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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Effect of Shear on Performance and Microbial Ecology of Continuously Stirred Anaerobic Digesters Treating Animal Manure

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ABSTRACT: We determined the effect of different mixing intensities on the performance, methanogenic population dynamics, and juxtaposition of syntrophic microbes in anaerobic digesters treating cow manure from a dairy farm. Computer automated radioactive particle tracking in conjunction with computational fluid dynamics was performed to quantify the shear levels locally. Four continuously stirred anaerobic digesters were operated at different mixing intensities of 1,500, 500, 250, and 50 revolutions per min (RPM) over a 260-day period at a temperature of $34 \pm 1^{\circ}C$. Animal manure at a volatile solids (VS) concentration of 50 g/L was fed into the digesters daily at five different organic loading rates between 0.6 and 3.5 g VS/L day. The different mixing intensities had no effect on the biogas production rates and yields at steady-state conditions. A methane yield of 0.241 ± 0.007 L CH_4/g VS fed was obtained by pooling the data of all four digesters during steady-state periods. However, digester performance was affected negatively by mixing intensity during startup of the digesters, with lower biogas production rates and higher volatile fatty acids concentrations observed for the 1,500-RPM digester. Despite similar methane production yields and rates, the acetoclastic methanogenic populations were different for the high- and low-intensity mixed digesters with *Methanosarcina* spp. and *Methanosaeta concilii* as the predominant methanogens, respectively. For all four digesters, epifluorescence microscopy revealed decreasing microbial floc sizes beginning at week 4 and continuing through week 26 after which no microbial flocs remained. This decrease in size, and subsequent loss of microbial flocs did not, however, produce any long-term upsets in digester performance.

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Introduction

Interest in more environmentally sound waste management practices in the livestock industry has intensified, while the traditional waste management practices of storage in noncovered anaerobic lagoons and unrestricted land application is being discouraged due to their negative impacts on the environment. Rather than the atmospheric release from anaerobic lagoons (not covered), methane (a potent greenhouse gas) from anaerobic bioreactors (covered) is oxidized to carbon dioxide during controlled flaring or generation of bioenergy. The potential of bioenergy generation has recently re-established interest in farm-based digesters to treat animal waste (Marsch and LaMendola, 2006). Anaerobic digestion of animal manure also reduces odor, controls ammonia release, and produces a fertilizer as nutrient-enriched biosludge (Angelidaki et al., 2003). Although industries have successfully utilized anaerobic digestion to reduce organic pollutants in high-strength waste streams for over 30 years (Verstraete et al., 1996), implementation of anaerobic digesters on US farms for the purpose of treating animal manure has had high failure rates. Such high failure rates are believed to be due to poor design and construction (Lusk, 1998), and inadequate acclimation of biomass to, for example, high ammonia levels (Angenent et al., 2002a).

Mixing plays several essential roles during anaerobic digestion of sludges, including enhancing substrate contact with the microbial community, improving pH and temperature uniformity, preventing stratification and scum accumulation, facilitating the removal of biogas from the digestant, and aiding in particle size reduction (Stafford et al., 1980). The most conventional method of mixing in full-scale reactors treating sludges is through mechanical methods using an impeller, liquid recirculation, or gas lift/gas recirculation. The intensity at which mixing occurs (the shear rate) has an effect on performance of anaerobic digestion of municipal sludges, such as waste activated sludge (Strenstrom et al., 1983). Research has shown that high mixing intensities resulted in particle size reduction and diffusion limitation reduction, which increased processing capacity for a digester treating municipal sludge (Lanting, 2003). In contrast, several other studies have shown that high mixing intensity and duration had a detrimental effect on reactor performance (Angenent et al., 2001; Dague et al., 1970; Hansen et al., 1999; McMahon et al., 2001; Speece et al., 2006; Stroot et al., 2001; Vavilin and Angelidaki, 2005). It has been hypothesized that a high shear rate may be harmful to anaerobic digestion because it disrupts spatial associations between syntrophic volatile fatty acid (VFA) oxidizing bacteria and hydrogen-utilizing methanogens. This has not been experimentally verified, however.

In understanding the role of mixing in anaerobic digestion with the ultimate goal to reduce failure rates of farm-based digesters, we studied the effect of mixing intensity (i.e., applied shear) on digester performance, methanogenic population dynamics, and syntrophic relationships in continuously stirred anaerobic digesters treating cow manure (i.e., water, urine, feces, and bedding material). Impeller speeds of 1,500, 500, 250, and 50-RPM, were applied to four identical 4.5-L digesters in parallel. Because we planned to study the break up of microbial flocs and the possible loss of a juxtaposed bacterial and archaeal community, we were interested in the local maximum shear intensity within the reactor rather than the spatially-averaged velocity gradient. Therefore, computer automated radioactive particle tracking (CARPT) in conjunction with computational fluid dynamics (CFD) was utilized to map shear distribution throughout the reactor and estimate local shear intensities. The performance of each digester was monitored with parameters indicating performance and stability, while molecular techniques, such as membrane hybridization and fluorescent in situ hybridization (FISH), were used to track changes in the methanogenic populations and the juxtaposition of bacteria and archaea in microbial flocs.

Materials and Methods

Experimental Apparatus

The reactor experiment was conducted with four laboratory-scale digesters made from clear PVC with a wet volume of 4.5 L. The digesters had a 25° slope angle hopper bottom (Fig. S1 in the supplementary materials) and were placed in a temperature-controlled chamber to maintain a temperature of 34 ± 1°C. Cold water recirculation was used to cool the digester contents of the 1,500 RPM digester to 34 ± 1°C, because heat from the mixer was conducted through the mixing shaft. The gas collection system of each digester setup consisted of a foam separation bottle, a pressurized ball used to eliminate air from being suctioned into the digesters during the decanting of effluent, a bubbler to allow visual determination of gas production, a biogas sampler, and a gas meter (Model 1 liter, Actaris Meterfabriek bv, Delft, The Netherlands). Each digester was continuously mixed with a mixer (Model 5vb, EMI, Inc., Clinton, CT) equipped with a 62-mm diameter axial flow impeller (Fig. S1; Lightnin A-310, Rochester, NY).

Reactor Operation

The digesters were inoculated with 4.5-L primary anaerobic digester sludge collected from the Metropolitan Sewer District's Coldwater Creek facility, St. Louis, MO. The anaerobic digester at this facility stabilizes a blend of primary sludge and waste activated sludge. After inoculation of the digesters, a 24-h acclimation period was allowed before the commencement of mixing. Raw dairy cow manure was collected fresh (less than 6 h after excretion) from the Martin Dairy Farm, Pevely, MO, twice throughout the study and stored at −20°C. The feed slurry was prepared from the collected raw manure by first screening through a 2-mm sieve and then by diluting with tap water to achieve a VS concentration of 50 g VS per liter (5% solids content based on VS). The first batch of cow manure was fed from day 0 until day 217, while the second batch was fed from day 218 until the end of the operating period. The reactors were fed manually every 24 ± 1 h by first removing an appropriate amount of reactor effluent and then adding the same volume of prepared manure feed (mixers were on during decanting and feeding). To avoid overloading of the reactors at startup, the initial loading rate was 0.6 g VS/L day, which was 16% of the target loading rate of 3.5 g VS/L day. The loading rate was increased in a step-wise manner after steady-state biogas production levels had been achieved with a minimum time period of one hydraulic retention time (HRT), except during the initial 0.6 g VS/L day loading period. Steady-state biogas production conditions were achieved when daily biogas production rates were within 10% of their average values after the operating period of at least one HRT time period. The loading rates used for the different loading steps throughout the study were 0.6 (days 1–49), 1.0 (days 50–121), 1.7 (days 122–196), 2.5 (days 197–234), and 3.5 g VS/L day (days 235–259), corresponding to a HRT (and sludge retention time [SRT] since they are the same in completely-stirred reactors) of 83, 50, 30, 20, and 15 days, respectively. To estimate the methane yields, we first calculated the specific methane production rates by pooling the daily methane production rates from a period of at least 10 days at the end of a steady-state period and correcting them to specific temperatures of 0°C and 1 atm.

Mixing Intensities

The impeller rotational speeds were set to a predetermined applied RPM by using a tachometer to calibrate at the start of the operating period (Bex-O-Meter, Model 38, The Bex Company, San Francisco, CA). This device was also used as a rotating torque meter to measure the spatially-averaged velocity gradient (g) as described by Sajjad and Cleasby (1995). For CARPT, a 150-μm Sc-46 particle was first sized and then coated with paralyne by Para Tech Coating, Inc. (Middletown, CT) after which it was activated at the University of Missouri-Columbia's reactor. Subsequently, we enclosed the particle in a 1-mm polypropylene ball with an adjusted overall density equal to the density of the digester contents. An array of 16 scintillating NaI detectors (Saint-Gobain, Newbury, OH) was used to track the Sc-46 particle. We calibrated the system setup by placing the tracer particle at 427 known locations and acquiring the counts at a frequency of 50 Hz. Tracing experiments were conducted over a time frame of 24 h for each digester. All the relevant dimensions, such as the vessel shape and diameter and the type of impeller, were the same as those used in the performance experiment. A more detailed discussion of the CARPT technique can be found elsewhere (Karim et al., 2004). Commercial CFD software (CFX 5.7.1, ANSYS, Inc., Canonsburg, PA) was used to model the geometry and to generate the body-fitted grids. In total, 43,000 computational cells were used for these CFD simulations. Details of the CFD experiments are given in the supplementary materials.

Physical and Chemical Analysis

Feed and effluent samples were analyzed for pH, total solids (TS), VS, total VFAs, soluble chemical oxygen demand (SCOD), and alkalinity according to procedures in *Standard Methods* (APHA, 1998). The amount of TS and VS in the inoculum was also determined. Total ammonium-N (ammonia and ammonium) was measured using an ammonia electrode (Model 95-12, Thermo Electron Corporation, Beverly, MA). Methane content of the biogas was determined weekly using a gas chromatograph (Series 350, Gow-Mac Instruments, Co., Lehigh Valley, PA) with a thermal conductivity detector. The temperature of the injection port and the packed column (HayesepQ, Supelco, Bellefonte, PA) was 20°C, and that of the detector was 40°C. Helium at a flow rate of 60 mL/min was used as the carrier gas.

Membrane Hybridization

Digester samples were centrifuged at 9,300 *g* and a temperature of 4°C, the supernatant was removed, and samples were immediately stored at −80°C. RNA was extracted from these samples by a low-pH hot-phenol extraction method, denatured, applied to Magna Charge membranes (GE Osmonics, Minnetonka, MN), and hybridized with [γ-^{32}P] ATP-labeled oligonucleotide hybridization probes targeting small subunit ribosomal RNA (SSU rRNA) (Raskin et al., 1997; Table I). The hybridization signal was quantified using a Phosphor Imager (Bio-Rad, Hercules, CA). Each population was expressed as a percentage of the total SSU rRNA determined by using a universal probe (Table I). We corrected the relative SSU rRNA levels of the methanogenic populations for nonspecific binding by subtracting the signal obtained from methanogenic probes that hybridized to *Escherichia coli* rRNA.

Fluorescent In Situ Hybridization

Digester samples were fixed with 4% paraformaldehyde for 2 h at 20°C and stored with phosphate buffer saline solution and ice-cold ethanol at −20°C. Hybridization was performed with 16S rRNA-targeting oligonucleotide DNA probes specific for archaea (S-D-Arch-0915-a-A-20) and bacteria (S-D-Bact-0338-a-A-18) (Table I) according to de los Reyes et al. (1998). Specimens were viewed with an epifluorescence microscope (BX41, Olympus, Melville, NY) and digital images were taken with a CCD camera (QImaging, Burnaby, Canada) and saved in Openlab 3.5 software (Improvision, Inc., Lexington, MA).

Table I. Oligonucleotide probes used in hybridizations.

Probes[a]	Target group	Characteristics[b]	References
S-*-Univ-1390-a-A-18	Virtually all organisms		Zheng et al. (1996)
S-D-Bact-0338-a-A-18	Virtually all bacteria		Amann et al. (1990)
S-D-Arch-0915-a-A-20	Virtually all Archaea		Stahl and Amann (1991)
S-O-Mmic-1200-a-A-21	Methanomicrobiales	Most use H_2–CO_2 and formate	Raskin et al. (1994b)
S-F-Mbac-0310-a-A-22	Methanobacteriaceae	Most use H_2–CO_2, some also use formate	Raskin et al. (1994b)
S-F-Mcoc-1109-a-A-20	Methanococcaceae	Most use H_2–CO_2 and formate	Raskin et al. (1994b)
S-G-Msar-0821-a-A-24	*Methanosarcina* spp.	Use acetate and other substrates (H_2–CO_2, methanol, and methylamines)	Raskin et al. (1994b)
S-S-M.con-0381-a-A-22	*Methanosaeta concilii*	Uses only acetate	Zheng and Raskin (2000)

[a]Probe nomenclature according to Alm et al. (1996).
[b]According to Raskin et al. (1994a).

Results

Mixing Intensity

Impeller mixing in the digesters produced a spatially-averaged velocity gradient (g) of 3,500, 630, 210, and 17 s^{-1} for the 1,500, 500, 250, and 50-RPM digesters, respectively. The 1,500 and 50-RPM intensities were close to the highest and lowest operational RPM achievable by the type of impeller motor that was used. With such a broad range of applied shear (and the absence of baffles) the vortices in the 1,500 and 500-RPM digesters created a 35% and 10% increase in maximum height of the liquid inside the digester, respectively, while only a small vortex developed in the 250-RPM digester, and no vortex was noticed in the 50-RPM digester. The absence of baffles promoted a high shear due to azimuthal (plane circles around the vertically-oriented impeller shaft) velocities. To quantify these azimuthal velocities, and also axial (vertical) and radial (horizontal) velocities, spatially throughout the bioreactor, CARPT was performed on the 500 and 250-RPM digesters. Limitations of the CARPT technique prevented analysis of the 1,500-RPM digester due to large standard error values because of excessive mixing. However, a CFD model, which was verified for the 500 and 250-RPM experiments, was used to estimate the 1,500-RPM mixing velocities. CARPT was also not successful for the 50-RPM digester, because the radioactive particle became fixed in an area with a low mixing intensity. CFD simulations for this RPM were not performed.

The velocity vector plots obtained in the 500 and 250-RPM digesters using CARPT show the direction and magnitude of the velocities inside the digesters, as well as the distinct circulation loops (Fig. S3 in the supplementary materials). The radial profiles (time and azimuthally averaged in cm [*x*-axes]) of the axial (vertical), radial (horizontal), and azimuthal (plane circles around the mixing shaft) velocities for the 1,500, 500, and 250-RPM operating conditions were plotted for three different axial locations: at the impeller plane ($z = 5$ cm—panels C); at the center ($z = 13$ cm—panels B) height of the liquid column; and at the top of the liquid column ($z = 23$ cm—panels A) (Fig. 1 and Figs. S4 and S5 in the supplementary materials). The radial profile of the axial velocity at the plane of the impeller showed the lowest axial velocities (between ~ -5 and $+15$ cm/s) compared to the center and the top of the liquid column (between ~ -35 and $+20$ cm/s), because the impeller pushed the liquid radially outwards (Fig. 1). For the middle and top of the liquid column, the axial velocities were upwards (positive) near the wall and downwards (negative) near the center following the velocity vectors (Fig. S3 in the supplementary materials). For all three axial locations, the mixing intensity affected the downward velocities relatively more than the upward velocities (Fig. 1). Results for the radial velocities and azimuthal velocities are found in the supplementary materials (Figs. S4 and S5, respectively).

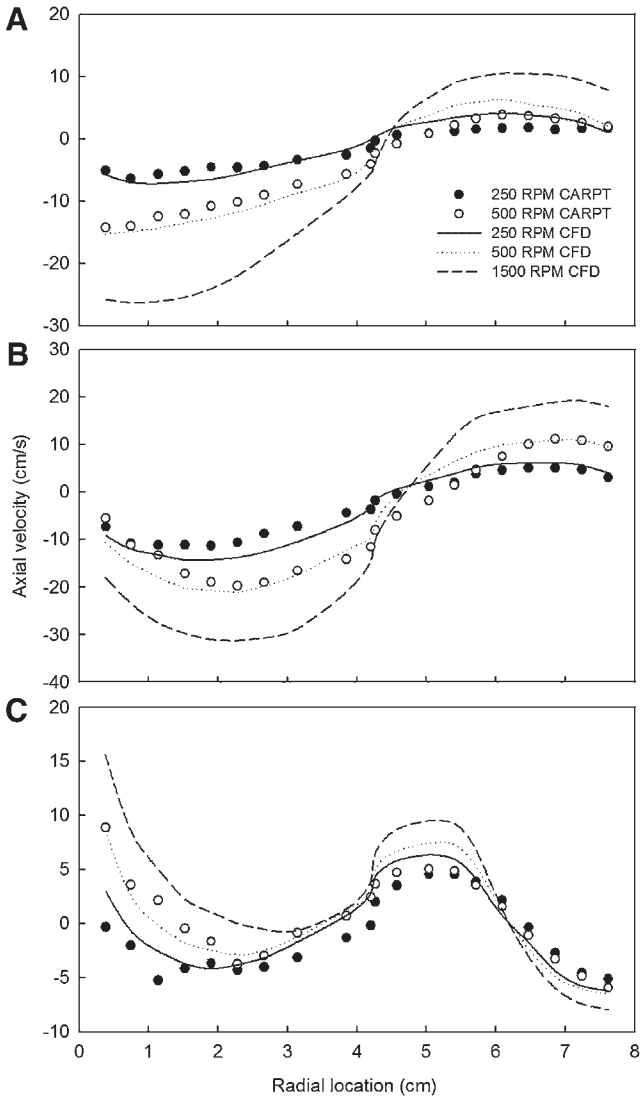

Figure 1. Radial profile of azimuthally averaged axial (vertical) velocities for the 1,500, 500, and 250-RPM digesters for the: (**A**) top of the liquid column ($z = 23$ cm); (**B**) center of the liquid column ($z = 13$ cm); and (**C**) impeller plane ($z = 5$ cm). Results from CARPT and CFD are shown.

To obtain quantitative shear stresses, the CARPT data from the 500 and 250-RPM operating conditions were first processed to instantaneous velocities, and then further converted to azimuthally averaged axial or radial shear stresses in dynes/cm^2 (Fig. 2). The axial and radial shear stresses were the highest in the region near the impeller for both the 500 and 250-RPM digesters (Fig. 2). The maximum value of the axial shear stress was $\sim$1,000 dynes/cm^2 and $\sim$800 dynes/cm^2, respectively, and this was $\sim$14,000 dynes/cm^2 and $\sim$9,000 dynes/cm^2, respectively, for the radial shear stress at 500 and 250 RPM (Fig. 2). These maximum values were, thus, $\sim$10 times higher than the maximum values of axial shear stress. However, such high radial shear stresses were only present

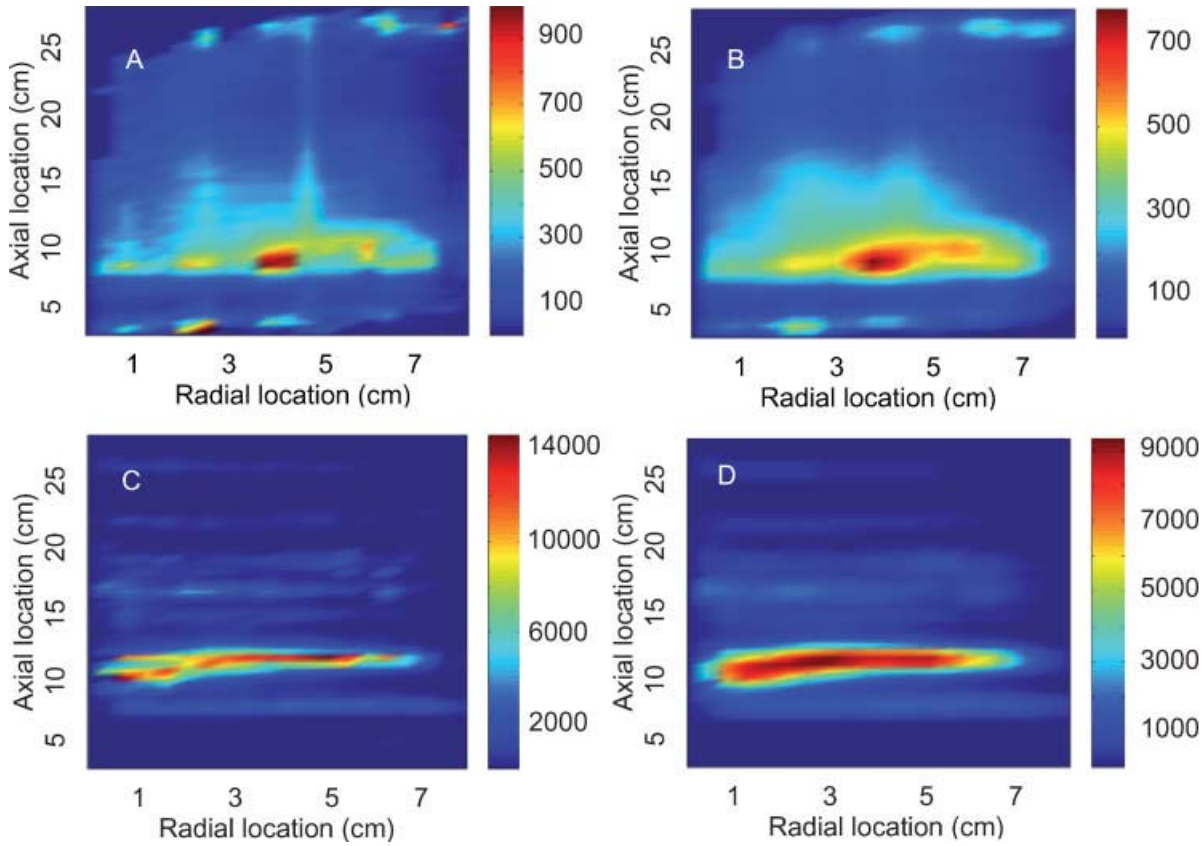

Figure 2. Maps from the center toward the tank wall (radial profile) over the height of the liquid column (axial profile) of the azimuthally-averaged axial (**A** and **B**) and radial (**C** and **D**) shear stress in dynes/cm^2 for the: (A and C) 500-RPM digesters and (B and D) 250-RPM digester.

near the impeller region, while the lower maximum axial shear stresses were more uniformly present (Fig. 2).

Reactor Performance and Stability

Despite a large range of shear stresses, the biogas production for all four digesters was found to be similar during steady-state periods (Fig. 3A). The methane yield for each digester during the steady-state periods was statistically not different between treatments (analysis of variance [ANOVA]: $n = 20$, $P = 0.73$, $\alpha = 0.05$). The overall methane yield, based on the amount of VS fed, was obtained by pooling the data for each digester and was 0.241 L CH_4/g VS fed with a standard error of ± 0.007 (Fig. 4). The methane yield based on the amount of VS consumed was found to be 0.541 L CH_4/g VS consumed with a standard error of ± 0.01 (Fig. 4). The first data point for the methane yields at a VS loading rate of 0.6 g VS/L day was not used because steady-state conditions had not been reached. The percentage of methane in the biogas for each digester over the entire operating period was similar, with a pooled average of $67.4 \pm 5.0\%$.

Differences in performance were seen during the initial startup period, when the most intensely mixed digester (1,500 RPM) produced little or no biogas between days 10 and 25 (Fig. 3A), and accumulated VFAs greater than 4,000 mg/L as acetic acid (Fig. 3B), while the other digesters showed a constant biogas production rate between ~0.6 and 1.0 L/day with low VFA concentrations. The 1,500-RPM digester began to recover between days 29 and 45, showing peaks in biogas production, which corresponded with drops in VFA levels (Fig. 3A and B). Similar observations were made for the 500-RPM digester after the first increase in loading rate from 0.6 to 1.0 g VS/L day. Between days 52 and 78, the biogas production for the 500-RPM digester remained lower than that of the 250 and 50-RPM digesters (Fig. 3A and B). On day 78, the VFA levels in the 500-RPM digester reached a peak and then began to rapidly decrease, causing the biogas production to rise to a level that was similar to the other digesters (Fig. 3A and B).

The inoculum was identical for each digester and consisted of TS and VS concentrations of 18.5 g/L and 10.2 g/L, respectively. The dairy cow manure feed had an average TS and VS concentration of 59.7 g/L and 50.9 g/L, respectively, during the operating period. Because of the solids being fed to the digesters, the initial VS concentration in the digesters increased from 10.2 g/L, up to an average for all digesters of 22.3 ± 0.9 g/L on day 89, where it began to

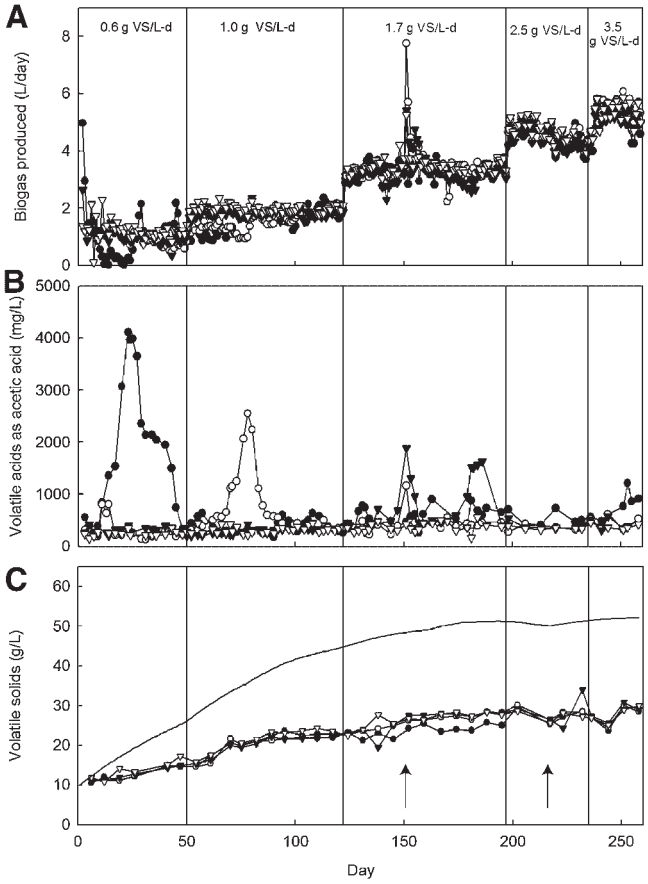

Figure 3. Performance data for the digesters throughout the entire operational period: (**A**) daily biogas production; (**B**) volatile acid levels; and (**C**) volatile solids for the: 1,500-RPM digester (●); 500-RPM digester (○); 250-RPM digester (▼); and the 50-RPM digester (▽). The line in (**C**) shows the theoretical volatile solids level if no degradation had occurred. Vertical lines divide the operating periods with different loading rates. The left arrow indicates the accidental shock and the right arrow identifies the change in the batch of cow manure.

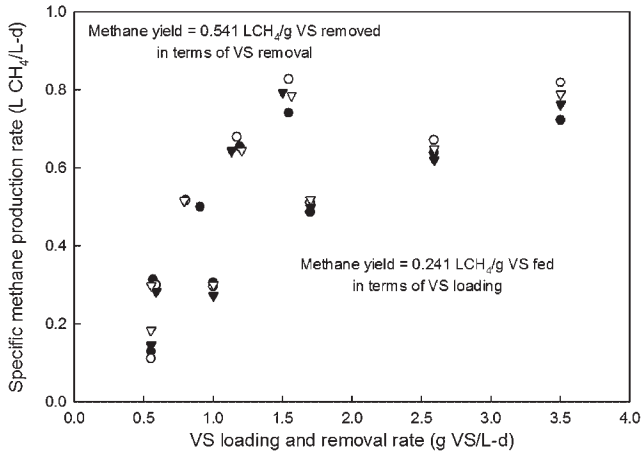

Figure 4. Specific methane production rates over the VS loading rates for the: 1,500-RPM digester (●); 500-RPM digester (○); 250-RPM digester (▼); and the 50-RPM digester (▽). The methane yields for the volatile solids removed and the volatile solids fed were obtained by linear regression analysis on data that was pooled from all digesters during each steady-state operational period except for 0.6 g VS/L day.

level off (Fig. 3C). This increase in VS in the digesters was due to gradual replacement of the initial, low VS inoculum with incoming cow manure feed that had a higher VS concentration of 50 g/L. This is apparent from the increase in VS concentration that would have occurred if no degradation had taken place (Fig. 3C). In addition, some of the increase was due to biomass growth. The effect of VS loading rate was statistically significant on the VS removal efficiencies in all digesters (ANOVA; $n = 16$; $P = 0.0013$; $\alpha = 0.05$). After each increase in VS loading rate (by feeding an increased volume of cow manure feed while keeping the VS concentration for the feed constant), the VS concentration in the digesters (and thus the effluent) increased. This is anticipated because of the shorter HRTs, allowing less time for the organic material (including nonsoluble VS) to be degraded. A shorter HRT at the higher VS loading rates also negatively affected the methane yield that was based on the VS loading rate, which started to level off at the highest VS loading rate (Fig. 4). In addition, the SCOD concentrations were also higher at the shortest HRT; with a consistently higher SCOD level for the 1,500-RPM digester compared to the 50-RPM during the entire operating period (e.g., ~14,000 mg/L and ~9,000 mg/L, respectively, at a loading rate of 3.5 g VS/L day). Ammonia concentrations in the effluent of all the reactors were similar over the entire operating period with an average of 1.24 ± 0.04 g NH_4-N/L. The steady-state VS removal efficiencies for all digesters during the loading rates of 1.0, 1.7, 2.5, and 3.5 g VS/L day were between 52% and 58%. During steady-state conditions, no statistically significant differences in VS removal efficiencies were found between the digesters (ANOVA: $n = 16$; $P = 0.84$; $\alpha = 0.05$). This result verifies the statistically similar methane yields from all digesters during steady-state periods.

Accidental addition of 3.3 times the normal volume of cow manure occurred on day 150 for the 500 and 250-RPM digesters, yielding information regarding the differences in the ability of the digesters to handle a transient hydraulic and organic shock load. The 500-RPM digester was able to handle the shock better than the 250-RPM digester by consuming almost all of the excess substrate in 4 days with only a small increase in VFA concentration (Fig. 3A and B). In contrast, the 250-RPM digester required almost 30 days to stabilize, and showed a much higher VFA accumulation. A planned transient shock load was performed at the end of the operating period by decanting and feeding twice the normal amount, corresponding to a doubling of the VS loading rate to 7.0 g VS/L day for 1 day (day 260). The digesters continued to be operated for 4 days after this shock load occurred (at a VS loading rate of 3.5 g VS/L day) while biogas production and VFA concentrations were monitored (Fig. S6). During these 4 days, the biogas production spiked, and some differences between the digesters were observed.

The 500-RPM digester showed the largest spike in biogas production with an increase of 48%, followed by the 250 and 50-RPM digesters with 47% and 28% increases, respectively. The 1,500-RPM digester showed the lowest spike in biogas production with an increase of only 22%, however, this digester had been operating at slightly higher VFA levels before the transient shock load. A large peak in gas production shows the capacity of a digester to absorb extra organic loads, which leads to lower VFA accumulation, indicating resilience to transient shock loads. The spike in VFA levels on day 262, 2 days after the shock, were 67%, 60%, 131%, and 200% of pre-shock values for the 1,500, 500, 250, and 50-RPM mixing intensities, respectively (Fig. S6).

Microbial Community Structure

Membrane hybridization results showed that 30–40% of the total SSU rRNA in the digester biomass generally consisted of bacteria and that mixing intensity did not appear to have an effect on the bacterial abundance (Fig. 5A). For archaea, the levels of the hydrogenotrophic methanogenic family Methanobacteriaceae increased in the intense-mixed digesters for the first 50 days of the operating period (from 1.5% of the SSU rRNA in the inoculum to 5% in the 1,500 and 500-RPM digester on day 48), while they remained somewhat constant for the 250 and 50-RPM digesters (2% on day 48) (Fig. 5B). The differences between the digesters disappeared during the rest of the operating period and the relative level of Methanobacteriaceae was 1% in all digesters on day 252 (Fig. 5B). Members of the hydrogenotrophic methanogenic family Methanococcaceae were 1.5% of the total SSU rRNA in the inoculum and remained in the biomass for the first 77 days at levels between 0.5% and 2.5%. The maximum level of 2.5% was found in the intense-mixed digesters and coincided with VFA peaks around days 20 and 77 for the 1,500 and 500-RPM digesters, respectively, during which we anticipated somewhat higher hydrogen partial pressures due to unstable conditions. The levels of Methanococcaceae decreased over the operating time and at day 252 they were virtually absent (Fig. S7). Hydrogenotrophic methanogens of the order Methanomicrobiales were between 0.1% and 1.2% of the total SSU rRNA during the first 50 days of the operating period (0.5% in the inoculum), but remained below 0.5% during the rest of the operating period (Fig. S7).

Considerable differences in the digester biomass were seen with respect to the levels of *Methanosaeta concilii* and *Methanosarcina* spp., both usually in competition for acetate (i.e., acetoclastic methanogens). The relative levels of SSU rRNA for *Methanosaeta concilii* were 2% in the inoculum and, after an initial increase to 4% on day 6, decreased to virtually zero for the most intense-mixed (1,500-RPM) digester over the remainder of the operating period. For the first 75 days of the operating period, relative levels of *M. concilii* were between 3.2% and 4.8% in the 500-RPM digester. These levels were below 1%, however, for the remainder of the operating period. Levels of *M. concilii* in the 250 and 50-RPM digesters were generally higher than that for the 1,500 and 500-RPM digesters from day 117 to the end of the operating period (Fig. 5C). *Methanosarcina* spp. was virtually absent in the inoculum and increased for the more intense-mixed digesters (1,500, 500, and 250-RPM digesters) during the first 77 days, while they remained at very low levels for the 50-RPM digester. At the end of the operating period, the levels of these acetoclastic methanogens were the highest for the bioreactors with the highest mixing intensity (1,500 RPM). The relative SSU rRNA levels for *Methanosarcina* spp. were between 2% and 4.5% for the 1,500-RPM digester and between 1% and 1.5% for the 500-RPM digester during the final 60 days of the

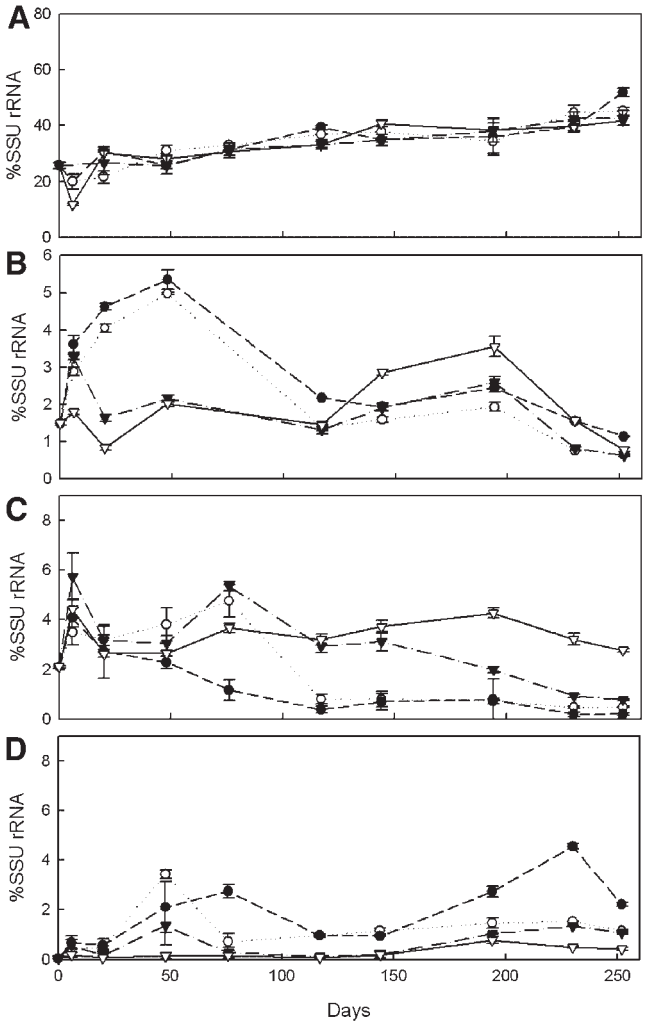

Figure 5. Relative hybridization signals of various probes versus operating time: (**A**) universal bacterial probe; (**B**) Methanobacteriaceae probe; (**C**) *M. concilii* probe; and (**D**) *Methanosarcina* spp. probe for the: 1,500-RPM digester (●); 500-RPM digester (○); 250-RPM digester (▼); and the 50-RPM digester (▽). The standard errors were calculated for three relative SSU rRNA signals. Data points at $t=0$ are for the inoculum from a primary anaerobic digester treating waste activated sludge.

operating period (Fig. 5D). For cow manure that was fed to the digesters from days 218 to the end of the operating period, the relative SSU 16S rRNA levels of bacteria and Methanobacteriaceae were 68% and 0.9%, respectively, with Methanococcaceae, Methanomicrobiales, *Methanosaeta concilii*, and *Methanocarcina* spp. being virtually absent.

Microbial Flocs

FISH analysis showed that the microbial flocs in all digesters were similar in size between days 6 and 28. However, the average floc size decreased between days 28 and 47 with very few large flocs left by day 47, but with numerous smaller flocs present. On day 75, only these smaller flocs remained (data not shown). On day 182, no flocs were documented in all digesters, only single cells and small clusters of cells up to 10 μm in diameter.

Discussion

High Mixing Intensities Favored *Methanosarcina* spp. Over *M. concilii* as the Predominant Acetoclastic Methanogen

We combined conventional methods to analyze reactor performance with molecular methods to quantify mixing intensity and methanogenic composition of the biomass in four lab-scale, continuously mixed digesters treating cow manure at different mixing intensities. The in situ mixing intensity measurements showed large differences in shear forces, especially at a location close to the impeller mixer. For example, the local velocity gradients, and thus shear stresses, were considerably higher in the 1,500-RPM digester compared to the 500 and 250-RPM digesters (Fig. 1). However, these differences in mixing intensity did not seem to effect the long-term operating performance at steady-state conditions (e.g., biogas production rates and VS removal efficiencies). The performance during startup of the intense-mixed digester was, however, negatively affected. In addition, the methanogenic community structure was different between the four digesters. We showed that *Methanosarcina* spp. was more abundant in the 1,500 and 500-RPM digesters while *M. concilii* was more abundant in the 250 and 50-RPM digesters, and therefore an effect of mixing intensity emerged with respect to the most abundant acetoclastic methanogen. In fact, the higher the mixing intensity in our digesters, the lower the relative levels of *M. concilii* and the higher the relative levels of *Methanosarcina* spp. It is generally accepted that *M. concilii* and *Methanosarcina* spp. compete for acetate in anaerobic digesters, which explains the inverted correlation between them. For conventional anaerobic digestion, *Methanosarcina* spp. wins the competition at stable, but high acetate concentrations (>250 mg/L) and *M. concilii* at low acetate concentrations (Huser et al., 1982; Jetten et al., 1992; Smith and Mah, 1978). In contrast, the link between shear and acetoclastic methanogen abundunce supports earlier findings that besides acetate concentrations, the competition between *Methanosarcina* spp. and *M. concilii* may be affected by other factors, such as adhesion (Grotenhuis et al., 1992) and feast and famine conditions (Angenent et al., 2002b; Conklin et al., 2006). The effect of mixing intensity on the competition between the two acetoclastic methanogens may be due to the difference in cell morphology and its susceptibility to shear. While individual *M. concilii* cells form long filaments in anaerobic biomass, *Methanosarcina* cells grow as cocci. High levels of shear in the 1,500 and 500 RPM digesters may, thus, have been a negative selection pressure on the formation of filaments of *M. concilii*, allowing the cocci shaped *Methanosarcina* spp. to achieve a competitive advantage. The effect of shear on this competition would especially be important after the disintegration of the microbial flocs in all digesters between days 28 and 47. Indeed, between these days the relative SSU levels for the competing *Methanosarcina* spp. increased considerably for all digesters except for the 50-RPM digester (Fig. 5D).

However, the competition between *M. concilii* and *Methanosarcina* spp. may not have been influenced by only the direct inhibition of *M. concilii* caused by the local high shear forces greater than 14,000 dynes/cm^2 in the 1,500-RPM digester (Fig. 2). An indirect effect of unstable conditions during startup of the 1,500-RPM digester (which resulted in a spike in VFAs concentrations) is the competitive advantage of *Methanosarcina* spp. during accumulation of acetate. McMahon et al. (2001) showed enriched microbial communities of *Methanosarcina* spp. in previously unstable digesters with periodic peaks of high acetate concentration. Specifically, they found that *Methanosarcina* spp. (a generalist) were abundant in digesters with a history of high concentrations of VFAs, whereas digesters with no past VFA build up showed high concentrations of *M. concilii* (a specialist). We observed a similar pattern with the emergence of *Methanosarcina* spp. on day 48 after a large rise in the VFA concentration on day 25 for the 1,500-RPM digesters (although levels rose also for the 500-RPM digester) (Figs. 3B–5D). In contrast, the 50-RPM digester without any VFA accumulation during the entire operating period mainly harbored *M. concilii* and never experienced a dramatic raise in *Methanosarcina* spp. From this experiment we cannot completely discern the direct (cell morphology) and indirect effect (acetate accumulation) of high mixing intensities in regards to the competition between *M. concilii* and *Methanosarcina* spp. It is likely that both effects have played a role in their competition, however, further studies are necessary to investigate this.

The performance of the most intense-mixed digester (1,500-RPM digester) was severely retarded during startup during which no considerable volume of methane was produced between days 10 and 25 (Fig. 3A). Using an organic loading rate of 3.1 g VS/L day, Griffin et al. (1998) found that for both mesophilic and thermophilic conditions, the digesters (treating a mixture of the organic

fraction of municipal solid waste, primary sludge, and waste activated sludge) performed poorly during the initial startup when systems were vigorously mixed. They found that the disappearance of *M. concilii* in the initial period (within the first week in Griffin et al. (1998)) and the virtual absence of *Methanosarcina* spp. in the inoculum of the vigorously mixed mesophilic digester caused an upset with high concentrations of VFAs. Our results, however, do not show such an immediate decrease in *M. concilii* populations, and the unstable performance in the 1,500-RPM digester cannot be explained by changes in the methanogenic community. In addition, the methanogenic community was similar for the 1,500 and 500-RPM digesters while the latter digester did not fail during this initial operating period (Figs. 3 and 5). Other important members of the community, such as acetogens, may have been inhibited or absent from the biomass, explaining the unstable performance in the beginning of the 1,500-RPM digester. The startup phase is often considered to be the critical step in anaerobic digester operation. The findings presented in this study and in Griffin et al. (1998) suggest that high mixing intensities should be avoided during startup periods.

Reactors That had an Unstable Period Became More Resilient to Shock Loads

Digesters that have been upset once were more capable of handling future upsets. The shock load that occurred on the 500 and 250-RPM digesters on day 150 revealed that the 500-RPM digester, which had survived an upset during the increase in VS loading rate from 0.6 to 1.0 g VS/L day, was more capable of handling the shock load than the 250-RPM digester, which had not experienced an upset. In addition, the transient shock load at the end of the study showed that the 50-RPM digester (which never had experienced an upset) showed the least resilience based on VFA accumulation. McMahon et al. (2001) argued that the development of enriched microbial communities with syntrophic bacteria and certain methanogens (and therefore with an increased capacity to remove intermediates) in previously upset digesters rendered them resilient to future shock loads. For example, the generalist *Methanosarcina* spp. were abundant in digesters with a history of high concentrations of VFAs and this may explain the faster recovery after shock loads. Conklin et al. (2006) reported that a digester with a *Methanosarcina*-enriched biomass was more stable during a simulated shock load than with a *Methanosaeta*-enriched biomass. Thus, the dominance of *Methanosarcina* spp. in the vigorously mixed digesters would make them more stable, which is, thus, an advantage of a high mixing intensity.

Continuous Mixing Disrupted Microbial Flocs Regardless of the RPM

Continuous shear affected the microbial flocs negatively. We showed that between days 6 and 28, all digesters contained similar sized flocs. However, the average floc size began to decrease around day 35. Others had speculated that vigorous mixing disrupts the spatial juxtaposition of microorganisms (Conrad et al., 1985; Dolfing, 1992; Whitmore et al., 1987). Not only have we found this to be true, we also found that it can completely break syntrophic relationships, even at low mixing intensities when digesters are completely mixed. This result was observed in all digesters by day 182 of the operating period. However, we did not find that the disruption of microbial flocs caused digester instability, VFA build-up, or digester failure at the conditions studied. Syntrophic bacteria and archaea present within this biologically diverse and interdependent system are usually viewed as utilizing close, physical associations to transfer metabolic products. However, it is possible that the continuous mixing applied to the digesters studied here was sufficient enough to overcome diffusion limitations, and thus remove the need for close spatial associations.

Despite no effect of mixing on long-term performance of our continuously mixed digesters, we anticipate that disruption of microbial flocs in high-rate anaerobic systems (i.e., reactor configurations that rely on biomass settling for operation at a shorter HRT compared to the SRT) will result in severe performance problems, because of two conditions: (1) when mixing is intermittent, serious diffusion limitations may arise if the juxtaposed relationship between the syntrophs is disrupted. Indeed, differences in mixing duration for intermittently mixed digesters have shown an effect on digester performance (Dague et al., 1970; Stroot et al., 2001); and (2) when settling of biomass is an integral part of the operating conditions to maintain high biomass concentrations, disruption of microbial flocs (and the resulting loss of biomass settleability) may lead to an excessive washout of active biomass. ASBRs are an example of a high-rate system by employing a settling step to maintain high levels of active biomass. A considerable increase in mixing intensity in an ASBR treating swine waste impeded digester performance because of the disruption of microbial flocs (Angenent et al., 2001). Discrepancies between anaerobic digester studies in terms of the effect of mixing, such as between the results of Lanting (2003) and Stroot et al. (2001) or Angenent et al. (2001), may, therefore, be explained by the different types of digester configuration and mixing conditions used. Varying mixing intensities with continuously stirred anaerobic digesters seem to affect digester performance considerably less than with intermittently mixed anaerobic digesters.

Conclusions

The results of this study support the following conclusions:

(1) Different mixing intensities had no effect on continuously stirred digester performance at steady-state conditions. During steady-state periods, all digesters performed similarly by producing equal amounts of

biogas. Intense mixing showed a negative effect during the initial startup. Thus, to prevent digester failure during the initial startup period, intense mixing should be avoided.

(2) Mixing intensity affected the competition between the acetoclastioc methanogens *M. concilii* and *Methanosarcina* spp. with the latter becoming important in the intensely mixed digesters. Since the presence of *Methanosarcina* spp. resulted in more stable digesters, the long-term stability may have been positively affected by increased mixing intensities.

(3) Disruption of the juxtaposed interaction between bacteria and archaea (methanogens) did not affect stable digestion of animal manure in continuously stirred bioreactors.

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