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Characterization of microbial trophic structures of two anaerobic bioreactors processing sulfate-rich waste streams

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ABSTRACT

A multi-compartment anaerobic bioreactor, designated the anaerobic migrating blanket reactor (AMBR), did not perform well in terms of chemical oxygen demand (COD) removal after an increase in sulfate load, compared to a conventional upflow anaerobic sludge blanket (UASB) reactor. The trophic structures of the bioreactors were characterized by analyzing the electron flows, formation and consumption of fermentation intermediates and terminal product (methane and hydrogen sulfide) formation. Critical performance parameters were linked to operational perturbations such as increase in sulfate load and changes in flow reversal schemes in the AMBR. Both of these manipulations affected the microbial communities, which were monitored by terminal restriction fragment length polymorphism (T-RFLP) analysis targeting the bacterial and archaeal domains. The less stable AMBR did not produce granular biomass, and in response to increased sulfate concentrations, experienced a reversal in the distribution of hydrogenotrophic methanogens that correlated with a shift in electron flow from butyrate to propionate. As this shift occurred, bacterial populations such as butyrate-producing clostridia, became predominant, thus leading to reactor imbalance. The stable UASB reactor developed and retained granules and maintained a relatively stable archaeal community. Sulfate perturbation led to the selection of a novel bacterial group (*Thermotogaceae*), which was most likely well adapted to the increasingly sulfidogenic conditions in the bioreactor.

1. Introduction

The microbial communities present in most anaerobic bioreactors consist of at least three functional groups: primary fermenting bacteria, secondary fermenting (syntrophic) bacteria and methane-producing archaea (MPA). The system gains in complexity when fed a sulfate-rich influent, such as waste streams produced in pulp and paper, brewing, edible oil, and corn wet-milling industries. The presence of sulfate in these wastewaters promotes competition between sulfate-reducing bacteria (SRB) and MPA (Elferink et al., 1994), and the outcome of this competition decides to what extent hydrogen sulfide or methane, the end products of the respective functional groups, are present in the biogas produced by these bioreactors. Hydrogen sulfide is malodorous, toxic against a wide range of microorganisms, and can serve as the precursor for corrosive

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sulfur compounds. During the last decade, a variety of advances in design and operation of anaerobic bioreactors for the treatment of sulfate-containing wastewaters have been made in order to address these concerns (Schenk et al., 1999; Speece, 1996; Khanal and Huang, 2003). From economic and environmental standpoints, the most attractive option is to recover the sulfur from the waste streams, while generating a biogas rich in methane and low in hydrogen sulfide. Treatment of sulfate-rich waste streams in an anaerobic migrating blanket reactor (AMBR) (Angenent and Sung, 2001; Angenent et al., 2002) was proposed to accommodate sulfur recovery by virtue of its staged design: the AMBR consists of multiple (at least three) compartments, horizontally connected by small openings through which water and biomass are allowed to flow. This configuration creates a substrate gradient in the direction of flow, and promotes the partial separation of acidogenic/sulfidogenic and methanogenic activities. Flow is reversed at regular time intervals to prevent biomass accumulation in the final compartments. The initial, outside compartments selectively promote fermentation and sulfidogenesis, leaving the inner compartment predominantly methanogenic (Angenent and Sung, 2001; Angenent et al., 2002; Barber and Stuckey, 1999). Since sulfidogenesis should primarily take place in the initial compartments, the biogas collected from these compartments should be high in hydrogen sulfide, facilitating sulfur recovery, for example through oxidation of hydrogen sulfide to sulfur (Janssen et al., 2001). As a result, the methanogens in the middle compartment should not be exposed to high hydrogen sulfide concentrations and the biogas collected from this compartment should be rich in methane.

During a previous study, we compared the performances of an AMBR and a conventional upflow anaerobic sludge blanket (UASB) reactor treating a sulfate-rich synthetic wastewater at mesophilic (35 °C) conditions (Briones et al., 2007). Because of the single-compartment design of a conventional UASB reactor, it has been reported to be prone to problems associated with sulfide toxicity (Elferink et al., 1994). Contrary to our expectations, the AMBR failed to achieve the performance level of the UASB reactor when challenged with a high sulfate load. We attributed these results to the failure to achieve sufficient staging of microbial communities across the AMBR compartments, as determined by comparing bacterial community profiles among compartments. Moreover, evaluating the dynamics of the predominant bacterial populations revealed key species that could be associated with the contrasting performance of the two reactors. In particular, a novel bacterial population affiliated to the *Thermotogaceae* became predominant in the UASB reactor after shifting from a low to a high sulfate load. The members of the *Thermotogaceae* that have been characterized so far are fermentative, but also capable of reducing elemental sulfur and thiosulfate and are either thermophilic or hyperthermophilic (Stetter et al., 1990; Ravot et al., 1995). While the predominance of this bacterial population suggested a shift toward a sulfur-dependent trophic structure, the ecological relationships giving rise to its prevalence are not known. Further characterization of the archaeal population dynamics in the two bioreactors found a more stable community structure in the UASB reactor compared to the AMBR (Briones et al., 2007). This stability may have been a critical factor in maintaining syntrophic interactions in the UASB reactor, including hydrogen-producing *Thermotogaceae* and hydrogenotrophic methanogens.

The objective of the present study was to examine additional microbiological and process parameters that contributed to the contrasting performance and microbial community characteristics of the two bioreactors. We report that reactor performance can be linked to consumption and production activities of MPA and secondary fermenting, or syntrophic bacteria. The activities of these two groups of microorganisms either maintained (UASB reactor) or created a bottleneck (AMBR) in methanogenic electron flow, which could also be related to the dynamics of the primary fermenting bacteria.

2. Materials and methods

2.1. Configuration and operating conditions of reactors

The configuration and operating conditions of the laboratory-scale AMBR and UASB reactor were described in detail by Briones et al. (2007). In brief, the AMBR consisted of five 4–l, intermittently mixed compartments fed by four peristaltic pumps (the middle compartment was never fed directly). From days 1 to 128, the flow was reversed four times per day, with the initial compartment fed for four hours, followed by two hours of feeding into the adjacent, inner compartment. The flow was then reversed using the same feeding regime in the opposite direction. After 128 days, the frequency of flow reversals was reduced to twice per day (days 129–144, outermost compartments fed for 10 h) and then to once a day (days 145–149, outermost compartments fed for 20 h). The alkalinity in the feed was reduced by 25% for three days starting on day 119. These departures from the routine feeding regime (days 1–128) were intended to reduce the pH and enhance staging in the reactor. The UASB reactor had a working volume of 4.9 l and was operated to achieve an upflow velocity of 1 m h^{-1}.

The reactors were operated at 35 °C and were inoculated with crushed granular biomass collected from a UASB reactor operated by the Anheuser Busch Brewery (St. Louis, MO). The initial mixed liquor volatile suspended solids concentration in both reactors was approximately 30 g l^{-1} and the initial volumetric loading rate (VLR) was approximately 5 g chemical oxygen demand (COD) l^{-1} d^{-1}. The VLR was increased until attaining the target VLR of approximately 15 g COD l^{-1} d^{-1}. The reactors were fed with a solution simulating wastewater from a corn wet-milling plant and trace elements according to Zehnder et al. (1980). The operational time for both reactors was divided into two periods with different influent sulfate concentrations. During Period 1 (days 0–99), the COD/SO_4^{2-} mass ratio in the influent was maintained at 24.4 (7000 mg l^{-1} COD; 287 mg l^{-1} SO_4^{2-}), while during Period 2 (days 100–149), the influent sulfate concentration was increased to reach a COD/SO_4^{2-} mass ratio of 5.0 (1400 mg l^{-1} SO_4^{2-}).

2.2. Analytical methods

Samples were collected from the AMBR and UASB reactor, processed, and characterized as described previously (Briones

et al., 2007). Briefly, individual volatile fatty acids (VFAs) were measured using high performance liquid chromatography and a UV detector, sulfate and sulfite concentrations were determined with ion chromatography and a conductivity detector, and methane and hydrogen levels in the biogas were measured using gas chromatography equipped with a flame ionization detector and a reduction gas detector, respectively. Measurements of total suspended solids, volatile suspended solids, total VFAs, pH, alkalinity, total and soluble COD, and total sulfides were conducted according to standard methods (Greenberg et al., 1992). Aqueous H_2S was calculated based on the following equation (Speece, 1996): H_2S fraction $= 1/(1 + (K_1/10^{-pH}))$, where K_1 is the first ionization constant of H_2S and is equal to $10^{-6.83}$ at 35 °C.

2.3. DNA extraction, PCR, construction of clone libraries, and phylogenetic analysis

Duplicate biomass samples (at least 100 mg wet weight) were collected on days 2, 35, 85, 127, and 148 from the AMBR and UASB reactor. DNA extraction from biomass samples, PCR of bacterial and archaeal 16S rRNA genes (using primers 27f and 1392r (Lane, 1991) and Ar109f and Ar912r (Lueders and Friedrich, 2000; Grosskopf et al., 1998), respectively), and construction and screening of clone libraries were performed as described by Briones et al. (2007). Clone libraries were constructed from samples collected on days 2 and 127 from both reactors. The source template for 16S rRNA gene clone library construction for the AMBR consisted of pooled DNA extracts from all five compartments.

The sequences of selected clones (selections based on library screening described by Briones et al. (2007)) were identified using the "Simrank" tool in Greengenes (http://greengenes.lbl.gov) (DeSantis et al., 2006) and the "Classifier" tool in the Ribosomal Database Project (RDP)-II Release 9.60 (http://rdp.cme.msu.edu/index.jsp) (Cole et al., 2005). Both of these tools are applicable for bacterial sequences, while the Simrank tool was used to identify the archaeal sequences, since RDP currently does not support archaeal classification. As of April 2008, the size of the aligned archaeal and bacterial database in Greengenes was 184,782 16S rDNA sequences, while the size of the bacterial RDP database was 511,847. In most cases, the bacterial Family classifications in RDP and Greengenes agreed. The exceptions were two clostridial sequences (AY648563 and temp1087727), which were therefore classified at the Order level. The GenBank accession numbers of the sequences generated for this study are provided in Briones et al. (2007).

2.4. T-RFLP and data analysis

Terminal restriction fragment length polymorphism (T-RFLP) and data analysis were performed as described by Briones et al. (2007). In brief, PCR prior to T-RFLP used the bacterial primers 27f* and 1392r (Lane, 1991) and the archaeal primers Ar109f and Ar912r* (Lueders and Friedrich, 2000; Grosskopf et al., 1998). The asterisk after the primer name indicates that this primer was labeled with 6-carboxyfluorescein (FAM). Amplicons from two replicate reaction mixtures were pooled and digested with restriction enzyme *Hae*III (New England Biolabs, Inc., Beverly, MA). The fluorescently labeled terminal restriction fragments (T-RFs) were resolved with an automated sequencer and T-RF fragment data were evaluated and converted to text format using Genescan 2.1 analysis software (Applied Biosystems, Foster City, CA). We considered a minimum T-RF peak height of 50 fluorescent units (FU) to represent an operational taxonomic unit (OTU). Data sets exported from Genescan 2.1 were converted to spreadsheet format (Microsoft Excel, Microsoft Corp., Redmond, WA) and normalized by obtaining the ratio of each peak height to the cumulative peak height.

3. Results and discussion

3.1. Percent electron flow

In order to assess the relative contribution of either methanogenic or sulfidogenic pathways to anaerobic digestion, we calculated the percent electron flow as described previously (Isa et al., 1986). This involved calculating the moles of methane produced and moles of sulfate consumed at particular days, then converting these values to their electron equivalents (expressed as COD). Since the bulk (97%) of methane is in the gas phase, assuming equilibrium between gas and liquid phases (Khanal and Huang, 2005), we considered only this fraction as relevant to the calculations. Sulfate reduction was measured by obtaining the difference between the sulfate fed to the systems and the sulfate remaining in the effluents. During Period 1, methanogenesis accounted for 92–98% of soluble COD removal from the influent in both reactors (Fig. 1). During this period, soluble COD removal averaged $77.5 \pm 10\%$ and $88.8 \pm 7.8\%$ in the AMBR and UASB reactor, respectively. After raising the sulfate load to 1400 mg SO_4^{2-} l^{-1} (Period 2), methanogenesis remained an important route for electron flow in both reactors. However, its contribution decreased from 86% one day after increasing sulfate load to 48% after 44 days into Period 2 in the AMBR. A partial recovery in the methanogenic pathway was observed during the final phase of the AMBR operation (days 145–149; flow reversal frequency was once a day), when the electron flow through MPA rose from 47% to 56%. On the other hand, the stability of

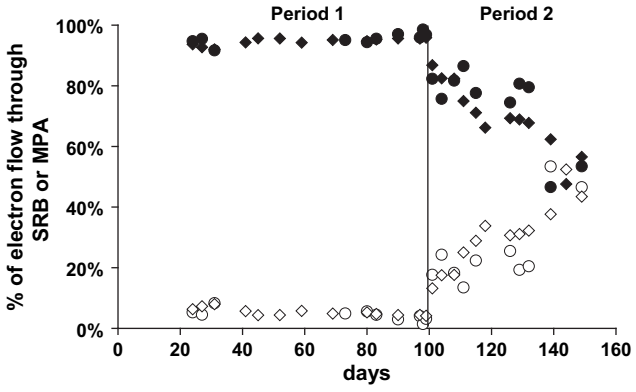

Fig. 1 – Methane production rates (A), effluent H_2S concentrations (B), and effluent sulfate concentrations (C) for the AMBR (◆) and UASB reactor (○).

methanogenesis in the UASB reactor was maintained between 74 and 86% up to day 132. Thereafter, the trend of electron flow was similar to that observed in the AMBR. The results indicate less resiliency in methane production in the AMBR compared to the UASB reactor in response to an increase in sulfate load. It should be noted that although analysis of percent electron flows provides a good measure of the importance a particular energetic pathway, it provides little indication of reactor performance. For this, evaluation of anaerobic digestion products and fermentation intermediates is required.

3.2. *Changes in methane production and VFA levels*

During startup, the volumetric loading rates (VLRs) in both bioreactors were gradually increased to a target VLR of 15 g COD l^{-1} d^{-1}, which was achieved on day 72 (for details of startup, refer to Briones et al. (2007)). The VLR in the UASB reactor was slightly higher during startup, which was reflected by the consistently higher methane production rates during this time (Fig. 2A). However, during maximum loading, both reactors maintained an average VLR of 15.9 g COD l^{-1} d^{-1}, with no statistical difference ($p > 0.05$) between the reactors. At this time, the UASB reactor displayed a substantially greater methane production rate than the AMBR (Fig. 2A), indicating that the UASB reactor out-performed the AMBR in terms of methane production at an influent COD/SO_4^{2-} mass ratio of 24.4.

The higher methane production in the UASB reactor compared to the AMBR just prior to the increase in sulfate load is supported by the lower UASB VFA levels at this time (Table 1 and Fig. 3B and C). VFA accumulation (an important indication of reactor imbalance (Schink and Stams, 2006)) in the AMBR likely resulted in inhibition of methanogenesis. The accumulation of hydrogen is an equally important indicator of reactor imbalance (Schink and Stams, 2006). In this case, the UASB reactor displayed elevated levels of hydrogen just prior to the increase in sulfate load (Table 1 and Fig. 3A). To better understand these differences in reactor performance, it was necessary to conduct a more detailed analysis of the reactor conditions and metabolism of the microbes involved.

3.3. *Changes in sulfur species concentrations*

Hydrogen sulfide levels in the effluents (Fig. 2B) showed similar trends in both reactors. Although effluent sulfate levels (Fig. 2C) during Period 1 showed no significant ($p > 0.05$) changes from a mean value of 16 mg l^{-1} in both reactors, H_2S levels in the effluents declined significantly ($p < 0.01$) during Period 1. This suggests that through time, sulfide was being utilized in both reactors. It is recognized that a majority of MPA lack assimilatory sulfate reductases (Daniels et al., 1986) and therefore require sulfide supplementation in the range of 1–25 mg S l^{-1} (Scherer and Sahm, 1981). The H_2S–S levels during Period 1 in both reactors averaged 18 mg S l^{-1}. Thus, the use of sulfide produced by the SRB for growth requirements of methanogens provides a likely explanation for the decline in sulfide levels.

During Period 2, the H_2S effluent levels in both reactors increased following a similar general trend (Fig. 2B), although the H_2S levels in the AMBR effluent tended to be more variable,

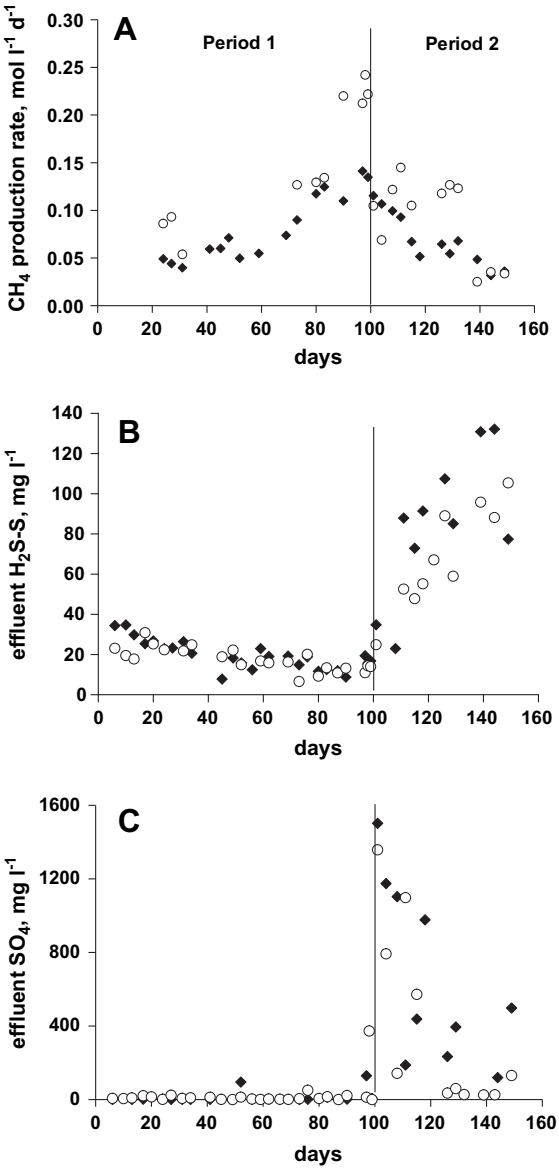

Fig. 2 – Percent electron flow through the bioreactors. The filled shapes correspond to electron flow through methanogens while the empty shapes correspond to electron flow through sulfate reducers. Diamonds and circles refer to the AMBR and UASB reactor, respectively.

which is probably a reflection of the continuous perturbations (alternating flow regimes) imposed on this system. The effluent sulfate levels were consistent with these trends (Fig. 2C). After a once a day flow reversal frequency was implemented (day 145), the effluent H_2S levels in the AMBR dropped from 140 to 82 mg l^{-1}. This observation suggest that either sulfide levels of around 140 mg H_2S–S l^{-1} (total sulfide of around 250 mg l^{-1}) are inhibitory for the *Desulfovibrio*-type SRB detected in our systems, or other factors relevant to this period had an impact on growth or activity of the SRB. A recent report suggests that members of the *Desulfovibrionaceae* remain highly competitive at total sulfide loading rates ranging from 500 to 1500 mg l^{-1} (Icgen and Harrison, 2006). On

Table 1 – Operational and performance data for AMBR and UASB reactor.

Operational time (days)[a]	Operational parameter[b]	AMBR Compartments					UASB
		1	2	3	4	5	
99 (before increasing SO_4^{2-} load)	SCOD	2813	2072	1450	1418	1538	474
	pH	6.5	6.8	6.9	6.8	6.8	6.9
	Acetate	1050	604	270	218	283	19
	Propionate	472	546	517	513	543	54
	Butyrate	251	107	38	27	24	48
	Methane	36	57	64	54	53	58
	Hydrogen	184	25	11	8	n.d.	983
	TS	44	36	42	43	37	34
118 (after increasing SO_4^{2-} load)	SCOD	4618	3807	3482	4043	4574	1101
	pH	6.5	6.8	7.1	6.9	6.8	7.6
	Acetate	1181	1102	641	961	1223	312
	Propionate	942	1170	807	987	1145	150
	Butyrate	130	111	51	105	128	26
	Methane	18	49	46	19	24	40
	Hydrogen	75	11	13	12	14	1
	TS	172	195	234	221	214	184
144 (flow reversal frequency = 2)	SCOD	5846	5272	4928	5025	5567	3152
	pH	6.5	6.6	6.7	6.7	6.8	7.0
	Acetate	1353	1340	1905	1185	1794	n.m.
	Propionate	414	428	566	369	539	n.m.
	Butyrate	695	604	604	n.d.	611	n.m.
	Methane	7	23	28	25	19	36
	Hydrogen	207	11	n.d.	n.d.	13	183
	TS	142	144	193	238	247	223
149 (flow reversal frequency = 1)	SCOD	6350	6245	5960	5833	5960	4978
	pH	6.2	6.3	6.4	6.4	6.4	6.7
	Acetate	1763	1592	1536	1503	1127	1772
	Propionate	609	543	498	498	433	513
	Butyrate	1126	1085	1157	1253	1148	384
	Methane	4	14	23	29	32	29
	Hydrogen	965	116	42	83	55	158
	TS	97	66	146	98	106	201

n.d., not detected; n.m., not measured. The total UASB VFAs at day 144 = 825 mg l^{-1}.
a In all cases, samples were collected from the AMBR when flow direction was from compartment 1 to compartment 5, at the midpoint between flow reversals, i.e., at flow reversal frequency (frf) = 4, samples collected three hours after feeding into compartment 1. Before feeding compartment 1, compartment 5 was fed for four hours followed by two hours of feeding into compartment 4. At frf = 2, samples were collected six hours after feeding into compartment 1. At frf = 1, samples were collected 12 h after feeding into C1.
b SCOD and VFA units, mg l^{-1}; hydrogen units, Pa; total sulfides (TS) units, mg l^{-1}-S.

the other hand, this study found that these levels of total sulfide are toxic to SRB belonging to *Desulfobulbus* spp., which may explain why this group was not detected in our study. Since *Desulfovibrionaceae* are highly tolerant of sulfides, large fluctuations in sulfide at constant sulfate loading may reflect their response to differences in electron donor availability. Specifically, the growth rate of *Desulfovibrio* spp. on organic substrates such as lactate is expected to be lower compared to their growth rate on hydrogen (Colleran et al., 1995). This relates to our observation of the predominance of *Streptococcaceae* in the AMBR during the end of Period 2 (Table 2 and Briones et al., 2007) (flow reversal frequency of once a day). Streptococci are well recognized lactic acid bacteria that grow well under high nutrient conditions, including bioreactors selecting for an acetate–propionate-based food chain (Dollhopf et al., 2001). This bacterial population could have been involved in cross-feeding relationships with *Desulfovibrio* spp., which utilize hydrogen or fermentation intermediates such as lactate as electron donors (Rabus et al., 2006). Therefore the drop in H_2S levels observed in the AMBR effluent at the end of Period 2 (Fig. 2B) is consistent with *Desulfovibrio* spp. utilizing lactate over hydrogen as electron donor when streptococci became a dominant group.

3.4. *Utilization of hydrogen*

Between MPA and SRB, the latter are better able to compete for hydrogen based on both thermodynamic and kinetic parameters (Colleran et al., 1995). When sulfate is limiting, as was observed during Period 1 (Fig. 2C), the growth and activity of the SRB are expected to be limited, which was reflected by their low abundance in the bioreactors during Period 1 (Table 2 and Briones et al., 2007). This can also be gleaned from the pattern of hydrogen production observed during the reactors operation (Fig. 3A). Limited SRB growth resulted in less competition for hydrogen, and therefore the levels of this

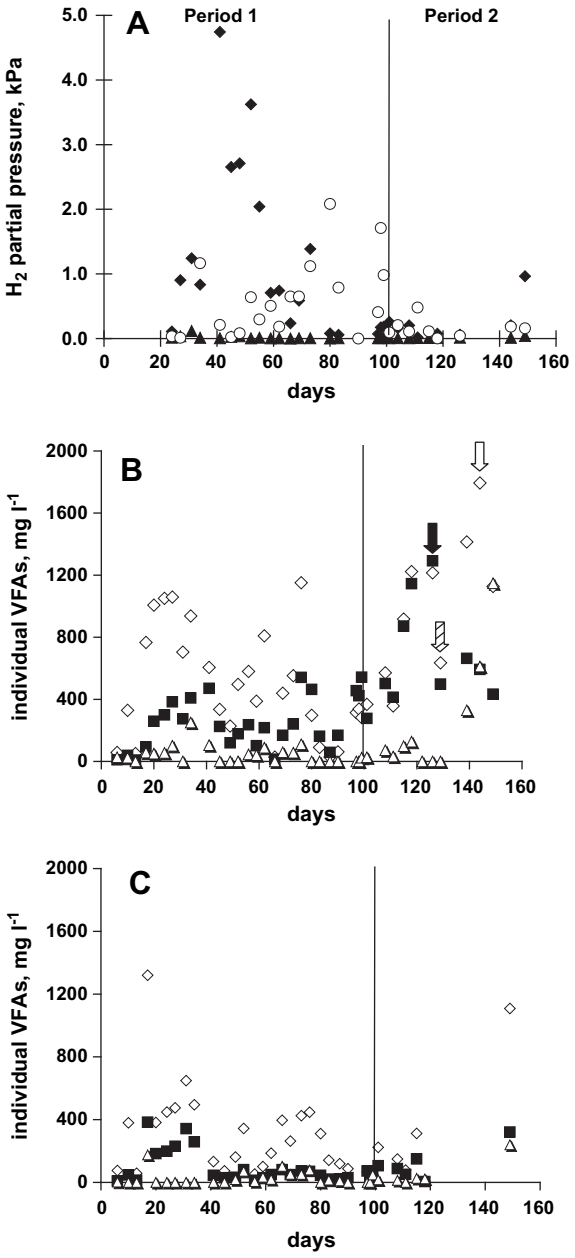

Fig. 3 – Fermentation intermediates in the bioreactors. A, hydrogen partial pressures. Filled diamonds and triangles correspond to maximum and minimum concentrations in a particular AMBR compartment. Empty circles correspond to H_2 concentrations in the UASB reactor. B, AMBR effluent VFAs. The filled arrow indicates 127 d, when the reversal of the distribution of hydrogenotrophic methanogens was first detected by T-RFLP analysis. The shaded arrow refers to day 129, when flow reversal frequency was reduced to two. The empty arrow refers to day 144, when flow reversal frequency was reduced to one. C, UASB reactor effluent VFAs. B and C, ◇ = acetate; ■ = propionate; Δ = butyrate.

intermediate were mostly dependent on growth and activity of hydrogenotrophic MPA.

The population dynamics of MPA (summarized in Table 2 and detailed in Briones et al., 2007) indicated less stability in the AMBR. During Period 1, the major MPA in both bioreactors in decreasing order of dominance were: *Methanosaetaceae*, *Methanobacteriaceae*, and *Methanospirillaceae*. This community structure was maintained in the UASB reactor throughout the experimental period. In the AMBR, however, *Methanospirillaceae* became the dominant hydrogenotrophic MPA during Period 2. Since *Methanobacteriaceae* and *Methanospirillaceae* are hydrogenotrophic MPA, we expect that this change would be reflected by the interspecies hydrogen transfer, or syntrophic interactions that these microorganisms mediate.

During Period 2, sulfate was in excess (Fig. 2C), thus favoring the SRB. As expected, hydrogen became limiting, which is supported by the relatively low levels of hydrogen detected in both reactors during this period (Fig. 3A). Since some SRB species are able to utilize hydrogen to below the minimum threshold required by MPA (Colleran et al., 1995), the competition for hydrogen between MPA and SRB during Period 2 impacted methanogenesis. Under these conditions, the juxtapositioning of hydrogenotrophic MPA and hydrogen-producing syntrophic bacteria in consortia provides a selective advantage. We had reported earlier (Briones et al., 2007) that conditions within the AMBR did not favor granule formation but instead formed amorphous flocs, in contrast to the situation in the UASB reactor where true granular structures developed. Current evidence suggests that butyrate-degrading granules are stable when the methanogenic partner is *Methanobacterium formicicum*, while in suspended cultures, *Methanospirillum hungatei* is often the dominant hydrogen utilizer (Schink and Stams, 2006; Wu et al., 1996), which relates to the change in dominant hydrogenotrophic MPA we observed in the AMBR. The microbial flocs in this system can be more prone to disintegration in the presence of sulfide, especially at higher shear rate (Nielsen and Keiding, 1998). On the other hand, well-formed granules may respond well to sulfate in the presence of iron by forming smaller sized particles with higher granule strength (van Hullebusch et al., 2007). In either case, the conditions during Period 2 could have led to selective washout of flocs involved in butyrate oxidation, where the likely syntrophic partner was *Methanobacteriaceae*. This should have had a direct impact on butyrate consumption, which we inferred from compartmental analysis described below.

3.5. *Inferring consumption and production of VFAs*

The most comprehensive data sets were collected from each AMBR compartment and the UASB reactor on days 99, 118, 144, and 149. These days represent the end point of Period 1 (day 99), 18 days after increasing the sulfate load (day 118), 15 and 5 days after lowering the flow reversal frequencies in the AMBR to twice a day (day 144) and once a day (day 149), respectively (Table 1). Acetate, propionate, and butyrate comprised the most consistently detected VFAs in the bioreactors, and the discussion is limited to these three major fermentation intermediates. To better understand the changes in VFA production (primary fermentation) and consumption (syntrophic or secondary fermentation) it is useful to look at the relative changes in concentration through a feeding cycle and the relative proportions of each VFA out of the total SCOD. We inferred the net production/consumption

Table 2 – Summary of major microbial groups monitored in bioreactors using T-RFLP.

Bioreactor	Period 1		Period 2	
	Bacteria	Archaea	Bacteria	Archaea
AMBR	*Btd* > *Tht* > *Clo* > *Stp* > *Spc*	*Mst* > *Mbt* > *Msp*	*Clo* > *Dsv* > *Stp* > *Btd* > *Spc* > *Tht*	*Mst* > *Msp* > *Mbt*
UASB	*Btd* > *Clo* > *Tht* > *Dsv*	*Mst* > *Mbt* > *Msp*	*Tht* > *Btd* > *Dsv* > *Spc* > *Clo* > *Stp*	*Mst* > *Mbt* > *Msp*

Details of microbial groups and their population dynamics are found in Briones et al. (2007).
Abbreviations: *Btd* = *Bacteroidaceae*; *Clo* = *Clostridiaceae*; *Dsv* = *Desulfovibrionaceae*; *Spc* = *Spirochaetceae*; *Stp* = *Streptococcaceae*; *Tht* = *Thermotogaceae*; *Mbt* = *Methanobacteriaceae*; *Msp* = *Methanospirillaceae*; *Mst* = *Methanosaetaceae*.

of an individual VFA by comparing their amounts in the outermost compartments during specific sampling periods. If we assume that the outermost compartments of the AMBR have similar degradative properties, then we may further assume that the quantity of an intermediate in compartment 1 (compartment being fed at sampling time) closely approximates the quantity in compartment 5 during the equivalent time of the feed cycle (three hours direct feeding). Therefore, the difference between intermediates in compartment 5 and compartment 1 reflects the net production/consumption occurring after the elapsed time (e.g., six hours when flow reversal frequency [frf] equals four) within a specific feed cycle. Lower quantities of a VFA in compartment 5 relative to compartment 1 (negative slope of the line connecting compartment 1 and compartment 5) suggests a net consumption of the intermediate, while a positive slope would suggest a net production. Note that a level slope would not necessarily indicate zero consumption/production, since the pre-existing pools of intermediates before sampling were unknown. Thus, comparison of compartment 1 and compartment 5 intermediates provide only an indication of net production/consumption, and does not allow determination of absolute production and consumption rates.

During day 99 (frf = 4; one day before increasing sulfate load), Fig. 3B shows that only propionate and acetate were rising at appreciable rates in the AMBR effluent at 38 and 29 mg l^{-1} d^{-1}, respectively. Fig. 4 shows, however, that despite the net accumulation of acetate, there was significant consumption activity occurring during one feeding cycle (negative slope). The low levels of butyrate can also be attributed to active consumption (negative slope) of this intermediate. Propionate, on the other hand, displayed a net accumulation during a feeding cycle (positive slope). It was also the most predominant VFA in four compartments. Therefore, at the end of Period 1, electron flow in the AMBR (predominantly methanogenic) coursed mainly through acetate and butyrate.

Using the same reasoning throughout the entire time period, we find that by the end of Period 2, the pattern of VFA consumption gradually shifted to an acetate–propionate trophic structure: at day 149, acetate and propionate consumption were apparent in the outer compartments, as evidenced by their negative compartment 1–compartment 5 slopes in Fig. 4. On the other hand, butyrate was the most abundant VFA accumulating in the AMBR with a positive compartment 1–compartment 5 slope. Thus, by the end of Period 2, a shift occurred in methanogenic electron flow from acetate–butyrate to acetate–propionate pathways. This correlates to the shift in hydrogenotrophic MPA populations described above, which involved the loss of *Methanobacteriaceae* which are known to interact with butyrate oxidizers in granules (Schink and Stams, 2006; Wu et al., 1996). Moreover, it is now recognized that all syntrophic propionate oxidizing bacteria are also capable of sulfate reduction (Schink and Stams, 2006). In fact, most syntrophic propionate oxidizers (excluding *Smithella propionica*) can now be isolated in pure culture with propionate and sulfate (Wallrabenstein et al., 1995, 1994; Harmsen et al., 1993; Harmsen et al., 1995). Therefore, the re-channeling of electrons toward propionate oxidation would be expected under increased sulfate loads. The disabling of the butyrate oxidation route of methanogenesis in the AMBR was further exacerbated by the selection of well-known butyrate-producing fermenting bacteria during Period 2, namely the clostridia (Wiegel et al., 2006). During Period 2, populations affiliated with clostridia became major bacterial groups in the AMBR (Table 2 and Briones et al., 2007). Numerous genera in these groups have been associated with butyrate production (Lawson et al., 2004; Schwiertz et al., 2002; Miller and Jenesel, 1979; Diez-Gonzalez et al., 1999). Therefore, during the latter part of Period 2, the bacterial and archaeal community structures in the AMBR along with their metabolic characteristics are consistent with the accumulation of butyrate with limited capacity for its breakdown. On the other hand, we previously reported that a novel population affiliated with *Thermotogaceae* became predominant in the UASB reactor at the end of Period 2 (Table 2 and Briones et al., 2007). While members of this group are widely known as hyperthermophiles, mesophilic thermotogas have recently been reported (Nesbo et al., 2006). Most cultured representatives of the *Thermotogaceae* ferment complex and simple carbohydrates to lactate, acetate, ethanol, L-alanine, carbon dioxide, and hydrogen as major end products (Huber and Hannig, 2006). The other major bacterial populations in the UASB reactor were representatives of the *Bacteroidaceae* and *Desulfovibrionaceae*, both of which would be expected to produce propionate, acetate, carbon dioxide, and hydrogen as major end products (Rabus et al., 2006; Smith et al., 2006). Therefore, the microbial community structure in the UASB reactor routed electrons through parallel pathways involving acetate, carbon dioxide, and hydrogen, with *Bacteroidaceae* likely contributing propionate. This trophic structure, which routes most electron flow through acetate, carbon dioxide, and hydrogen is one that is inherently more balanced, since it shifts the burden of electron flow away from reduced fermentation intermediates which require syntrophic interactions for their breakdown. However, under increasingly sulfidogenic conditions, we would expect the accumulation of acetate to become a problem in the UASB reactor.

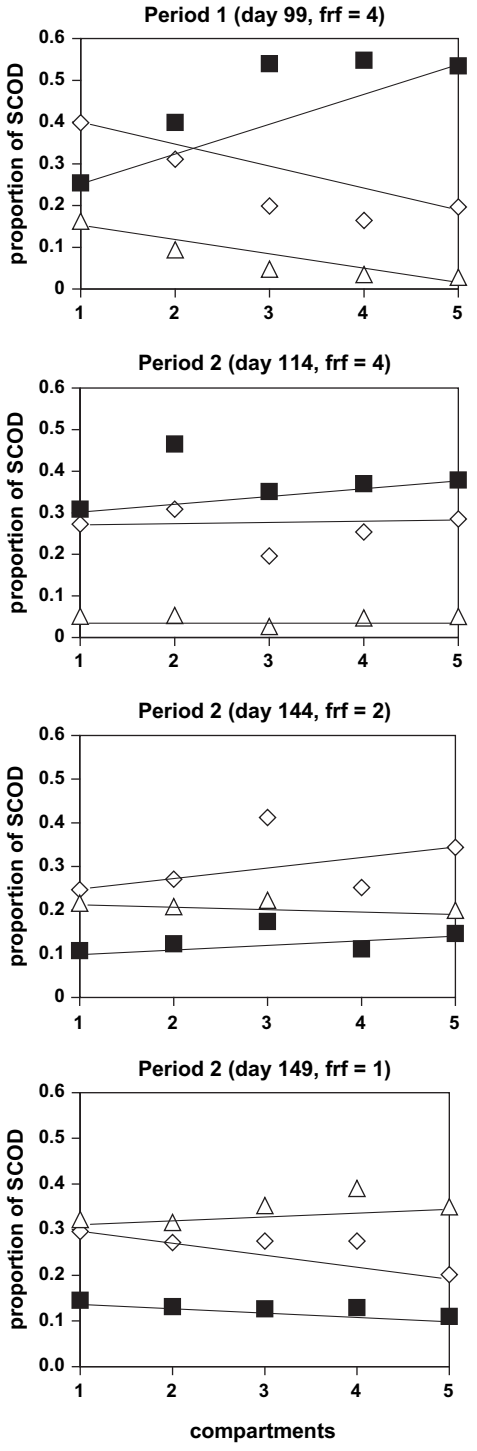

Fig. 4 – Proportion of the major VFAs in terms of SCOD in individual AMBR compartments during days 99, 114, 144, and 149. The lines were drawn to connect VFA levels in compartments 1 and 5, to infer production and consumption activities. ◇ = acetate; ■ = propionate; Δ = butyrate.

3.6. *Propionate:acetate ratios*

The propionate:acetate (Pr/Ac) ratio has been suggested as a useful ratio for predicting the stability of anaerobic digestion (Aiyuk et al., 2006; Wang et al., 1997; Hill et al., 1987). A Pr/Ac ratio greater than 1.4 has been suggested as a signal of reactor imbalance (Aiyuk et al., 2006; Hill et al., 1987), while Wang et al. (1997) suggests that an abrupt change in Pr/Ac ratio (e.g., shifts from <1 to >3.5) is a reliable indicator of reactor failure. Fig. 5 shows the highest Pr/Ac ratios of 3.9–4.0 (and the steepest rates of change) in both reactors in the 20 days prior to shifting sulfate load (days 80–99), which also corresponded to the peak times of methane production (Fig. 1A). Since the UASB reactor ultimately performed better during higher sulfate load, the Pr/Ac ratio by itself was not a good predictor of reactor performance. It should be noted, however, that during the entire operational periods, the more stable bioreactor also maintained a more stable Pr/Ac ratio consistently below one (Fig. 5).

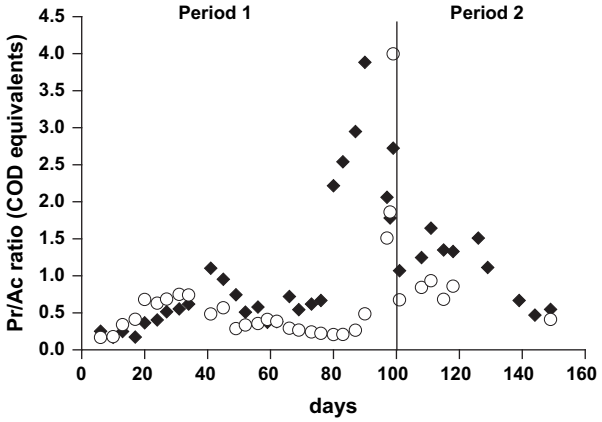

Fig. 5 – Propionate to acetate ratios in terms of COD equivalents observed in the effluent during reactor operation. AMBR (◆) and UASB reactor (○).

4. Conclusions

1. High strength waste streams containing sulfate need to maintain both methanogenic and sulfidogenic routes of carbon and electron flow to degrade organic matter and remove sulfate. The proportions of electrons flowing through either methanogenic or sulfidogenic routes depend mainly on the COD/SO_4^{2-} ratio.
2. At a COD/SO_4^{2-} ratio of 5, the competition for hydrogen between MPA and SRB is more intense than at a higher COD/SO_4^{2-} ratio. Propionate oxidation becomes more favored, since all known propionate degraders are also sulfate reducers. Propionate oxidation may or may not involve interspecies hydrogen transfer in the presence of sulfate. When granule formation is not favored, *Methanospirillaceae* are more likely to constitute the major hydrogen-consuming methanogen, while *Methanobacteriaceae* may be more vulnerable to sulfides or washout. This would mainly impact butyrate degradation.
3. Maintenance of butyrate degradation likely requires stable granules or flocs, which were present in the UASB reactor. A mechanism for retaining butyrate-degrading consortia in the AMBR needs to be developed under high sulfate loads.

One possible solution is to implement biofilm formation on a support matrix.

4. The UASB reactor maintained granular biomass as well as more stable Pr/Ac ratios and MPA community structure. Based on performance parameters and microbial community structure, carbon and electron flow during Period 2 coursed predominantly through acetate, carbon dioxide, and hydrogen, leading to lower build-up of reduced fermentation intermediates. However, acetate accumulation would eventually become a concern in the UASB reactor.

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