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ANAEROBIC CODIGESTION OF MUNICIPAL SOLID WASTE AND BIOSOLIDS UNDER VARIOUS MIXING CONDITIONS—II: MICROBIAL POPULATION DYNAMICS

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Abstract—Microbial population dynamics were evaluated in anaerobic codigesters treating municipal solid waste and sewage sludge. Ribosomal RNA based oligonucleotide probes were used to characterize changes in population abundance of syntrophic volatile fatty acid degrading bacteria and methanogens. Changes in community structure were linked to traditional performance parameters during the recovery of previously unstable codigesters induced by a reduction in mixing levels. *Methanosarcina* spp. were the most abundant aceticlastic methanogens in unstable codigesters with high acetate concentrations, while *Methanosaeta concilii* was dominant in stable systems with low levels of acetate. Growth of *Syntrophobacter wolinii* was enhanced during stabilization of a codigester with a well-developed population of *Methanobacteriaceae*, possibly because the presence of adequate numbers of these hydrogenotrophic methanogens encouraged the syntrophic oxidation of propionate. Mesophilic saturated fatty acid beta-oxidizing syntrophs were most abundant in previously unstable codigesters. One minimally mixed reactor became unstable after switching to continuously mixed conditions. After the switch, total archaeal abundance decreased sharply, though *Methanobacteriaceae* and *Methanosarcina* spp. levels increased as the fermentation became unbalanced. Based on the results presented here, mixing appears to inhibit the syntrophic oxidation of volatile fatty acids, possibly by disrupting the spatial juxtaposition of syntrophic bacteria and their methanogenic partners.

Key words—anaerobic digestion, municipal solid waste, biosolids, sewage sludge, mixing, methanogens, syntrophic bacteria, oligonucleotide probes, ribosomal RNA, 16S rRNA

NOMENCLATURE

CM	continuously mixed
F/M	food to microorganism ratio
HAc	acetic acid
MM	minimally mixed
OFMSW	organic fraction of municipal solid waste
PCR	polymerase chain reaction
SFAS	saturated fatty acid-β-oxidizing syntrophs
SPOB	syntrophic propionate-oxidizing bacteria
SRB	sulfate-reducing bacteria
SSU rRNA	small-subunit ribosomal RNA
T_w	wash temperature
VFA	volatile fatty acid
VS	volatile solids
VS_{feed}	volatile solids in feedstock
$V_{Sinoculum}$	volatile solids in inoculum
WAS	waste activated sludge

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INTRODUCTION

The feasibility of codigestion of two or more organic waste streams (e.g., organic fraction of municipal solid waste (OFMSW), sewage sludge or biosolids, animal waste, agricultural solid waste) has been demonstrated at both the laboratory-scale (Poggi-Varaldo and Oleszkiewicz, 1992; Rivard *et al.*, 1990; Saunders *et al.*, 1990; Stenstrom *et al.*, 1983) and the full-scale level (Ahring, 1994; Cecchi *et al.*, 1988). A variety of startup strategies, operating conditions, and reactor configurations were evaluated, but microbial community structure generally was not linked to digester performance. Until recently, such analyses were difficult due to a lack of adequate tools to study microbes directly in their natural habitats. Culture-based methods have been especially difficult to use in anaerobic systems because syntrophic interactions, low growth rates, unknown growth requirements, and obligate anaerobiosis make anaerobic microorganisms difficult to isolate and identify. Molecular tools based on sequence comparison of

small-subunit (SSU) ribosomal RNA (rRNA) molecules have made it possible to study complex microbial communities without the need to culture microorganisms, thereby reducing the widely acknowledged biases associated with culturing (Ward *et al.*, 1992). Oligonucleotide probes targeting SSU rRNAs of phylogenetically defined groups of microbes (methanogens, sulfate-reducing bacteria (SRB), fiber digesting microbes) already have been used for the quantification of population abundance in a variety of anaerobic environments (e.g., gastrointestinal environments (Lin *et al.*, 1997; Stahl *et al.*, 1988), sediments (Devereux *et al.*, 1992; MacGregor *et al.*, 1997), and bioreactors (Hansen *et al.*, 1999; Harmsen *et al.*, 1996; Raskin *et al.*, 1995)). We previously used oligonucleotide probe hybridizations to evaluate methanogen population dynamics in anaerobic codigesters (Griffin *et al.*, 1998), and demonstrated how this technology can be used to link microbial community structure and digester performance.

The performance of an anaerobic codigestion system is tied closely to the structure of its microbial community. Stable anaerobic digestion is carried out by representatives of four major metabolic groups: hydrolytic-fermentative bacteria, proton-reducing acetogenic bacteria, hydrogenotrophic methanogens, and aceticlastic methanogens (Zinder *et al.*, 1984). During balanced carbohydrate fermentation, the majority of electrons are channeled through acetate, hydrogen, and formate (Schink, 1992). If these intermediates build up, more reduced organic intermediates (e.g., propionate, butyrate, lactate, ethanol) accumulate, often resulting in a drop in pH. The accumulation of volatile fatty acids (VFA) during anaerobic digester overload is well documented (e.g., Ahring *et al.*, 1995; Harper and Pohland, 1986; Hickey and Switzenbaum, 1991; McCarty *et al.*, 1963; McCarty and Mosey, 1991) and it has been demonstrated that VFA toxicity effects are exacerbated at low pH values (Barredo and Evison, 1991; Fukuzaki *et al.*, 1990). Without adequate levels of populations that can remove hydrogen and other intermediates, VFA continue to accumulate, inhibiting methanogenesis and causing further imbalance. Since the microorganisms responsible for VFA consumption (i.e., proton-reducing acetogens) are very sensitive to the accumulation of their own metabolites (hydrogen, formate, and acetate) (Stams, 1994), the inhibition of methanogenesis by these products causes further VFA build up (Schink, 1997). Therefore, the rapid acidification of an overloaded digester can bring VFA oxidation and methanogenesis to a complete halt, preventing digester recovery (Kaspar and Wuhrmann, 1978; Zinder, 1993). Ironically, recovery can be difficult for digesters with a history of very stable operation, since propionate-degrading organisms are thought to be virtually absent from such systems, due to a lack of steady substrate (propionate) supply (McCarty and Mosey, 1991). A successful recovery from overload requires adequate levels of VFA-degrading microbes to metabolize the surplus intermediates, sufficient quantities of methanogens to consume the hydrogen and acetate produced during VFA oxidation, and environmental conditions which encourage their close association.

The recent phylogenetic characterization of several syntrophic propionate-oxidizing bacteria (SPOB) and saturated fatty acid-β-oxidizing syntrophs (SFAS) resulted in the development of SSU rRNA-based oligonucleotide probes for these organisms (Hansen *et al.*, 1999; Harmsen *et al.*, 1995; McMahon *et al.*, in preparation; Stams, 1994; Zhao *et al.*, 1993). Herein, we present the application of these probes and previously designed probes for methanogens (Raskin *et al.*, 1994; Zheng and Raskin, 2000) to study the population dynamics in mesophilic anaerobic digesters treating OFMSW, primary sludge, and waste activated sludge (WAS). Operating conditions and detailed digester performance data are presented in the accompanying paper (Stroot *et al.*, 2001), in which we report that propionate and acetate accumulated to high levels in continuously mixed digesters that were subjected to aggressive startups and overloading. While acetate was eventually consumed, propionate persisted throughout system operation. When the mixing level was reduced, propionate was degraded and digester operation was stabilized, indicating that adjustment of the mixing level could potentially serve as an effective operational tool for stabilizing unstable digesters. To help explain these observations, we here emphasize the importance of linking digester performance results to microbial population dynamics, with a focus on syntrophic bacteria and their methanogenic partners.

MATERIALS AND METHODS

Digester operation and chemical analyses

In Experiment 1, four laboratory-scale digesters were operated in a semi-continuous mode (daily feeding and wasting) at mesophilic conditions (37°C) with an initial target retention time of 20 days, as described in detail by Stroot *et al.* (2001). One digester was started without an exogenous inoculum (Digester 1), while the three other digesters were seeded with cattle manure and anaerobic digester sludge (Digester 2), cattle manure only (Digester 3), and anaerobic digester sludge only (Digester 4), at a food to microorganism (F/M) ratio of 0.33 g volatile solids $(VS)_{feed}/gVS_{inoculum}$ (Stroot *et al.*, 2001). The digesters were fed a mixture of OFMSW, primary sludge, and WAS, combined according to typical US production rates (Griffin *et al.*, 1998). Initially, the design organic loading rate was 3.7 g VS/l active volume/day. If necessary, the pH was controlled by chemical addition or by reducing the daily feed rate (Stroot *et al.*, 2001). The digesters were continuously and vigorously mixed on a shaker table for two weeks, and subsequently switched to minimally mixed conditions (thoroughly shaken by hand for 2 min each day).

In Experiment 2, six laboratory-scale digesters were used to compare digester performance under continuously mixed

(Digesters 5–7) and minimally mixed (Digesters 8–10) conditions for three different organic loading rates (3.5, 7.6, and 9.4 g VS/l active volume/day). The digesters also were operated in a semi-continuous mode at 37°C with an initial target retention time of 20 days (Stroot *et al*., 2001). Anaerobic digester sludge ($F/M = 0.25\,g\ VS_{feed}/gVS_{inoculum}$) was used to inoculate all six digesters.

For Experiment 3, the contents of two digesters, operated for 65 days in Experiment 2 (Digesters 7 and 8), were divided into two separate digesters each (designated Digesters 7-CM and 7-MM, and Digesters 8-CM and 8-MM, respectively). Digesters 7-CM and 8-MM were operated under the same conditions as Digester 7 (continuously mixed, organic loading rate of 9.4 g VS/l active volume/day) and Digester 8 (minimally mixed, organic loading rate of 3.5 g VS/l active volume/day), respectively. Digester 7-MM was operated under minimally mixed conditions, while Digester 8-CM was mixed continuously.

Total VFA concentrations, pH, alkalinity, and biogas production were measured daily, and individual VFA concentrations, biogas composition, solids, and fiber content were measured two to three times per week (Griffin *et al*., 1998; Stroot *et al*., 2001).

Quantification of microbial populations

Samples were collected for rRNA analyses at least twice a week (Griffin *et al*., 1998) and nucleic acids were extracted in duplicate from selected samples using a low-pH hot-phenol protocol (Griffin *et al*., 1998; Stahl *et al*., 1988). The quality of extracted rRNA was routinely inspected using polyacrylamide gel electrophoresis (Alm and Stahl, 2000). Membrane hybridizations were conducted using Magna Charge membranes (Micron Separation Inc., Westboro, MA) as previously described (Griffin *et al*., 1998; Raskin *et al*., 1997) with minor modifications. Nucleic acids were denatured with 1.5% glutaraldehyde for 10 min at room temperature, diluted to 0.4 or 0.25 ng total rRNA/μl (as quantified with probe S-*-Univ-1390-a-A-18) for hybridization with specific probes, and blotted (100 μl per slot). The diluted nucleic acids were diluted further to 0.1 ng rRNA/μl (100 μl blotted per slot) for hybridization with S-*-Univ-1390-a-A-18. Total nucleic acids (rRNA and DNA) blotted per slot were generally less than 60 ng, well below the estimated membrane saturation point of 160 ng (Alm *et al*., 2000).

Dilution series of pure culture rRNAs were applied in triplicate on independent membranes (reference membranes), rather than on sample membranes. These reference membranes were prehybridized, hybridized, washed, and exposed together with sample membranes to reduce possible variability between the two sets of membranes (Raskin *et al*., 1997). Pure culture rRNA was available for all methanogens, for *Syntrophobacter fumaroxidans* MPOB (grown in pure culture on fumarate (Harmsen, 1996; Stams *et al*., 1993) and provided by A. Stams (Wageningen University, The Netherlands), and for *Syntrophomonas wolfeii* LYB (grown in pure culture on crotonate (Boone *et al*., 1989; Zhao *et al*., 1990) and obtained from the Oregon Collection of Methanogens (http://naual.ese.ogi.edu/ocm). Since pure cultures of *Syntrophobacter wolinii*, *S. pfennigii*, and strain LYP were not available, the SSU rRNA genes of these organisms were cloned and rRNAs were transcribed *in vitro* (McMahon *et al*., 1998) for use as standards. Cocultures of *S. wolinii* and *S. pfennigii* (Harmsen *et al*., 1995) were provided by A. Stams. *Syntrophobacter wolinii* was grown on fumarate and *S. pfennigii* was grown on propionate with sulfate (Stams *et al*., 1993; Wallrabenstein *et al*., 1995). Both organisms were grown in coculture with a *Methanospirillum* species. Strain LYP was obtained from the Oregon Collection of Methanogens, grown in coculture with *Methanospirillum hungateii* with 20 mM propionate and 20 mM sulfate at 37°C (Boone *et al*., 1989). This organism was recently proposed to be the type strain of *Smithella propionica* gen. nov., sp. nov. (Liu *et al*., 1999). Genomic DNA was extracted from cocultures and the SSU rDNA gene of the SPOB was amplified using the polymerase chain reaction (PCR). The gene was cloned into a plasmid containing a T7 RNA polymerase promoter and transcripts were produced using T7 RNA polymerase and the Ampliscribe Transcription Kit (Epicentre Technologies, Madison, WI) (McMahon *et al*., 1998).

The oligonucleotide probes employed in this study are listed in Table 1. Probes were 5′ end labeled with ($\gamma-^{32}P$) ATP using bacteriophage T_4 polynucleotide kinase (Raskin *et al*., 1994). Membranes were washed at the previously determined wash temperature (T_W) (Table 1). The hybridization signals were quantified using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and the abundance of each target group was calculated using a standard curve constructed using the pure culture rRNA dilution series. For each membrane hybridized with a specific probe, an identical membrane was hybridized with a universal probe, S-*-Univ-1390-a-A-18 (Zheng *et al*., 1996), to be able to express the abundance of target SSU rRNA as a fraction of total SSU rRNA (Griffin *et al*., 1998; Raskin *et al*., 1997).

Table 1. Oligonucleotide probes used in hybridizations

Probe[a]	T_w	Target organism(s)	RNA standard	Original reference
S-*-Univ-1390-a-A-18	44	Virtually all Organisms		Zheng *et al*. (1996)
S-D-Arch-0915-a-A-20	58	Virtually all Archaea	*Methanosarcina acetivorans*	Stahl and Amann (1991)
S-D-Bact-0338-a-A-18	55	Virtually all Bacteria	*S. fumaroxidans* MPOB	Amann *et al*. (1990)
S-G-Msar-0821-a-A-21	60	*Methanosarcina* spp.	*Methanosarcina acetivorans*	Raskin *et al*. (1994)
S-S-Mst.co-0381-a-A-22	54	*Methanosaeta concilii*	*Methanosaeta concilii* GP6	Zheng and Raskin (2000)
S-F-Mbac-0310-a-A-22	57	*Methanobacteriaceae*	*Methanobacterium wolfeii*	Raskin *et al*. (1994)
S-S-S.fum-0464-a-A-19	52	*Syntrophobacter fumaroxidans* MPOB	*S. fumaroxidans* MPOB	Harmsen *et al*. (1995) and McMahon *et al*. (in preparation)
S-S-S.pfn-0460-a-A-21	53	*Syntrophobacter pfennigii*	*S. pfennigii* transcript	Harmsen *et al*. (1995) and McMahon *et al*. (in preparation)
S-S-S.wol-0223-a-A-19	57	*Syntrophobacter wolinii*	*S. wolinii* transcript	Harmsen (1996); McMahon *et al*. (in preparation)
S-G-Dsbb-0660-a-A-20	57	*Desulfobulbus propionicus*	*D. propionicus* transcript	Devereux *et al*. (1992)
S-*-LYP-0450-a-A-23	54	Strain LYP[b]	Strain LYP transcript	McMahon *et al*. (in preparation)
S-F-Synm-0700-a-A-23[c]	54	*Syntrophomonadaceae*	*S. wolfeii* LYB	Hansen *et al*. (1999)

[a] Probe names are standardized according to the oligonucleotide probe database (Alm *et al*., 1996).

[b] Strain LYP was recently proposed to be the type strain of *Smithella propionica* gen. nov., sp. nov. (Liu *et al*., 1999). Therefore, probe S-*-LYP-0450-a-A-23 can be renamed as S-S-S.prop-0450-a-A-23.

[c] In order to eliminate the potential binding of this probe to non-target species, an unlabeled version of a competitive probe (S-F-Synm-0700-p-A-23) was always used together with the labeled probe S-F-Synm-0700-a-A-23 in a 1:1 ratio (Hansen *et al*., 1999).

Table 2. Microbial community analysis of inoculum sources (% SSU rRNA ± standard deviation)

Probe	Target organisms	Cattle manure	Sludge	Sludge
		Experiment 1	Experiment 1	Experiment 2
		Digesters 2, 3	Digesters 2, 4	Digesters 7, 8
S-D-Bact-0338-a-A-18	Virtually all *Bacteria*	84.7 ± 8.5	73.9 ± 9.0	63.7 ± 8.7
S-D-Arch-0915-a-A-20	Virtually all *Archaea*	0.47 ± 0.10	4.52 ± 0.46	8.33 ± 0.95
S-G-Msar-0821-a-A-21	*Methanosarcina* spp.	< 0.17[a]	< 0.12[a]	0.24 ± 0.13
S-S-Mst.co-0381-a-A-22	*Methanosaeta concilii*	0.42 ± 0.04	1.54 ± 0.10	3.32 ± 0.38
S-F-Mbac-0310-a-A-22	*Methanobacteriaceae*	0.19 ± 0.06	< 0.12[a]	< 0.15[a]
S-S-S.fum-0464-a-A-19	*Syntrophobacter fumaroxidans* MPOB	0.44 ± 0.12	0.27 ± 0.14	0.37 ± 0.12
S-S-S.pfn-0460-a-A-19	*Syntrophobacter pfennigii*	0.37 ± 0.07	0.33 ± 0.08	0.48 ± 0.08
S-S-S.wol-0223-a-A-19	*Syntrophobacter wolinii*	0.22 ± 0.11	0.44 ± 0.12	0.43 ± 0.11
S-G-Dsbb-0660-a-A-20	*Desulfobulbus* spp.	0.34 ± 0.06	0.53 ± 0.08	0.43 ± 0.06
S-*-LYP-0450-a-A-23	Strain LYP	0.25 ± 0.31	< 0.20[a]	0.28 ± 0.29
S-F-Synm-0700-a-A-23	*Syntrophomonadaceae*	0.49 ± 0.16	0.42 ± 0.16	1.76 ± 0.25

[a] The relative hybridization signals from some samples were below the detection limit. Detection limits vary with probe labeling efficiency, nucleic acid loading, and length of exposure in PhosphorImager cassettes and are therefore different for each sample and probe.

Table 1 also indicates whether native or transcribed rRNA was used for quantification.

RESULTS

Microbial community structure in inocula

Table 2 presents the hybridization results for the two inoculum sources used in Experiment 1 (cattle manure and anaerobic digester sludge) and for the anaerobic digester sludge inoculum used in Experiment 2. Anaerobic sludge for Experiments 1 and 2 was obtained from the same sewage sludge digester, but on separate occasions. Methanogens (*Archaea*) were significantly more abundant in anaerobic sludge than in manure, though their levels in the sludge for Experiment 1 were lower than those in the sludge for Experiment 2 and in other sludge samples (Griffin *et al*., 1998; Raskin *et al*., 1995). *Methanosaeta concilii* was particularly abundant, while *Methanosarcina* spp. were virtually absent. This observation is consistent with the low acetate concentrations present in the anaerobic sludge digester from which this inoculum was taken (Griffin *et al*., 1998). The hydrogenotrophic *Methanobacteriaceae* were not detectable in the sludge samples, but were present at low levels in the manure. *Syntrophobacter fumaroxidans* MPOB was slightly more abundant in the manure and *S. wolinii* was found at higher levels in the sludge samples. *Syntrophobacter pfennigii* was present at comparable levels in the manure and the sludge used for Experiment 1, but was somewhat more abundant in the sludge used for Experiment 2. *Desulfobulbus* spp. were more prevalent in the sludge than in the manure, especially in the sludge used for Experiment 1. The SSU rRNA levels of strain LYP were close to the detection limit in all three inoculum sources. Finally, *Syntrophomonadaceae* were present in all three samples, but were more abundant in the sludge used for Experiment 2.

Population dynamics during stabilization of Digesters 1–3

During the startup of the digesters in Experiment 1, Digester 4 performed well, while Digesters 1–3 experienced very high concentrations of VFA and low biogas production (Stroot *et al*., 2001). Acetate accumulated in these three digesters to approximately 4500 mg/l as acetic acid (HAc), though it was effectively metabolized starting around days 10–12 (Stroot *et al*., 2001). Propionate also built up to high concentrations (above 1500 mg/l as HAc), and persisted for more than 2 weeks after day 14 (i.e., the day that the mixing level was reduced). The subsequent decline in propionate concentration between days 30 and 40 was attributed to the reduced mixing levels (Stroot *et al*., 2001). Therefore, we analyzed microbial population dynamics in the three digesters before, during, and after propionate removal to determine which organisms were most active during this stabilization period.

To be able to link digester performance with microbial population dynamics in Digesters 1–3, selected chemical performance data are presented in Fig. 1A–C along with hybridization results for various target populations (Fig. 1D–L). Additional chemical results are presented in the companion paper (Stroot *et al*., 2001). The rapid consumption of propionate resulted in a temporary increase in specific gas production in all three digesters. Acetate and butyrate levels were low in the digesters after day 25, though a slight increase in acetate concentration was observed when propionate was consumed (Fig. 1A–C; Stroot *et al*., 2001).

The results of quantitative hybridizations indicated that the relative rRNA levels of *Archaea* increased substantially in all three digesters between days 25 and 39 (Fig. 1D–F). *Methanosarcina* spp. and *Methanobacteriaceae* were the most abundant methanogen populations targeted by the probes used in this study (Fig. 1D–F), while *M. concilii* SSU rRNA generally was present at around 0.5% (Fig. 1J–L). In

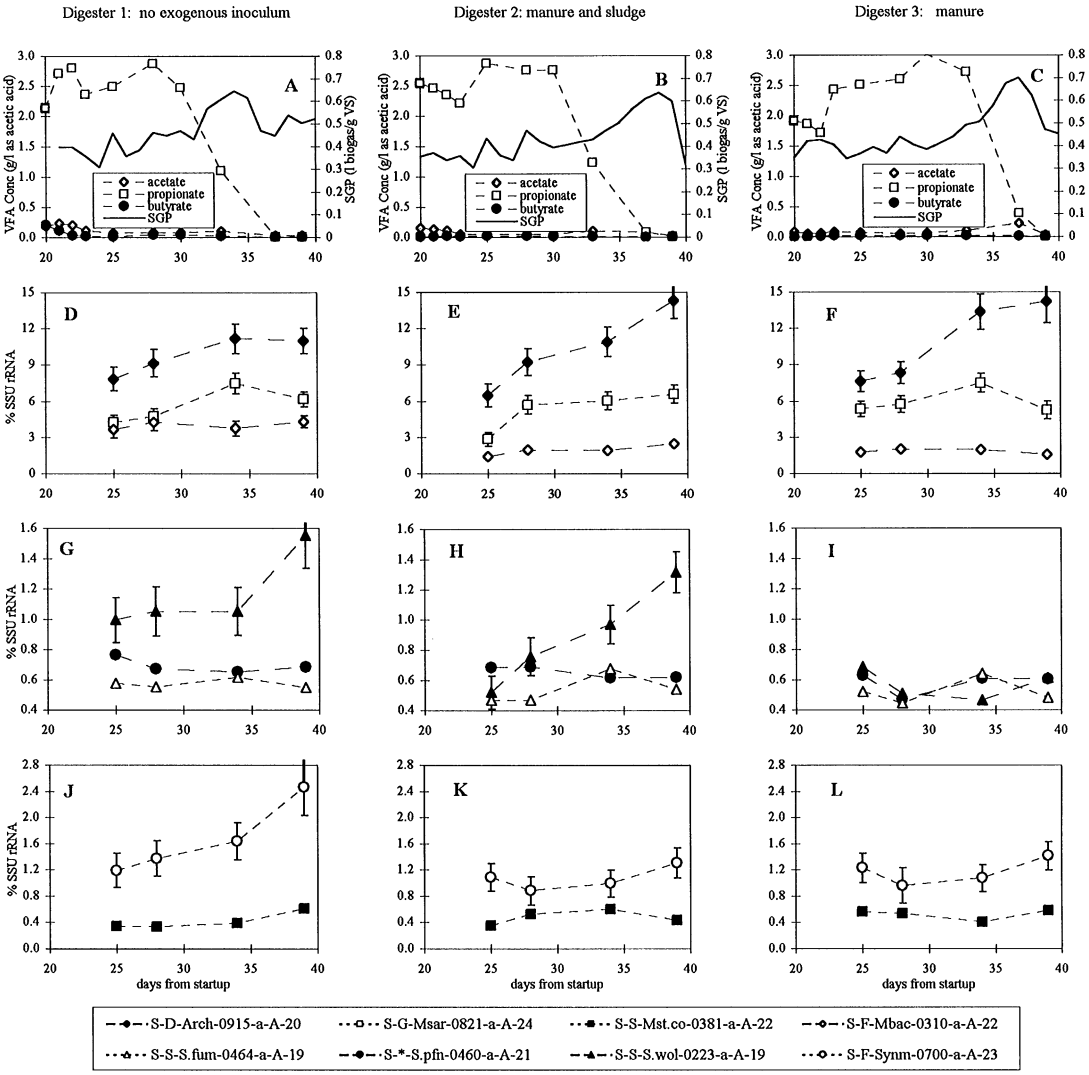

Fig. 1. VFA concentrations, specific gas production (SGP), and microbial population dynamics in Digesters 1 (A, D, G, J), 2 (B, E, H, K), and 3 (C, F, I, L) during digester stabilization (days 20–40). The organic loading rate was 2.6 g VS/l active volume/day for all three digesters during the time period shown. Error bars indicate standard deviations. Some standard deviations are not reported in panels (G)–(I) to improve the clarity of presentation; coefficients of variation were generally between 20 and 30%.

contrast, in Digester 4, which exhibited a rapid startup even under continuously mixed conditions, *M. concilii* were present at levels an order of magnitude higher (4.8% SSU rRNA) and *Methanosarcina* spp. were virtually absent. *Methanosarcina* spp. abundance increased in Digesters 1–3 and stabilized or decreased slightly after propionate levels had been reduced. The relative levels of *Methanobacteriaceae* did not change appreciably in any of the three digesters during or after propionate turnover, though Digester 1 contained higher levels of these hydrogenotrophic methanogens throughout this time period.

Syntrophobacter wolinii appeared to be involved in propionate consumption in Digesters 1 and 2, since its relative abundance increased greatly during this period (Fig. 1G and H). However, *S. wolinii* levels remained lower in Digester 3 and did not increase during propionate turn over (Fig. 1I). It is thus unclear which organisms were degrading propionate in Digester 3. *Syntrophobacter pfennigii* and *S. fumaroxidans* strain MPOB were present at all times, but their levels did not change appreciably in any digester between days 25 and 39 (Fig. 1G–I). *Desulfobulbus* spp. were present at low levels in Digesters 1–3 (0.23–0.35% SSU rRNA) during this same period. Strain LYP was not detectable in any of the samples. *Syntrophomonadaceae* were present at significant levels in all three digesters, but their relative abundance increased only in Digester 1 (Fig. 1J).

Population dynamics in Digesters 7-MM and 8-CM

To be able to relate microbial population dynamics to digester performance, some chemical performance

data for Digesters 7-MM and 8-CM (Experiment 3) are presented in Fig. 2A and B. Additional chemical results are presented in the companion paper (Stroot *et al.*, 2001). Propionate had reached very high levels (2000 mg/l as HAc) in Digester 7 during Experiment 2 (Stroot *et al.*, 2001) and, consequently, the propionate concentration was high in Digester 7-MM at the start of Experiment 3 (Fig. 2A). After 18 days of operation under minimally mixed conditions, propionate was almost completely consumed and a

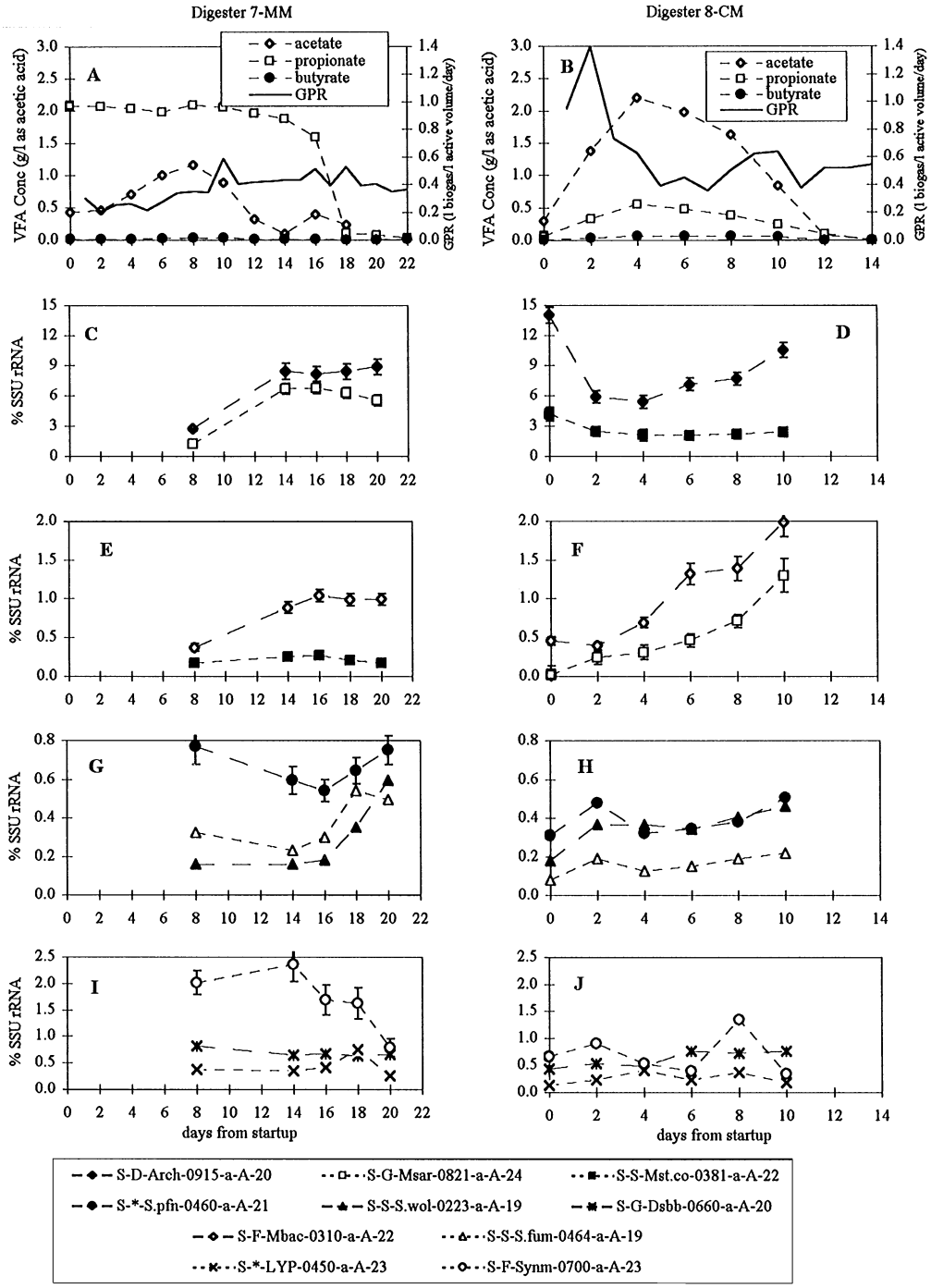

Fig. 2. VFA concentrations, gas production rate (GPR), and microbial population dynamics in Digesters 7-MM (A, C, E, G, I) and 8-CM (B, D, F, H, J). The organic loading rates for these digesters are given in the companion paper (Stroot *et al.*, 2001). Error bars indicate standard deviations. Some standard deviations are not reported in panels (G)–(J) to improve the clarity of presentation; coefficients of variation were generally between 20 and 30%, except for probe S-S-S.wol-0223-a-A-19 in panel (G), which had very high coefficients of variation on days 8, 14, and 16. The detection limit for these samples was approximately 0.2%, indicating that the hybridization response for these days was below the detection limit.

temporary elevation in acetate levels was observed. In Digester 8-CM, acetate and propionate levels initially were low due to the stable conditions in Digester 8 (Experiment 2), but began to accumulate immediately after continuous mixing was initiated (Fig. 2B).

Microbial population dynamics in Digesters 7-MM and 8-CM are shown in Fig. 2C–J. *Archaea* were present initially at markedly different levels in the two digesters (Fig. 2C and D). *Archaea* levels likely were low in Digester 7 since this digester exhibited low biogas production (Stroot *et al*., 2001). When the mixing level was reduced at the start of Experiment 3, digester performance improved and *Archaea* levels increased substantially within 14 days (Fig. 2C). Relative archaeal concentrations decreased dramatically in Digester 8-CM within 2 days after the switch to continuous mixing, and subsequently increased again when digester feeding was suspended in an attempt to maintain stable conditions (Fig. 2D). The *Archaea* in Digester 7-MM consisted mostly of *Methanosarcina* spp. (Fig. 2C), though *Methanobacteriaceae* levels also increased substantially during propionate turnover (Fig. 2E). *Methanosaeta concilii* was present in Digester 7-MM at levels at least an order of magnitude lower than in Digester 8-CM (Fig. 2E and D, respectively). *Methanosarcina* spp. were initially very low in Digester 8-CM (Fig. 2F), since they were virtually absent in Digester 8 at the end of Experiment 2 (McMahon *et al*., in preparation). However, as acetate became available for these fast-growing aceticlastic methanogens, their levels increased sharply (Fig. 2F). *Methanosaeta concilii* concentrations in Digester 8-CM decreased from 4.1 to 2.0% as acetate and propionate accumulated, but remained relatively steady (between 2.0 and 2.5%) while these VFAs were consumed (Fig. 2D). In contrast, *M. concilii* levels remained high (between 4.3 and 4.4%) in Digester 8-MM during the same period (data not shown). During VFA degradation, a substantial increase in *Methanobacteriaceae* activity also was observed in Digester 8-CM (Fig. 2F).

As the propionate concentration fell dramatically in Digester 7-MM, several changes were observed in the SPOB populations. After day 14, the relative levels of *S. fumaroxidans* strain MPOB and *S. wolinii* increased sharply (Fig. 2G) and the levels of strain LYP roughly doubled (Fig. 2I). In contrast, their levels did not change appreciably in Digester 7-CM as propionate persisted (data not shown). The activity of *S. pfennigii* was highest in Digester 7-MM on day 8 (Fig. 2G), at almost twice the level observed in Digester 8-CM (Fig. 2H). The abundance of this SPOB decreased from day 8 to 16 in Digester 7-MM, but then increased steadily as propoionate was consumed. In Digester 8-CM, levels of *S. fumaroxidans* strain MPOB, *S. wolinii*, and *S. pfennigii* all increased initially, but then remained relatively constant until day 10 (Fig. 2H). *Desulfobulbus* spp. were present at similar concentrations in both reactors (Fig. 2I and J) and their relative abundance increased slightly in Digester 8-CM during propionate consumption. *Syntrophomonadaceae* levels were initially high in Digester 7-MM, but decreased as the digester stabilized (Fig. 2I). By day 20, their relative abundance in Digester 7-MM ($0.79 \pm 0.17\%$) was similar to the levels observed in Digester 8-MM ($0.75 \pm 0.17\%$). In contrast, these SFAS remained active in Digester 7-CM ($2.34 \pm 0.33\%$ on day 20). In Digester 8-CM, *Syntrophomonadaceae* levels fluctuated, rising on day 8 to $1.34 \pm 0.43\%$ (Fig. 2J).

DISCUSSION

The methanogenic population dynamics observed in this study support previous hypotheses about the importance of these organisms in anaerobic digestion processes (Griffin *et al*. , 1998; Novaes, 1986; Raskin *et al*., 1994; Zinder, 1984). Methanogen (archaeal) abundance increased dramatically as VFA were consumed during digester stabilization following a reduction in the mixing level. The most dramatic increases were observed during propionate turnover in Digesters 2 (Fig. 1E) and 7-MM (Fig. 2C). These increases were likely the combined result of an increase in substrate availability as propionate was degraded to acetate and reducing equivalents (hydrogen and/or formate), and the decrease in inhibition caused by elevated levels of propionate (Ahring *et al*., 1995; Barredo and Evison, 1991; Henson *et al*., 1986). The increase in methanogen abundance was consistent with the concurrent rise in specific gas production (Fig. 1A–C) and gas production rate (Fig. 2A).

Methanosarcina spp. were the most abundant aceticlastic methanogens in digesters with a history of high acetate levels, while *M. concilii* levels were highest in the most stable digesters (Digesters 4 and 8-MM), confirming previous hypotheses (e.g., Zinder, 1993): *Methanosarcina* (generalists with high growth rates at elevated acetate concentrations) should be favored in systems with significant acetate accumulation, while *Methanosaeata* (specialists with a higher affinity for acetate) should have a competitive advantage in much more stable habitats, in which acetate levels are low. Previous work on codigestion produced similar results (Griffin *et al*., 1998; McMahon *et al*., in preparation). *Methanobacteriaceae* levels increased substantially as VFAs were rapidly degraded in Digester 7-MM, when hydrogen and formate were presumably plentiful. However, *Methanobacteriaceae* levels did not increase during propionate turnover in Digesters 1–3 (Fig. 1). In these systems, *Methanobacteriaceae* were already abundant just prior to propionate turnover, suggesting that their levels may have been sufficient to consume the hydrogen/formate produced. Alternatively, the increase in total archaeal abundance

indicates that other hydrogenotrophic methanogens may have been more active in these systems. In general, the ecological significance of different hydrogen and formate utilizing methanogens and the competitiveness among these populations have not been studied in as much detail as for aceticlastic methanogens.

During periods of rapid propionate consumption, SPOB were dependent on aceticlastic methanogens (*Methanosarcina* spp. and *Methanosaeta* spp.) and hydrogenotrophic methanogens (such as *Methanobacteriaceae*) to consume their metabolic products. The long lag period before SPOB began to metabolize large quantities of propionate in Digesters 1–3, and 7-MM may have occurred because adequate syntrophic interactions between SPOB and methanogens had not been established. Notably, on day 25, *S. wolinii* levels were significantly higher in Digester 1, compared to those in Digesters 2 and 3, possibly because the hydrogenotrophic *Methanobacteriaceae*, which may have served as the syntrophic partners of *S. wolinii*, were comparatively more established in Digester 1 prior to the switch to minimally mixed conditions. As a result, a significant reduction in propionate levels was observed earlier in this reactor than in Digester 2 or 3 (Fig. 1A–C). Table 3 summarizes the levels of *Methanobacteriaceae* and *S. wolinii* in all three digesters before and after the reduction in mixing level. A well-established hydrogen consuming population likely allowed the syntrophic VFA oxidizers to grow more quickly and to consume propionate more rapidly, thereby stabilizing Digester 1 sooner. Other researchers have also observed the dependence of SPOB on well-developed hydrogenotrophic populations (Stams *et al*., 1992; van Lier *et al*., 1993). These observations underscore the importance of inoculum selection for the long-term stability of anaerobic digesters. Digester 1 was started without an inoculum, whereas Digesters 2 and 3 were started with a mixture of manure and anaerobic sewage sludge and anaerobic sewage sludge, respectively. Thus, Digesters 2 and 3 contained significant levels of methanogens from the start. However, the dominant hydrogenotrophic methanogens in anaerobic sewage sludge are *Methanomicrobiales,* while *Methanobacteriaceae* are present only at very low levels (Griffin *et al*., 1998; Raskin *et al*., 1995). It is possible that *Methanobacteriaceae* were not able to compete with the abundant *Methanomicrobiales* during startup of Digesters 2 and 3, whereas they were able to increase to significant levels in Digester 1 immediately after startup. If *Methanobacteriaceae* are the preferred syntrophic partners for SPOB, the low abundance of these hydrogenotrophs could have hindered SPOB activity.

In general, SPOB were present at low levels in most digesters analyzed in this study. Other researchers noted that levels of propionate-degrading populations are probably especially low in stable digesters which do not produce propionate, especially since the range of substrates that can be used by SPOB is limited (Harper and Pohland, 1986; McCarty and Mosey, 1991; Xing *et al*., 1997a,b). This hypothesis could have implications for protecting against overload, since digesters with a history of very stable operation may be particularly susceptible to failure during a sudden influx of organic material. The effects of digester history on system performance during overload conditions is further discussed in a related study (McMahon *et al*., in preparation).

In the present study, low levels of SPOB were observed in the continuously mixed Digester 8-CM, even though substrate (propionate) was plentiful. Propionate was degraded when the organic loading rate was reduced to prevent failure, but it was consumed more slowly than in Digester 7-MM. The SPOB apparently did not thrive under these conditions, but managed to maintain a small population and eventually consume the accumulated propionate. This apparent inhibition could have been the result of high acetate levels or could have been caused by the physical disruption of syntrophic interactions. Previous studies document the inhibitory effects of acetate and hydrogen accumulation on propionate degradation (Ahring and Westerman, 1985; Fukuzaki *et al*., 1990; Hickey and Switzenbaum, 1991), but a disruption of the spatial juxtaposition of syntrophs and their partners by vigorous mixing may also have caused such inhibition (Conrad *et al*., 1985; Dolfing, 1992; Whitmore *et al*., 1987). Also, *Desulfobulbus* spp. may have played some role in propionate consumption in this reactor.

No direct evidence is available to confirm that the SPOB were utilizing propionate syntrophically in our systems since several of these organisms are known to

Table 3. Percent abundance of *Methanobacteriaceae* and *Syntrophobacter wolinii* before (day 12) and after (day 25) the switch to minimally mixed conditions for Experiment 1[a]

	Methanobacteriaceae % SSU rRNA ± SD		*Syntrophobacter wolinii* % SSU rRNA ± SD	
Digester	Day 12	Day 25	Day 12	Day 25
1	2.97 ± 0.30	3.62 ± 0.70	0.44 ± 0.08	0.99 ± 0.14
2	0.82 ± 0.06	1.41 ± 0.12	0.42 ± 0.12	0.52 ± 0.11
3	0.44 ± 0.05	1.73 ± 0.11	0.41 ± 0.13	0.68 ± 0.12

[a] All three digesters were switched to minimally mixed conditions after day 14. SD is standard deviation.

grow by sulfate-reducing or fermentative metabolisms (Stams *et al*., 1993; van Kuijk and Stams, 1995; Wallrabenstein *et al*., 1994, 1995). However, we speculate that the SPOB were not reducing sulfate in the digesters, since sulfate was shown to be limiting (Griffin *et al*., 1998). Even if enough sulfate was present to serve as electron acceptor, it would probably be utilized by SRB who have a higher affinity for sulfate (van Kuijk and Stams, 1995), such as *Desulfobulbus* spp. It is also deemed unlikely that substrates such as fumarate or pyruvate would have much ecological importance (Schink, 1992; Stams, 1997). In addition, most fluctuations in SPOB population abundance coincided with changes in VFA concentrations and methanogen activity, suggesting that these organisms were indeed syntrophically degrading propionate.

Data for *S. wolinii*, *S. pfennigii*, *Desulfobulbus propionicus*, and strain LYP were obtained using *in vitro* transcribed rRNA as standards for quantitative hybridizations (Table 1). We recently determined that the abundance of target groups can be underestimated when *in vitro* transcribed rRNA is used as a standard in hybridizations (McMahon *et al*., 1998). However, we demonstrated that the use of transcripts still made it possible to monitor changes in population levels over time and to compare population abundance between systems. Thus, the data presented here should be interpreted with this limitation in mind.

Digester 7-MM, which had a history of unstable operation, initially supported SFAS populations at levels more than twice those observed in digesters which had been stable for long periods of time, such as Digester 8-MM and other minimally mixed reactors described in a related study (McMahon *et al*., in preparation). This may be attributed to the diverse substrate pool present during unbalanced fermentation and the wide substrate utilization range of SFAS (McCarty and Mosey, 1991; Roy *et al*., 1986; Zhao *et al*., 1993). As Digester 7-MM became more stable after the decrease in mixing levels, SFAS abundance decreased to around 1.0%. Their levels remained high in Digester 7-CM (data not reported) presumably because substrates remained plentiful. SFAS abundance was low initially in Digester 8-CM though their levels increased transiently as fermenters began to produce longer chain VFAs in response to the switch to continuous mixing in Digester 8-CM. SFAS concentrations did not increase significantly possibly because the continuously mixed conditions inhibited syntrophic associations with methanogens. In contrast to the decrease in SFAS abundance in Digester 7-MM the SFAS concentration increased during stabilization of Digester 1. One explanation for this observation is that hydrogenotrophic methanogen populations (*Methanobacteriaceae*) were much better developed in Digester 1 than in Digesters 2, 3, and 7-MM (Table 3 and Fig. 2E), allowing a higher rate of syntrophic growth on longer chain acids while they were still present. As Digester 1 continued to stabilize beyond day 39, SFAS levels decreased to approximately 1.0% on day 85, at which point the system had been stable for about 45 days (Stroot *et al*., 2001). However, microbial data are not available for the period between days 39 and 85, so this hypothesis cannot be evaluated in more detail. In any case, the data suggest that elevated SFAS concentrations indicate a history of recent instability.

In summary, population dynamics of SPOB, SFAS, and methanogens were used to evaluate the stabilization of anaerobic co-digesters, which had been operated with high levels of VFAs characteristic for overload conditions. *Methanosarcina* spp. were the dominant aceticlastic methanogens present in digesters with high rates of propionate turnover, while *Methanosaeta concilii* were more abundant in stable systems maintaining low concentrations of acetate. During destabilization due to an increase in mixing levels, *Methanosarcina* spp. and *Methanobacteriacea* increased dramatically. *Syntrophobacter wolinii* appeared to be the SPOB responsible for propionate turnover during stabilization. *Syntrophobacter wolinii* and SFAS were particularly abundant in the two digesters that suffered from high VFA accumulation during startup under continuously mixed conditions. Continuously mixed conditions appeared to inhibit the syntrophic interactions between SPOB or SFAS and their aceticlastic and hydrogenotrophic partners, possibly by disrupting spatial juxtaposition. Other studies have documented the formation of close associations between syntrophs and methanogens (e.g., granules and artificially constructed cocultures) effectively degrading propionate or butyrate (Harmsen *et al*., 1996; Schmidt and Ahring, 1995; Stams *et al*., 1992); the current study suggests that maintaining such associations can be critical to the stable operation of anaerobic digesters. The results presented here offer experimental evidence for a number of widely accepted theories of competition and population dynamics in anaerobic digesters. However, the complexity of these systems makes it difficult to conclusively describe all microbial interactions with currently available techniques.

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