 ± 69	< 0.05
Phylogenetic diversity	90 ± 6	71 ± 4	< 0.05

phenol-degrading digester (Ju and Zhang, 2014). The same study found W22-affiliated, shotgun metagenomic sequences closely related to *Candidatus* Cloacamonas acidaminovorans. Hence, although there is limited knowledge of the functioning of W22 and *Candidatus* Cloacamonas in anaerobic digesters, it suggests that the two genera share similar roles and that an unknown environmental factor favors one over the other in the digesters studied here.

Last, SR1 was differentially abundant, with higher abundance in FFD. Davis et al. (2009) hypothesized that bacteria belonging to SR1 have a sulfur-based metabolism and have a competitive advantage over other sulfur-metabolizing bacteria, such as *Proteobacteria*, when there is a constant supply of *fairly high* levels of sulfur and sulfide. High feeding frequency may have supplied sulfate and sulfide consistently as compared to feeding once per day and thus favored SR1. Although no significant difference in the relative abundance of *Proteobacteria* was found using LEfSe, the relative abundance in DFD was approximately 0.1% while in FFD it was approximately 0.01% (data not shown).

3.4. Practical implications

The findings presented here raise concerns about the extrapolation of results from laboratory-scale, daily-fed digesters to the operation of full-scale digesters that will be fed continuously. Black box modelling of anaerobic digesters based on kinetics constants derived from once-daily fed digesters will not correctly represent reaction kinetics in more frequently fed digesters, and hence they will not accurately model the methane yields of full-scale digesters. In the case of feeding once daily, the digester showed signs of overloading and poor process performance. The use of results from laboratory-scale, daily-fed digesters can therefore lead to oversized full-scale plants, unnecessarily increasing their cost.

4. Conclusion

This study demonstrates that feeding frequency can affect both process performance and microbial communities in food waste AD. By comparing within-day process performance, it was shown that a daily-fed digester exhibits greater fluctuation in variables such as pH and acetate. These variables characterize the digester environment and thus could account for differences in the microbial community composition.

Table 6
Taxa that are more abundant in DFD or FFD, LDA-score in parenthesis. The mean relative abundance ± standard deviation for differentially abundant taxa in each digester is presented in separate columns to the right of the taxa name.

Taxa more abundant in DFD	Abundance in DFD (%)	Abundance in FFD (%)	Taxa more abundant in FFD	Abundance in DFD (%)	Abundance in FFD (%)
***Firmicutes* (4.79)**	31 ± 1	17 ± 3	***Tenericutes* (5.14)**	16 ± 4	42 ± 7
Clostridia (4.61)	24 ± 2	16 ± 3	*Mollicutes* (5.13)	15 ± 4	42 ± 7
Clostridiales (4.75)	20 ± 2	9.5 ± 0.4	*Acholeplasmatales* (5.14)	15 ± 4	42 ± 7
			Acholeplasmataceae (5.11)	15 ± 4	42 ± 7
Erysipelotrichichales (4.52)	6.3 ± 0.7	0.08 ± 0.03	unassigned genus (5.26)	0.003 ± 0.002	38 ± 7
RFN20 (4.50)	6.3 ± 0.7	0.08 ± 0.03			
			SR1		
***Bacteroidetes* (4.80)**	26 ± 3	14 ± 6	unassigned family (4.51)	0.4 ± 0.5	7 ± 2
Bacteroidia (4.78)	26 ± 3	14 ± 6			
Bacteroidales (4.80)	26 ± 3	14 ± 6			
unassigned family (4.78)	13 ± 4	2.3 ± 0.7			
unassigned genus (4.76)	13 ± 4	2.3 ± 0.7			
WWE1					
Candidatus Cloacamonas (4.53)	6.6 ± 0.4	0.03 ± 0.8			
Tenericutes					
Acholeplasma (4.79)	15.2 ± 4	3.3 ± 0.9			

Additionally, once-per-day feeding led to inhibition of β-oxidation and propionic acid degradation. This in turn results in reduced overall methane yields. Finally, high OLR in combination with a labile feedstock was postulated to result in process instability under once-per-day feeding.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.biortech.2018.08.096.

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