Appl Microbiol Biotechnol
DOI 10.1007/s00253-016-7967-9

ENVIRONMENTAL BIOTECHNOLOGY

Unexpected competitiveness of *Methanosaeta* populations at elevated acetate concentrations in methanogenic treatment of animal wastewater

Si Chen[1] · Huicai Cheng[2] · Jiang Liu[3] · Terry C. Hazen[1,3,4] · Vicki Huang[5] · Qiang He[1,4]

Received: 19 July 2016 / Revised: 16 September 2016 / Accepted: 26 October 2016

Abstract Acetoclastic methanogenesis is a key metabolic process in anaerobic digestion, a technology with broad applications in biogas production and waste treatment. Acetoclastic methanogenesis is known to be performed by two archaeal genera, *Methanosaeta* and *Methanosarcina*. The conventional model posits that *Methanosaeta* populations are more competitive at low acetate levels (<1 mM) than *Methanosarcina* and vice versa at higher acetate concentrations. While this model is supported by an extensive body of studies, reports of inconsistency have grown that *Methanosaeta* were observed to outnumber *Methanosarcina* at elevated acetate levels. In this study, monitoring of anaerobic digesters treating animal wastewater unexpectedly identified *Methanosaeta* as the dominant acetoclastic methanogen population at both low and high acetate levels during organic overloading. The surprising competitiveness of *Methanosaeta* at elevated acetate was further supported by the enrichment of *Methanosaeta* with high concentrations of acetate (20 mM). The dominance of *Methanosaeta* in the methanogen community could be reproduced in anaerobic digesters with the direct addition of acetate to above 20 mM, again supporting the competitiveness of *Methanosaeta* over *Methanosarcina* at elevated acetate levels. This study for the first time systematically demonstrated that the dominance of *Methanosaeta* populations in anaerobic digestion could be linked to the competitiveness of *Methanosaeta* at elevated acetate concentrations. Given the importance of acetoclastic methanogenesis in biological methane production, findings from this study could have major implications for developing strategies for more effective control of methanogenic treatment processes.

Keywords *Methanosaeta* · *Methanosarcina* · Anaerobic digestion · Acetate · Acetoclastic methanogenesis

Electronic supplementary material The online version of this article (doi:10.1007/s00253-016-7967-9) contains supplementary material, which is available to authorized users.

✉ Qiang He
qianghe@utk.edu

[1] Department of Civil and Environmental Engineering, University of Tennessee, Knoxville, TN 37996, USA

[2] Biology Institute, Hebei Academy of Sciences, Shijiazhuang, Hebei, China

[3] Department of Microbiology, University of Tennessee, Knoxville, TN, USA

[4] Institute for a Secure and Sustainable Environment, University of Tennessee, Knoxville, TN, USA

[5] Department of Biomedical Engineering, University of Virginia, Charlottesville, VA, USA

Introduction

Biogas is a clean fuel that can be produced from renewable feedstock in methanogenic treatment processes such as anaerobic digestion, which exploit methanogenesis as an exclusively microbial process. Despite the broad application of methanogenic processes in waste treatment, process instability remains a major challenge to process control and optimization. To improve process efficiency and stability, considerable efforts have been made to delineate the complex microbial communities involved in methanogenesis, which remains inadequately characterized.

Among pathways of methanogenesis, acetoclastic methanogenesis is typically the most important in methanogenic treatment processes (Ferry 1992; Jetten et al. 1990), contributing to more than 60% of methane production (Bridgham et al. 2013; Ferry 1992; Penning et al. 2006). Recent findings also indicate that syntrophic acetate oxidation

Published online: 17 November 2016

coupled with hydrogenotrophic methanogenesis may constitute another potentially important pathway of methane production from acetate (Dolfing 2014; Sun et al. 2014). To date, *Methanosaeta* and *Methanosarcina* represent the only archaeal genera known to carry out acetoclastic methanogenesis, converting acetate into methane and carbon dioxide. In previous studies, *Methanosarcina* populations have been reported to exhibit higher maximum growth rates, μ_{max}, and half-saturation coefficients, K_S, than those of *Methanosaeta* populations (Conklin et al. 2006; De Vrieze et al. 2012). Subsequently, *Methanosarcina* populations are expected to be more competitive than *Methanosaeta* populations in methanogenic processes with elevated acetate levels. Accordingly, *Methanosaeta* populations are anticipated to predominate over *Methanosarcina* populations at low acetate levels due to the higher affinity of *Methanosaeta* to acetate (De Vrieze et al. 2012; Smith and Ingram-Smith 2007).

Consistent with these observations, *Methanosaeta* have been found to be the dominant acetoclastic methanogen populations at low acetate concentrations (i.e., <1 mM), which is typical for methanogenic treatment processes with stable performance (McHugh et al. 2003; Zheng and Raskin 2000). At elevated acetate levels, particularly during process instability, *Methanosarcina* populations were found to outcompete *Methanosaeta* populations (De Vrieze et al. 2012; Ma et al. 2013), which has been attributed to the greater μ_{max} and K_S of *Methanosarcina* as compared with *Methanosaeta*. However, other studies of methanogenic treatment processes have reported the dominance of *Methanosaeta* over *Methanosarcina* despite elevated acetate concentrations (Chen and He 2015b; Franke-Whittle et al. 2014; Leite et al. 2015; Schmidt et al. 2014; Ziganshin et al. 2016). These observations are inconsistent with the reported growth kinetics of *Methanosaeta* and *Methanosarcina* (Conklin et al. 2006; De Vrieze et al. 2012), suggesting the possibility of unidentified attributes shaping the competitive interactions between *Methanosaeta* and *Methanosarcina*.

In this study, we investigated anaerobic digesters unexpectedly dominated by *Methanosaeta* at elevated acetate levels resulting from organic overloading. Subsequent enrichment of acetoclastic methanogens by acetate (20 mM) in batch reactors confirmed the dominance of *Methanosaeta* over *Methanosarcina* at elevated acetate concentrations. To further examine the competition between *Methanosaeta* and *Methanosarcina*, high acetate levels (>20 mM) were established in continuous anaerobic digesters by the direct addition of extraneous acetate, which again led to the dominance of *Methanosaeta* over *Methanosarcina*. To the best of our knowledge, this study for the first time reproducibly demonstrated the competitiveness of *Methanosaeta* over *Methanosarcina* at elevated acetate concentrations, providing much needed insight into the ecophysiology of acetoclastic methanogens. Given the importance of acetoclastic methanogenesis, findings from this study could have major implications for strategies to improve the performance of methanogenic treatment processes.

Materials and methods

Setup and operation of continuous anaerobic digesters treating animal wastewater

Triplicate continuous mesophilic anaerobic digesters were established prior to this study using dairy wastewater as the feedstock at a constant organic loading rate (OLR) of 1.0 g volatile solids (VS)/L/day as described previously (Chen et al. 2012; Zhang et al. 2011). All three completely mixed digesters had a working volume of 3.6 L. The temperature of the anaerobic digesters was controlled at 35 °C and the hydraulic retention time was maintained at 20 days. All anaerobic digesters exhibited stable operation with consistent pH, methane yield, and volatile fatty acid level. Following the establishment of stable anaerobic digestion performance, an episode of organic overloading was initiated by increasing the OLR from 1.0 to 1.8 g VS/L/day with the addition of poultry waste as an organic-rich co-substrate as previously described (Chen et al. 2012), which resulted in the disruption of stable operation characterized by the rapid accumulation of organic acids and inhibition of biogas production. The use of poultry waste enabled the increase in OLR without changing the hydraulic loading. It was also shown previously that the addition of poultry waste did not lead to ammonia inhibition at the OLR tested (Zhang et al. 2011). Subsequently, stable process performance was reestablished by suspending the use of poultry waste as a co-substrate. Process parameters, including pH, methane production, and volatile fatty acids, were monitored daily or more frequently during unstable process operation as described previously (Chen et al. 2012).

Enrichment of acetoclastic methanogens in batch reactors

To further identify methanogen populations thrived at elevated acetate levels during organic overloading in the continuous anaerobic digesters, batch enrichment reactors with acetate as the only substrate in a defined medium were set up using previously described procedures (Chen and He 2015a). Initial enrichment reactors were seeded with a 10% (*v*/*v*) inoculum of the digestate from the continuous anaerobic digesters with 20 mM acetate as the sole substrate, which was replenished when depleted. At the completion of 10 feedings of 20 mM acetate, secondary enrichment reactors were established by transferring a 10% (*v*/*v*) inoculum from the initial enrichment reactors into fresh medium followed by repeated feedings of 20 mM acetate

for 10 times when previous feedings were depleted. All enrichment reactors were set up in triplicates and maintained in a shaking incubator at 80 rpm and 35 °C. Methane production was monitored periodically. Biomass samples from secondary enrichment reactors were preserved at −80 °C for further analysis.

To eliminate the impact of pH, trace metal, and ammonia on the competitiveness of distinct methanogen populations during cultivation, the enrichment batch reactors used a defined anaerobic basal medium prepared according to the following recipe (per liter) as previously described (He and Sanford 2002): NaCl, 1.0 g; $MgCl_2 \cdot 6\ H_2O$, 0.5 g; KH_2PO_4, 0.2 g; NH_4Cl, 0.3 g; KCl, 0.3 g; $CaCl_2 \cdot 2H_2O$, 0.015 g; trace element solution, 1.0 mL; Se/Wo solution, 1.0 mL; and resazurin, 1.0 mg. The trace element solution contained the following (per liter): 1.5 g of $FeCl_2 \cdot 4H_2O$, 0.19 g of $CoCl_2 \cdot 6H_2O$, 0.1 g of $MnCl_2 \cdot 4H_2O$, 70 mg of $ZnCl_2$, 6 mg of H_3BO_3, 36 mg of $Na_2MoO_4 \cdot 2H_2O$, 24 mg of $NiCl_2 \cdot 6H_2O$, and 1.0 mg of $CuCl_2 \cdot 2H_2O$. The selenium-tungsten solution contained 6 mg of Na_2SeO_3 per liter, 8 mg of $Na_2WO_4 \cdot 2H_2O$ per liter, and 0.54 g of NaOH per liter. L-cysteine (0.031 g/L) and $Na_2S \cdot 9\ H_2O$ (0.048 g/L) were added as reductants into the basal medium after it had been boiled and cooled to room temperature under an oxygen-free N_2 atmosphere. $NaHCO_3$ (2.52 g/L) was added to the medium as the buffer. The headspace of the medium container was continuously flushed with an oxygen-free N_2/CO_2 gas mix, and the pH was adjusted to 7.0 ± 0.1 by varying the CO_2 composition of the gas mix. The medium was subsequently dispensed into serum bottles flushed with pure N_2 and sealed with butyl rubber stoppers and aluminum caps. A sterile vitamin solution (1%) was added after autoclaving (Wolin et al. 1963).

Testing the response to elevated acetate in continuous anaerobic digesters

Following attempts to enrich acetoclastic methanogens in batch reactors fed with 20 mM acetate in defined medium, the impact of elevated acetate was further evaluated in continuous anaerobic digesters treating animal wastewater. An episode of elevated acetate level was introduced to the anaerobic digesters with the direct addition of acetate. To maintain acetate at elevated levels, concentrated sodium acetate stock solution (5 M) was added to the triplicate continuous anaerobic digesters with stable performance. Acetate level was closely monitored in the anaerobic digesters, and adjustments in acetate loading were made accordingly to keep the acetate level above 20 mM. The loading rate of animal wastewater remained unchanged at 1.0 g VS/L/day with or without the addition of extraneous acetate.

To verify the reproducibility of the response of the continuous anaerobic digesters to elevated acetate, a second episode of elevated acetate was initiated 9 days after the conclusion of the first episode of elevated acetate. Following the same acetate feeding strategy as used in the first episode, acetate was kept above 20 mM for 6 days in the anaerobic digesters. Process parameters of anaerobic digestion performance, including pH, methane production, and acetate concentration, were monitored as described previously (Chen et al. 2012).

Biomass samples were taken from the triplicate anaerobic digesters at the following five-time points (Supplementary Fig. S1): A) immediately before the initiation of the first episode of elevated acetate; B) before the end of the first episode of elevated acetate; C) after the conclusion of the first episode of elevated acetate but immediately before the initiation of the second episode of elevated acetate; D) before the end of the second episode of elevated acetate; and E) after the conclusion of the second episode of elevated acetate. Samples were preserved at −80 °C for further analysis.

Monitoring microbial population abundance with real-time quantitative PCR (qPCR)

The 16S rRNA gene copy numbers in the continuous anaerobic digesters and batch enrichment reactors were quantified by qPCR assays specifically targeting *Methanosaeta*, *Methanosarcina* and total *Archaea* populations. All qPCR assays were performed with previously validated primer/probe sets using the TaqMan chemistry (Table 1). Following the extraction and purification of whole community DNA from biomass samples using previously described protocols (Zhang et al. 2009), qPCR assays were performed in 25-μL reaction volume with 15 pmol of the primers, 5 pmol of the probe, Brilliant II QPCR Master Mix (Agilent, Santa Clara, California, USA) and 1 μL DNA sample. Thermal cycling consisted of a starting incubation at 50 °C for 2 min and an initial denaturation at 95 °C for 10 min, followed by up to 45 cycles at 95 °C for 30 s, and 60 °C (for total *Archaea*) or 57 °C (for *Methanosaeta*) or 61 °C (for *Methanosarcina*) for 45 s. The qPCR procedures were performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) as previously described (Chen et al. 2014). Fluorescence response data were processed with the software provided by the manufacturer (Bio-Rad). Gene copy numbers were determined from standard curves based on the log transformation of known concentrations versus the threshold cycle (C_T). DNA templates used as the standards for qPCR were partial 16S rRNA genes of representative archaeal populations cloned from the continuous anaerobic digesters in a previous study (Chen et al. 2012), including *Methanosaeta* (GenBank Accession No. JN052761) and *Methanosarcina* (GenBank Accession No. JN052757). Standard curves of the qPCR assays were obtained from the C_T-Log[Template] plots derived from the quantification of 10-fold dilution series of 16S rRNA gene templates.

Table 1. List of primer and probe sets for 16S rRNA gene qPCR assays in this study

Target	Primer/probe	Sequence (5′-3′)	Position *E. coli* No.	Amplicon Size (bp)	T_m[a] (°C)	Reference
	Forward Primer					
Archaea	Arc-787F	ATTAGATACCCSBGTAGTCC	787–806	273	61.0	(Yu et al. 2005)
	Probe					
	Arc-915P	AGGAATTGGCGGGGGAGCAC	915–934		70.1	
	Reverse Primer					
	Arc-1059R	GCCATGCACCWCCTCT	1044–1059		62.3	
	Forward Primer					
Methanosaeta	Mst-702F	TAATCCTTGAAGGACCACCA	702–721	161	61.0	
	Probe					
	Mst-753P	ACGGCAAGGGACGAAAGCTAGG	753–774		70.0	(Yu et al. 2005)
	Reverse Primer					
	Mst-862R	CCTACGGCACCGACAAC	846–862		62.0	
	Forward Primer					
	Msc-586F	CGGTTTGGTCAGTCCTCCG	586–604		61.6	
	Probe					
Methanosarcina	Msc-743P	AACGGGTTCGACGGTGAGGGACGA	743–766	257	70.6	(Chen and He 2015b)
	Reverse Primer					
	Msc-842R	ACCAGACACGGTCGCGC	826–842		59.8	

[a] Melting temperatures were determined using the Oligo Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html)

Clone library analysis of methanogen populations enriched by elevated acetate

Clone library analysis was performed to examine the composition of methanogen communities enriched by acetate in batch enrichment reactors. DNA extracts from triplicate enrichment reactors were pooled for PCR amplification of the 16S rRNA genes using the following *Archaea*-specific primers (DeLong 1992): Arch21F (5′-TTCCGGTTGATCCYGCCGGA-3′) and Arch958R (5′-YCCGGCGTTGAMTCCAATT-3′) following previously described protocols (Chen et al. 2012). Amplicons of 16S ribosomal RNA (rRNA) gene sequences were subsequently purified and cloned into plasmid vectors as described previously (Chen et al. 2012). Twenty cloned plasmid inserts were randomly selected for sequencing with the ABI Prism BigDye chemistry (Applied Biosystems, Foster City, CA, USA) using M13 forward and reverse primers. The obtained sequences were checked for chimeric artifacts using the Chimera Check program in the Ribosomal Database Project II (Cole et al. 2003). High quality 16S rRNA gene sequences were deposited at GenBank under the following accession numbers: KJ504229—KJ504247.

Subsequently, phylogenetic analysis was conducted on the 16S rRNA gene sequences retrieved from the methanogen communities enriched with acetate by searching the NCBI GenBank database for closely related sequences. The phylogenetic positions of these 16S rRNA gene sequences were assessed by phylogenetic trees constructed with the neighbor-joining algorithm (1000 bootstrap resamplings) using MEGA 4.0 (Tamura et al. 2007).

High-throughput sequencing of anaerobic digester microbial communities during episodes of elevated acetate

In addition to qPCR analysis, high-throughput sequencing was also used to characterize the methanogen populations in the continuous anaerobic digesters during the two episodes of elevated acetate. Firstly, whole community DNA extracts were PCR amplified with 515-F and 806-R Golay barcoded primers (Supplementary Table S1) targeting the V4 region of the 16S rRNA gene (Caporaso et al. 2012) using the following thermal cycling program: 94 °C for 3 min; 25 cycles at 94 °C for 45 s followed by 50 °C for 60 s and 72 °C for 90 s; and 72 °C for 10 min at the end. PCR products were confirmed by gel electrophoresis and then purified with the Wizard® SV Gel and PCR Clean-Up system (Promega, Madison, WI, USA). DNA concentrations of the amplicons were quantified by the Invitrogen Quant-It Pico Green DNA quantification kit (Life Technologies, Carlsbad, CA, USA) and samples were pooled at equimolar ratios. Subsequently, the amplicon libraries were subjected to pair-end sequencing (2 × 250 bp) on the Illumina MiSeq platform (Illumina, San Diego, CA, USA).

All sequence reads retrieved from the Illumina MiSeq platform were analyzed by the Mothur (v.1.35) platform following procedures outlined by previous studies (Zhang and He 2013). After sequence quality processing including barcode and primer trimming, denoising, and chimera checking, RDP Classifier was used to assign valid sequences from each sample to taxonomic ranks with confidence threshold of 80% as previously described (Cole et al. 2003). Sequences assigned to archaeal taxa were retrieved for the characterization of methanogen populations. Sequences were deposited at the Sequence Read Archive (SRA) of GenBank with accession number SRP071225.

Chemical analysis

Biogas production from the anaerobic digesters was determined using a water displacement method described previously (Zhu et al. 2011). Methane content in biogas was analyzed using a Hewlett Packard 5890 Series II gas chromatograph equipped with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carbonxen®-1000; Sigma-Aldrich, St Louis, MO, USA). Argon was used as the carrier gas with a flow rate of 5 mL/min and the following temperature scheme: oven 125 °C, injection port 150 °C, and detector 170 °C. Volatile fatty acids (VFAs) including acetate were analyzed with an Agilent 1200 series high-performance liquid chromatography (HPLC) system (Santa Clara, CA, USA) equipped with an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA) using 4 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL/min. Prior to HPLC analysis, samples were prepared by filtration and acidification to 0.1 N H_2SO_4 by adding 25 μL of 2 N H_2SO_4 to 475 μL of sample as previously described (He and Sanford 2004). Detection of VFAs was at 210 nm with a multiple wavelength detector.

Statistical analysis

To evaluate the statistical significance of the differences between *Methanosaeta* and *Methanosarcina*, including population abundance and relative ratio, qPCR results were analyzed with one-way analysis of variance (ANOVA). Significant differences were indicated by a probability value (p) less than 0.05 in ANOVA analysis. Correlations between acetate concentration and the 16S rRNA gene copy numbers of *Methanosaeta* or *Methanosarcina* were evaluated with the Pearson's correlation coefficient (r) and p value, which were determined as measures of the strength and significance of the correlation, respectively. Statistical analysis was performed with JMP Pro 10 for Windows (SAS Institute Inc., Cary, North Carolina, USA).

Results

Dominance of *Methanosaeta* during process instability

The abundance of acetoclastic methanogens was monitored by qPCR when the anaerobic digesters experienced an episode of elevated acetate due to substrate overloading (Fig. 1). Prior to the sharp rise in acetate concentration, process performance was stable in the anaerobic digesters with the acetate level consistently below 1 mM. Accordingly, *Methanosaeta* populations were more abundant than *Methanosarcina* populations, which was expected as values of K_S for *Methanosaeta* reported in the literature are typically 1 mM or below while those for *Methanosarcina* are greater than 4 mM (Yilmaz et al. 2014), thus favoring the growth of *Methanosaeta* over *Methanosarcina* at acetate levels below 1 mM in the anaerobic digesters with stable operation.

However, when acetate reached 44 ± 3 mM at the height of process perturbation, *Methanosaeta* remained as the dominant acetoclastic methanogen population, averaging 163% more abundant than *Methanosarcina* (Fig. 1). The dominance of *Methanosaeta* was inconsistent with the prediction from reported values of growth kinetics that high acetate concentrations would instead support the competitiveness of *Methanosarcina* over *Methanosaeta* (Conklin et al. 2006; De Vrieze et al. 2012). Nonetheless, it was possible that other process factors other than acetate concentration might have contributed to the unexpected dominance of *Methanosaeta* over *Methanosarcina*, such as pH or availability of trace metals (Demirel and Scherer 2011).

Enrichment of *Methanosaeta* at elevated acetate levels

To exclude the potential impact of unfavorable pH or deficiency in trace metals on the competition between *Methanosaeta* and *Methanosarcina*, batch reactors with neutral pH and

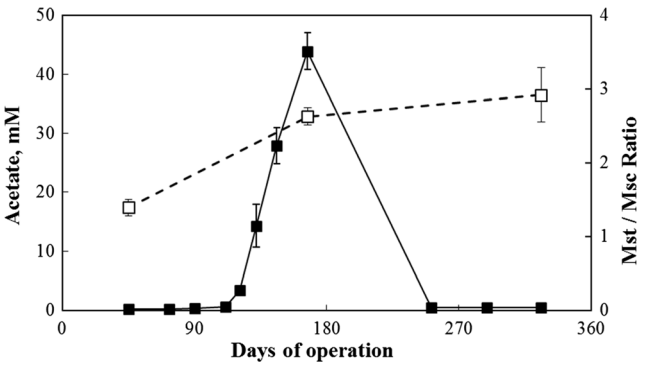

Fig. 1 Dynamics of acetate and acetoclastic methanogens (as measured by qPCR as the *Methanosaeta* to *Methanosarcina* ratio of 16S rRNA gene copy numbers—Mst/Msc ratio) in response to organic overloading in anaerobic digesters treating animal wastewater. Acetate: *closed squares*; Mst/Msc ratio: *open squares*. Data points were means of triplicates with the *error bars* showing standard deviations

excess trace metals were set up using acetate as the sole substrate. The initial concentration of acetate was 20 mM, much higher than the reported range of K_S for *Methanosaeta* (0.36–1.2 mM) or *Methanosarcina* (4.0–6.5 mM) (Yilmaz et al. 2014). Given the greater μ_{max} values reported for *Methanosarcina*, ranging from 0.2 to 1.5 d^{-1}, than those of *Methanosaeta*, typically between 0.07 to 0.69 d^{-1}, *Methanosarcina* would be expected to be more abundant than *Methanosaeta* in these batch reactors following repeated feeding of 20 mM acetate.

The methanogenic populations in the batch reactors were subsequently characterized by 16S rRNA gene clone library and qPCR analyses. Clone library analysis revealed that all archaeal 16S rRNA gene clones in the batch reactors were affiliated with *Methanosaeta* instead of *Methanosarcina* (Fig. 2), indicative of the enrichment of *Methanosaeta* instead of *Methanosarcina* by elevated acetate. Admittedly, clone library analysis is not quantitative; nevertheless, the dominance of *Methanosaeta* over *Methanosarcina* was evident. More importantly, all of the clones classified as *Methanosaeta* were closely related to known *Methanosaeta* populations, particularly *Methanosaeta concilii* (Fig. 2), suggesting that the *Methanosaeta* populations prospered at elevated acetate levels were not distinctively different from those previously reported to thrive only at low acetate concentrations.

The abundance of acetoclastic methanogens was also quantified with greater accuracy using qPCR. In support of the results from clone library analysis, qPCR quantification showed that *Methanosaeta* were enriched in the batch reactors fed with high concentrations of acetate, accounting for over 80% of the archaeal community (Table 2). In comparison, the presence of *Methanosarcina* was nearly negligible, consistent with the lack of detection of *Methanosarcina* by clone library analysis.

The enrichment of *Methanosaeta* as the predominant acetoclastic methanogens in the batch reactors provided direct evidence of the competitiveness of *Methanosaeta* over *Methanosarcina* at elevated acetate levels, which was divergent from predictions from reported growth kinetics parameters for acetoclastic methanogens (Conklin et al. 2006; De Vrieze et al. 2012). Since these observations were made in reactors with defined medium, subsequent efforts were made to characterize the response of *Methanosaeta* populations to elevated acetate concentrations in continuous anaerobic digesters treating dairy wastewater.

Process performance of continuous anaerobic digesters in response to elevated acetate

Two episodes of elevated acetate were introduced in the continuous anaerobic digesters. To achieve acetate levels above 20 mM throughout the testing periods, concentrated acetate was added to the anaerobic digesters along with the animal wastewater. During the first episode of elevated acetate, the acetate loading rate was raised gradually, eventually reaching 12.5 mmol/L/day, which was kept for 5 days before acetate feeding stopped (Supplementary Fig. S1).

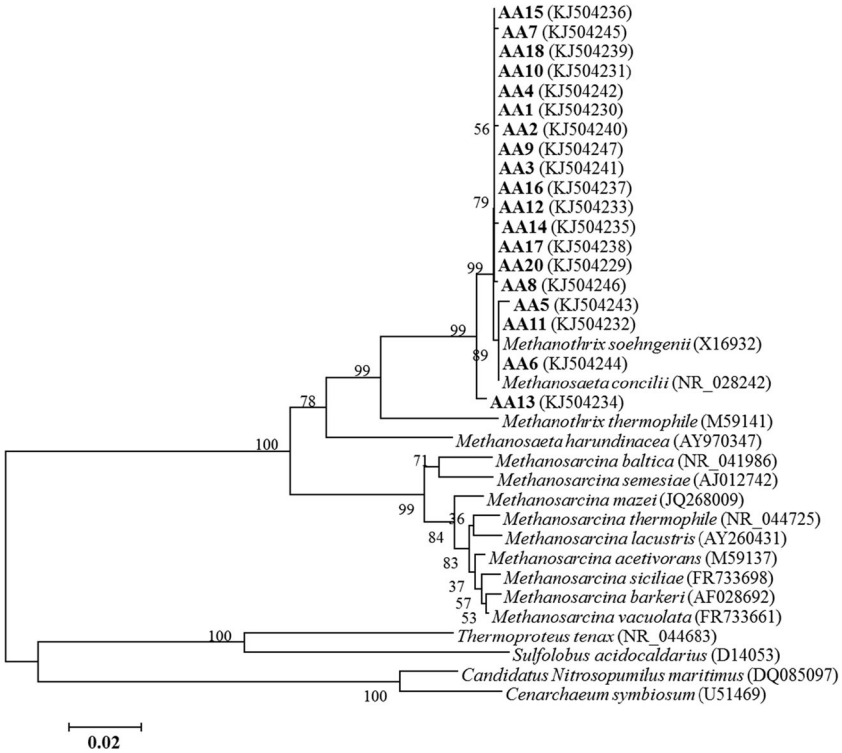

Fig. 2 Neighbor-joining phylogenetic tree showing the relationships of partial archaeal 16S rRNA gene sequences cloned from the anaerobic populations enriched by 20 mM acetate (*boldface type*) to reference archaeal strains. The numerical values at branch nodes indicate bootstrap values per 1000 resamplings. The scale bar represents the number of substitutions per sequence position. GenBank accession numbers of the 16S rRNA gene sequences are shown in the parentheses

Table 2. qPCR quantification of acetoclastic methanogens enriched by acetate

16S rRNA Gene target	No. of copies/mL[a]	Relative abundance (% of total *Archaea*)[a]
Methanosaeta	$(8.9 \pm 0.7) \times 10^8$	80.4 ± 13.5
Methanosarcina	$(3.6 \pm 0.9) \times 10^6$	0.3 ± 0.0
Total *Archaea*	$(1.1 \pm 0.1) \times 10^9$	–

Enrichments were established by a 10% (*v*/*v*) inoculum of anaerobic digestate from continuous anaerobic digesters treating animal wastewater to anaerobic medium followed by 10 feedings of 20 mM acetate and subsequently a 2nd 10% (*v*/*v*) transfer to anaerobic medium followed by another 10 feedings of 20 mM acetate

[a] Values are the means of triplicates ± standard deviation

During the first episode of elevated acetate, with the increases in acetate loading rate, acetate concentration rose above 20 mM but with large fluctuations, with the highest level reaching 54.4 ± 15.0 mM (Fig. 3a). When acetate loading rate reached the maximum, the acetate level in the anaerobic digesters stabilized between 34.2 ± 6.2 and 36.6 ± 5.7 mM, resulting in an extended period of operation with elevated acetate. Following the pause in acetate feeding, acetate level declined gradually to around 1 mM (Fig. 3a), when the second episode of elevated acetate was introduced. During the second episode of elevated acetate, the acetate loading rate was raised directly to the maximum of 12.5 mmol/L/day (Supplementary Fig. S1), resulting in a jump in acetate level to above 20 mM (Fig. 3a). It should be noted that while the acetate level did rise above 20 mM in response to the increase in acetate loading rate, it never climbed above 30 mM, which was reached in the first episode of elevated acetate with the same acetate loading rate of 12.5 mmol/L/day, indicating that a greater capacity of acetate utilization was established following the first episode of elevated acetate. Subsequent to the termination of acetate feeding, acetate level again declined gradually to around 1 mM, similar to the trend in the first episode of elevated acetate (Fig. 3a).

Corresponding to the increases in acetate loading, methane production rose significantly (Fig. 3b). When acetate level was kept above 30 mM during the first episode of elevated acetate, the methane production rate averaged 426 ± 6 mL/L/day, significantly higher than the average of 204 ± 3 mL/L/day prior to the increases in acetate loading. Similarly, during the second episode of elevated acetate, methane production increased considerably, averaging 437 mL/L/day, before declining upon the termination of acetate addition (Fig. 3b). Notably, response of the pH in the anaerobic digesters to elevated acetate was minimal, fluctuating within a narrow range of 7.7–8.0 (Fig. 3a). These results indicate that despite the dramatic changes in acetate loading, the anaerobic digesters remained consistently stable with increased methane production upon exposure to elevated acetate.

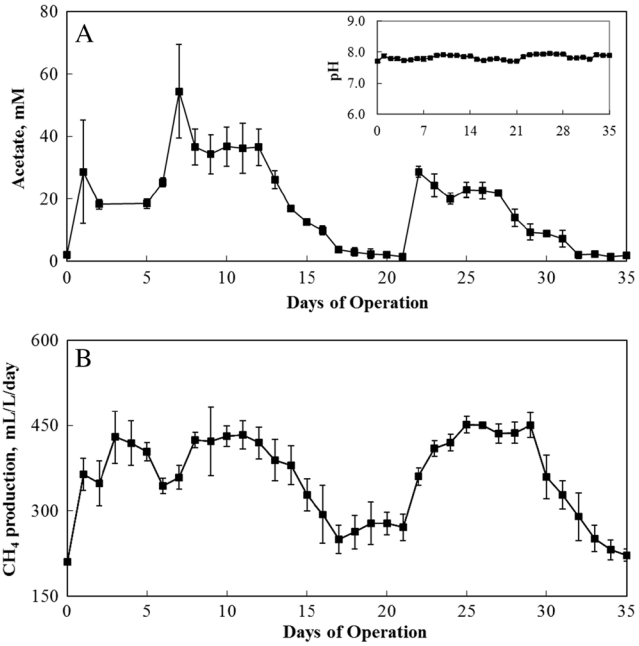

Fig. 3 Methane production **a** and acetate concentration **b** during two episodes of elevated acetate resulting from the addition of extraneous acetate to continuous anaerobic digesters treating animal wastewater. The *inset* shows the dynamics of pH during episodes of elevated acetate. Data points were means of triplicates with the *error bars* indicating the standard deviation

Response of acetoclastic methanogens to elevated acetate in continuous anaerobic digesters

While process performance was not affected by elevated levels of acetate, the acetoclastic methanogen populations, particularly *Methanosaeta*, were highly dynamic in response to the addition of extraneous acetate. Monitoring of the 16S rRNA gene copy number with qPCR showed that average *Methanosaeta* abundance rose sharply from the baseline level of 2.5×10^9 copies/mL before acetate addition to 9.0×10^9 copies/mL during the first episode of elevated acetate (Fig. 4). Following the pause in acetate addition and subsequent drop in acetate concentration back to the baseline level of approximately 1 mM (Fig. 3a), the abundance of *Methanosaeta* declined accordingly to the baseline level. The resumption of acetate addition for the second episode of elevated acetate resulted in the rebound of the *Methanosaeta* population abundance from the baseline level, similar to the trend observed during the first episode of elevated acetate. The reproducibility of the sharp increase in *Methansaeta* population abundance in response to elevated acetate indicates that the *Methanosaeta* populations in these anaerobic digesters were indeed very competitive at high acetate levels.

The growth of the other acetoclastic methanogen population, *Methanosarcina*, also responded positively to elevated acetate levels (Fig. 4), albeit at a smaller magnitude. The baseline abundance of *Methanosarcina* prior to acetate addition

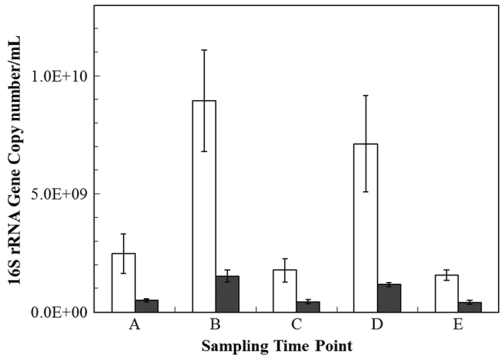

Fig. 4. Population abundance of *Methanosaeta* (*white*) and *Methanosarcina* (*black*), measured as 16S rRNA gene copy numbers by qPCR, in response to elevated acetate resulting from the addition of extraneous acetate to continuous anaerobic digesters treating animal wastewater. Description of the sampling points is provided in Supplementary Fig. S1. Data points were means of triplicates with the *error bars* indicating the standard deviation

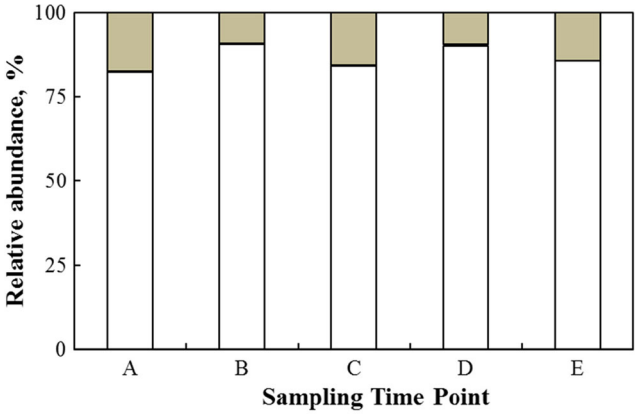

Fig. 5. Relative abundance of *Methanosaeta* (*white*) and *Methanosarcina* (*black*) in the archaeal community in the continuous anaerobic digesters in response to elevated acetate resulting from the addition of extraneous acetate to continuous anaerobic digesters treating animal wastewater. Results are from the high-throughput sequencing of 16S rRNA gene amplicon libraries. Description of the sampling time points is provided in Supplementary Fig. S1

averaged 5.0×10^8 copies/mL, which increased to 1.5×10^9 copies/mL during the first episode of elevated acetate. Subsequently, *Methanosarcina* abundance declined to the baseline level once acetate addition stopped and acetate concentration declined to the baseline level (Fig. 3a). As expected, *Methanosarcina* abundance increased again upon the resumption of acetate addition during the second episode of elevated acetate (Fig. 4). These results show that *Methanosarcina* populations favored high acetate concentrations similarly as *Methanosaeta* did.

Competitiveness of *Methanosaeta* over *Methanosarcina* at elevated acetate

It should be noted that the abundance of *Methanosaeta*, as monitored by qPCR, was much greater than that of *Methanosarcina* throughout the study period, irrespective if acetate concentration (Fig. 4). High-throughput sequencing of the microbial communities in the anaerobic digesters revealed that the relative abundance of *Methanosaeta* remained above 82% at all sampling points (Fig. 5), supporting the competitiveness of *Methanosaeta* over *Methanosarcina*. These results further illustrated that the dominance of *Methanosaeta* over *Methanosarcina* was augmented by elevated acetate. For example, the *Methanosaeta* to *Methanosarcina* ratio of 16S rRNA gene copy number averaged 4.1 at baseline acetate levels, which increased to 6.0 at elevated acetate levels (Fig. 6a), suggesting the greater competitiveness of *Methanosaeta* than *Methanosarcina* at higher acetate concentrations.

A closer examination of the relationship between acetate concentration and population abundance indicated that higher levels of acetate was associated with greater population abundance for both *Methanosaeta* and *Methanosarcina* (Fig. 6b). However, the slope of the linear regression between acetate concentration and population abundance was much greater for *Methanosaeta* than *Methanosarcina*, suggesting greater competitiveness of *Methanosaeta* over *Methanosarcina* with regard to acetate.

Discussion

Acetoclastic methanogenesis has been considered as the dominant pathway for microbial methane production in natural and engineered processes (Ferry 1992; Jetten et al. 1990). As the only genera representing acetoclastic methanogens, *Methanosarcina* and *Methanosaeta* have been studied extensively to elucidate the physiology of acetoclastic methanogenesis. Since growth kinetics are the key parameters for the control of methanogenic processes, studies have focused on the maximum growth rate, μ_{max}, and half-saturation coefficient, K_S, of *Methanosarcina* and *Methanosaeta*. The reported K_S values range from 0.36 to 1.2 mM for *Methanosaeta* and between 4.0 and 6.5 mM for *Methanosarcina* (Yilmaz et al. 2014). For μ_{max}, values in the literature vary from 0.2 to 1.5 d^{-1} for *Methanosarcina* and from 0.07 to 0.69 d^{-1} for *Methanosaeta* (Yilmaz et al. 2014). A general trend is that *Methanosaeta* populations tend to have lower values of μ_{max} and K_S than *Methanosarcina*.

According to these growth kinetics parameters, one could expect that *Methanosaeta* would be more competitive than *Methanosarcina* when acetate concentration is low, i.e., less than 1 mM, while *Methanosarcina* would outcompete *Methanosaeta* at higher acetate levels (De Vrieze et al. 2012; Smith and Ingram-Smith 2007). Indeed, *Methanosaeta* have been found to be the dominant acetoclastic methanogen populations in methanogenic treatment processes with stable

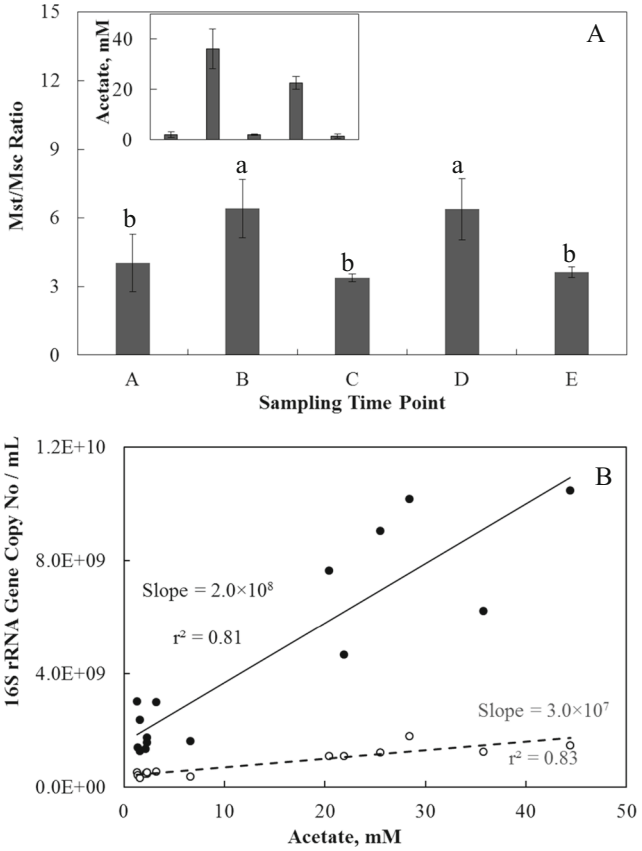

Fig. 6. Linkage between acetate concentration and abundance of *Methanosaeta* and *Methanosarcina* in continuous anaerobic digesters treating animal wastewater. **a** Correspondence between elevated acetate level (*inset*) and relative abundance of acetoclastic methanogens, measured as the *Methanosaeta* to *Methanosarcina* ratio of 16S rRNA gene copy numbers (Mst/Msc ratio); data points were means of triplicates with the *error bars* showing standard deviations. The means are not significantly different from each other in columns labeled with the same *italicized lowercase letters* (ANOVA, Tukey's HSD test, $p < 0.05$). **b** Linear regression of the abundance of *Methanosaeta* (*closed circle*) and *Methanosarcina* (*open circle*) with regard to acetate concentration

performance, which typically have acetate concentrations less than 1 mM (McHugh et al. 2003; Zheng and Raskin 2000). Accordingly, *Methanosarcina* populations were found to outcompete *Methanosaeta* at elevated acetate level characteristic of unstable methanogenic processes (De Vrieze et al. 2012; Ma et al. 2013). These observations are consistent with the growth characteristics expected from the reported kinetics parameters (Conklin et al. 2006; De Vrieze et al. 2012).

However, there are other studies of methanogenic treatment processes that have reported the dominance of *Methanosaeta* over *Methanosarcina* at elevated acetate concentrations when *Methanosarcina* would instead be expected to be more competitive (Chen and He 2015b; Franke-Whittle et al. 2014; Ito et al. 2011; Leite et al. 2015; Moertelmaier et al. 2014; Narihiro et al. 2015; van Haandel et al. 2014). Various process conditions, such as the lack of certain essential nutrients, have been suggested to be attributable to these seemingly inconsistent observations but without thorough validation. One possibility remained unexplored until this study was the potential divergence of the competitive relationship between uncharacterized populations of *Methanosaeta* and *Methanosarcina* from those reported in the literature.

In this study, elevated acetate levels, i.e., >20 mM, were established in both batch and continuous anaerobic digesters via organic overloading with animal waste (Fig. 1) or direct addition of acetate (Fig. 3). The dominance of *Methanosaeta* over *Methanosarcina* was confirmed at these elevated acetate levels in both batch and continuous operational modes, using three microbial community analysis tools—clone library, qPCR, and high-throughput sequencing (Figs. 2, 4 and 5). To the best of our knowledge, this study represents the first time that the competitiveness of *Methanosaeta* over *Methanosarcina* at elevated acetate concentrations was reproducibly demonstrated, providing much needed insight into the ecophysiology of acetoclastic methanogens. Given the importance of acetoclastic methanogenesis, findings from this study could have major implications for developing strategies to improve the performance of methanogenic treatment processes. Further efforts are needed to identify the genetic and physiological differences between *Methanosaeta* populations competitive at elevated acetate levels and those that are not. Equally important is the need to determine the distribution of these less characterized *Methanosaeta* populations capable of high activity at elevated acetate levels.

Acknowledgements This work was partly supported by a U.S. Environmental Protection Agency Grant XA-83539201 and the Foundation of Top Talents of Hebei Province, China Grant E2015100006. SC was partly supported by the Institute for a Secure and Sustainable Environment at the University of Tennessee, Knoxville.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Bridgham SD, Cadillo-Quiroz H, Keller JK, Zhuang Q (2013) Methane emissions from wetlands: biogeochemical, microbial, and modeling perspectives from local to global scales. Glob Chang Biol 19:1325–1346

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J 6:1621–1624

Chen S, He Q (2015a) Enrichment and specific quantification of *Methanocalculus* in anaerobic digestion. J Biosci Bioeng 120: 677–683

Chen S, He Q (2015b) Persistence of *Methanosaeta* populations in anaerobic digestion during process instability. J Ind Microbiol Biotechnol 42:1129–1137

Chen S, Zamudio Cañas EM, Zhang Y, Zhu Z, He Q (2012) Impact of substrate overloading on archaeal populations in anaerobic digestion of animal waste. J Appl Microbiol 113:1371–1379

Chen S, Zhu Z, Park J, Zhang Z, He Q (2014) Development of *Methanoculleus*-specific real-time quantitative PCR assay for assessing methanogen communities in anaerobic digestion. J Appl Microbiol 116:1474–1481

Cole JR, Chai B, Marsh TL, Farris RJ, Wang Q, Kulam SA, Chandra S, McGarrell DM, Schmidt TM, Garrity GM, Tiedje JM (2003) The ribosomal database project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic Acids Res 31:442–443

Conklin A, Stensel HD, Ferguson J (2006) Growth kinetics and competition between *Methanosarcina* and *Methanosaeta* in mesophilic anaerobic digestion. Water Environ Res 78:486–496

De Vrieze J, Hennebel T, Boon N, Verstraete W (2012) *Methanosarcina*: the rediscovered methanogen for heavy duty biomethanation. Bioresour Technol 112:1–9

DeLong EF (1992) Archaea in coastal marine environments. Proc Natl Acad Sci U S A 89:5685–5689

Demirel B, Scherer P (2011) Trace element requirements of agricultural biogas digesters during biological conversion of renewable biomass to methane. Biomass Bioenerg 35:992–998

Dolfing J (2014) Thermodynamic constraints on syntrophic acetate oxidation. Appl Environ Microbiol 80:1539–1541

Ferry JG (1992) Methane from acetate. J Bacteriol 174:5489–5495

Franke-Whittle IH, Walter A, Ebner C, Insam H (2014) Investigation into the effect of high concentrations of volatile fatty acids in anaerobic digestion on methanogenic communities. Waste Manag 34:2080–2089

He Q, Sanford RA (2002) Induction characteristics of reductive dehalogenation in the *ortho*-halophenol-respiring bacterium, *Anaeromyxobacter dehalogenans*. Biodegradation 13:307–316

He Q, Sanford RA (2004) Acetate threshold concentrations suggest varying energy requirements during anaerobic respiration by *Anaeromyxobacter dehalogenans*. Appl Environ Microbiol 70: 6940–6943

Ito T, Yoshiguchi K, Ariesyady HD, Okabe S (2011) Identification of a novel acetate-utilizing bacterium belonging to *Synergistes* group 4 in anaerobic digester sludge. ISME J 5:1844–1856

Jetten MSM, Stams AJM, Zehnder AJB (1990) Acetate threshold values and acetate activating enzymes in methanogenic bacteria. FEMS Microbiol Lett 73:339–344

Leite AF, Janke L, Lv Z, Harms H, Richnow H-H, Nikolausz M (2015) Improved monitoring of semi-continuous anaerobic digestion of sugarcane waste: effects of increasing organic loading rate on methanogenic community dynamics. Int J Mol Sci 16:23210–23226

Ma J, Zhao B, Frear C, Zhao Q, Yu L, Li X, Chen S (2013) *Methanosarcina* domination in anaerobic sequencing batch reactor at short hydraulic retention time. Bioresour Technol 137:41–50

McHugh S, Carton M, Mahony T, O'Flaherty V (2003) Methanogenic population structure in a variety of anaerobic bioreactors. FEMS Microbiol Lett 219:297–304

Moertelmaier C, Li C, Winter J, Gallert C (2014) Fatty acid metabolism and population dynamics in a wet biowaste digester during re-start after revision. Bioresour Technol 166:479–484

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

Penning H, Claus P, Casper P, Conrad R (2006) Carbon isotope fractionation during acetoclastic methanogenesis by *Methanosaeta concilii* in culture and a lake sediment. Appl Environ Microbiol 72:5648–5652

Schmidt T, Ziganshin AM, Nikolausz M, Scholwin F, Nelles M, Kleinsteuber S, Pröter J (2014) Effects of the reduction of the hydraulic retention time to 1.5 days at constant organic loading in CSTR, ASBR, and fixed-bed reactors–performance and methanogenic community composition. Biomass Bioenerg 69:241–248

Smith KS, Ingram-Smith C (2007) *Methanosaeta*, the forgotten methanogen? Trends Microbiol 15:150–155

Sun L, Müller B, Westerholm M, Schnürer A (2014) Syntrophic acetate oxidation in industrial CSTR biogas digesters. J Biotechnol 171:39–44

Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24:1596–1599

van Haandel A, De Vrieze J, Verstraete W, dos Santos VS (2014) *Methanosaeta* dominate acetoclastic methanogenesis during high-rate methane production in anaerobic reactors treating distillery wastewaters. J Chem Technol Biotechnol 89:1751–1759

Wolin EA, Wolin MJ, Wolfe RS (1963) Formation of methane by bacterial extracts. J Biol Chem 238:2882–2886

Yilmaz V, Ince-Yilmaz E, Yilmazel YD, Duran M (2014) Is aceticlastic methanogen composition in full-scale anaerobic processes related to acetate utilization capacity? Appl Microbiol Biotechnol 98:5217–5226

Yu Y, Lee C, Kim J, Hwang S (2005) Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol Bioeng 89:670–679

Zhang Y, He Q (2013) Characterization of bacterial diversity in drinking water by pyrosequencing. Water Sci Technol Water Supply 13:358–367

Zhang Y, Zhang X, Zhang H, He Q, Zhou Q, Su Z, Zhang C (2009) Responses of soil bacteria to long-term and short-term cadmium stress as revealed by microbial community analysis. Bull Environ Contam Toxicol 82:367–372

Zhang Y, Zamudio Canas EM, Zhu Z, Linville JL, Chen S, He Q (2011) Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. Bioresour Technol 102:779–785

Zheng D, Raskin L (2000) Quantification of *Methanosaeta* species in anaerobic bioreactors using genus- and species-specific hybridization probes. Microb Ecol 39:246–262

Zhu Z, Hsueh MK, He Q (2011) Enhancing biomethanation of municipal waste sludge with grease trap waste as a co-substrate. Renew Energy 36:1802–1807

Ziganshin AM, Schmidt T, Lv Z, Liebetrau J, Richnow HH, Kleinsteuber S, Nikolausz M (2016) Reduction of the hydraulic retention time at constant high organic loading rate to reach the microbial limits of anaerobic digestion in various reactor systems. Bioresour Technol 217:62–71