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Methanosarcinaceae and Acetate-Oxidizing Pathways Dominate in High-Rate Thermophilic Anaerobic Digestion of Waste-Activated Sludge

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This study investigated the process of high-rate, high-temperature methanogenesis to enable very-high-volume loading during anaerobic digestion of waste-activated sludge. Reducing the hydraulic retention time (HRT) from 15 to 20 days in mesophilic digestion down to 3 days was achievable at a thermophilic temperature (55°C) with stable digester performance and methanogenic activity. A volatile solids (VS) destruction efficiency of 33 to 35% was achieved on waste-activated sludge, comparable to that obtained via mesophilic processes with low organic acid levels (<200 mg/liter chemical oxygen demand [COD]). Methane yield (VS basis) was 150 to 180 liters of CH_4/kg of VS_{added}. According to 16S rRNA pyrotag sequencing and fluorescence in situ hybridization (FISH), the methanogenic community was dominated by members of the *Methanosarcinaceae*, which have a high level of metabolic capability, including acetoclastic and hydrogenotrophic methanogenesis. Loss of function at an HRT of 2 days was accompanied by a loss of the methanogens, according to pyrotag sequencing. The two acetate conversion pathways, namely, acetoclastic methanogenesis and syntrophic acetate oxidation, were quantified by stable carbon isotope ratio mass spectrometry. The results showed that the majority of methane was generated by nonacetoclastic pathways, both in the reactors and in off-line batch tests, confirming that syntrophic acetate oxidation is a key pathway at elevated temperatures. The proportion of methane due to acetate cleavage increased later in the batch, and it is likely that stable oxidation in the continuous reactor was maintained by application of the consistently low retention time.

Anaerobic digestion (AD) is a biological process which has been widely used for waste-activated sludge and primary sludge stabilization. It offers substantial advantages over alternative stabilization techniques, including low energy requirements, good-quality biosolids product, energy recovery in the form of methane (CH_4) gas, and the possibility of nutrient recovery (1, 2). Conventional anaerobic digesters are operated at a mesophilic temperature (35°C) with long hydraulic retention times (HRTs) of 15 to 20 days on waste-activated sludge (WAS), which is excess bacterial material and residual organics from combined aerobic treatment of wastewater, and more than 20 days on primary sludge (PS), which is the settled fraction from raw wastewater (1, 3, 4). Long treatment times are a key disadvantage of anaerobic digestion, requiring large tank volumes and, consequently, high capital cost, as well as increased mixing and maintenance costs. Therefore, reducing digester volume would strongly enhance the competitiveness of anaerobic technologies.

Thermophilic anaerobic digestion (55°C to 70°C) is an alternative to conventional mesophilic anaerobic digestion (35°C). The majority of systems are operated at 55°C (5, 6), as higher temperatures can result in instability due to ammonia inhibition and reduced operability (2). Thermophilic anaerobic digestion offers higher rates of destruction of pathogens and organic solids as well as potentially enhancing methane production. Several studies have shown that improvements in performance in thermophilic digestion are mainly due to an increase in hydrolysis coefficient (7–9), which determines speed of degradation, rather than to an increase in the fraction of degradable material. In addition to this advantage, the growth rates of thermophilic methanogens are 2 to 3 times higher than those of mesophilic methanogens (10). Therefore, the HRT can theoretically be reduced to 5 to 8 days at 55°C. Recent studies have reported substantial methanogenic activities at a short HRT of 2 to 4 days in the thermophilic pretreatment stage of a two-stage temperature-phased anaerobic digestion (TPAD) (11) when the system is fed activated sludge.

The main risk with a short HRT is washout of methanogens as well as other functional groups, causing a build-up of organic acids and a decreased pH (12). A key concern regarding elevated temperatures is the decrease in pK_a of the ammonium/ammonia (NH_4^+/NH_3) acid-base pair, which means that the NH_3 concentration will be 2 to 3 times higher for a given pH, potentially causing ammonia inhibition (1 to 2 mM) (2).

The microbial composition and distribution in anaerobic digesters have been the focus of recent studies using high-throughput molecular techniques. The bacterial and archaeal community structures are highly diverse and affected by substrate composition (6, 12–14), HRT (12), and operating temperature (6, 15). Two methanogenic pathways from acetate have been reported, including (i) direct acetate cleavage in acetoclastic methanogenic archaea, such as *Methanosarcina* and *Methanosaeta*, and (ii) nonacetoclastic oxidation, i.e., the syntrophic association of acetate-oxidizing organisms and hydrogenotrophic methanogens, including *Methanobacteriales* (5, 16). The latter involves interspecies electron transfer, in which acetate oxidizers transfer electrons through an electron carrier such as hydrogen or formate (17)

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TABLE 1 Characteristics of thickened waste-activated sludge (WAS) from the Elanora wastewater treatment plant

Characteristic	Value[a]
Total solids (g/liter)	28.2 ± 0.8
Volatile solids (g/liter)	19.3 ± 0.5
pH	7.1 ± 0.1
Chemical oxygen demand (g/liter)	28.7 ± 2.5
Volatile fatty acid (g/liter)	0.2 ± 0.1
NH_4^+-N (g/liter N)	0.08 ± 0.03

[a] Values are means ± standard deviations for 10 different feed collections used in the study over 12 months.

while keeping the concentration of the electron carrier low (18). An increase in temperature leads to thermodynamic favorability of the oxidation component of the process (19, 20). Hypothesized mechanisms such as direct interspecies electron transfer (17, 21) would be similarly enhanced through enhancement of the oxidation component. Next-generation pyrosequencing has further clarified methanogenic populations in thermophilic systems (6, 15), confirming broad diversity but generally an increased dominance of *Methanosarcina* over *Methanosaeta* compared to mesophilic systems.

The relative contribution to methanogenesis via the different metabolic pathways can be determined using the stable isotopic signatures of $^{13}C/^{12}C$. The technique has been used mostly to study biogenic methane formation in anoxic environments and has shown that production of δCH_4 from reduction of CO_2 exhibits larger fractionation than that from cleavage of acetate (22, 23). In the context of an anaerobic digester, it can be used to study the relative contribution from acetate cleavage and oxidation, with oxidation resulting in a lower $^{13}CH_4$ isotopic signature ($\delta^{13}CH_4$) due to differential partitioning of bicarbonate to methane during hydrogenotrophic methanogenesis (23).

In this study, we determined the potential of high-rate, high-temperature methanogenic digestion as a replacement technology for conventional mesophilic digestion of organic solids at the most commonly applied thermophilic temperature of 55°C. The analysis was based on operation of laboratory-scale continuous anaerobic digesters operating on waste-activated sludge (WAS) for approximately 1 year. The HRT was reduced to 2 to 4 days in order to determine the limitation of methanogenesis. Analytical techniques focused on identifying kinetic capacity, limits, mechanism, and identity of the functional digester and its community for thermophilic anaerobic digestion.

MATERIALS AND METHODS

Substrate. The substrate was biological nutrient removal (BNR) waste-activated sludge (WAS) (10-day aerobic sludge age) collected from the Elanora wastewater treatment plant, located at Gold Coast, Australia. The feed was thickened to obtain total solids (TS) of 2 to 3% and stored at approximately 4°C prior to feeding to minimize degradation and preserve integrity of the material. Seed sludge was collected from a mesophilic anaerobic digester from the Oxley Creek wastewater treatment plant in Brisbane, Australia. Thickened WAS was fed to each single stage reactor in a short pulse every 4 h. The average characteristics of WAS are shown in Table 1.

Experimental setup and operational protocol. The experimental platform consisted of two single-stage thermophilic reactors (1-liter working volume) operated in a semicontinuous mode at 55°C and neutral pH (Fig. 1). One reactor served as a control and was operated at a constant hydraulic retention time (HRT) of 4 days throughout the experimental program; the second reactor acted as an experimental system with HRT as the experimental variable. Reactor temperature was controlled by circulating hot water inside the reactors' water jackets from a water bath maintained at 55°C. Reactors were fed at intervals of 4 h (6 times daily). During feeding events, fresh feed sludge was added to the reactors and an equal volume of effluent was withdrawn simultaneously using a multihead peristaltic pump. The experimental reactors were operated at three HRTs and ran for a total of 350 days: (i) 4-day HRT (200 days), (ii) 3-day HRT (100 days), and (iii) 2-day HRT (50 days).

Biogas and volumetric methane production rates, pH, volatile fatty acids (VFAs), and volatile solid (VS) destruction were closely monitored to evaluate process performance.

Analytical methods. Digestate was collected from each reactor three times per week for chemical analyses, including total solids (TS), volatile solids (VS), volatile fatty acid (VFA), chemical oxygen demand (COD), and ammonium-nitrogen (NH_4^+-N). Analysis followed standard methods (24). Biogas volume was measured daily from each reactor using tipping bucket gas meters and continuously logged online, while gas composition (H_2, CO_2, and CH_4) was determined using a PerkinElmer gas chromatograph (GC) equipped with a thermal conductivity detector (GC-TCD).

(i) VS destruction. The two calculation methods used to determine VS destruction were the Van Kleeck equation and the mass balance equation. The Van Kleeck equation assumes that the amount of mineral solids is conserved during digestion (25) and uses the volatile fractions (VS/TS − VS_{frac}) in the inlet and outlet as references:

$$\%\ \text{VS destruction} = \frac{VS_{frac_i} - VS_{frac_o}}{VS_{frac_i} - (VS_{frac_i} \times VS_{frac_o})} \times 100$$

where VS_{frac_i} and VS_{frac_o} are the volatile fractions (VS/TS) in the inlet and outlet solids, respectively.

The mass balance equation uses VS concentrations in the inlet (feed) and outlet (effluent), expressed as

$$\%\ \text{VS destruction} = \frac{VS_{conc_i} - VS_{conc_o}}{VS_{conc_i}} \times 100$$

where VS_{conc_i} and VS_{conc_o} are VS concentrations (g/liter) of inlet and outlet, respectively.

In addition, batch biochemical degradability tests (biochemical methane potential [BMP] tests) were conducted on the inlet and outlet to provide an independent assessment of process performance, while specific methanogenic activity (SMA) tests were performed to evaluate the specific function of microbial communities within the continuous digesters. The test protocols are listed below.

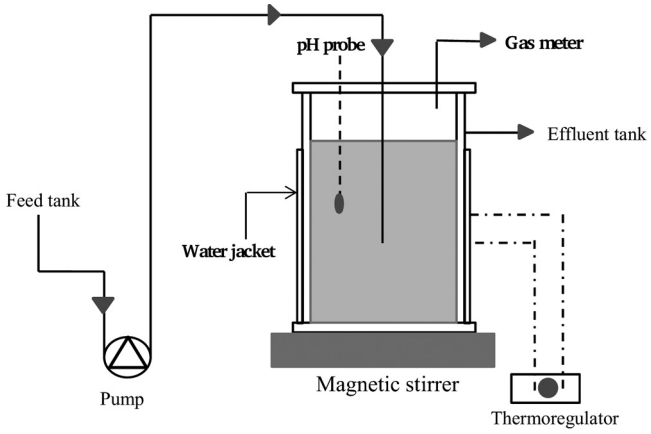

FIG 1 Schematic diagram of the bench scale thermophilic reactor.

(ii) Assay for residual biochemical methane potential. Biochemical methane potential (BMP) tests were employed to determine the residual methane potential from digestion residues and assess the stability of the effluent. The method is based on the work of Angelidaki et al. (26). Batch digestions were performed in 160-ml nonstirred glass serum bottles (100-ml working volume) at 37°C. The inoculum was material collected from a mesophilic anaerobic digester operating at a municipal wastewater treatment plant in Brisbane, Australia. Substrates used in the BMP tests were digestates collected from the thermophilic reactors on day 137, day 249, and day 323, respectively. The inoculum to substrate ratio used in the BMP test was 1:1 (VS basis). All tests were carried out in triplicate, and triplicate blanks were conducted to correct for background methane from the inocula. Error bars in the figures represent 95% confidence in error based on the average of the triplicate (two-tailed *t* test).

The key kinetic parameters of BMP test are degradation extent (f_d), which is the fraction of the substrate that may be converted to methane, and the apparent first-order hydrolysis rate coefficient (k_{hyd}), which determines speed of degradation. The parameters f_d and k_{hyd} were estimated by optimizing f_d and k_{hyd} in a simple first-order model through Aquasim 2.1d, as was done in the batch tests described by Batstone et al. (27).

(iii) Continuous modeling. Continuous reactor modeling was done to determine that measures of degradability sourced through BMP were representative of reactor performance. The test continuous reactor was modeled using the IWA anaerobic digestion model 1 (ADM1) (28) in Aquasim 2.1d, the WAS interface model of Nopens et al. (29), the observed COD/VS ratios given in Table 1, and measured biomass nitrogen content. Input material degradability (f_d) and hydrolysis coefficient (k_{hyd}) were simultaneously estimated using both gas flow and effluent solids concentration (two-parameter searches were done) using secant searches, with parameter uncertainty being reported as linear uncorrelated estimates of 95% confidence regions based on two-tailed *t* tests. Model source files are available on request.

(iv) Specific methanogenic activity (SMA) tests. Methanogenic activity tests were performed in nonstirred, 160-ml serum bottles incubated at 55°C. Each bottle contained 10 ml inoculum and 90 ml basic anaerobic (BA) medium (100-ml total liquid volume). BA medium was prepared as described by Angelidaki et al. (26), while inoculum was harvested from the continuous thermophilic reactors during the steady state, particularly at day 137 (period I), day 270 (period II), and day 342 (period III). Sodium acetate and sodium formate were added as the substrates to concentrations of 3 g/liter and 6 g/liter, respectively, to determine acetoclastic and hydrogenotrophic activity. Blanks contained inoculum and medium without the substrate. Formate was chosen as an alternative source of electron donor to H_2 in the reduction of CO_2, since they are thermodynamically and stoichiometrically similar (30). All bottles were flushed with high-purity N_2 gas for 3 min (1 liter/min), sealed with a rubber stopper retained with an aluminum crimp cap, and stored in temperature-controlled incubators at 55°C (±1°C). All tests were carried out in triplicate, and all error bars in the figures indicate 95% confidence in the average of the triplicates. Gas production, composition, and substrate contents were monitored throughout the experimental period. Specific methanogenic activity was determined by regression against the linear section of the methane evolution curve.

(v) Stable carbon isotopic signature. The gas samples for stable carbon isotopic analysis of $\delta^{13}CH_4$ and $\delta^{13}CO_2$ were collected in a time series in 5-ml Exetainers during the SMA tests. The principle of $^{13}C/^{12}C$ fractionation is discussed by Conrad (31). Isotopic fractionation evaluates enrichment of one isotope relative to another in specific products and infers the generation pathway based on partitioning through the metabolic process. The fraction of CH_4 produced from the CO_2 reduction pathway to total CH_4, f_{mc}, is calculated as $(\delta CH_4 - \delta_{ma})/(\delta_{mc} - \delta_{ma})$, where δCH_4 is the measured variable and δ_{ma} and δ_{mc} are isotopic values of acetate-derived CH_4 and CO_2-derived CH_4, respectively.

Generally, microbes preferentially utilize ^{12}C compounds over ^{13}C compounds (23). According to the literature, hydrogenotrophic methanogenesis exhibits a significantly larger fractionation factor than that of acetate-dependent methanogenesis due to the ability to select ^{12}C preferentially during methanogenesis (31, 32). The fractionation values measured in pure cultures of CO_2 reducers (hydrogen utilizers) range between 1.031 and 1.077 and from 1.007 to 1.027 for the acetoclastic pathway (31). The apparent fractionation (α) can be calculated as $(\delta^{13}CO_2 + 1{,}000)/(\delta^{13}CH_4 + 1{,}000)$.

The relative contributions of acetate-derived CH_4 (δ_{ma}) and CO_2-derived CH_4 (δ_{mc}) to total CH_4 (δCH_4) can be quantified using the following mass balance equation: $\delta CH_4 = f_{mc}\delta_{mc} + (1 - f_{mc})\delta_{ma}$.

Thus, f_{mc} is solved as follows: $(\delta CH_4 - \delta_{ma})/(\delta_{mc} - \delta_{ma})$.

δ_{ma} was chosen at an average value of −33‰ according to reference 31, whereas δ_{mc} was chosen based on the dominant acetoclastic methanogens obtained from the pyrotag sequencing results.

The analyses of isotopic signatures were performed using an Isoprime gas chromatograph combustion isotope ratio mass spectrometer (GC-c-IRMS) linked to an Agilent 7890A gas chromatograph with a CP-Poraplot Q column (50 m × 0.32 mm), with helium as the carrier gas. A CO_2-CH_4 (1:1) gas mixture standard ($\delta^{13}CO_2$ = −18.76‰ and $\delta^{13}CH_4$ = −40.14‰) was injected before and after gas analysis. Each sample was run in duplicate, and all reported $\delta^{13}C$ values have a precision with an average standard deviation of 0.3‰.

(vi) Molecular analysis. The analysis of microbial community structure and diversity were carried out during stable operation using fluorescence in situ hybridization (FISH) and 16S rRNA gene pyrosequencing. FISH was performed as described by Amann et al. (33). FISH preparations

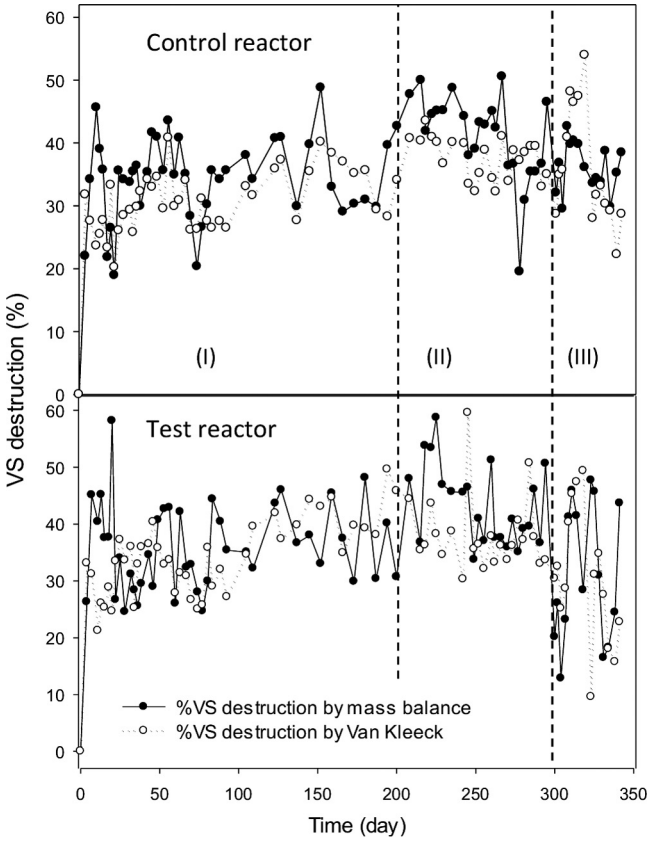

FIG 2 Volatile solid destruction for mass balance (●) and Van Kleeck (○) calculation in the control reactor (top) and test reactor (bottom). VS destruction in the test reactor when operated at 3- and 4-day HRTs was stable and comparable to that of the control reactor, but it varied strongly at 2-day HRT (vertical lines indicate transitions from a 4- to a 3- to a 2-day HRT in the test reactor).

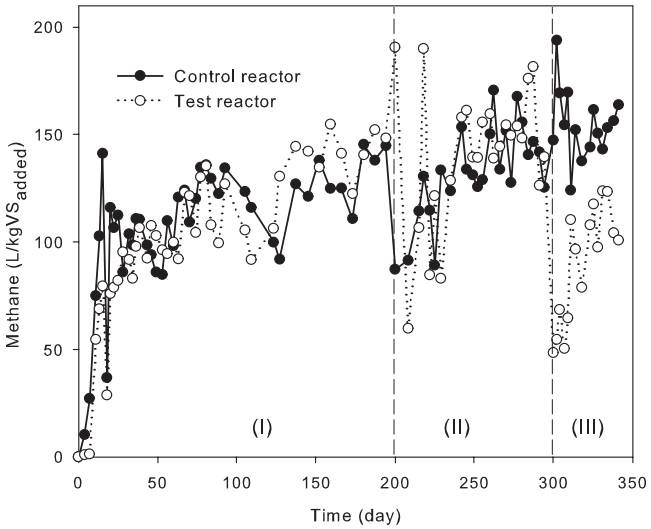

FIG 3 Gas production in control and experimental reactors.

were visualized with a Zeiss LSM 510 Meta confocal laser scanning microscope (CLSM) using three excitation channels (488 nm, green emission; 545 nm, red emission; and 633 nm, blue emission). FISH probes used in this study included $Eub338_{mix}$ (5′-GCTGCCTCCCGTAGGAGT-3′) probes for most members of the *Bacteria* (34), Arc915 (5′-GTGCTCC CCCG CCAATTCCT-3′) probes for most *Archaea* (35), Sarci551 (5′-GA CCCAATAATCACGATCAC-3′) for members of the genus *Methanosarcina* (36), and Ttoga660 (5′-GTTCCGTCTCCCTCTACC-3′) for members of the order *Thermotogales* (37).

Genomic DNA was extracted from the dewatered pellets using a FastDNA spin kit for soil. The quantity and quality of the extracted DNA were measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technology, Rockland, DE) and agarose gel (0.8%, wt/vol) electrophoresis. The primers used for pyrotag sequencing were 926f (5′-AAAC TYAAAKGAATTGACGG-3′) and 1392r (5′-ACGGGCGGTGTGTAC-3′) (33). 16S rRNA gene pyrosequencing was carried out according to Roche 454 protocols using a Roche 454 GS FLX sequencer (Roche, Switzerland). Sequences that were shorter than 350 bp and reads containing any unresolved nucleotides were removed from the pyrosequencing-derived data sets. Operational taxonomic units (OTUs) were determined for each denoised sequence data set by using the uclust OTU picker version 1.2.21q of the QIIME software pipeline (38).

RESULTS

Reactor performance. The process performance of both control and experimental reactors was evaluated by VS destruction, production of methane, and organic acid levels. Figure 2 shows fluctuating VS destruction for the control reactor at an average of 33 to 35% using both mass balance and Van Kleeck equations, indicating consistency between the two measures. While VS destruction showed some variation, this is to be expected where the analysis is momentary. The performance of the test reactor was comparable to that of the control across the first two periods when HRT was reduced from 4 days to 3 days. However, at a 2-day HRT, VS destruction varied greatly between the two methods, indicating non-steady-state issues.

In most cases, H_2 was not detected (and was therefore below the detection limit of 0.1%), while CH_4 accounted for more than 60% of biogas composition and CO_2 for 30%. Total methane production levels obtained during the 4-day and 3-day HRT periods were similar and ranged from 150 to 180 liter CH_4/kg VS_{added}, as shown in Fig. 3. However, gas production decreased by 30% when the HRT was shortened to 2 days. The key measure to determine process stability was VFA concentration, as shown in Table 2. The concentrations of C_2-C_4 VFAs were low (<200 mg/liter COD) at a 4-day HRT in both the control and test reactors and then increased consistently to approximately 500 mg/liter COD at shorter HRTs (Table 2). pH values of the digesters decreased from 7.04 during the 4-day HRT to 6.89 during the 3-day HRT and varied in the range of 6.74 to 7.04 during the 2-day HRT. The ammonia concentrations in both digesters varied in the range of 700 to 1,000 mg/liter and were not affected by changes in HRT.

Digestate degradability. The digestates produced at different HRTs were assessed using BMP tests. A summary of the hydrolysis rate coefficients and degradability fractions determined using the nonlinear parameter estimation method is shown in Table 3, while the cumulative methane production is shown in Fig. 4. The degradability of feed sludge was estimated at approximately 41%,

TABLE 3 Comparison of degradation parameters of feed WAS and short-HRT digester effluent fitted using model-based analysis[a]

Material or HRT	k_{hyd} (day^{-1})	f_d	B_0 (liters CH_4/kg VS_{added})
Feed material (WAS)	0.19 ± 0.040	0.41 ± 0.18	222 ± 12
4-day HRT	0.18 ± 0.010	0.20 ± 0.011	131 ± 9
3-day HRT	0.19 ± 0.014	0.20 ± 0.010	128 ± 10
2-day HRT	0.17 ± 0.010	0.31 ± 0.012	170 ± 7

[a] k_{hyd} is the apparent first-order hydrolysis coefficient; f_d is the degradation fraction; B_0 is the methane production potential. Samples from 4-day, 3-day, and 2-day HRTs were collected on day 137, day 249, and day 323, respectively. Values are means ± standard deviations for triplicates.

TABLE 2 Comparison of levels of volatile fatty acids between the control and test reactors across three operating periods

Period	HRT	Value (mg/liter COD)[a]			
		Total VFAs	Acetate	Propionate	C_{4+} acids
I	Control	175 ± 68	97 ± 28	65 ± 24	13 ± 7
	4 day	104 ± 53	67 ± 30	32 ± 23	5 ± 2
II	Control	164 ± 43	104 ± 18	51 ± 28	10 ± 3
	3 day	254 ± 82	171 ± 49	77 ± 30	5 ± 3
III	Control	98 ± 19	79 ± 17	11 ± 4	8 ± 2
	2 day	497 ± 224	301 ± 182	142 ± 67	54 ± 28

[a] Values are means ± standard deviations for different measurements over each period.

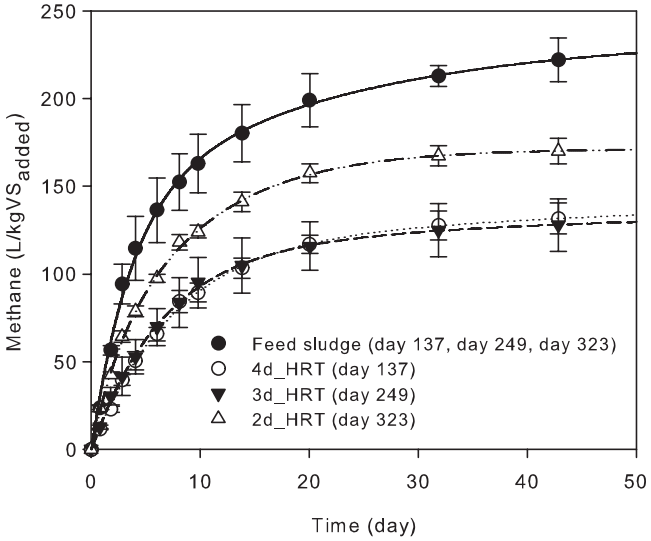

FIG 4 Cumulative methane production from the residual degradation tests. Points represent experimental measurements, error bars show 95% confidence intervals based on triplicate analyses, and lines represent model fit.

with an average gas production of 210 to 230 liter CH_4/kg VS_{added} over a 40-day incubation. The values were in agreement with previous studies (4, 7).

Figure 5 shows an analysis of the residual methane potential of the digestates collected from each reactor compared to that of feed sludge. Recalcitrant component is based on the material not converted to methane ($1 - f_d$). The material calculated as destroyed in reactors is the difference between feed and digestate potentials. This showed that the 4-day and 3-day HRT continuous thermophilic reactors degraded 80% of the available material, while the 2-day HRT system converted only 50% of the available material, leaving a less stabilized product. The thermophilic reactors themselves made an additional 10% available for degradation, as seen by the drop in the recalcitrant component. Model-based analysis results in Fig. 5 show that the BMP was a good estimate of in-reactor performance to determine f_d, as the input degradability estimated based on model outputs aligned well with material as analyzed by BMP. Uncertainty in model-based estimates of f_d was high due to correlation with k_{hyd} and the limited amount of dynamic operation. k_{hyd} was estimated at 1.5 and 2.5 day^{-1} (solids and gas, respectively), though with an uncertainty on the order of ± 1 day^{-1}.

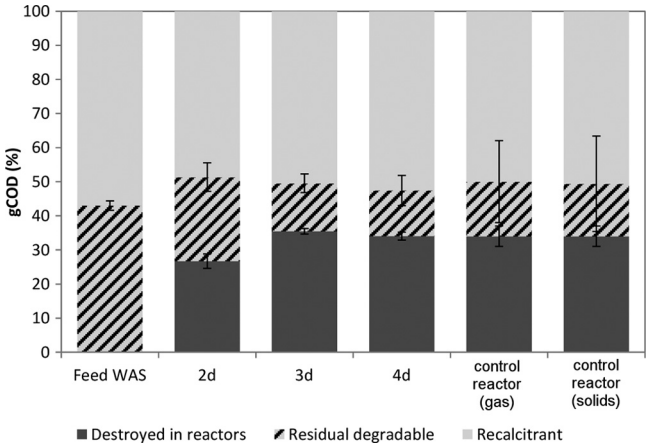

FIG 5 Comparison of degradable fractions of feed WAS and short HRT digesters (error bars show 95% confidence in mean VS destruction, based on triplicate analyses of the BMP tests). Potentials for 4, 3, and 2 days were taken from the experimental reactor on day 137, day 249, and day 323, respectively. Estimates of in-reactor performance were based on averages ± errors of two data sets, i.e., gas data and VS data, for 6 measurements around these days. Continuous results based on parameter estimation (k_{hyd} and f_d) with simulation of whole data set.

TABLE 4 Summary of kinetic parameters for the acetate-utilizing ($k_{m,ac}$) and hydrogenotrophic ($k_{m,for}$) methanogenesis[a]

HRT	Value (g COD · g VS^{-1} · day^{-1})	
	$k_{m,ac}$	$k_{m,for}$
4 day	0.77 ± 0.022	1.4 ± 0.13
3 day	0.69 ± 0.018	1.5 ± 0.17
2 day	0.81 ± 0.069	1.4 ± 0.20

[a] Samples from 4-day, 3-day, and 2-day HRTs were collected on day 137, day 270, and day 342, respectively. Values are means ± standard deviation for triplicates.

Specific methanogenic activity (SMA) tests. The acetate uptake rate was in the range of 0.69 to 0.81 g COD · g VS^{-1} · day^{-1}, while formate uptake was double that, at 1.4 to 1.5 g COD · g VS^{-1} · day^{-1} (Table 4). There were no significant differences in the substrate uptake rates between digesters with different retention times. Blanks are also shown (Fig. 6), demonstrating that endogenous methane production was negligible compared with substrate-derived methane production.

The stable carbon isotopic signatures of CH_4/CO_2 and the fractionation factors of acetate and formate degradation are presented in Fig. 6. The results indicate that formate was degraded, and as expected, δCO_2 and δCH_4 were both enriched in ^{12}C (−18‰ and −60‰, respectively). The isotopic signatures became more negative (i.e., further enriched in ^{12}C) with increasing uptake of formate. In contrast, the produced methane become increasingly heavier during acetate conversion, suggesting a gradual shift of the dominant pathway from hydrogenotrophic to acetoclastic methanogenesis as the reaction proceeded. In all cases, except at the end of the acetate batch (last two points), the majority of the methane was apparently hydrogen derived.

Community structure. FISH analyses were carried out to determine the active methanogenic and bacterial populations. Fluorescence microscopy of FISH samples collected at different time points always found that the archaea were dominated by *Methanosarcina* (Fig. 7, pink) with a complete overlap between total archaea (Arc915-cy3 probe; Fig. 7, red) and *Methanosarcina*-specific (Sarci551-cy5 probe; Fig. 7, blue). However, during the 2-day HRT period, the portion of *Methanosarcina* was much lower, suggesting a decrease in its relative abundance. Instead, we found thermophilic bacteria in the order *Thermotogales* (Fig. 7, yellow, representing the overlap between the $Eub338_{mix}$-FITC [fluorescein isothiocyanate] and Ttoga660-cy3 probes).

Pyrotag sequencing results confirmed the dominance of members of the archaeal family *Methanosarcinaceae*, with members of the *Methanobacteriaceae* also being present at approximately 15 to 20% of the methanogenic population (Fig. 8). The bacterial community was composed mainly of the phyla *Proteobacteria* (notably *Alphaproteobacteria* and *Betaproteobacteria*), *Bacteroidetes*

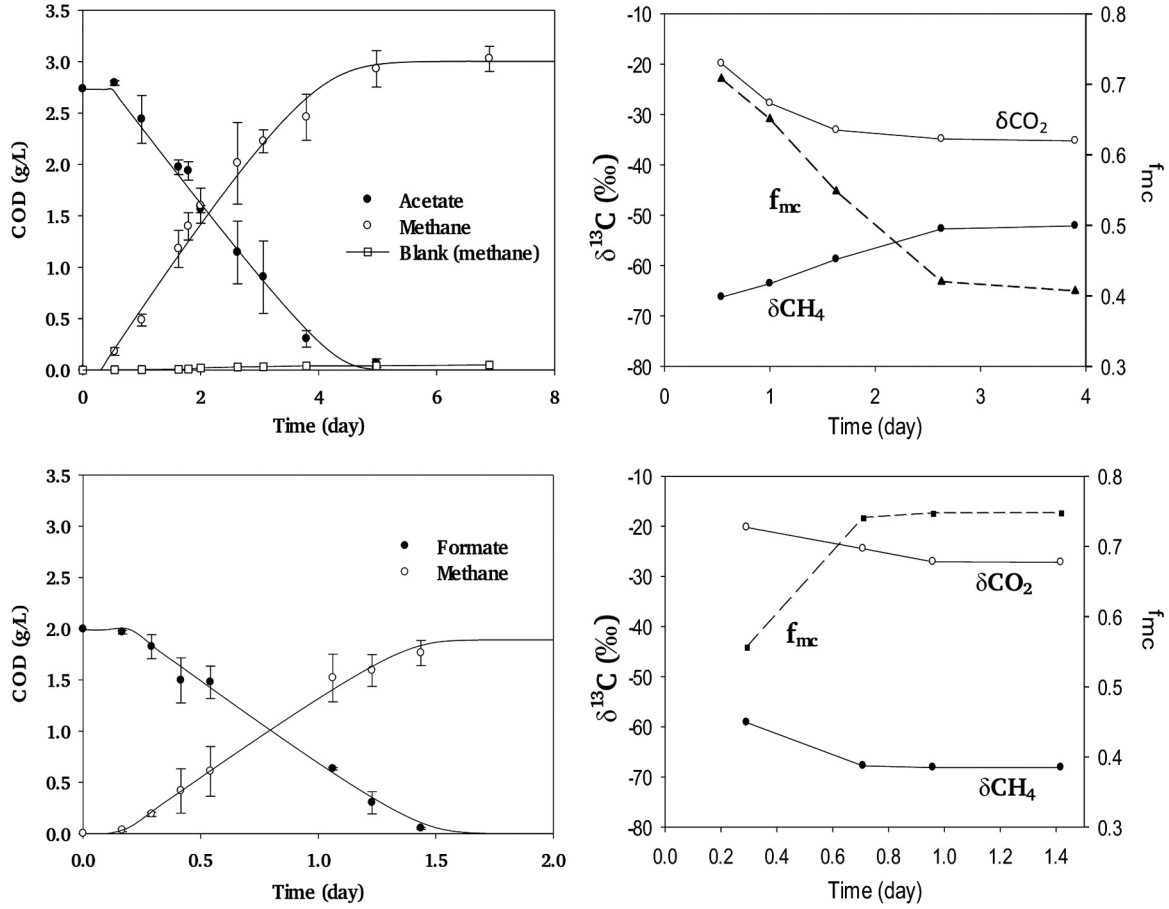

FIG 6 Evolution of $\delta^{13}C$ of CH_4 and CO_2 and the fraction of CH_4 produced from CO_2 reduction pathway (f_{mc}) during acetate (top) and formate (bottom) conversion (note that the time scales for acetate and formate are different). Error bars show 95% confidence based on triplicate analyses; solid lines represent δCO_2 and δCH_4; dashed lines represent f_{mc}. SMA based on samples taken on day 137 (4-day HRT).

(mainly members of the class *Sphingobacteriia*), and *Chloroflexi* (dominated by members of the class *Anaerolineae*), which were abundant in the feed sludge. There was a significant increase in members of the phylum *Firmicutes* (20 to 30%), affiliated at the order level with *Clostridiales*, in the digester samples and a noticeable presence of members of the order *Thermotogales* (5 to 10%) in the microbial communities harvested from the digesters at shorter HRTs. This is consistent with FISH analyses and previous findings (6, 20).

The methanogenic population dropped from 25 to 30% to less than 5% of the total microbial population as the HRT was shortened from 4 to 2 days. The bacterial community was mostly influenced by the feed microbial composition, but the level of diversity varied in response to operating conditions, in this case HRT. The microbial communities obtained at 4-day (day 25 to 200) and 3-day (day 245 to 287) HRTs were similar and remained consistent throughout the operating periods. During the 2-day HRT period (day 300 to 350), the bacterial population changed strongly over time, which likely contributed to the large variation in VS destruction and process instability under these conditions. The key functional groups were still prevalent, with a noticeable shift in abundance toward thermophilic members of the *Firmicutes* and *Thermotogae* and a drop in the phylum *Chloroflexi*.

DISCUSSION

The overall performance of the high-rate thermophilic anaerobic digesters in this study was comparable to the results obtained from mesophilic digesters operated at 15 to 20 day HRT reported by Ge et al. (7) and Bolzonella et al. (39) and better than that of digesters operated at reduced HRTs (8 to 12 days) reported by Lee et al. (12) and Nges and Liu (40). Contradictory to these previous studies, methanogenesis of the high-rate system in the current study remained relatively stable, with a consistently low VFA content of <200 mg/liter at 3- and 4-day HRTs. In previous studies, a short HRT of 2 to 4 days was used mainly in the thermophilic pretreatment stage to intensify hydrolysis rather than methanogenesis; thus, gas production was relatively low (11, 39). This is not the case for our system, and reasons for this may include the consistent and stable start-up and operation at a low HRT, which selected from the start for competent organisms in this context. More than 80% of the available material was degraded and subsequently converted to methane at the HRT of 3 to 4 days, which is consistent with a high hydrolysis coefficient.

The stable methanogenesis achieved in this study can be attributed to higher reaction rates at elevated temperatures and changes in microbial community structure and their metabolic pathways in response to thermodynamic changes and short-retention-time pres-

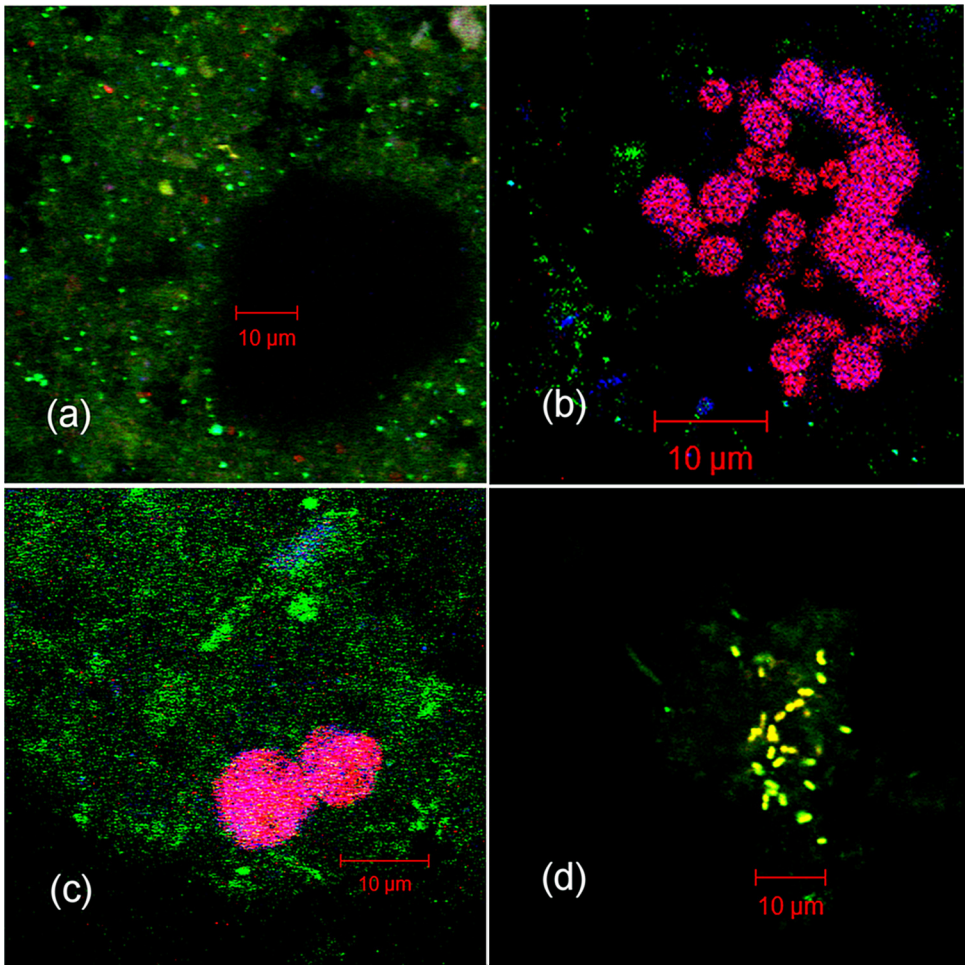

FIG 7 FISH images of the microbial consortia derived from waste-activated sludge (a), the thermophilic digester at day 200 (4-day HRT) (b), the thermophilic digester at day 285 (3-day HRT) (c), and the digester at day 330 (2-day HRT) (d). Bacteria (detected with the probe Eub338$_{mix}$) are in green, aggregates of *Methanosarcina* (Sarci551) are in pink, and rod-shaped *Thermotogales* (Ttoga660) are in yellow.

sure. As reported earlier (3, 7), the hydrolysis rate was almost double when the temperature was increased from 35°C to 55°C, and the improvement was transferred to the subsequent processes. Likewise, apparent methanogenic coefficients increased from 0.37 day^{-1} under mesophilic conditions (3) to 0.77 day^{-1} for acetoclastic methanogenesis and 1.4 day^{-1} for hydrogenotrophic methanogenesis under thermophilic digestion. Uptake on formate (as a proxy for hydrogenotrophic methanogenesis) was higher than on acetate, which is in agreement with previous studies (3, 14). The results therefore indicated that hydrolysis and methanogenesis were both improved at 55°C. With a 2-day HRT, methanogenic coefficients were similar to the values obtained with 3- and 4-day HRTs, yet the overall performance of the continuous digesters decreased. The main failure mode with a low HRT was loss of methanogenesis, rather than loss of the primary hydrolysis, evidenced by a drop in gas flow and accumulation of acetate and C_{3+} acids. This indicates that methanogenesis is the rate-limiting step at high temperatures (rather than hydrolysis) and that unlike in many mesophilic systems, hydraulic failure can be determined by an increase in intermediates rather than unhydrolyzed material simply washing out.

When examining the methanogenic pathways in particular using isotopic fractionation analysis (4 day HRT), we observed a shift from CO_2-reducing methanogenesis to acetate cleavage at the end of the batch test. However, the continuous system was consistently dominated by oxidation, with f_{mc} being larger than the theoretical 33% (41), possibly due to constant washout and replacement of the microbial communities in the continuous digesters. This observation suggests that cleavage may dominate again at longer retention times. The thermodynamic shifts in methanogenic pathways with respect to increase in temperature have also been reported (16, 42), suggesting that high temperature and short HRT favor the syntrophic association between acetate oxidizers and hydrogenotrophic methanogens, which may include *Methanosarcinaceae* and *Methanobacteriaceae*. *Methanobacteriaceae* have previously been found only to be hydrogen and formate utilizing, while *Methanosarcinaceae* can also cleave acetate, with the metabolic possibility also for acetate oxidation to hydrogen (43, 44). The high level of formate activity may be due to activity by *Methanobacteriaceae*, since *Methanosarcinaceae* have not been previously observed to grow on formate (though the genes for this do exist [45]). We believe part of the reason the reactors were stable at low HRT is that applying a short HRT from the start enabled a stable dominance of oxidation capacity, which was further favored by the higher temperature.

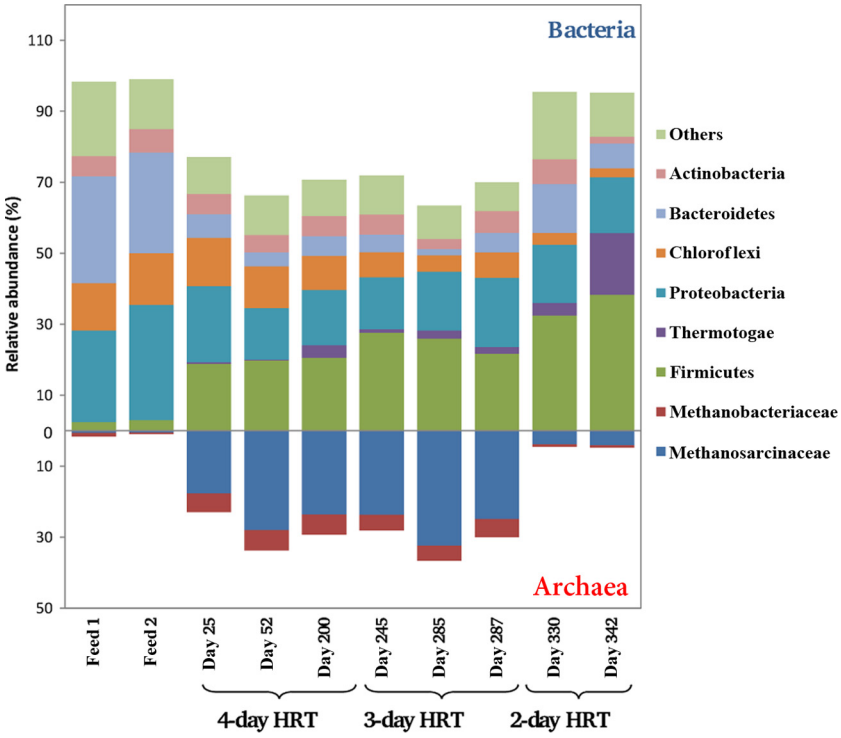

FIG 8 Relative abundances of phylogenetic groups in the thermophilic digesters collected at different operating times and then grouped in terms of HRTs. Bacterial communities are presented by the dominating phyla, while the archaeal *Euryarchaeota* are presented by the dominant families *Methanosarcinaceae* and *Methanobacteriaceae*. Phylogenetic groups accounting for ≤0.5% of all classified sequences are summarized in the artificial group "others."

The microbial community structures of mesophilic anaerobic digesters have been well documented with the dominant bacterial groups of *Chloroflexi*, *Bacteroidetes*, and *Firmicutes* and archaeal members of *Methanomicrobiales* and *Methanosaetaceae* (13, 46). However, the pyrotag sequencing results showed the exclusive dominance of *Methanosarcinaceae* and the hydrogenotrophic methanogens *Methanomicrobiaceae*, including the genera of *Methanobrevibacter*, *Methanothermobacter*, and *Methanobacterium*, in the thermophilic digesters. *Methanosarcinaceae*, which dominate in the thermophilic digester, are capable of either hydrogenotrophic or acetoclastic methanogenesis. The absence of obligate acetoclastic *Methanosaetaceae* could be attributed to a high ammonia level (5) and short HRT, which imposes selective pressure on the slow-growing microorganisms (the maximum growth rates were 0.2 day^{-1} for *Methanosaetaceae* and 0.6 day^{-1} for *Methanosarcinaceae* [3, 47]).

Only a limited number of syntrophic acetate oxidation bacteria have been isolated and characterized, including the thermophilic bacteria *Thermacetogenium phaeum* (48), belonging to the phylum *Firmicutes*, *Thermotoga lettingae* strain TMO (49), belonging to the phylum *Thermotogae*, and two mesophilic bacteria both belonging to the phylum *Firmicutes* (50). These phyla were dominant in the thermophilic biogas reactors, with increasing abundance of the orders *Clostridiales* and *Thermotogales* at shorter HRTs. While hypothetical acetate-oxidizing members of the *Chloroflexi* were lost at a 2-day HRT, this appears to have been offset by the increase in other acetate-oxidizing candidates. In fact, the drop in *Methanosarcinaceae* (from almost 20% to <5%) was the most distinguishable microbial characteristic of the change from pre- to postfailure. It suggests that the system collapsed mainly due to loss of methanogens rather than the reduced activity of the syntrophs. Indeed, if syntrophic bacterial acetate oxidizers were lost while *Methanosarcinaceae* were retained, one would expect that methanogenesis would be maintained through acetoclastic methanogenesis rather than acetate oxidation, which was not the case.

A significant reduction of HRT from 15 to 20 days down to 3 to 4 days means that the daily sludge flow rate could be increased up to five times or the digester volume could be likewise reduced. The heating demand for thermophilic digesters operated at 55°C can be provided from methane production using cogeneration. Furthermore, where semibatch operation is applied, the material meets the standards for pathogen destruction (51). The VS destruction rate of the thermophilic digester was 33 to 35%, which is comparable to what would be achieved at approximately 12 to 15 days in a mesophilic digester (based on BMP results). Residual degradability at 4 days and 3 days was 0.20, which is comparable to the commonly applied standard of 17% (52) and indicates that the material is suitable for reuse either immediately or after additional minor conditioning.

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