



# Microbial community adaptation influences long-chain fatty acid conversion during anaerobic codigestion of fats, oils, and grease with municipal sludge

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## ABSTRACT

Codigesting fats, oils, and greases with municipal wastewater sludge can greatly improve biomethane recovery at wastewater treatment facilities. Process loading rates of fats, oils, and greases have been previously tested with little knowledge of the digester microbial community structure, and high transient fat loadings have led to long chain fatty acid (LCFA) accumulation and digester upsets. This study utilized recently-developed quantitative PCR assays for syntrophic LCFA-degrading bacteria along with 16S amplicon sequencing to relate changes in microbial community structure to LCFA accumulation during transient loading increases to an anaerobic codigester receiving waste restaurant oil and municipal wastewater sludge. The 16S rRNA gene concentration of the syntrophic  $\beta$ -oxidizing genus *Syntrophomonas* increased to ~15% of the *Bacteria* community in the codigester, but stayed below 3% in the control digester that was fed only wastewater sludge. *Methanosaeta* and *Methanospirillum* were the dominant methanogenic genera enriched in the codigester, and together comprised over 80% of the *Archaea* community by the end of the experimental period. Constrained ordination showed that changes in the codigester *Bacteria* and *Archaea* community structures were related to measures of digester performance. Notably, the effluent LCFA concentration in the codigester was positively correlated to the specific loading rate of waste oil normalized to the *Syntrophomonas* 16S rRNA concentration. Specific loading rates of  $0\text{--}1.5 \times 10^{-12}$  g VS oil/16S gene copies-day resulted in LCFA concentrations below 30 mg/g TS, whereas LCFA accumulated up to 104 mg/g TS at higher transient loading rates. Based on the community-dependent loading limitations found, enhanced biomethane production from high loadings of fats, oils and greases can be achieved by promoting a higher biomass of slow-growing syntrophic consortia, such as with longer digester solids retention times. This work also demonstrates the potential for controlling the loading rate of fats, oils, and greases based on the analysis of the codigester community structure, such as with quantitative PCR measurements of syntrophic LCFA-degrading bacteria abundance.

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## 1. Introduction

Anaerobic digestion is commonly used at municipal wastewater treatment plants (WWTPs) to process waste sludge and recover

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renewable energy as biomethane. Fats, oils, and greases (FOG) are desirable substrates for enhancing biomethane recovery through codigestion because they have a methane yield potential per g VS that is 250%–350% greater than the wastewater sludge typically fed to municipal digesters (Davidsson et al., 2008; Girault et al., 2012; Luostarinen et al., 2009). Reported increases in digester methane production from 140% to 620% during FOG codigestion with wastewater sludge (Wan et al., 2011; Wang et al., 2013) have demonstrated the potential to significantly improve economics and

reduce energy footprints of municipal WWTPs with FOG codigestion.

When fats and oils are added to the anaerobic digestion process, they are rapidly hydrolyzed into their major constituents of glycerol and long-chain fatty acids (LCFA) (Hanaki et al., 1981). After lipid hydrolysis, most of the energy content resides in LCFA, which can comprise over 90% of the chemical oxygen demand of the original lipid molecule (Sousa et al., 2009). The formation of methane from LCFA involves a syntrophic partnership of proton-reducing acetogenic bacteria, which utilize the  $\beta$ -oxidation pathway to convert LCFA into acetate and formate/hydrogen, along with acetoclastic and hydrogenotrophic methanogenic archaea (Schink, 1997; Sousa et al., 2009; Weng and Jeris, 1976). All of the isolated bacterial species known to  $\beta$ -oxidize LCFA syntrophically belong to two families, *Syntrophomonadaceae* and *Syntrophaceae* (Hatamoto et al., 2007; Jackson et al., 1999; McInerney, 1992; Sousa et al., 2007b; Wu et al., 2007). Generally, the conversion of LCFA into methane is considered the rate-limiting step for lipid degradation in anaerobic digesters (Angelidaki and Ahring, 1992; Cirne et al., 2007; Hanaki et al., 1981).

Process failures observed at elevated FOG loading rates have impeded the ability to fully exploit higher biomethane production during FOG codigestion with municipal wastewater sludge (Davidsson et al., 2008; Girault et al., 2012; Luostarinen et al., 2009; Noutsopoulos et al., 2013; Wan et al., 2011; Wang et al., 2013). Specifically, the LCFA released during lipid hydrolysis can inhibit anaerobic microorganisms at high concentrations (Angelidaki and Ahring, 1992; Koster and Cramer, 1987; Lalman and Bagley, 2000; Rinzema et al., 1994), thereby limiting their bioconversion into methane. Causes of inhibition have been attributed to LCFA adsorption onto cell surfaces, which can lead to direct toxicity (Hanaki et al., 1981; Rinzema et al., 1994) and/or substrate transport limitations (Pereira et al., 2005). While acetoclastic methanogens are believed to be the most sensitive group to LCFA toxicity (Koster and Cramer, 1987; Lalman and Bagley, 2000, 2001; Rinzema et al., 1994), the inhibition of hydrogenotrophic methanogens and syntrophic bacteria by LCFA has also been suggested (Hanaki et al., 1981; Lalman and Bagley, 2002; Pereira et al., 2005; Roy et al., 1985). Reported threshold values for FOG loading that led to decreased methane yields during codigestion with municipal wastewater sludge ranged from  $-0.4$  to  $2.1$  g VS/L-d (Girault et al., 2012; Luostarinen et al., 2009; Noutsopoulos et al., 2013; Silvestre et al., 2011; Wan et al., 2011; Wang et al., 2013). However, these empirical FOG loading thresholds do not account for digester microbial populations and their role in LCFA conversion, and are thus of limited use for predicting the response of a digester following transient increases in FOG loading. An improved understanding of the relationship between the digester biomass composition and LCFA accumulation is needed to develop strategies for stable codigester operation with increased FOG loadings and enhanced methane recovery.

The importance of biomass adaptation for stable FOG digestion has been indicated by previous studies. Silvestre et al. (2011) observed that stepwise increases in FOG loading led to the development of biomass with higher LCFA  $\beta$ -oxidation and methanogenic activities during codigestion with municipal sludge. Alves et al. (2001) found that both the tolerance to LCFA toxicity as well as the LCFA-biodegradation activity increased with long-term exposure to lipids in an anaerobic fixed-bed bioreactor. Similarly, long-term acclimation was identified as a key factor influencing the resilience to LCFA toxicity in a series of digester sludges exposed to skim milk and oleate based wastewaters (Silva et al., 2014). While these results collectively indicated that biomass adaptation could affect the efficiency of FOG conversion, the microbial community structures of these digester sludges were not assessed. The

dynamics of LCFA-degrading microbial communities have been previously studied using highly enriched systems with LCFA as the primary carbon source (Salvador et al., 2013; Shigematsu et al., 2006; Sousa et al., 2007a; Ziels et al., 2015). However, the relationship between the LCFA conversion efficiency and microbial community structure during FOG codigestion with municipal wastewater sludge has received little attention. The changes in LCFA-degrading community structure caused by transient increases in FOG loading therefore need further study to enable better predictions of acceptable FOG loadings during codigestion.

The main objective of this study was to elucidate the relationship between digester biomass composition and LCFA conversion rates and removal efficiency during FOG codigestion with municipal wastewater treatment sludge. Quantitative PCR targeting LCFA-degrading syntrophic bacteria and methanogenic archaea was conducted along with Illumina 16S rRNA gene amplicon sequencing of *Bacteria* and *Archaea* communities to monitor microbial population structure changes in a FOG codigester and control digester treating municipal wastewater solids. The specific goals were to: (1) determine the effects of FOG addition on the digester microbial community structure, and (2) examine relationships between microbial community structure and LCFA removal following transient variations in FOG loading.

## 2. Materials and methods

### 2.1. Digester operation

Two semi-continuous complete-mix anaerobic digesters (4 L working volume) were operated at  $37^\circ\text{C}$  with a 20-day hydraulic retention time (HRT) for 198 days. The digesters were mixed with axial flow impellers at 275–325 rpm. They were started with anaerobic digester sludge collected from Henriksdal WWTP in Stockholm, Sweden, and were fed with a mixture of waste primary sludge (WPS) and waste activated sludge (WAS) collected from the same plant throughout the experiment. The WAS+WPS was collected biweekly and stored at  $4^\circ\text{C}$ . The digesters were manually fed once daily by withdrawing the volume of reactor liquid corresponding to the volume of the feed prior to addition. The average feed WPS+WAS volatile solids (VS) concentration was  $28 \pm 2$  g VS/L and the feed sludge VS loading rate (VSLR) for both digesters averaged  $1.4 \pm 0.1$  g VS/L-day over the course of the experiment. After an initial startup period of 53 days of only feeding WPS+WAS, waste cooking oil (hereby referred to as FOG) from a nearby restaurant was added to one of the digesters for codigestion. The start of FOG codigestion was defined as day 1 of the experimental period (Table 1). The FOG VS content was  $\sim 99\%$ , and its addition to the codigester was increased in a stepwise manner over time to  $1.5$  g VS/L-d (52% of the total feed VS) by day 94 (Table 1).

Digester performance was monitored with daily biogas production, methane content, pH, effluent volatile fatty acids (VFA), effluent LCFA, total solids (TS), and VS. Biogas production was measured with tipping bucket displacement gas meters (Milli-Gascounters, Ritter, Germany). The biogas composition was analyzed weekly for methane, carbon dioxide, oxygen, and hydrogen sulfide using a portable gas analyzer (Biogas Check, Geotech, UK). All measured gas volumes are reported at standard temperature and pressure (1 atm pressure and  $0^\circ\text{C}$ ). The TS and VS contents of the sludge were determined according to Swedish Standard Method SS028311. The pH of the digesters was measured using an Inolab pH 7310 meter (InoLab, Wissenschaftlich-Technische Werkstätten, Germany) immediately after withdrawing sludge from the reactors. VFA (acetate, propionate, butyrate, iso-butyrate, valerate, iso-valerate, capronate and iso-capronate) were analyzed by GC-FID (HP 6890, Hewlett Packard),

**Table 1**

Total influent volatile solids loading rate (VSLR), FOG VSLR, and the percent of FOG VS in the feed versus time for the FOG codigester. Values in parentheses indicate one standard deviation.

Days	Total VSLR (g VS/L-day)	FOG VSLR (g VS/L-day)	% FOG in feed (VS-basis)
–53–0	1.4 (0.2)	0	0
1–9	1.9 (0.1)	0.25	13
10–37	1.8 (0.1)	0.5	27
38–72	2.1 (0.1)	0.75	36
73–79	2.4 (0.0)	1.0	41
80–93	2.6 (0.1)	1.25	47
94–145	2.9 (0.1)	1.5	52

and were separated with a BP21 (FFAP) column (30 m × 0.32 mm × 0.25 µm, SGE Analytical Science) as described previously (Jonsson and Borén, 2002). LCFA were measured according Ziels et al. (2015) with minor modifications. Briefly, 10 mL of digester sludge was centrifuged at 10,000 × g for 10 min, and immediately decanted. Approximately 0.2 g of pelleted sludge was transferred to a pre-weighed glass extraction vial, which was then dried at 80 °C for 15 h, allowed to cool, and reweighed. 1 mL of water was then added to the sample, and the subsequent LCFA extraction and quantification was conducted as described by Ziels et al. (2015). The average recovery of palmitate (C<sub>16:0</sub>), stearate (C<sub>18:0</sub>), and oleate (C<sub>18:1</sub>) spiked to digester sludge samples was 100%, 92%, and 83%, respectively.

## 2.2. Batch methanogenic activity assays

Batch methanogenic activity tests were conducted with biomass from both digesters in order to determine the maximum conversion kinetics of acetate and oleate on days 0, 65, and 135, according to the protocol by Karlsson et al. (2012). Briefly, 15 mL digester sludge aliquots (0.17–0.19 g VS) were directly transferred into 160 mL glass serum bottles containing 130 mL of anaerobic basal medium prepared according to Karlsson et al. (2012), and were then sealed with butyl rubber septa. The bottles were flushed with a mixture of O<sub>2</sub>-free 80:20 N<sub>2</sub>:CO<sub>2</sub> to ensure anaerobic conditions. Substrate (either sodium acetate or oleic acid) was then added to an initial concentration of 5 mM. Control vials without any added substrate were also included in each batch test. All treatments were run in triplicate, and were maintained at 37 °C for approximately 260 h without mixing. The gas production was measured based on the pressure increase in the bottles using a handheld pressure transducer (Testo 3123, Testo, Sparta, New Jersey). The methane content of the headspace was measured in triplicate by GC-FID (Hewlett Packard, 5880 A) at each gas pressure sampling point. The methane production in the control vials was subtracted from the substrate-amended vials to determine the methane production attributed to substrate conversion.

A modified Gompertz equation was used to describe the inoculum-corrected methane production curves in the batch kinetic assays, as proposed by Palatsi et al. (2012) and Silva et al. (2014):

$$M(t) = P \cdot \exp \left[ - \exp \left[ \frac{q_{\max} \cdot e}{P} (\lambda - t) + 1 \right] \right]$$

where  $M$  is the accumulated methane (mL CH<sub>4</sub>/L) at time =  $t$  (days),  $P$  is the maximum cumulative methane production (mL CH<sub>4</sub>/L),  $q_{\max}$  is the maximum methane production rate (mL CH<sub>4</sub>/L-day),  $e$  is 2.7182818, and  $\lambda$  is the lag-phase time (days). All methane generation terms are in reference to standard temperature and pressure. The model fitting and parameter standard error estimation was conducted with nonlinear regression in R version 3.0.2.

## 2.3. Analysis of microbial community structure

### 2.3.1. DNA extraction and quantification

Digester biomass samples were collected for DNA analysis on days 0, 37, 51, 64, 114 and 138 from the FOG codigester and on days 0, 64, 86 and 138 from the control. The samples were prepared by transferring 10 mL of digester sludge aliquots directly into sterile 15 mL tubes, immediately centrifuging at 10,000 × g for 10 min at 4 °C, carefully decanting the supernatant, and storing the remaining pellet at –20 °C. DNA was isolated from approximately 0.2 g of wet solids using the PowerSoil® RNA/DNA Isolation Kit (MO BIO, Inc, Carlsbad, California) according to the manufacturer's instructions. The concentration of extracted DNA was immediately measured with the Quant-IT dsDNA High Sensitivity Assay Kit with a Qubit 2.0 fluorometer (Invitrogen, Carlsbad, California). Triplicate DNA extractions were analyzed at each sampling date to estimate variance in population abundances and ensure that observed changes in community structure were not due to technical error. Extracted DNA was stored in nuclease-free water at –20 °C.

### 2.3.2. Quantitative PCR (qPCR)

Reaction contents and thermocycling conditions for qPCR analysis were as described by Ziels et al. (2015). qPCR analysis on syntrophic LCFA β-oxidizing bacteria was conducted targeting 16S rRNA genes of the genera *Syntrophomonas* and *Syntrophus* using the primers and probes developed by Ziels et al. (2015), as these primers/probes were the only established TaqMan qPCR assays targeting these syntrophic β-oxidizing bacterial groups at this time. Additionally, qPCR analysis was conducted targeting the domain *Bacteria*, the methanogenic archaeal orders of *Methanomicrobiales*, *Methanobacteriales*, *Methanococcales*, and the methanogenic archaeal families of *Methanosarcinaceae* and *Methanosetaeaceae* using previously developed primer/probe sets (Yu et al., 2005). Further details on the qPCR primer/probe sets used in this study are provided in Supplementary Table 1. All samples were analyzed in duplicate. No-template controls (NTCs) were included with each qPCR run. Extracted DNA from the digester biomass samples was diluted 1:10 in nuclease-free water to prevent PCR inhibition. Calibration standards for the qPCR assays were prepared as described by Ziels et al. (2015), and were included in duplicate in each qPCR run for all target groups. The strains from which 16S rRNA gene sequences were used to construct the calibration standards are given in Supplementary Table 1, along with the average slopes and intercepts of the qPCR calibration curves.

### 2.3.3. High-throughput amplicon sequencing of 16S rRNA genes and statistics

Selected DNA extracts were processed for high-throughput amplicon sequencing on the Illumina MiSeq platform, using the protocol described by Ziels et al. (2015). Briefly, two-step nested PCR was conducted prior to sequencing to enhance sensitivity. *Bacteria* and *Archaea* sequence libraries were generated separately

for each sample by using different primer sets in the initial PCR. The primers used in the initial PCR for the *Bacteria* sequence library generation were a modified 341F (5'-CCTAYGGGRBGCASCAG-3') and a modified 806R (5'-GGACTACNNGGTATCTAAT-3') (Sundberg et al., 2013), and for the *Archaea* sequence library the primers Arch-349F (5'-GYGCASCAGKCGMGA AW-3') and Arch-915R (5'-GTGCTCCCCGCCAATTCCT-3') were used. PCR amplification, purification, and library construction were conducted in accordance to Ziels et al. (2015). Libraries were sequenced with an Illumina MiSeq at the University of Copenhagen Molecular Microbial Ecology Lab. Sequences were submitted to the NCBI Sequence Read Archive as BioProject PRJNA301747.

Paired-end sequences were joined using the fastq-join method (Aronesty, 2013) with a minimum overlap of 100 bp and a zero percent difference allowed in the overlap region. The UPARSE method was used to trim sequences to 298 bp and filter sequences based on a maximum estimated error of 0.05 using USEARCH61 (Edgar, 2010; Edgar et al., 2011). Sequence chimeras were identified against the RDP Gold reference database (v.9) included in the UCHIME distribution (Edgar et al., 2011) within the QIIME pipeline v.1.8.0 (Caporaso et al., 2010). Filtered sequences were clustered into operational taxonomic units (OTUs) based on 3% sequence divergence using USEARCH61 (Edgar, 2010). Representative sequences of each OTU were identified based on the cluster seed, and were classified using a naïve Bayesian algorithm with the Ribosomal Database Project (RDP) Classifier program version 2.2 (Wang et al., 2007). For the *Bacteria* sequence libraries, *Archaea* sequences were removed prior to further analysis, and similarly *Bacteria* sequences were filtered and removed from *Archaea* sequence libraries. A total of 1,442,363 quality-filtered reads were obtained from the Illumina sequencing of *Bacteria* 16S rRNA gene amplicons ( $n = 21$  samples), and 1,312,584 quality-filtered reads were obtained from sequencing of *Archaea* 16S rRNA gene amplicons ( $n = 21$  samples).

Multivariate statistical analysis and diversity metrics were calculated using the vegan library version 2.0.10 (Oksanen et al., 2007) in R version 3.0.2. Canonical correspondence analysis (CCA) was performed using sequence counts (scaled to equal reads per sample) for OTUs that were present above 1% of the population in at least one sample. The environmental parameters that maximized the model significance were identified using forward-backward selection based on P-values. Bray-Curtis distance matrices were calculated after rarefying samples to an equal number of reads. Diversity calculations were performed on entire sample sequence libraries (rarefied to equal reads) excluding singleton OTUs. Richness was calculated as the number of OTUs after rarefying to the smallest number of reads per sample. Hill numbers were used to measure diversity based on effective species numbers ( ${}^qD$ ) at varying orders ( $q = 0, 1, 2$ ) (Hill, 1973; Jost, 2006). Diversity of order 0 (i.e.  ${}^0D$ ) is equal to species richness, while  ${}^1D$  is equal to  $\exp(\text{Shannon entropy})$ , and  ${}^2D$  is equivalent to  $1/(\text{Simpson concentration})$  (Hill, 1973; Jost, 2006). Diversity of order  ${}^1D$  weights species frequencies equally, while diversity of order  ${}^2D$  disproportionately emphasizes dominant species (Jost, 2006; Vuono et al., 2015).

### 3. Results

#### 3.1. FOG codigestion led to enhanced methane production and higher LCFA conversion kinetics

Two parallel anaerobic digesters were operated to compare differences in performance between FOG codigestion with WPS+WAS and the digestion of only WPS+WAS for 145 days (Table 1). By day 94 after FOG codigestion was commenced, the FOG

VSLR reached 1.5 g VS/L-d (52% of the total VSLR w/w), corresponding to an increase in the total VSLR to the codigester of 110% relative to the control (2.9 versus 1.4 g/L-d) (Fig. 1A). Accordingly, the daily methane production significantly increased by 170% in the FOG codigester (5200 mL  $\text{CH}_4/\text{d} \pm 560$ ) relative to the control (1900 mL  $\text{CH}_4/\text{d} \pm 200$ ) at the highest VSLR ( $p < 1e-3$ , unpaired  $t$ -test; Fig. 1B). Due to the high VS concentration of the added FOG, the HRT of the codigester was only decreased by 3% to 19.4 days at the highest VSLR. Thus, codigesting FOG at a VSLR of 1.5 g FOG VS/L-d resulted in about 170% higher daily methane production with no significant process penalty in the digester HRT.

The specific methane yield on a VS-basis also increased by 31% in the FOG codigester (420 mL  $\text{CH}_4/\text{g VS}_{\text{fed}} \pm 40$ ) relative to the control (320 mL  $\text{CH}_4/\text{g VS}_{\text{fed}} \pm 70$ ) at the highest VSLR ( $p < 1e-3$ ), indicating that the added FOG was more biodegradable than the WPS+WAS. The higher degree of biodegradability of the added FOG was further supported by the significantly higher VS reduction in the FOG codigester over the control throughout the experimental period, achieving  $63\% \pm 4$  versus  $51\% \pm 5$  ( $p < 1e-3$ , unpaired  $t$ -test). Assuming that the VS reduction of the feed WPS+WAS in the FOG codigester was similar to the control digester (i.e. 51%), the VS reduction of the feed FOG in the codigester was estimated to be 74% during the highest FOG VSLR. Consequently, the specific LCFA concentration in the codigester effluent increased to a maximum of  $104 \pm 11$  mg LCFA/g TS on day 114 of FOG addition, in comparison to  $4 \pm 0.5$  mg LCFA/g TS in the control (Fig. 1D). Thus, the 74% reduction in FOG VS at the highest loading rate resulted in LCFA accumulation within the codigester.

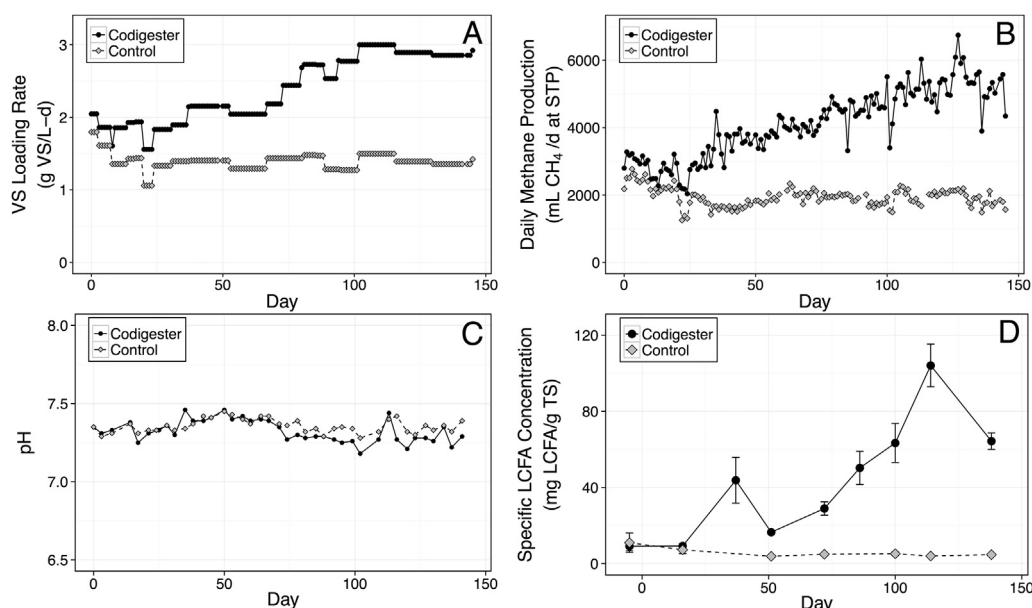
The pH was slightly reduced (Fig. 1C) after day 50 of FOG codigestion when the LCFA concentration increased (Fig. 1D). The VFA concentration never exceeded 100 mg/L in both digesters (data not shown), indicating that the codigester had sufficient VFA consumption capacity at the prevailing LCFA conversion rates. LCFA were therefore the major intermediate metabolites that accumulated during FOG degradation (Fig. 1D). The LCFA in the codigester effluent were comprised of 38% palmitic, 16% stearic, and 46% oleic acids on average (Supplementary Fig. 1).

The maximum methane production rates ( $q_{\text{max}}$ ) of both digester sludges were determined in batch assays (on days 0, 65, and 135) fed with either acetate or oleate to compare the kinetic capacities of the digester populations over time. The  $q_{\text{max}}$  of oleate-fed batch kinetic assays were significantly increased for the FOG codigester sludge from  $45 \pm 5$  on day zero to  $110 \pm 5$  mL  $\text{CH}_4/\text{L-d}$  by day 65 (Fig. 2A), corresponding to an increase of 2.4-times relative to the control. Yet, the oleate  $q_{\text{max}}$  for the FOG codigester on day 135 was similar to day 65 ( $p > 0.1$ ; Fig. 2A). Similar  $q_{\text{max}}$  values in the acetate-fed batch assays were also observed between the codigester and control for all batch tests (Fig. 2B); the acetate  $q_{\text{max}}$  increased between days zero and 65 and then stabilized for both systems. These results indicate that FOG codigestion resulted in elevated oleate degradation kinetics, and that the codigester community reached its maximum rate of LCFA conversion by day 65.

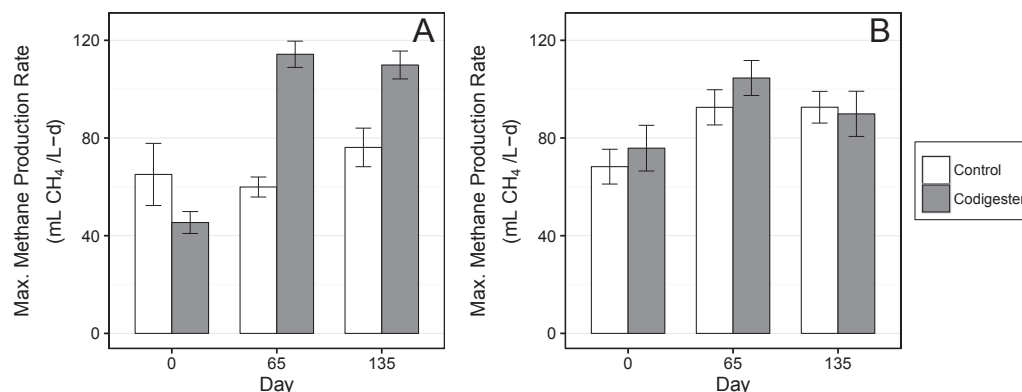
#### 3.2. FOG codigestion selected for syntrophic $\beta$ -oxidizing bacteria and specific methanogenic archaea partners

Analysis of the *Bacteria* community diversity showed that Hill numbers of orders  ${}^1D$ , and  ${}^2D$  substantially increased in the FOG codigester by the end of the experimental period (Supplementary Fig. 2). The taxonomic richness ( ${}^0D$ ) of the *Bacteria* community in the FOG codigester did not change by day 138 ( $p > 0.1$ , paired  $t$ -test), while diversities of order  ${}^1D$  and  ${}^2D$  in the FOG codigester increased by 3.0-times and 4.6-times relative to the control, respectively ( $p < 0.001$ , unpaired  $t$ -test; Supplementary Fig. 2). While it is apparent that the diversity of *Bacteria* was substantially elevated at





**Fig. 1.** Comparison of the FOG codigester and the control digester performance over experimental period, based on (A) daily volatile solids loading rate, (B) daily methane production, (C) digester pH, and (D) effluent LCFA concentrations. The LCFA concentrations shown in (D) were calculated as the sum of palmitate, stearate, and oleate in the digester effluent solids (error bars represent one standard deviation with  $n = 4$ ).

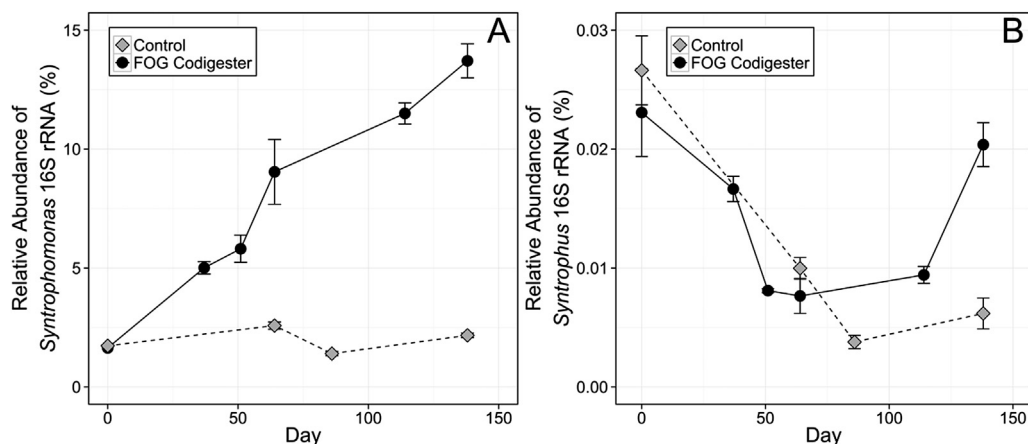


**Fig. 2.** Maximum methane production rate in batch assays fed with (A) oleate and (B) acetate, as determined by fitting the Gompertz growth model to observed methane production in triplicate biological replicates. Error bars represent the standard error based on the non-linear regression model fitting ( $n = 3$ ).

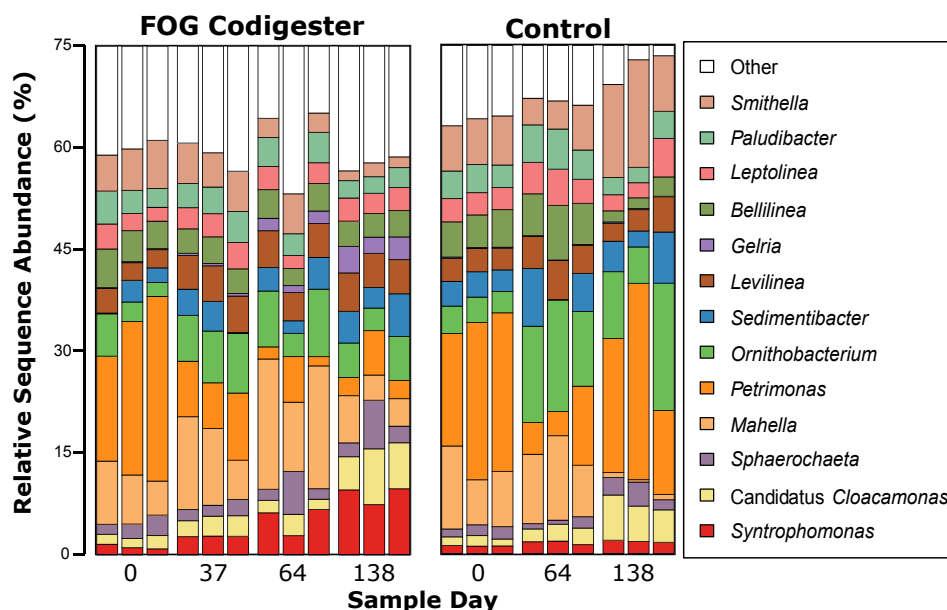
the orders  $^1D$  and  $^2D$  in the FOG codigester versus the control, the larger relative increase in diversity of order  $^2D$  indicates that the addition of FOG had a larger influence on the diversity of dominant bacterial species.

The increase in diversity of *Bacteria* in the FOG codigester occurred concomitantly with the significant growth of  $\beta$ -oxidizing *Syntrophomonas* within the *Bacteria* community. *Syntrophomonas* relative abundance in the *Bacteria* community increased to  $14\% \pm 0.7$  in the FOG codigester over the experimental period but remained below 3% relative abundance in the control digester, as measured by qPCR (Fig. 3A). In contrast, the other known LCFA  $\beta$ -oxidizing genus, *Syntrophus*, was measured by qPCR at less than 0.03% of the *Bacteria* community in both digesters (Fig. 3B). The *Syntrophus* abundance significantly decreased from day zero to 86 in both digesters ( $p < 1e-3$ ), but returned to its initial level in the FOG codigester by day 138. The enrichment of *Syntrophomonas* within the *Bacteria* community was corroborated by 16S rRNA gene amplicon sequencing, which showed that *Syntrophomonas* became the dominant genus in the *Bacteria* sequence library by day 138 (Fig. 4). *Syntrophomonas* significantly increased in the FOG

codigester *Bacteria* sequence library from  $1.2\% \pm 0.3$  initially to  $9.0\% \pm 1.0$  by day 138 ( $p = 0.009$ , paired  $t$ -test), but stayed below 2% in the control (Fig. 4). *Gelria* was another genus that also increased in the FOG codigester *Bacteria* sequence library relative to the control (Fig. 4). On the other hand, *Petrimonas* was initially the dominant bacterial genus in both digesters with a relative sequence abundance of  $\sim 20\%$ , but had a significantly lower relative sequence abundance in the FOG codigester at  $4.0\% \pm 2.2$  by day 138 ( $p = 0.03$ , paired  $t$ -test; Fig. 4). *Smithella* also significantly decreased from  $6.2\% \pm 0.9$  to  $1.7\% \pm 0.3$  relative sequence abundance in the FOG codigester *Bacteria* community ( $p = 0.01$ , paired  $t$ -test), but increased from  $6.7\% \pm 0.1$  to  $12\% \pm 4.5$  in the control (Fig. 4). Bray-Curtis dissimilarity values relative to the initial *Bacteria* community structure (day zero) significantly increased between days 37 ( $0.44 \pm 0.04$ ) to 138 ( $0.61 \pm 0.03$ ) in the FOG codigester ( $p < 1e-3$ , paired  $t$ -test), whereas no significant changes in Bray-Curtis dissimilarity values occurred in the control *Bacteria* community over time ( $p > 0.1$ ; Supplementary Fig. 3). These results demonstrate that FOG codigestion resulted in changes in the *Bacteria* community structure, primarily attributed to the growth of the



**Fig. 3.** Relative fraction of 16S rRNA genes of the  $\beta$ -oxidizing bacterial genera, (A) *Syntrophomonas* and (B) *Syntrophus*, within the total *Bacteria* 16S rRNA gene concentration in the FOG codigester and control digester over the experimental period, as determined by qPCR. Error bars represent a standard deviation based on DNA extraction replicates ( $n=3$ ) and qPCR technical replicates ( $n=2$ ) for each time point.



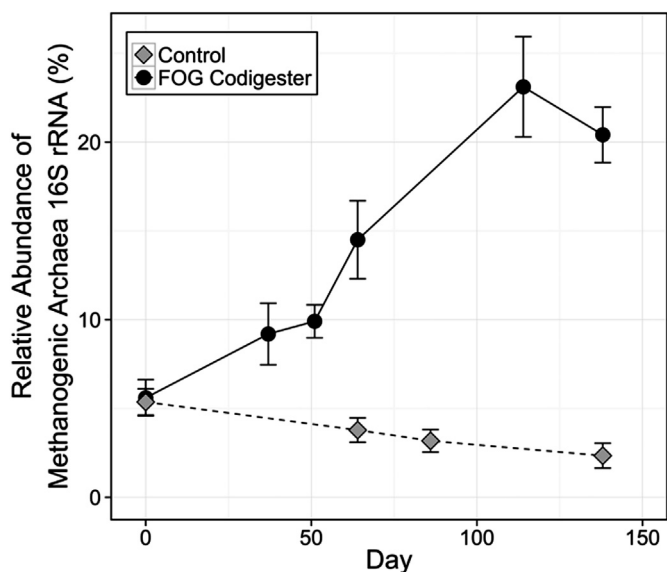
**Fig. 4.** Relative sequence fraction of the 13 most abundant genera within the FOG codigester and control digester libraries produced from Illumina MiSeq sequencing of *Bacteria* 16S rRNA gene amplicons. The triplicate bars shown for each sample day represent replicate DNA extractions. The relative sequence fraction of all other genera were summed, and are shown here as "Other".

initially rare members of *Syntrophomonas* and *Gelria* and the washout of initially dominant bacterial members such as *Petrimonas* and *Smithella* (Fig. 4).

In contrast to changes in *Bacteria* diversity, Hill diversity of orders  $^1D$ , and  $^2D$  for the *Archaea* showed a decreasing trend in the FOG codigester over the experimental period (Supplementary Fig. 4). Notably, the diversity of orders  $^1D$ , and  $^2D$  for *Archaea* in the FOG codigester on day 138 were 6% and 8% of that for *Bacteria*, respectively. *Archaea* species richness ( $^0D$ ) for the FOG codigester and control were 34% and 51% of that for *Bacteria* on day 138, respectively. These results indicate that the diversity of *Archaea* was less than that of the *Bacteria* communities in both digesters.

Significant changes in the *Archaea* community structure and abundance were observed with FOG codigestion. The fraction of methanogenic archaea 16S rRNA genes in the prokaryotic community (calculated as the sum of all methanogen groups targeted by qPCR divided by total methanogens + *Bacteria*) increased 8-

times by day 138 in the FOG codigester relative to the control ( $p < 1e-3$ , unpaired  $t$ -test; Fig. 5). The methanogenic archaea 16S rRNA gene concentration comprised over 20% of the prokaryotic community by the end of the experimental period, while that of the control digester was 2.5% (Fig. 5). Bray-Curtis dissimilarity values relative to the initial *Archaea* community structure significantly increased in the FOG codigester between days 37 ( $0.29 \pm 0.08$ ) and 138 ( $0.62 \pm 0.10$ ) ( $p < 1e-3$ , paired  $t$ -test) (Supplementary Fig. 5). The increase in Bray-Curtis dissimilarity of the codigester *Archaea* community relative to its initial population reveals that the addition of FOG resulted in changes in the *Archaea* community structure. A significant increase in the hydrogenotrophic *Methanospirillum* relative sequence abundance from  $1.3\% \pm 0.4$  to  $34\% \pm 3.0$  occurred between day zero and 138 in the FOG codigester ( $p = 0.002$ , paired  $t$ -test; Fig. 6). In contrast, *Methanospirillum* stayed below 3% relative sequence abundance in the control digester (Fig. 6). *Methanosaeta* was the dominant acetoclastic genus

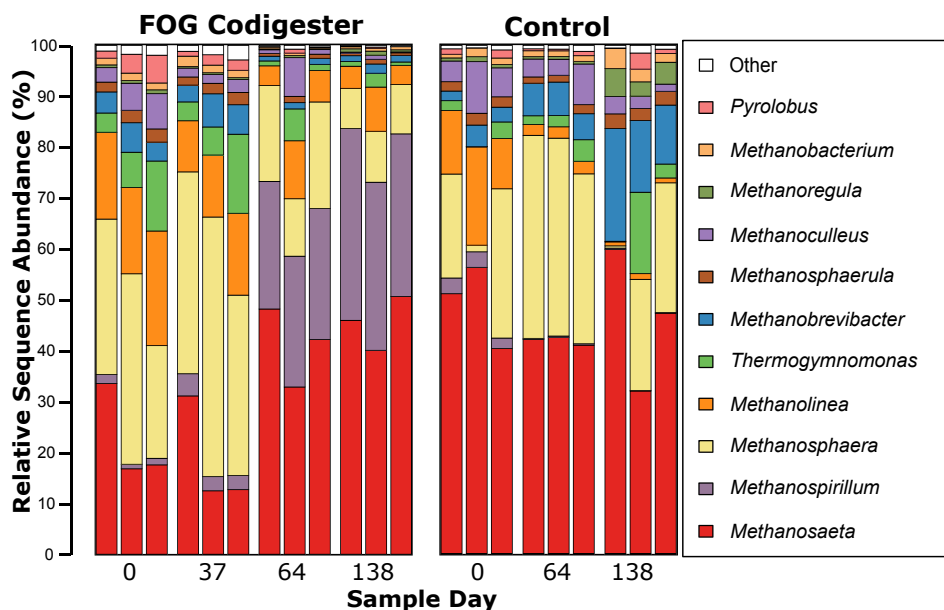


**Fig. 5.** Change in the relative fraction of methanogenic *Archaea* 16S rRNA genes in the prokaryotic community of the control digester and FOG codigester. The total methanogenic *Archaea* 16S rRNA gene concentration was determined as the sum of all methanogen groups targeted in qPCR, and the prokaryotic gene concentration was determined as the sum of methanogenic *Archaea* and total *Bacteria* 16S rRNA gene concentrations.

### 3.3. Microbial community structure was related to reactor performance during FOG codigestion

Canonical correspondence analysis (CCA) was utilized to elucidate potential relationships between environmental and operational digester parameters and *Bacteria* and *Archaea* OTU sequence abundances within the digester samples (Fig. 7). The four environmental gradients found to be most effective at explaining the community abundance data for *Bacteria* and *Archaea* were: daily methane production rate, maximum methane production rate from oleate ( $q_{max, oleate}$ ), maximum methane production rate from acetate ( $q_{max, acetate}$ ), and time (experimental days). Analysis of variance (ANOVA) on the CCA models showed that the selected environmental variables were significant in constraining both *Bacteria* and *Archaea* OTU abundances ( $p < 0.05$ ). The fractions of the total variability that was explained through the CCA models were 69% and 67% for the *Bacteria* and *Archaea* OTU abundance datasets, respectively.

To further evaluate whether microbial community structure was related to system performance during FOG degradation, a parameter was developed to relate the food-to-microorganism ratio ( $F:M$ ) for FOG loading to the LCFA-degrading microbial population within the codigester. Due to the significant increase in *Syntrophomonas* abundance in the codigester relative to the control (Figs. 3A and 4), we investigated whether *Syntrophomonas* abundance could serve as a predictor for LCFA-degradation activity. A parameter termed



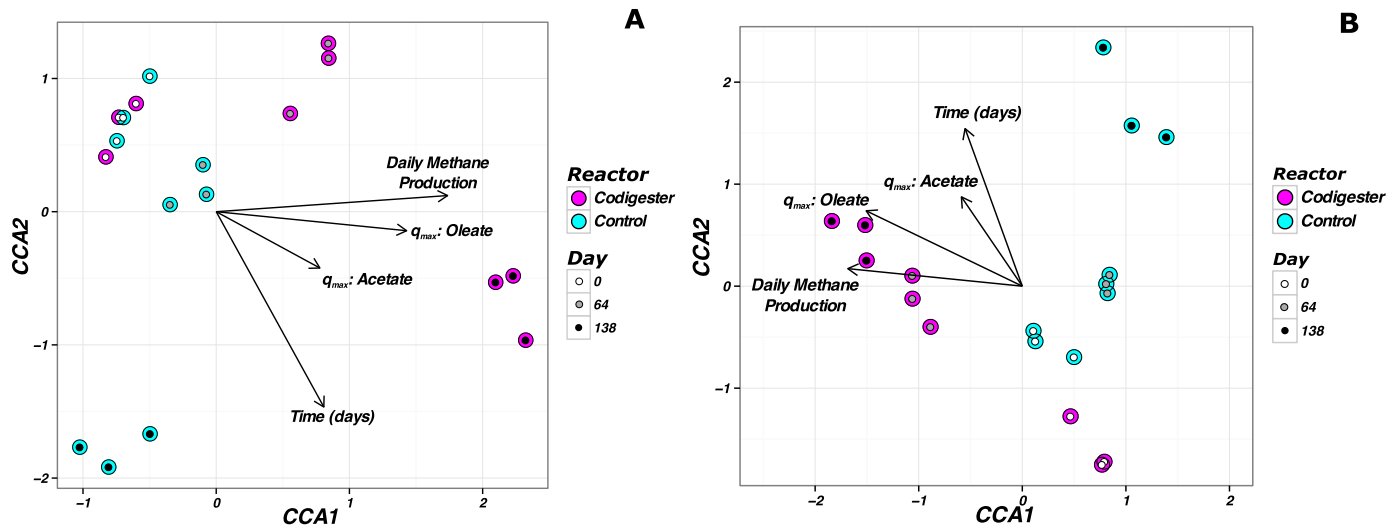
**Fig. 6.** Relative sequence fraction of the 11 most abundant genera within the FOG codigester and control digester libraries produced from Illumina MiSeq sequencing of *Archaea* 16S rRNA gene amplicons. The triplicate bars shown at each sample day represent replicate DNA extractions. The relative sequence fraction of all other genera were summed, and are shown here as "Other".

within the FOG codigester, increasing from a relative concentration of  $23\% \pm 9$  on day zero to  $46\% \pm 5$  by day 138 ( $p = 0.01$ ; Fig. 6). Pearson correlation coefficients between the average relative sequence abundances of *Methanospirillum* and *Methanosaeta* with *Syntrophomonas* in the FOG codigester were 0.90 and 0.96, respectively. *Methanospirillum* and *Methanosaeta* species were therefore key methanogenic groups growing along with *Syntrophomonas* in the codigester microbiome during the degradation of FOG.

the FOG-to-syntroph feed ratio ( $F:M_{Synt}$ ) was thereby developed by normalizing the FOG loading to the concentration of *Syntrophomonas* in the digester:

$$F:M_{Synt} = \frac{Q \times VS_{FOG}}{V \times X_{VS} \times f_{Synt}}$$

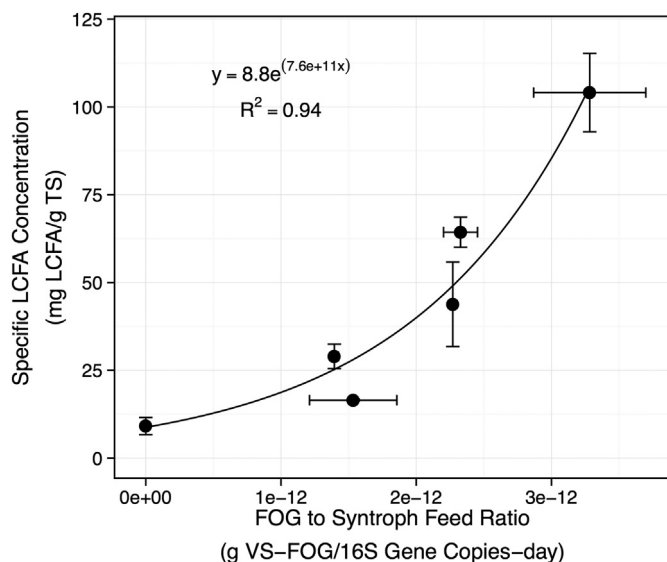
where:  $Q$  is the daily feed volume (L/d),  $VS_{FOG}$  is the influent FOG VS concentration (g VS/L),  $V$  is the reactor working volume (L),  $X_{VS}$  is the reactor VS concentration (g VS/L), and  $f_{Synt}$  is the specific



**Fig. 7.** Canonical correspondence analysis (CCA) plots based on (A) *Bacteria* and (B) *Archaea* scaled OTU sequence counts in the codigester and control digester samples, constrained by the four highest-scoring environmental gradients: daily methane production, maximum methane production from oleate ( $q_{max, oleate}$ ), maximum methane production from acetate ( $q_{max, acetate}$ ), and time (experimental days). The color of the outer circles represents the sample digester source and the color of the inner circles represents the sample collection day. The triplicate samples shown for each sample day are from replicate DNA extractions. The length and direction of each arrow represents the scaling used for that environmental gradient in the CCA model.

concentration of *Syntrophomonas* based on qPCR (16S rRNA copies/g VS). The  $F:M_{Synt}$  was calculated based on a 10-day average of daily FOG VS loading, a 10-day average of the reactor VS concentration, and the specific concentration of *Syntrophomonas* 16S rRNA genes per g VS on the day of the LCFA measurement.

The effluent LCFA concentration in the codigester was positively correlated to the  $F:M_{Synt}$  ( $R^2$  of nonlinear regression = 0.94), indicating that the LCFA removal efficiency was related to the abundance of *Syntrophomonas* biomass within the codigester (Fig. 8). Specific FOG loading rates of  $0\text{--}1.5 \times 10^{-12}$  g VS/16S gene copies-day resulted in effluent LCFA concentrations below 30 mg LCFA/g TS, whereas LCFA accumulated up to 104 mg LCFA/g TS at higher  $F:M_{Synt}$  values.



**Fig. 8.** Specific effluent LCFA concentration of the FOG codigester versus the FOG to syntroph feed ratio ( $F:M_{Synt}$ ). The exponential trend line was fitted with a non-linear regression method in R v. 3.0.2. Error bars represent one standard deviation.

#### 4. Discussion

High FOG loadings during codigestion with municipal sludge have led to process upsets (Davidsson et al., 2008; Girault et al., 2012; Luostarinen et al., 2009; Noutsopoulos et al., 2013; Wang et al., 2013) and even delayed process recovery (Wan et al., 2011). Threshold FOG loading limits for codigestion have been recommended (Noutsopoulos et al., 2013; Wang et al., 2013), but these loading limits have not considered the impacts of microbial community adaptation. In this study, changes in microbial community structure were closely monitored during transient increases in FOG loading, which showed that LCFA accumulation was related to both the FOG loading rate and the abundance of syntrophic  $\beta$ -oxidizing consortia. LCFA accumulated at higher specific loading rates of FOG relative to the abundance of *Syntrophomonas* 16S rRNA genes in the codigester. Thus, previous reports of unstable digester performance at high FOG loadings (Davidsson et al., 2008; Girault et al., 2012; Luostarinen et al., 2009; Noutsopoulos et al., 2013; Wan et al., 2011; Wang et al., 2013) could possibly have been attributed to an insufficient abundance of LCFA-degrading syntrophic bacteria, so that LCFA accumulated to a concentration high enough to inhibit methanogenic activity (Angelidaki and Ahring, 1992; Hanaki et al., 1981; Rinzeema et al., 1994). The significant growth of LCFA-degrading syntrophic bacteria and methanogenic archaea in the FOG codigester in this study supports previous observations that gradual stepwise increases in FOG loading stimulated higher rates of LCFA  $\beta$ -oxidation and methanogenesis (Silvestre et al., 2011) and that biomass resilience to LCFA was impacted by the exposure time to lipids (Alves et al., 2001; Silva et al., 2014). Our finding that LCFA conversion efficiency depends upon the digester biomass composition may also help to explain some of the variation in reported threshold limits of FOG loading during municipal sludge codigestion, which ranged from  $\sim 0.4$  to  $2.1$  g FOG VS/L-d (Girault et al., 2012; Luostarinen et al., 2009; Noutsopoulos et al., 2013; Silvestre et al., 2011; Wan et al., 2011; Wang et al., 2013). The highest FOG VSLR tested in this study was  $1.5$  g VS/L-d, which is close to the FOG VSLR threshold of  $1.6$  g VS/L-d reported previously during FOG codigestion with municipal sludge by Luostarinen et al. (2009). At our highest FOG VSLR, LCFA accumulated to  $104$  mg LCFA/g TS with



no appreciable increase in VFA. This phenomenon was also observed by *Silvestre et al. (2011)* and *Girault et al. (2012)* during FOG codigestion with municipal sludge, suggesting that acetogenesis from LCFA can be rate-limiting in such processes. The decrease in effluent LCFA in the codigester on day 138 in our study thus indicates that a higher FOG VSLR may be possible after further time for microbial community adaptation. Overall, these results implicate that the FOG loading threshold during codigestion may be considered a 'moving target' that relies upon the degree of microbial community adaptation and the activity within the digester microbiome to degrade LCFA.

This is the first study to monitor changes in syntrophic  $\beta$ -oxidizing bacteria abundance during FOG codigestion by qPCR, which highlighted a differential selection of *Syntrophomonas* over *Syntrophus* species. The family of *Syntrophomonadaceae* has been previously identified as a key syntrophic bacterial group enriched in anaerobic digester biomass during unsaturated LCFA degradation (*Baserba et al., 2012; Sousa et al., 2009, 2007a; Ziels et al., 2015*). In contrast, *Syntrophaceae* have been detected at higher abundances in anaerobic communities degrading saturated LCFA (*Sousa et al., 2009*) as well as long-chain alkanes (*Gray et al., 2011; Zengler et al., 1999*). While the saturated fatty acids of palmitate ( $C_{16:0}$ ) and stearate ( $C_{18:0}$ ) together comprised 54% of the LCFA in the codigester effluent on average (*Supplementary Fig. 1*), it is not known whether the LCFA composition of the influent FOG influenced the selection of *Syntrophomonas* over *Syntrophus*. It may be also possible that the enrichment of *Syntrophomonas* in the codigester was attributed to specific syntroph-methanogen pairings with *Methanospirillum* and *Methanosaeta*, which were the dominant methanogenic archaeal groups enriched with FOG addition and were strongly correlated with *Syntrophomonas* abundance (i.e. Pearson coefficients  $>0.9$ ).

The positive association of the FOG codigester *Bacteria* and *Archaea* community profiles with the constrained gradients of daily methane production and  $q_{max, oleate}$  further confirm that changes in microbial community structure were related to measures of codigester performance. Hill diversity numbers also proved to be effective metrics to capture the alpha and beta components of community structure changes within the digesters, as was similarly found with other biological wastewater treatment processes (*Vuono et al., 2015*). Diversity numbers of orders  ${}^1D$  and  ${}^2D$  for the *Bacteria* community increased along with measures of reactor performance in the FOG codigester, but the same was not true with species richness ( ${}^0D$ ). This agrees with previous findings indicating that higher order diversity (i.e. evenness) in digester *Bacteria* communities correlated positively with COD removal and methane production, whereas species richness did not (*Werner et al., 2011*). While the *Bacteria* species richness of the FOG codigester remained stable in this study, the community experienced changes in dominant members (captured by  ${}^2D$ ) that contributed to higher ecosystem function. The higher methane production of the codigester following shifts in dominant *Bacteria* community members (higher  ${}^2D$ ) coincided with the growth of the functionally specific *Syntrophomonas*. The increased biodiversity within the codigester was therefore attributed to the growth of initially rare syntrophic bacteria, which were also a driving force behind improvements in the codigester daily methane production and increased values of  $q_{max, oleate}$ . This finding agrees with previous observations that the abundance of syntrophic bacteria was linked to anaerobic digester resilience (*Regueiro et al., 2015; Werner et al., 2011*).

Evaluating the activity of syntrophic fatty acid-oxidizing bacteria has previously been proposed as an alternative approach to gauge the activity of methanogenic pathways, since the metabolism of syntrophic fatty acid-oxidizing bacteria is directly

coupled to the activity of methanogens (*Smith et al., 2015*). Our proposed relationship between LCFA removal and the *Syntrophomonas* 16S rRNA gene concentration was based on the assumption that *Syntrophomonas* was a major syntrophic bacterial group involved in LCFA  $\beta$ -oxidation, and that *Syntrophomonas* biomass could serve as a surrogate measure for the activity of the LCFA-degrading community (syntrophic bacteria + methanogens). This assumption was supported by the observation that LCFA were the primary metabolite of FOG degradation rather than acetate, indicating that LCFA  $\beta$ -oxidation was not likely limited by aceticlastic methanogenesis. Similar to our findings, the concentration of syntrophic bacterial biomass was recently found to be the limiting factor for butyrate conversion via  $\beta$ -oxidation in non-defined methanogenic enrichments (*Junicke et al., 2016*). *Ziels et al. (2015)* also found that the relative fraction of *Syntrophomonas* in the *Bacteria* community of oleate-fed anaerobic enrichments was positively correlated with the specific methane production rate. Since the thermodynamics of LCFA-degradation hinges upon the activity of methanogenic archaea and syntrophic  $\beta$ -oxidizing bacteria (*Schink, 1997*), we propose that the FOG-loading capacity of a given digester can be better predicted by closely monitoring the abundance and activity of those populations.

From a process engineering perspective, efficient turnover of LCFA is critical for maintaining the maximum kinetic capacity of the digester community (*Pereira et al., 2005*). The positive correlation observed between the effluent LCFA concentration and the codigester  $F:M_{synt}$  suggested that a higher biomass of syntrophic LCFA-degrading consortia could increase the threshold loading rate of FOG during codigestion. A higher biomass of slow growing *Syntrophomonas* has been associated with longer anaerobic digester solids retention times (*Hao et al., 2016; Lee et al., 2011*). Therefore, the methane production of FOG codigestion processes should be greater with longer solids retention times by allowing a higher mass loading of FOG while maintaining an  $F:M_{synt}$  that supports low effluent LCFA concentrations. This work also demonstrates the potential to minimize LCFA accumulation by controlling transient FOG loading rates based on the analysis of the codigester community structure, such as with qPCR measurements of syntrophic LCFA-degrading bacteria abundance.

## 5. Conclusions

Microbial adaptation was important for the conversion of FOG into methane during codigestion with municipal wastewater sludge. *Syntrophomonas* bacteria were the primary syntrophic  $\beta$ -oxidizing group that was enriched in the codigester microbiome. Increases in digester LCFA concentrations occurred when the FOG loading rate relative to *Syntrophomonas* abundance exceeded a threshold value. Changes in the FOG codigester and control digester microbiomes were best explained through constrained ordination by measures of digester function. These results collectively indicate that more efficient LCFA conversion into methane can be achieved by strategically managing transient FOG loadings to allow sufficient time for microbial community adaptation through higher abundances of LCFA-degrading consortia.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2016.07.043>.

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