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

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## 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](https://doi.org/10.1007/s00253-016-7967-9)) contains supplementary material, which is available to authorized users.

## 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 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,  $\mu_{\max}$ , 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  $\mu_{\max}$  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 that 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<sub>2</sub>·6 H<sub>2</sub>O, 0.5 g; KH<sub>2</sub>PO<sub>4</sub>, 0.2 g; NH<sub>4</sub>Cl, 0.3 g; KCl, 0.3 g; CaCl<sub>2</sub>·2H<sub>2</sub>O, 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<sub>2</sub>·4H<sub>2</sub>O, 0.19 g of CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.1 g of MnCl<sub>2</sub>·4H<sub>2</sub>O, 70 mg of ZnCl<sub>2</sub>, 6 mg of H<sub>3</sub>BO<sub>3</sub>, 36 mg of Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O, 24 mg of NiCl<sub>2</sub>·6H<sub>2</sub>O, and 1.0 mg of CuCl<sub>2</sub>·2H<sub>2</sub>O. The selenium-tungsten solution contained 6 mg of Na<sub>2</sub>SeO<sub>3</sub> per liter, 8 mg of Na<sub>2</sub>WO<sub>4</sub>·2H<sub>2</sub>O per liter, and 0.54 g of NaOH per liter. L-cysteine (0.031 g/L) and Na<sub>2</sub>S·9 H<sub>2</sub>O (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<sub>2</sub> atmosphere. NaHCO<sub>3</sub> (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<sub>2</sub>/CO<sub>2</sub> gas mix, and the pH was adjusted to 7.0 ± 0.1 by varying the CO<sub>2</sub> composition of the gas mix. The medium was subsequently dispensed into serum bottles flushed with pure N<sub>2</sub> 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  $-80\text{ }^{\circ}\text{C}$  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- $\mu\text{L}$  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  $\mu\text{L}$  DNA sample. Thermal cycling consisted of a starting incubation at  $50\text{ }^{\circ}\text{C}$  for 2 min and an initial denaturation at  $95\text{ }^{\circ}\text{C}$  for 10 min, followed by up to 45 cycles at  $95\text{ }^{\circ}\text{C}$  for 30 s, and  $60\text{ }^{\circ}\text{C}$  (for total *Archaea*) or  $57\text{ }^{\circ}\text{C}$  (for *Methanosaeta*) or  $61\text{ }^{\circ}\text{C}$  (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 <i>E. coli</i> No.	Amplification Size (bp)
	Forward Primer			
<i>Archaea</i>	Arc-787F	ATTAGATACCCSBGTAGTCC	787-806	273
	Probe			
	Arc-915P	AGGAATTGGCGGGGGAGCAC	915-934	
	Reverse Primer			
	Arc-1059R	GCCATGCACCCWCCTCT	1044-1059	
	Forward Primer			
<i>Methanosaeta</i>	Mst-702F	TAATCCTTGAAGGACCACCA	702-721	161
	Probe			



Target	Primer/probe	Sequence (5'-3')	Position <i>E. coli</i> No.	Amplification Size (bp)
	Mst-753P	ACGGCAAGGGACGAAAGCTAGG	753-774	
	Reverse Primer			
	Mst-862R	CCTACGGCACCGACAAC	846-862	
	Forward Primer			
	Msc-586F	CGGTTTGGTCAGTCCTCCG	586-604	
	Probe			
<i>Methanosarcina</i>	Msc-743P	AACGGGTTCGACGGTGAGGGAC GA	743-766	257
	Reverse Primer			
	Msc-842R	ACCAGACACGGTCGCGC	826-842	

<sup>a</sup>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<sub>2</sub>SO<sub>4</sub> 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<sub>2</sub>SO<sub>4</sub> by adding 25 µL of 2 N H<sub>2</sub>SO<sub>4</sub> 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).

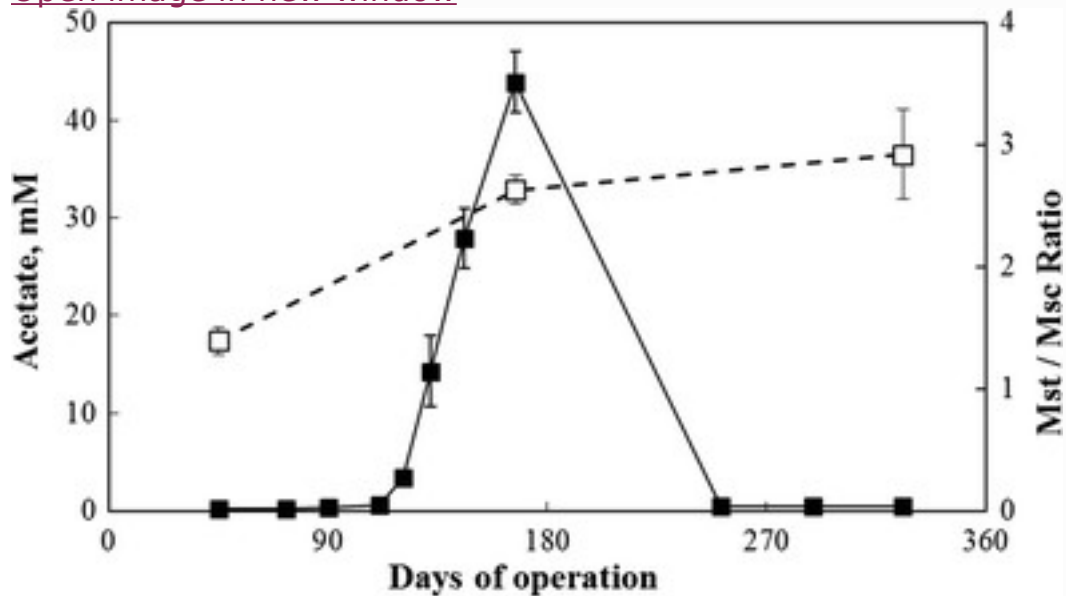
## Results

### 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<sub>s</sub>* 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.

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**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

However, when acetate reached  $44 \pm 3$  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).

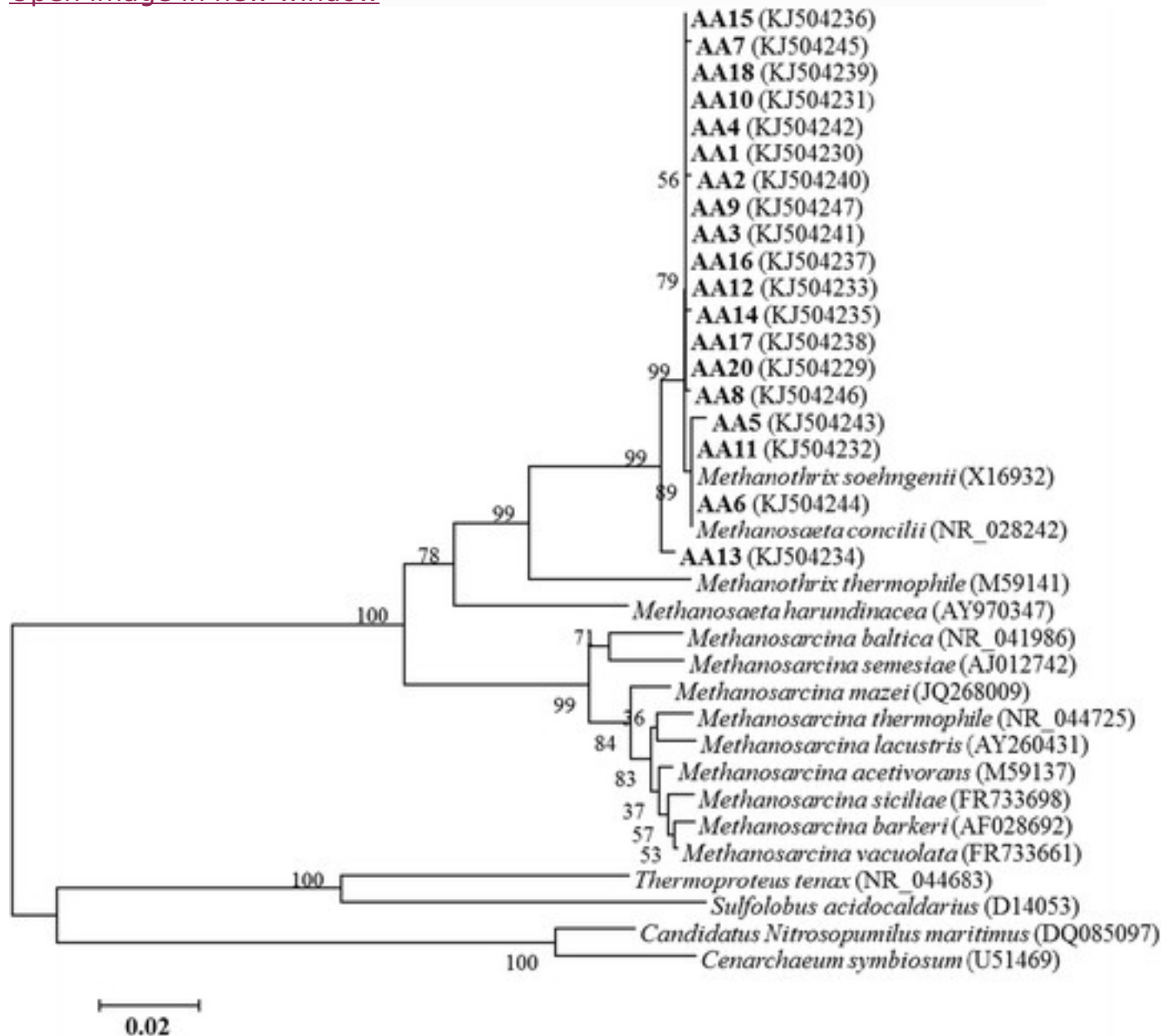
### Enrichment of *Methanosaeta* at elevated acetate levels

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 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  $\mu_{max}$  values reported for *Methanosarcina*, ranging from 0.2 to 1.5 d<sup>-1</sup>,

than those of *Methanosaeta*, typically between 0.07 to 0.69 d<sup>-1</sup>, *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.

[Open image in new window](#)



**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

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.

**Table 2.**

qPCR quantification of acetoclastic methanogens enriched by acetate

<b>16S rRNA Gene target</b>	<b>No. of copies/mL<sup>a</sup></b>	<b>Relative abundance (% of total Archaea)<sup>a</sup></b>
<i>Methanosaeta</i>	$(8.9 \pm 0.7) \times 10^8$	$80.4 \pm 13.5$
<i>Methanosarcina</i>	$(3.6 \pm 0.9) \times 10^6$	$0.3 \pm 0.0$
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

<sup>a</sup>Values are the means of triplicates  $\pm$  standard deviation

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).

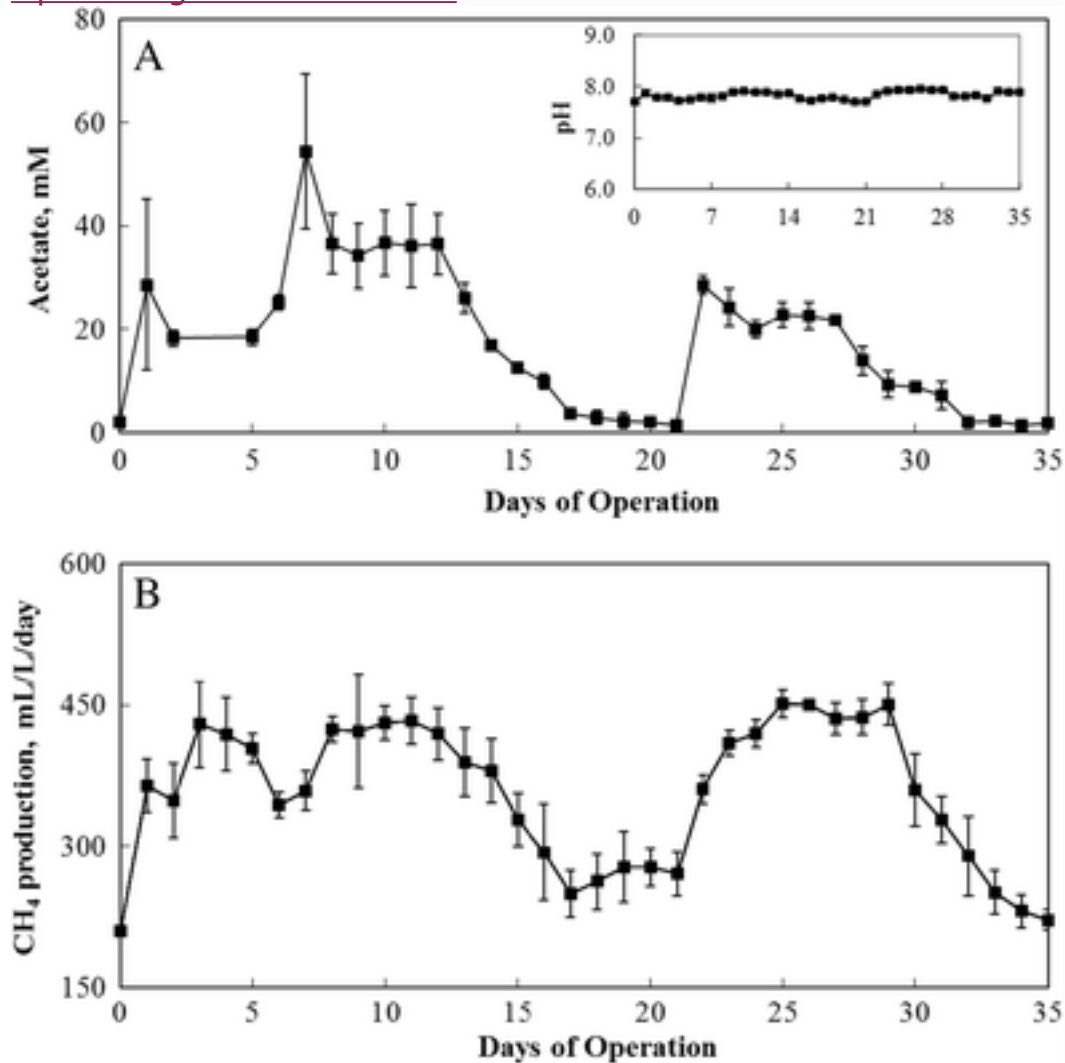
During the first episode of elevated acetate, with the increases in acetate loading rate, acetate concentration rose above 20 mM but with large



fluctuations, with the highest level reaching  $54.4 \pm 15.0$  mM (Fig. [3a](#)). When acetate loading rate reached the maximum, the acetate level in the anaerobic digesters stabilized between  $34.2 \pm 6.2$  and  $36.6 \pm 5.7$  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](#)).



[Open image in new window](#)



**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

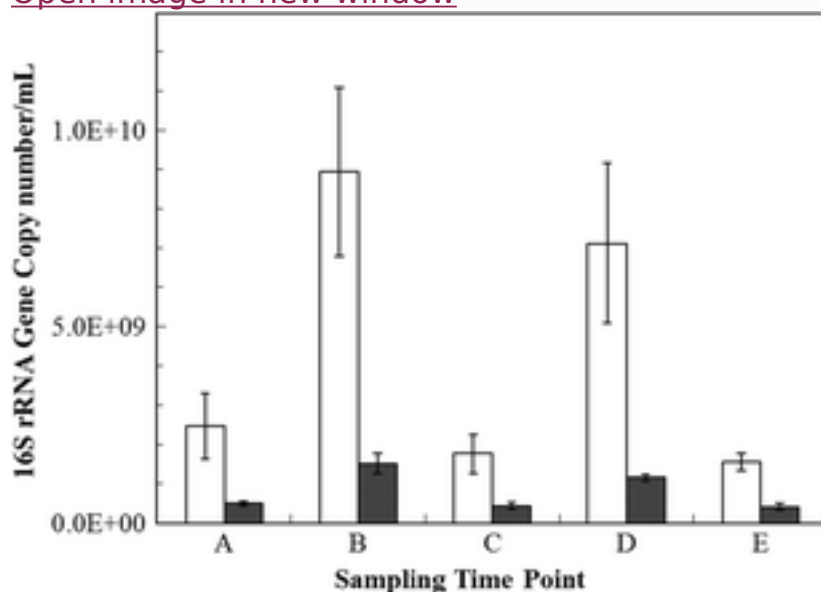
Corresponding to the increases in acetate loading, methane production rose significantly (Fig. [3b](#)). When acetate level was kept above 30 mM during the first episode of elevated acetate, the methane production rate averaged  $426 \pm 6$  mL/L/day, significantly higher than the average of  $204 \pm 3$  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.

### 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  $2.5 \times 10^9$  copies/mL before acetate addition to  $9.0 \times 10^9$  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 *Methanosaeta* population abundance in response to elevated acetate indicates that the *Methanosaeta* populations in these anaerobic digesters were indeed very competitive at high acetate levels.

[Open image in new window](#)



**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

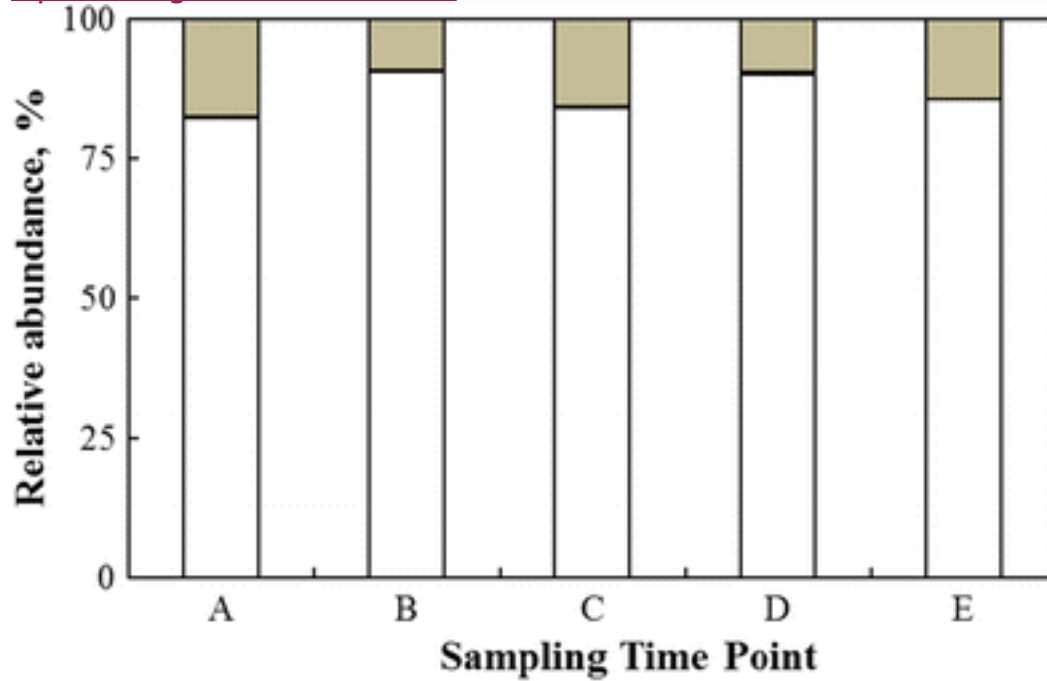
points is provided in Supplementary Fig. [S1](#). Data points were means of triplicates with the *error bars* indicating the standard deviation

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 averaged  $5.0 \times 10^8$  copies/mL, which increased to  $1.5 \times 10^9$  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

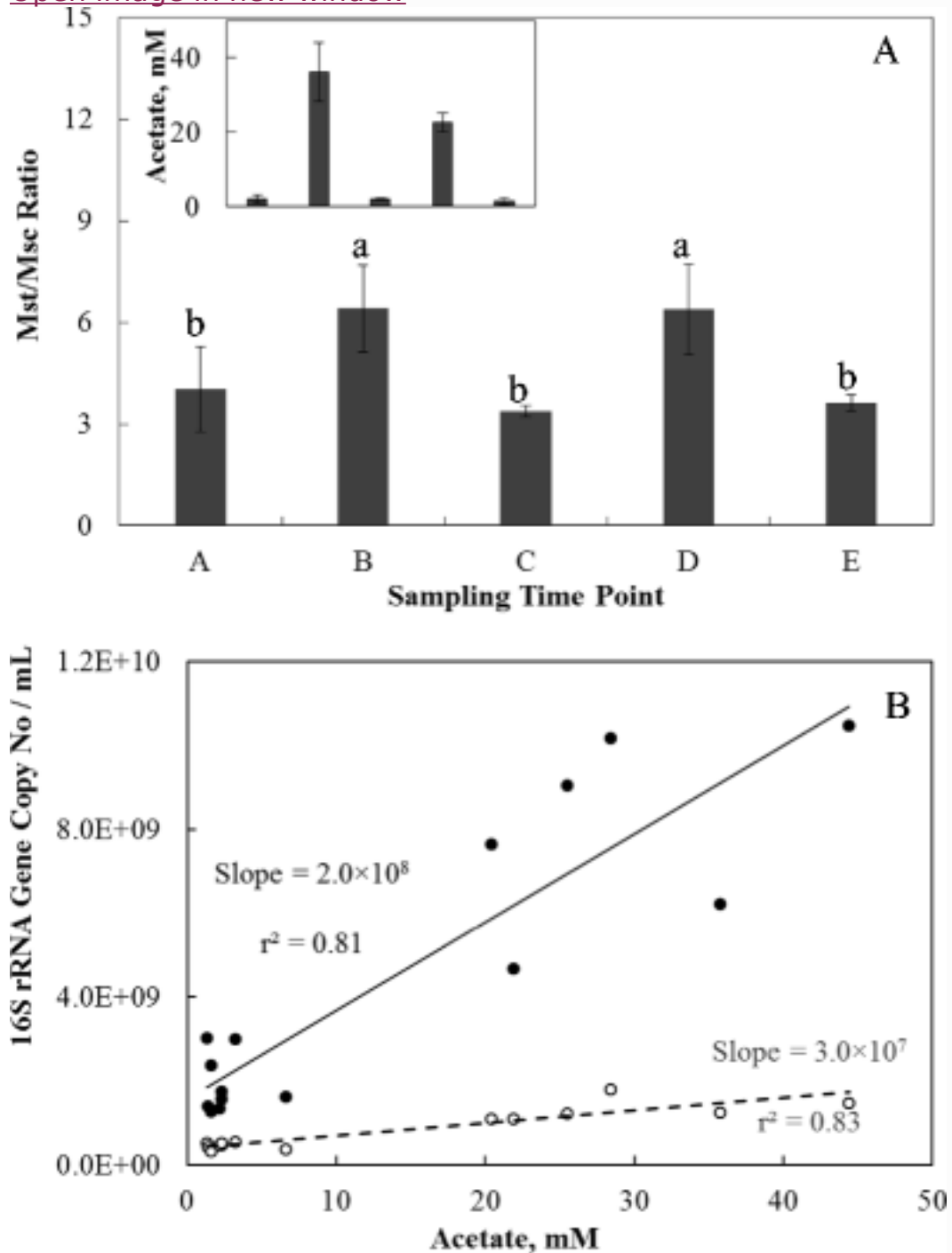
It should be noted that the abundance of *Methanosaeta*, as monitored by qPCR, was much greater than that of *Methanosarcina* throughout the study period, irrespective of acetate concentration (Fig. [4](#)). High-throughput sequencing of the microbial communities in the anaerobic digesters revealed that 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.

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**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](#)

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**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

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,  $\mu_{\max}$ , 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  $\mu_{\max}$  values in the literature vary from 0.2 to 1.5 d<sup>-1</sup> for *Methanosarcina* and from 0.07 to 0.69 d<sup>-1</sup> for *Methanosaeta* (Yilmaz et al. 2014). A general trend is that *Methanosaeta* populations tend to have lower values of  $\mu_{\max}$  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 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.

## Notes

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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.

## Supplementary material

[253\\_2016\\_7967\\_MOESM1\\_ESM.pdf](#) (64 kb)

ESM 1 (PDF 64 kb)

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