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Persistence of *Methanosaeta* populations in anaerobic digestion during process instability

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Abstract Anaerobic digestion is a sustainable technology for the treatment of organic waste and production of biogas. Acetoclastic methanogenesis accounts for the majority of methane production in anaerobic digestion. Therefore, sustaining robust acetoclastic methanogens is important for stable process performance. Due to faster growth kinetics at high acetate concentrations, it has been considered that *Methanosarcina* would be more prevalent than *Methanosaeta* in unstable anaerobic digestion processes which frequently experience high acetate levels. Methanogen population dynamics were monitored in multiple continuous anaerobic digesters for 500 days. Results from quantitative polymerase chain reaction analysis show that *Methanosaeta* dominated over *Methanosarcina* in anaerobic digestion at high acetate levels up to 44 mM, suggesting the potential of *Methanosaeta* as a robust and efficient acetoclastic candidate for resilient anaerobic methane conversion. Further efforts are needed to identify mechanisms contributing to the unexpected competitiveness of these methanogens at high acetate levels observed in this study.

Keywords Anaerobic digestion · Acetate · Methane · *Methanosaeta* · *Methanosarcina*

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Introduction

Anaerobic digestion has been long established as a sustainable technology for the treatment of organic waste and production of biogas as a renewable energy source [21]. The broader application of this technology requires improvement in process efficiency and stability to further enhance the technical and economic feasibility of anaerobic digestion. Given that anaerobic digestion is a predominantly microbial process, considerable research efforts have been devoted to understanding the microbial communities underlying the multitude of biochemical reactions involved in anaerobic digestion. Logically, methanogens have been the focus of these efforts as the key microbial populations directly responsible for methane production.

It is well known that hydrogenotrophic methanogenesis and acetoclastic methanogenesis are the two primary pathways of methane formation in anaerobic digestion, with the latter generally considered to be attributable for the majority of methanogenesis in anaerobic digestion processes [6, 14]. Despite the diversity of methanogens, *Methanosaeta* and *Methanosarcina* are the only two genera recognized as acetoclastic methanogens capable of methane production from acetate. However, *Methanosaeta* and *Methanosarcina* have been shown to differ significantly in certain physiological traits. While *Methanosaeta* is strictly acetoclastic using acetate as the only substrate for methane production [16], *Methanosarcina* is more versatile, capable of all three pathways of methane production, i.e., hydrogenotrophic, acetoclastic, and methylotrophic methanogenesis, using more diverse substrates [5]. Compared with *Methanosarcina*, *Methanosaeta* populations are known to have lower maximum specific growth rates (μ_{max}) and half saturation concentrations (K_S) for growth on acetate, indicative of the

higher affinity of *Methanosaeta* to acetate [4, 5]. Because of the divergence in acetate-dependent growth kinetics between *Methanosaeta* and *Methanosarcina*, it is commonly considered that *Methanosaeta* populations would be more kinetically competitive and abundant than *Methanosarcina* populations in environments with low acetate concentration (<1 mM), which is typical in anaerobic digestion processes with stable performance [4, 10, 11]. In contrast, *Methanosarcina* populations are expected to be more predominant than *Methanosaeta* populations at higher acetate levels, which are frequently encountered in unstable anaerobic digesters with organic acids accumulation [4, 5].

However, these patterns were not evident in continuous anaerobic digesters previously established for the treatment of animal waste, where *Methanosaeta* populations appeared to be more abundant than *Methanosarcina* populations during both stable and unstable operations with clone library analysis [2, 26]. In order to better understand this apparent inconsistency with the kinetic characteristics of *Methanosaeta* and *Methanosarcina*, in this study, the population dynamics of both populations were more frequently monitored using quantitative polymerase chain reaction (qPCR) assays at 15 time points throughout an episode of process perturbation and subsequent recovery. Results from this study show that *Methanosaeta* populations were able to dominate the acetoclastic methanogen communities at acetate concentrations as high as 44 mM (2.6 g/L) in anaerobic digesters treating animal waste, suggesting the need for more efforts to understanding the physiology of *Methanosaeta* as robust methanogen populations.

Materials and methods

Operation and sampling of anaerobic digesters

One set of triplicate mesophilic continuous anaerobic digesters was established as controls with dairy waste as the sole substrate and all operational conditions were kept constant in the control digesters throughout the study period. Another set of triplicate anaerobic digesters were initially set up using dairy waste as the sole substrate similarly as the control digesters. However, this set of anaerobic digesters, referred to as treatment digesters hereafter, were subsequently subjected to stepwise increases in organic loading rate (OLR) with the addition of poultry waste to the feedstock as an organic-rich co-substrate as previously described [26]. The increases in OLR eventually resulted in unstable performance in the treatment digesters characterized by the accumulation of organic acids and reduced methane production. Biomass samples were taken from both sets of anaerobic digesters, throughout unstable operation and the period when stable operation was restored, for the monitoring of specific methanogen populations in response to changes in process conditions using qPCR.

All six anaerobic digesters had a working volume of 3.6 L and were operated in a constant temperature room at 35 °C. The loading rate of dairy waste was kept constant for all six digesters at 1.0 g volatile solids (VS)/L/d. During normal operation, the digesters were fed at 4-h intervals and the hydraulic retention time was maintained at 20 days. After reaching stable process performance, poultry waste was added as the co-substrate into treatment digesters at the loading rate of 0.3 gVS/L/day, which was subsequently raised stepwise to 0.5 and 0.8 gVS/L/day, with the latter corresponding to a total OLR of 1.8 gVS/L/day accounting for both dairy and poultry wastes (Fig. 1). As a result of the elevated OLR, stable performance in the treatment digesters was disrupted as indicated by the accumulation of organic acids and reduction in methane production. Feeding had to be stopped for 21 days to prevent the complete collapse of process performance due to further accumulation of organic acids and inhibition of methanogenesis. To re-establish stable performance in treatment digesters, feeding was later restored stepwise to 0.5, and 1.0 gVS/L/day using dairy waste as the only substrate. Subsequently, poultry waste was introduced again as the co-substrate into treatment digesters to raise the total OLR from 1.0 to 1.5 gVS/L/day, while maintaining stable performance in the treatment digesters. Despite the changes in the treatment digesters, the control digesters were subjected to the constant feeding rate at 1.0 gVS/L/day and exhibited stable performance throughout the study period.

Biomass samples were taken from both control and treatment digesters at 15 time points representing stable performance before stepwise increases in OLR, unstable performance following increases in OLR, and the recovery of stable performance in treatment digesters, which corresponding to OLR ranging from 0.5 to 1.8 gVS/L/day. The samples were pelleted by centrifugation and preserved at −80 °C until analysis.

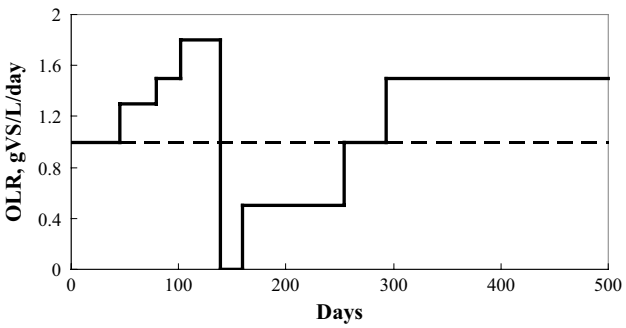

Fig. 1 Organic loading rate (OLR) applied in the control digesters (*dash line*) and treatment digesters (*solid line*) during the study period

Table 1 Characteristics of TaqMan qPCR primer/probe sets used in this study

Primer/probe name[a]	Target	Sequence (5′–3′)	Position *E. coli* no	T_m^b (°C)	GC (%)	Amplicon size (bp)	References
Arc-787F(F)	*Archaea*	ATTAGATACCCSBGTAGTCC	787–806	61.0	45	273	[23]
Arc-915P(P)		AGGAATTGGCGGGGGAGCAC	915–934	70.1	65		
Arc-1059R(R)		GCCATGCACCWCCTCT	1044–1059	62.3	63		
Mst-702F(F)	*Methanosaeta*	TAATCCTTGAAGGACCACCA	702–721	61.0	45	161	[23]
Mst-753P(P)		ACGGCAAGGGACGAAAGCTAGG	753–774	70.0	59		
Mst-862R(R)		CCTACGGCACCGACAAC	846–862	62.0	65		
Msc-586F(F)	*Methanosarcina*	CGGTTTGGTCAGTCCTCCG	586–604	61.6	63	257	This study
Msc-743P(P)		AACGGGTTCGACGGTGAGGGACGA	743–766	70.6	63		
Msc-842R(R)		ACCAGACACGGTCGCGC	826–842	59.8	71		
Mc-274F(F)	*Methanoculleus*	GGAGCAAGAGCCCGGAGT	274–291	60.8	67	223	[3]
Mc-361P(P)		CGTGATAAGGGAACCTCGAGTGCCT	361–385	69.1	56		
Mc-477R(R)		CCAATAAAAGTGGCCACCACT	477–497	59.5	48		

[a] Designations in the parentheses: *F* forward primer, *R* reverse primer, and *P* probe

[b] Melting temperature estimated using the Oligo Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html)

Chemical analysis

Production of biogas and accumulation of volatile fatty acids (VFAs) in the anaerobic digesters were used as the primary parameters to assess the performance of anaerobic digestion. Biogas production was determined using a previously described water displacement method [28]. Methane content in biogas was analyzed using a Hewlett Packard 5890 gas chromatograph (Agilent Technologies, Santa Clara, California, USA) equipped with a thermal conductivity detector (TCD) and a Supelco packing column (60/80 Carbonxen®-1000; Sigma-Aldrich, St Louis, Missouri, USA). Argon was used as the carrier gas with a flow rate of 5.0 ml/min and with the following temperature scheme: oven 125 °C, injection port 150 °C, and detector 170 °C. VFAs were quantified with a Agilent 1200 series High-Performance Liquid Chromatography (Agilent Technologies, Santa Clara, California, USA) equipped with a Bio-Rad Aminex HPX-87H ion exclusion column (Bio-Rad, Hercules, California, USA) as previously described [8].

TaqMan qPCR analysis

Genus-specific TaqMan qPCR assays were used to quantify major populations of acetoclastic methanogens, i.e., *Methanosarcina* and *Methanosaeta*, as well as hydrogenotrophic methanogens of the genus *Methanoculleus*. A domain-specific TaqMan qPCR assay was also performed to quantify total archaeal populations, which was used to determine the relative abundance of individual methanogen populations in the entire archaeal community. All qPCR assays used published TaqMan primer/probe sets except that for *Methanosarcina* (Table 1). A careful examination of existing *Methanosarcina*-specific TaqMan primer/probe sets revealed relatively low coverage and an improved *Methanosarcina*-specific primer/probe set was subsequently designed (Table 1).

The specificity and efficiency of all primer/probe sets used in this study were further validated as previously described [3]. DNA templates used as the standards for validation were 16S rRNA genes of specific archaeal populations cloned from the anaerobic digesters of this study, including *Methanosaeta* (GenBank Accession No. JN052761), *Methanosarcina* (GenBank Accession No. JN052757), and *Methanoculleus* (GenBank Accession No. JN052755). Amplification efficiency (*E*) of the qPCR assay was determined with the threshold cycle (C_T)-Log[Template] plot derived from the quantification of tenfold dilution series of 16S rRNA gene templates.

For all TaqMan qPCR assays, the primers and dual-labeled TaqMan probe, 5′-end labeled with 6-carboxyfluorescein (FAM) and 3′-end labeled with the Black Hole Quencher (BHQ), were purchased from Biosearch Technologies (Novato, California, USA). Whole community DNA was extracted and purified as previously described [25]. All qPCR assays were performed in 25-μL reaction volume with 15 pmol of the primers, 5 pmol of the probe, and Brilliant II QPCR Master Mix (Agilent, Santa Clara, California, USA). Thermal cycling included 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 all primer/probe sets) for 45 s. The qPCR procedure was performed with a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA) as previously described [3]. Gene copy numbers were determined from standard curves based on the log transformation of known concentrations versus C_T.

Statistical analysis

To evaluate anaerobic digestion performance with parameters including methane production, acetate concentration, and relative abundance of specific methanogen populations, the differences in these performance parameters under three operational conditions, i.e., treatment digesters with low acetate, treatment digesters with high acetate, as well as the controls, were analyzed using one-way analysis of variance (ANOVA). Significant differences in performance parameters between different operational conditions were indicated by a probability value (p) less than 0.05 in ANOVA analysis with Tukey's HSD test. Correlation between the relative abundance of *Methanosaeta* and *Methanosarcina* was 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. All statistical analyses were performed with JMP Pro 10.0 for Windows (SAS Institute Inc., Cary, North Carolina, USA).

Results and discussion

Performance of anaerobic digesters

The control digesters were subjected to a constant OLR at 1.0 gVS/L/day throughout the study period (Fig. 1). Accordingly, the control digesters maintained stable performance during the study period as indicated by the steady volumetric methane production rate averaging 206 ± 17 mL/L/day and low acetate concentration averaging 0.33 ± 0.15 mM (Fig. 2).

In contrast, the treatment digesters experienced higher OLR with stepwise increases to 1.3, 1.5, and 1.8 gVS/L/day following 44, 77, and 99 days of operation, respectively (Fig. 1). As expected, the OLR increases in the treatment digesters steadily boosted methane production, which peaked at 418 ± 9 mL/L/day on day 111 after the OLR was raised to 1.8 gVS/L/day (Fig. 2). During this period, the treatment digesters achieved relatively stable performance as suggested by the low acetate levels ranging between 0.22 and 0.57 mM, which were similar to those in the control digesters. However, process performance in the treatment digesters rapidly deteriorated consequently, with methane production dropping to 84 ± 4 mL/L/day and acetate concentration spiking to 44 mM, indicative of organic acids accumulation and process imbalance (Fig. 2). Evidently, substrate overloading led to increasing inhibition of methanogenesis and acetate utilization.

As a result, the treatment digesters underwent a period of unstable performance characterized by impaired methane production and elevated levels of acetate, which was reversed by lowering the OLR (Fig. 1). Following the stabilization of process performance in the treatment digesters, OLR was restored stepwise from 0 to 0.5, 1.0, and finally 1.5 gVS/L/day on day 295 to re-establish stable operation. Indeed, in the last 176 days of the study period, the treatment digesters experienced stable operation with methane production averaging 298 ± 39 mL/L/day and acetate concentration averaging 0.57 ± 0.14 mM, evidence that the accumulation of organic acids had subsided (Fig. 2).

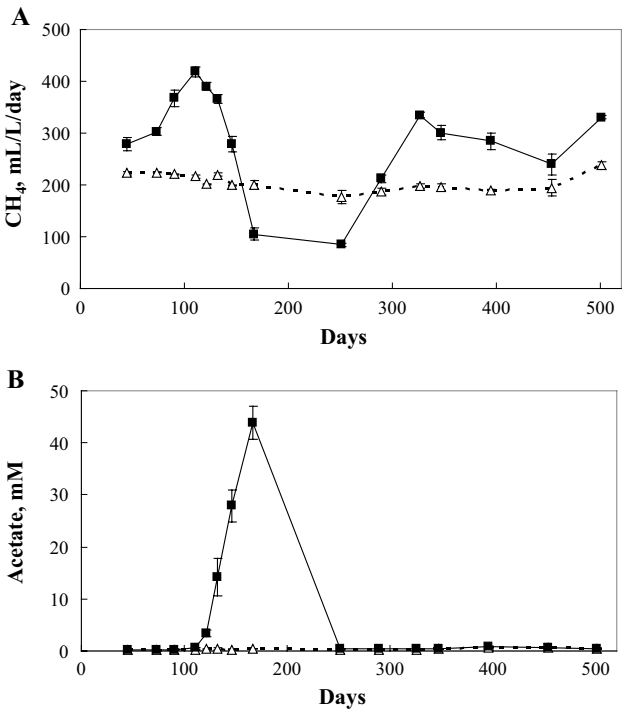

Fig. 2 Methane production (**a**) and acetate concentration (**b**) in control digesters (*dashed line with open triangle*) and treatment digesters (*solid line with filled square*). *Data points* were means of triplicates with the *error bars* showing standard deviations

Thus, during the study period, the methanogen populations in the treatment digesters were exposed to alternating conditions between stable process performance characterized by low acetate levels less than 1 mM and unstable process performance represented by high acetate levels greater than 40 mM, providing contrasting acetate concentrations to compare the competitiveness of acetoclastic methanogen populations of divergent kinetics for acetate utilization.

Validation of genus-specific qPCR assays for methanogens

Amplification efficiency, specificity, and reproducibility were the primary considerations in the validation of qPCR assays used in this study (Table 1). Amplification efficiency was evaluated experimentally using tenfold serial dilutions

Table 2 Validation of TaqMan qPCR assays used in this study

16S rRNA gene template/target	Template concentration (copy/μL)	R^{2a}	Slope[a]	Amplification efficiency[b] (%)
Archaea	8.1×10^2–8.1×10^9	0.995	−3.548	91.4
Methanosaeta	3.6×10^2–3.6×10^9	0.996	−3.382	97.6
Methanosarcina	5.6×10^2–5.6×10^9	0.988	−3.351	98.8
Methanoculleus	5.5×10^2–5.5×10^9	0.994	−3.490	93.4

[a] Coefficient of determination (R^2) and slope were determined with C_T-Log[Template] regression using data from qPCR quantification of ten-fold dilution series of 16S rRNA gene templates

[b] Amplification efficiency was calculated using the formula $[10^{(-1/\text{slope})} - 1] \times 100\ \%$

of 16S rRNA gene templates representing the genera of *Methanosaeta*, *Methanosarcina*, and *Methanoculleus*. Amplification with the primer/probe sets used in this study was highly reproducible with strong correlation ($R^2 > 0.98$) between template concentration and C_T (Table 2). The amplification efficiency for all assays ranged between 91 and 98 %, which are considered to be feasible for qPCR quantifications [24].

The specificity of the qPCR assays used in this study was also evaluated. Each genus-specific qPCR assay was tested with 16S rRNA gene templates representing *Methanosarcina*, *Methanosaeta*, and *Methanoculleus*. Amplification was only observed for DNA templates, correctly targeted by the corresponding genus-specific qPCR assay and no cross-amplification was detected after 45 PCR cycles (data not shown), demonstrating the specificity of the genus-specific qPCR Assays.

Dynamics of methanogen populations in control digesters

In the control digesters, qPCR results show that the relative abundance of all three methanogen populations monitored in this study, i.e., *Methanosaeta*, *Methanosarcina*, and *Methanoculleus*, remained steady with only marginal fluctuations (Fig. 3a), consistent with the stable process performance expected in the control digesters (Fig. 2). *Methanosaeta* populations constituted the dominant methanogens, averaging greater than 52 % of the archaeal community (Fig. 3a). In fact, the relative abundance of *Methanosaeta* remained above 40 % throughout the study period.

In contrast, *Methanosarcina* populations averaged less than 14 % of the archaeal community and never exceeded 20 % of the archaeal community, which was considerably less abundant than *Methanosaeta* (Fig. 3a). The dominance of *Methanosaeta* over *Methanosarcina* in the stable control digesters was similar to the observations made in previous studies of anaerobic digestion processes with stable performance [6, 10, 27].

The only hydrogenotrophic methanogens monitored in this study were populations associated with the genus

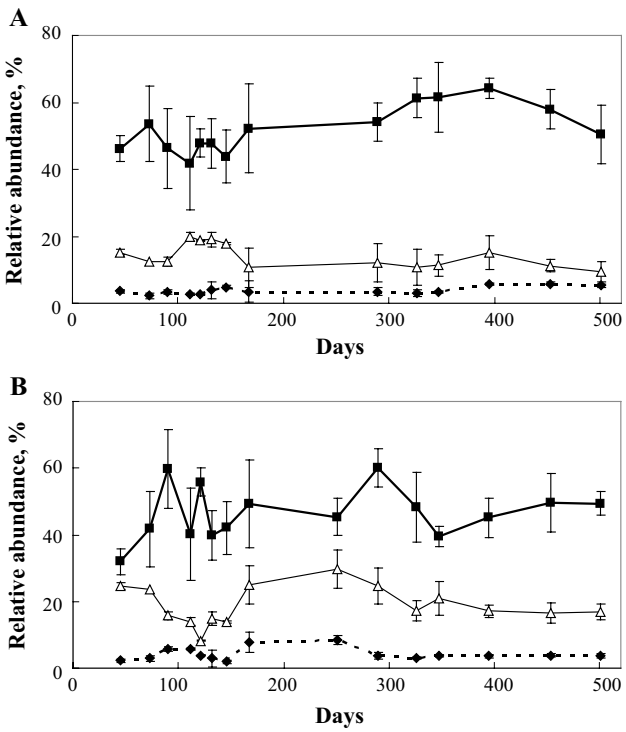

Fig. 3 Relative abundance of *Methanosaeta* (Mst), *Methanosarcina* (Msc), and *Methanoculleus* (Mc) in archaeal communities as determined by qPCR quantification of 16S rRNA gene copy numbers in control digesters (**a**) and treatment digesters (**b**). *Data points* were means of triplicates with the *error bars* showing standard deviations. Mst *solid line with filled square*, Msc *solid line with open triangle*, and Mc *dashed line with filled diamond*

Methanoculleus, which was present as a minor constituent of the methanogen community, representing 3.8 % of the archaeal community on average in the control digesters (Fig. 3a). Since *Methanoculleus* is only one of many genera of hydrogenotrophic methanogens, the low relative abundance of *Methanoculleus* did not necessarily suggest the low activity of hydrogenotrophic methanogenesis. Indeed, the sum of the relative abundance of acetoclastic methanogens *Methanosaeta* and *Methanosarcina* ranged between 60 and 73 % in the control digesters (Fig. 3a).

Thus, non-*Methanoculleus* hydrogenotrophic methanogens could be significant constituents of the rest of the archaeal community.

Dynamics of methanogen populations in treatment digesters

Despite an episode of unstable process performance in the treatment digesters (Fig. 2), the distribution of methanogen populations in the treatment digesters was similar to that in the control digesters, with *Methanosaeta* being the dominant methanogen population throughout the study period. A careful examination of qPCR results revealed that the abundance of *Methanosaeta* was more dynamic in the treatment digesters than that in the control digesters (Fig. 3b). Averaging 47 % of the archaeal community in the treatment digester, the relative abundance of *Methanosaeta* fluctuated between 32 and 60 %, which was greater than the range of 42–64 % observed for *Methanosaeta* in the control digesters. Contrasting the range of population abundance of *Methanosaeta* between the controls and treatments, it appeared that *Methanosaeta* became comparatively less abundant in treatment digesters than controls, despite remaining as the dominant methanogen.

A similar trend was also found for *Methanosarcina*, which remained consistently less abundant than *Methanosaeta* (Fig. 3b). The relative abundance of *Methanosarcina* varied between 8 and 30 % of the archaeal community in the treatment digesters, which was greater than the range of 9–20 % determined for *Methanosarcina* in the controls. Evidently, the population abundance of *Methanosarcina* increased, at least at some time points, in the treatment digesters as compared with the control digesters. Nonetheless, *Methanosarcina* was not able to outcompete *Methanosaeta* during the prolonged period of unstable process performance with high acetate concentration. The relative abundance of *Methanoculleus* was found to be higher at some time points in the treatment digesters than the controls, as shown by the greater range of *Methanoculleus* abundance in the treatment digesters (1.9–8.4 %) than in the controls (2.2–5.8 %).

The dominance of *Methanosaeta* over *Methanosarcina* in the treatment digesters was surprising, as *Methanosaeta* is considered to be less competitive than *Methanosarcina* in unstable processes, where higher acetate concentration would kinetically favor the dominance of *Methanosarcina* [4, 5, 11]. Thus, the continued dominance of *Methanosaeta* in the unstable treatment digesters throughout the study period was inconsistent with known kinetic characteristics of acetoclastic methanogens. The availability of trace metals may influence the growth kinetics of methanogens. Since trace metals such as iron, nickel, and cobalt are typically present in dairy manure at levels within the optimal concentration ranges for the anaerobic digestion of animal waste [1, 7, 13], it is likely that the impact of trace metals on methanogen growth was not substantial. The addition of nitrogen-rich poultry waste suggests the potential impact of ammonia toxicity on the growth kinetics of methanogens. At the highest organic loading rate in this study, i.e., 1.8 gVS/L/day with the addition of poultry waste, the total ammonia concentration reached the highest level of 1900 mg/L, which remained well below the inhibition thresholds around 7000 and 3000 mg/L for *Methanosarcina* and *Methanosaeta*, respectively [5]. Furthermore, *Methanosaeta* has been found to be more sensitive to ammonia toxicity than *Methanosarcina* [5]. Therefore, ammonia toxicity is not likely to have contributed to the competitiveness of *Methanosaeta* in these anaerobic digesters.

Given the importance of acetate in acetoclastic methanogenesis, the relationship between acetate concentration and the abundance of *Methanosaeta*/*Methanosarcina* was further examined.

Impact of acetate concentration on methanogen abundance

A close examination of process parameters showed that process performance in the treatment digesters consisted of distinct phases with significantly different levels of acetate (Fig. 4a), which was the primary VFA constituent detected in the digesters. The high acetate phase included a time period between day 132 and 167. The average acetate level during the high acetate phase reached 28.7 mM, significantly higher than that of 0.70 mM during the low acetate phase representing the rest of the study period (Fig. 4a). During the high acetate period, methane production declined rapidly from 366 ± 9–167 ± 11 mL/L/day (Fig. 2a), indicative of the onset of unstable operation. During the low acetate phase of the treatment digesters, the acetate level was 0.70 ± 0.88 mM, which was not significantly different from the acetate level of 0.33 ± 0.15 mM in the control digesters maintained under stable operation continuously. Thus, the anaerobic digestion process during the low acetate phase of the treatment digesters resembled the stable process maintained in the control digesters.

Impact of acetate concentration on *Methanosaeta*

Comparisons of the relative abundance of methanogens between control and treatment digesters showed the dominance of *Methanosaeta* over *Methanosarcina* during both low and high acetate phases in the treatment digesters. The relative abundance of *Methanosaeta* populations was not significantly different between the treatment digesters during the low acetate phase and the control digesters (Fig. 4b), which was expected as the low acetate level would favor the dominance of *Methanosaeta*. It was

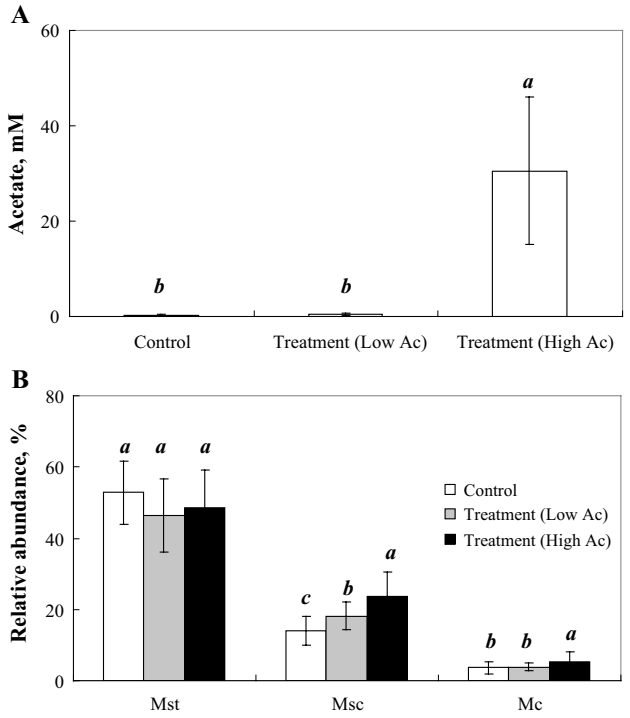

Fig. 4 Relationship between acetate concentration (**a**) and methanogen population abundance (**b**) in control digesters, treatment digesters with low acetate concentrations, and treatment digesters with high acetate concentrations. Methanogen populations included *Methanosaeta* (Mst), *Methanosarcina* (Msc), and *Methanoculleus* (Mc). Results 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, $p < 0.05$)

unexpected, however, that the abundance of *Methanosaeta* in the treatment digesters during the low acetate phase was not significantly different from that during the high acetate phase (Fig. 4b). The persistence of *Methanosaeta* as the dominant acetoclastic methanogens at high acetate levels is surprising, as *Methanosarcina* populations would be projected to dominate in environments with high acetate concentrations [4].

In fact, several previous studies also noted the unexpected dominance of *Methanosaeta* in anaerobic digestion processes at high acetate concentrations [12, 20, 22], suggesting that this occurrence might be widespread in anaerobic digestion. Evidently, the unexpected dominance of *Methanosaeta* over *Methanosarcina* might involve mechanisms that either enhance the competitiveness of *Methanosaeta* or hinder the growth of *Methanosarcina*.

Impact of acetate concentration on *Methanosarcina*

While acetate concentration had little impact on the abundance of *Methanosaeta*, high acetate concentrations did have a positive impact on *Methanosarcina*, as its population abundance was statistically higher during the high acetate phase than the low acetate phase in the treatment digesters (Fig. 4b). Nonetheless, the increase was relatively small and *Methanosarcina* remained as the minor acetoclastic methanogen during the high acetate phase. These observations indicate that *Methanosarcina* populations were indeed more kinetically competitive at higher acetate concentrations as found previously [4, 5]; however, the improvement in growth kinetics was not sufficient to enable *Methanosarcina* to outcompete *Methanosaeta* at the acetate levels examined in this study.

It should be noted that *Methanosarcina* was overall more abundant in the treatment digesters than the control digesters (Fig. 4b). Since the process performance fluctuated considerably in the treatment digesters undergoing multiple changes in OLR (Fig. 1), it is possible that the slight increase in *Methanosarcina* abundance could be attributed to the less stable process in the treatment digesters as compared to the stable operation in the control digesters.

Impact of acetate concentration on *Methanoculleus*

To evaluate the impact of acetate on hydrogenotrophic methanogenesis, *Methanoculleus* was monitored as a representative of hydrogenotrophic methanogens. *Methanoculleus* remained a minor methanogen population regardless of acetate concentration. The relative abundance of *Methanoculleus* was statistically higher during the high acetate phase than the low acetate phase in the treatment digesters (Fig. 4b). Since *Methanoculleus* is not able to directly utilize acetate for acetoclastic methanogenesis, it is possible that the pathway of anaerobic acetate oxidation might become feasible at elevated levels of acetate [9, 15], subsequently supporting the growth of hydrogenotrophic methanogens such as *Methanoculleus*.

Correlation between *Methanosaeta* and *Methanosarcina*

Given the contrasting acetate utilizing kinetics between *Methanosaeta* and *Methanosarcina* [4, 5, 19], these two populations are projected to exhibit patterns of intense mutually exclusive growth competition. An examination of the composition of the methanogen communities in the digesters monitored in this study revealed a modest but statistically significant negative correlation between *Methanosaeta* and *Methanosarcina* (Fig. 5), providing indications of growth competition. However, the magnitude of the negative correlation reflected a rather weak pattern of competition, likely due to the persistent dominance of *Methanosaeta* which was not reversed by high concentrations of acetate as it should have according to growth kinetics currently known for *Methanosaeta* (Fig. 4b).

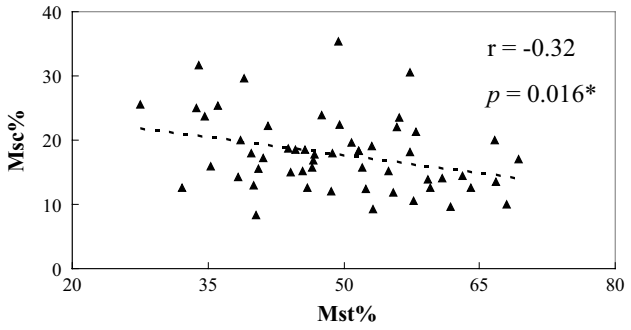

Fig. 5 Correlation of relative abundance between *Methanosaeta* (Mst) and *Methanosarcina* (Msc) in the treatment digesters

It is intriguing that *Methanosaeta* displayed divergent growth behaviors in anaerobic digestion processes. Many studies have shown that *Methanosaeta* are outcompeted by *Methanosarcina* in anaerobic digesters with high levels of acetate [11, 17, 18], which is consistent with growth kinetics known for acetoclastic methanogens [4, 5]. However, a number of studies have noted the lack of correlation between *Methanosaeta* and acetate concentration in some processes [12, 20, 22].

Results from qPCR quantification of acetoclastic methanogens in this study demonstrated the dominance of *Methanosaeta* over *Methanosarcina* at acetate concentrations up to 44 mM (Fig. 2). It is possible that the methanogen communities in the anaerobic digesters of this study were predominated by uncharacterized *Methanosaeta* strains with growth kinetics distinct from those of known *Methanosaeta* strains. Alternatively, deficiency of unidentified growth factors in these anaerobic digesters could have hindered the growth of *Methanosarcina* more than that of *Methanosaeta*. Nonetheless, the dominance of *Methanosaeta* over *Methanosarcina* at high acetate levels did not appear to be isolated, cases evidenced by similar observations made in previous studies [12, 20, 22]. Given the importance of acetoclastic methanogenesis in anaerobic digestion [6], these findings may have important implications for modeling and optimizing anaerobic digestion processes. Research efforts are needed to further elucidate mechanisms responsible for the divergent growth behaviors of *Methanosaeta*.

Conclusions

Acetoclastic methanogenesis accounts for the majority of methane production in anaerobic digestion processes. Therefore, sustaining robust acetoclastic methanogens is important for stable process performance. Due to faster growth kinetics at high acetate concentrations, *Methanosarcina* is assumed to dominate over *Methanosaeta* in unstable anaerobic digestion processes which frequently encounter high levels of acetate. Results from qPCR monitoring demonstrate that *Methanosaeta* could instead be more competitive than *Methanosarcina* at high acetate levels, which was inconsistent with known growth kinetics of acetoclastic methanogens. These findings may have important implications for the understanding and modeling of anaerobic digestion processes.

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Conflict of interest The authors declare that they have no conflict of interest. This article does not contain any studies with human participants or animals performed by any of the authors.

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