ASSESSMENT OF MICROBIAL ACTIVITY IN ANAEROBIC COLUMNS TREATING SYNTHETIC MINE DRAINAGE[1]

M. Logan[2], D. Ahmann, and L. Figueroa

Abstract. The importance of sulfate-reducing bacteria for metal precipitation in anaerobic passive treatment systems for remediation of acid mine drainage has been established. Conditions leading to decline of sulfate-reducing activity and failure of passive treatment systems are not well understood; however, this study hypothesizes that decline in performance is related to decline in substrate availability for sulfate-reducing bacteria. Other microbial functions break down complex organic material to provide the simple organic compounds required by sulfate reducers, and are essential for sustainability of passive mine drainage treatment systems. An understanding of relationships between microbial activities and system performance is thus essential to the design of anaerobic passive treatment systems for long-term performance. The objective of this research is to develop a method to: (1) assess the activities of important microbial functions that influence sulfate reduction in an anaerobic passive treatment system, and (2) apply the method to an anaerobic column system treating synthetic mine drainage to detect differences in activities as the system ages, for the purpose of determining the rate-limiting step(s) in the degradation of organic material as they relate to sulfate reduction. The approach involves the use of a long-term column study in conjunction with short-term batch studies, which add substrate supplements to the organic material from sacrificed columns in order to probe the activities of important microbial functions. The substrate supplements each target a distinct microbial function at a specific step in the anaerobic degradation of complex organic compounds. Activities measured in batch studies correlate to overall column performance in terms of sulfate-reducing activity. Results of gas analyses from the batch studies illustrate the usefulness of this approach in quantifying important microbial functions, as well as identifying the rate-limiting step(s) in the degradation of organic material as the system ages. Data suggest an overall stimulation of metabolic activities by cellobiose and glucose, and indicate the rate-limiting step(s) lie between cellulose and cellobiose.

Additional Key Words: mine drainage, passive treatment, sulfate reducers, methanogens

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Introduction

Thousands of abandoned mines that generate acidic metal-laden drainage exist throughout the western United States, many in remote locations. Cost-effective, low-maintenance, long-term technologies are desirable for remediation of these sites. Passive treatment systems promoting the activity of sulfate-reducing bacteria use inexpensive, readily available substrate materials and have shown promise in removing metals and acidity from mine drainage. A variety of substrates have been used in anaerobic passive treatment systems, with similar initial sulfate removal rates (Cocos et al. 2002, Wildeman et al. 1997, Wildeman et al. 1997). However, long-term performance of these systems remains inconsistent. Conditions leading to decline of sulfate-reducing activity and failure of passive treatment systems are not well understood.

This study hypothesizes that decline in system performance is related to decline in substrate availability for sulfate-reducing bacteria. Previous research has focused primarily on the activity of sulfate-reducing bacteria in anaerobic passive treatment systems. However, other microbial groups break down complex organic material to provide the simple organic compounds required by sulfate reducers and are essential for long-term sustainability of passive mine drainage treatment systems.

Anaerobic degradation of complex organic material to simple organic compounds is carried out by a consortium of microbial species in natural environments (Wildeman and Updegraff, 1997). The same is true in passive treatment systems. Sulfate-reducing bacteria require relatively simple compounds, such as H_2, lactate, acetate, ethanol, and other small organic compounds, as electron donors. Sulfate reducers are dependent on other microbial functions to provide the simple compounds they require for carbon and energy. This consortium of species, including those that perform hydrolysis and fermentation, is essential to the long-term sustainability of passive treatment systems. Conversely, methane-producing bacteria compete with sulfate reducers for fermentation products, such as acetate and H_2, and can divert the energy flow to an undesired pathway (Daly et al. 2000). A schematic of key carbon transformations in anaerobic passive treatment systems, adapted from Gottschalk, in which complex organic polymers are degraded to simple organic substrates, is presented in Fig. 1.

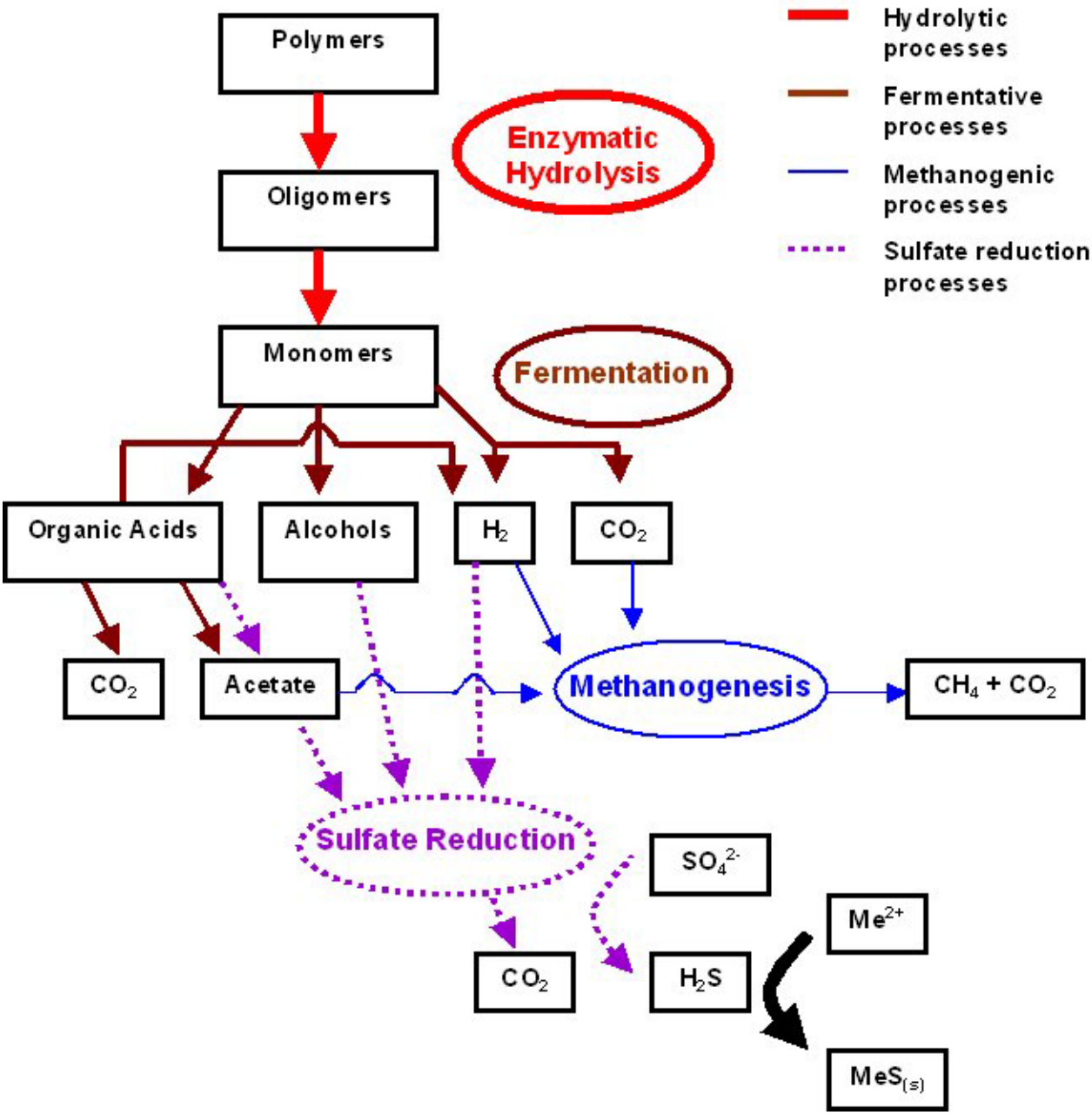

Figure 1. Schematic of key carbon transformations in anaerobic passive treatment systems. CO_2 from methanogens is added to account for the presence of acetoclastic methanogens. (Adapted from Gottschalk, 1986.)

An understanding of relationships between microbial activities and system performance is essential to the design of anaerobic passive treatment systems for long-term performance. An

approach based on microbial ecology that quantifies the functions of contributing populations, as well as the rate-limiting step(s) that control sulfate reduction, will lead to better designs and more robust passive treatment systems (Wildeman and Updegraff, 1997).

Molecular techniques provide information about the presence of bacteria in a system, but not about their activities (Daly et al. 2000, Raskin et al. 1996). While many microbial populations may be present in an anaerobic system treating mine drainage, it is their actual functions that are important when assessing system performance. Rather than identify specific bacteria, this research investigates a new method to quantify the activities of important microbial functions in an anaerobic column system treating synthetic mine drainage as the system ages and sulfate removal rates change.

Five model substrate supplements, based on the schematic of key carbon transformations presented in Fig. 1, were used in batch experiments with organic material from sacrificed columns as a way to probe the activities of important microbial functions. The substrate supplements chosen (cellulose, cellobiose, glucose, lactate, and acetate) range from a complex polymer to simple organic acids and are each meant to target a distinct microbial function at a specific step in the degradation process of complex organic compounds. Changes in activities following the addition of supplements can provide information about the rate-limiting step(s) in the degradation of organic material from complex to simple as they relate to sulfate reduction. If a specific substrate amendment is found to cause a significant decrease in sulfate concentrations and/or increase in headspace H_2S concentrations, concentrations of that particular component are present in limited amounts within the system.

Gas samples were analyzed throughout the batch experiments for CO_2, H_2S, and CH_4 to measure the activities stimulated by the supplements. A more simplified schematic of carbon flow illustrates the five model compounds used as substrate supplements and the gases produced by the microbial functions that degrade those compounds in Fig. 2. A detailed description is of the experimental system is given in the following section.

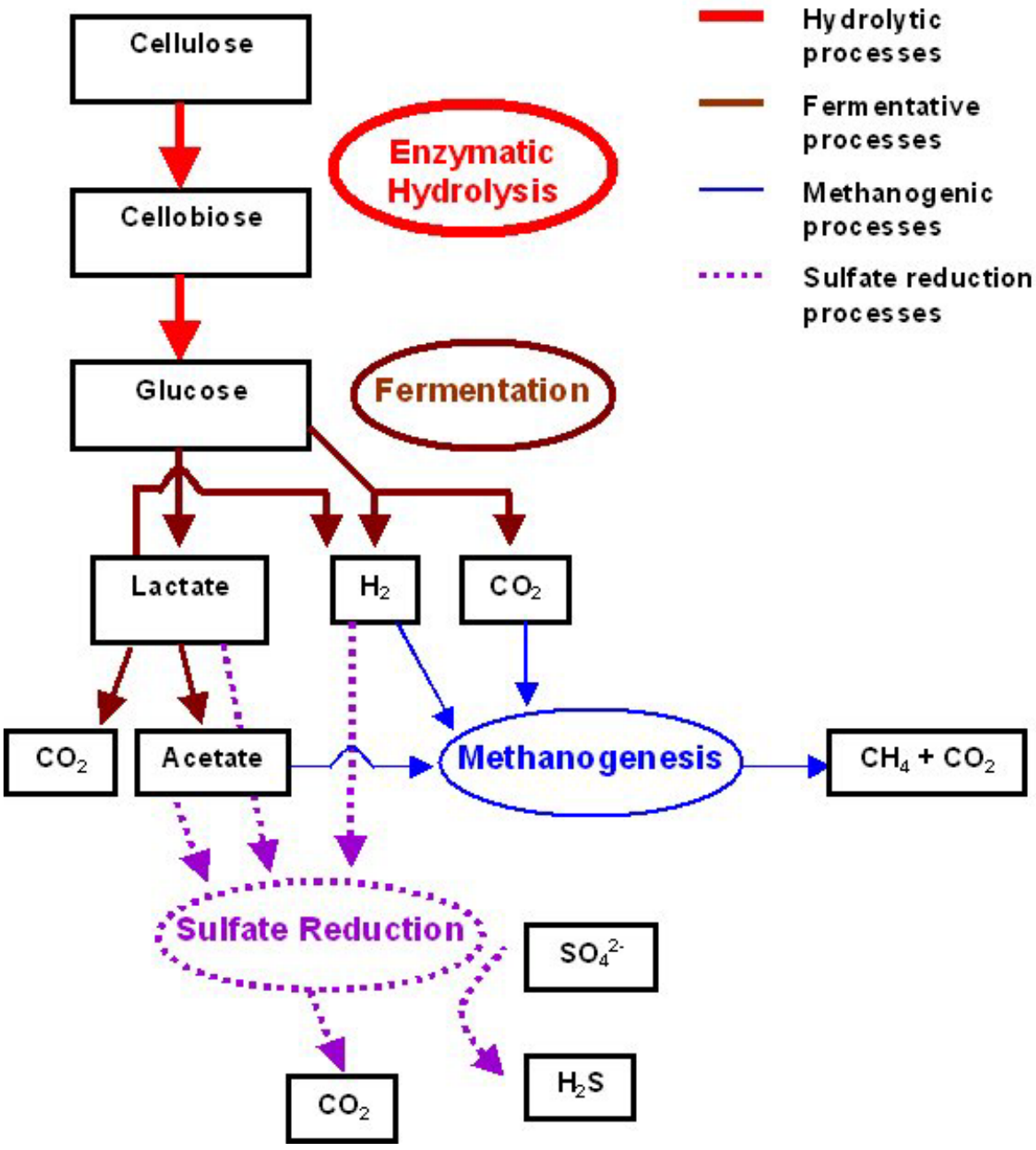

Figure 2. Simplified schematic of carbon transformations in anaerobic passive treatment systems distinguishing model compounds added and gases measured in the experimental system. (Adapted from Gottschalk, 1986.)

Methods

Columns. Each column set consisted of eight replicate glass columns (Table 1) packed with 150 g dry weight of a homogenized mixture of 15% walnut wood shavings, 20% dairy cattle manure, 10% alfalfa, 5% wetland sediment inoculum, 5% limestone, and 45% silica sand (subsequently referred to as the reactive mixture), with remaining space at the top of the column filled with silica sand. Dairy manure was collected from the agricultural research center at CSU in February 2002 and again in February 2003 and stored at 4)C. Walnut wood and alfalfa pellets were processed in a Wiley mill through a 4 mm sieve. Wetland sediment was obtained from the Big Five mine drainage treatment wetland by T. Wildeman in 1998 and stored at 4)C. Limestone and silica sand were purchased from local vendors. The materials for each column were mixed in separate plastic bags until homogenized.

Columns for Set 1were packed wet with 1000 mg/L sulfate solution and allowed to sit for five days before influent pumping began. A twelve-channel peristaltic pump was used to feed synthetic mine water through the columns from a common container (Table 2). Influent was fed up through each column bottom and removed from a port located approximately 22 cm from the bottom.

Columns for Set 2 were packed wet with DI water. Fresh manure collected in February 2003 was used in the reactive mixture for Column Set 2. Influent pumping began the day after packing, with a twelve-channel peristaltic pump used to feed sodium sulfate solution through the columns from a common container (Table 2). Influent was fed up through each column bottom and removed from a port located approximately 15 cm from the bottom. On day 27, when sulfate reduction had begun, two columns were sacrificed for batch study and influent for the remaining six columns was changed to simulated mine water and the flow rate was slowed.

Effluent was monitored for alkalinity (HACH digital titrator method #8203), pH (Orion probe and meter), and conductivity (YSI 35 probe and meter). Effluent from each column was filtered through 0.2 μm filters and analyzed for sulfate (Dionex ICS-90, AS14A column), with the remaining filtered portion frozen for future analyses of metals and sulfur (Perkin Elmer ICP-AES).

Table 1: Column characteristics

Specification	Column Set 1	Column Set 2
Column Volume (mL)	589	589
Reactive Volume (mL)	399	324
Diameter (cm)	5	5
Length (cm)	30	30
Reactive Mass (g)	150	150

Table 2: Mine water influent composition and flow

Constituent	Column Set 1	Column Set 2 (days 1-27)	Column Set 2 (> 27 days)
Sulfate (mg/L) as Na_2SO_4	1000	1000	1000
Zinc (mg/L) as $ZnSO_4 \cdot 7H_2O$	50	0	50
Manganese (mg/L) as $MnSO_4 \cdot H_2O$	50	0	50
Nickel (mg/L) as $NiSO_4 \cdot 6H_2O$	50	0	0
pH	6.0	6.0	6.0
Flow (mL/d)	90	90	30

Batch Studies

Batch studies for each column set were performed using a portion of the initial reactive mixture used to construct the columns and later using mixtures from a pair of sacrificed columns. Approximately 10 g of reactive mixture (dry weight) was placed into each serum bottle with 85 mL of liquid. Liquid consisted of 1000 mg/L sodium sulfate and a 10 mM substrate supplement, with pH approximately 6.5. Negative controls did not receive substrate supplements, only sodium sulfate, while other serum bottles received either 10 mM cellulose, cellobiose, glucose,

sodium lactate, or sodium acetate as the substrate supplement. Each was duplicated for a total of 12 serum bottles per batch study.

During the batch study of the initial mixture for Column Set 1, an anaerobic glovebox was used when transferring the mixture into serum bottles, which contained liquid previously sparged with N_2. For subsequent batch studies, the reactive mixture was transferred into serum bottles under ambient conditions. Liquid was then sparged with N_2 for 20 minutes and headspace for 7 minutes before sealing the bottles.

Serum bottles were incubated on their sides at room temperature on a shaker in the dark for 60 h. Gas and liquid samples were taken at 0, 6, 12, 24, 30, 36, 48, 54, and 60 h. Sterile needles were used for sampling and needles and syringes were flushed with N_2 before sampling to ensure air was not introduced into the serum bottles. Approximately 1.5 mL of gas and 3 mL of liquid were removed from each bottle when sampled. Gas samples were analyzed immediately for CO_2, H_2S, and CH_4 (Agilent P200 Micro GC with column A molesieve 5A 100m and column B ppu 8m). Each liquid sample was evenly divided between two 1.7 mL microcentrifuge tubes. One of the two tubes contained 100 μL of 0.1 M zinc acetate to trap any dissolved sulfide in the sample and prevent its oxidation to sulfate (Spear, 1999), allowing sulfur speciation to be determined. All tubes were spun in a microcentrifuge at maximum speed for three minutes. The supernatant from each sample was filtered through a 0.2 μm filter into a clean microcentrifuge tube and frozen. The samples that received zinc acetate will be used for sulfate analysis (Dionex ICS-90), while the samples without zinc acetate will be used for sulfur and metals (Perkin Elmer ICP-AES) and organic acid (HPLC) analyses.

Results and Discussion

Preliminary column experiments were performed in which a 24-h pulse of a substrate supplement was added to the mine water feed to the columns (data not shown). It was difficult to discern any visible trends in the total sulfur data from the column effluents after the addition of substrate supplements. Based on these preliminary experiments, it was determined that it was not possible to assess the effects of substrate supplements in column systems, largely due to their relative complexity. For example, heterogeneities and preferential flow paths influence the ability to obtain representative samples. It is also difficult to determine the ideal pulse duration

through a column, so as not to significantly change the microbial community composition within the system.

These issues can be minimized by performing batch studies in serum bottles incubated on a shaker to eliminate variations in exposure to the substrate supplement by ensuring that all the material is exposed to the same amount of substrate supplement for the same amount of time. Batch studies also allow multiple substrate supplements to be tested at a given time using the same reactive mixture. In addition, gas analyses are necessary to facilitate the identification of metabolically active communities within the system at a given time. Gas collection has proven difficult with the column systems and changes in gas composition would be best measured in batch systems.

Column Performance

After running Column Set 1 with synthetic mine drainage for nearly one month, it became clear based on effluent sulfate measurements that sulfate reduction was not occurring to the degree expected based on previous column studies (Figure 3). Effluent sulfate concentration was not significantly different than influent sulfate concentration, while previous column experiments (data not shown) exhibited measurable sulfate reduction in 14 to 21 days. Thus, Column Set 1 was terminated after 38 days. Column Set 2, made using fresh manure, was then assembled. No metals were added in the influent until sulfate reduction had begun to occur. These columns have exhibited sulfate-reducing activity similar to previous column experiments (Figure 4). The first pair of columns from Column Set 2 was sacrificed for batch study after 27 days of operation.

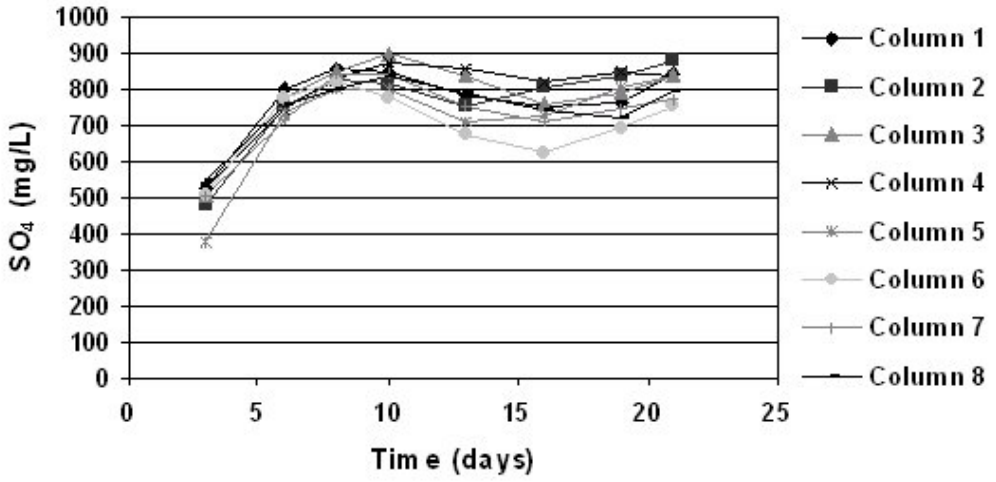

Figure 3. Sulfate in effluent from Column Set 1. (Influent sulfate concentration was 1000 mg/L.)

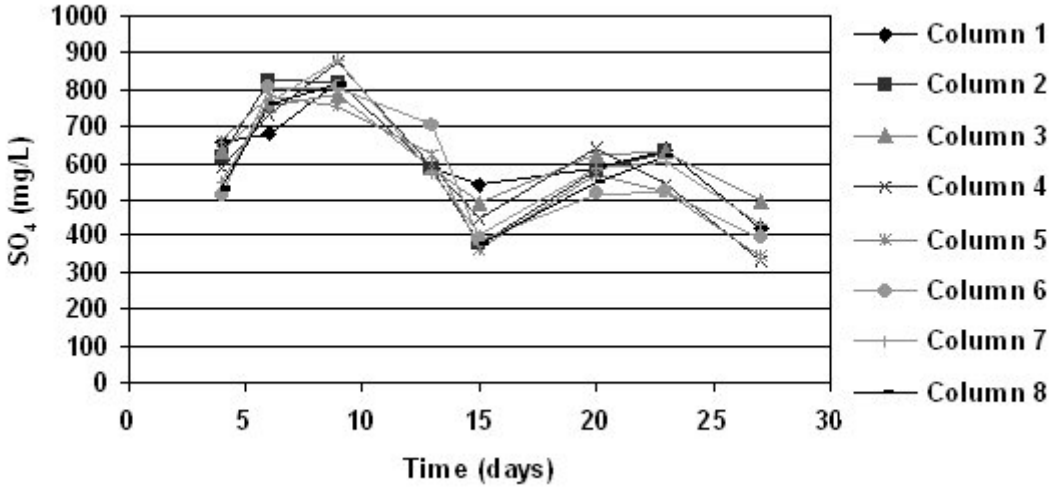

Figure 4. Sulfate in effluent from Column Set 2. (Influent sulfate concentration was 1000 mg/L.)

Column Experiments with Batch Analysis of Activity

Batch studies for each column set were performed using a portion of the initial reactive mixture used to construct the columns and later using mixtures from a pair of sacrificed columns. The percent CO_2, H_2S, and CH_4 measured in the headspace during the batch experiments are presented in Tables 5-8. H_2 was below detection in all samples. The remainder of the headspace

gas was composed of N_2, which was used to sparge the samples before the serum bottles were sealed.

Column Set 1. Data for Column Set 1 is presented below in Figure 5 through Figure 10. The batch study using the initial mixture for Column Set 1 shows the presence of a methanogen population and very little stimulation of sulfate reducers, with the exception of one of the samples that received acetate. Acetate was the only supplement that increased CH_4 and H_2S production above the control. In addition, CO_2 production measured 25% lower than the control with the addition of acetate. Thus, acetate stimulated both sulfate-reducing activity and methanogenesis, while possibly inhibiting overall activity based on CO_2 production. Overall activity in the initial mixture, as indicated by CO_2 production, was stimulated by cellobiose and glucose, suggesting a rate-limiting step lies between cellulose and cellobiose in the degradation of organic material.

The batch study performed using the reactive mixture from sacrificed columns after 38 days shows decreased overall activity as measured by CO_2 production, but it was greatly stimulated by cellobiose and glucose. The drop in activities suggests that by day 38 much of the easily consumed soluble organic material in the columns was all used up, or had washed away in the flow-through column system.

The mixture from sacrificed columns did not produce any H_2S in batch experiments without the addition of a substrate supplement. As these columns did not exhibit any significant sulfate reduction while operating, this result was not surprising. The fact that sulfate reduction could be stimulated in the mixture from Column Set 1 signifies that the population was present, but was not receiving the necessary carbon source to be active. Lactate stimulated sulfate reducers directly, while cellobiose and glucose stimulated sulfate reducers indirectly. With lactate as the substrate supplement, H_2S production increased to 1.15% in the headspace. Methanogenesis was stimulated by lactate and acetate, and possibly inhibited by cellobiose and glucose. This apparent inhibition could be explained by possible competition for nutrients or electron donors by another anaerobic population that was stimulated by cellobiose and glucose.

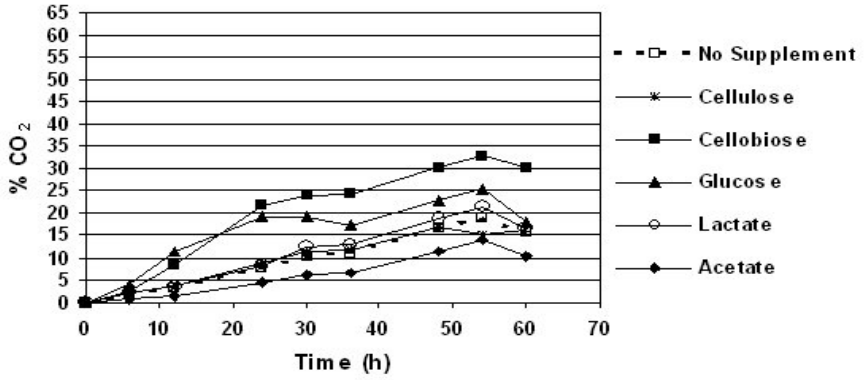

Figure 5. Headspace CO_2 in batch study of initial mixture from Column Set 1. (Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 1.60%, cellulose ! 5.34%, cellobiose ! 1.76%, glucose ! 0.81%, lactate ! 1.59%, and acetate ! 4.20%.)

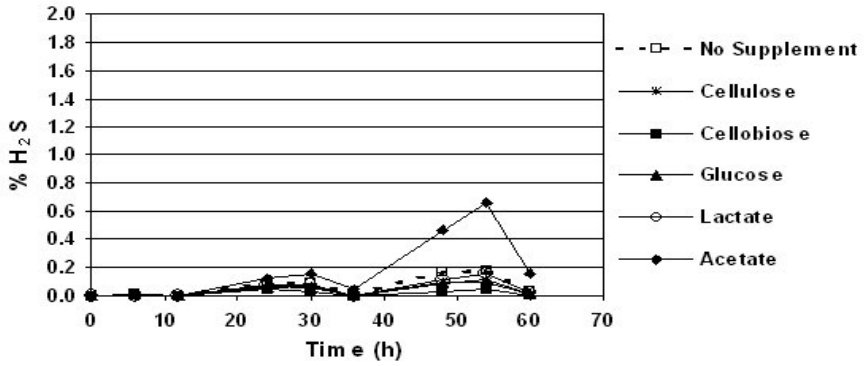

Figure 6. Headspace H_2S in batch study of initial mixture from Column Set 1. (Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 0.01%, cellulose ! 0.04%, cellobiose ! 0.01%, glucose ! 0.04%, lactate ! 0.04%, and acetate ! 0.40%.)

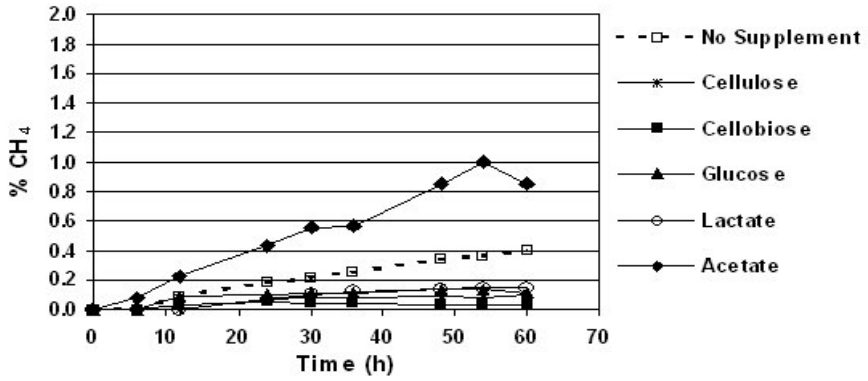

Figure 7. Headspace CH_4 in batch study of initial mixture from Column Set 1. (Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 0.04%, cellulose ! 0.02%, cellobiose ! 0.05%, glucose ! 0.06%, lactate ! 0.08%, and acetate ! 0.55%.)

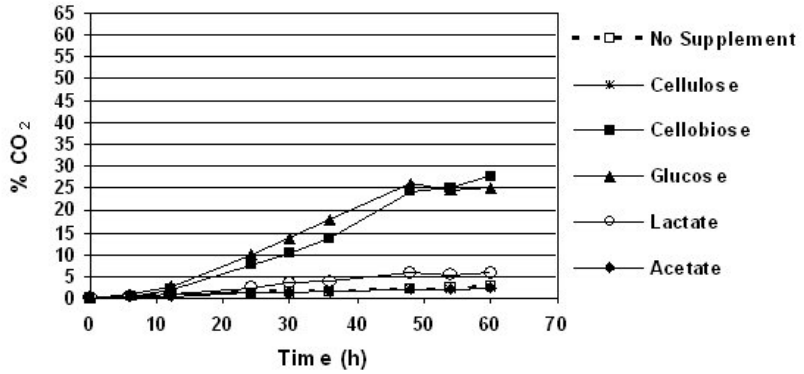

Figure 8. Headspace CO_2 in batch study of Column Set 1 after 38 days. (Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 0.35%, cellulose ! 0.27%, cellobiose ! 3.20%, glucose ! 3.78%, lactate ! 0.90%, and acetate ! 0.09%.)

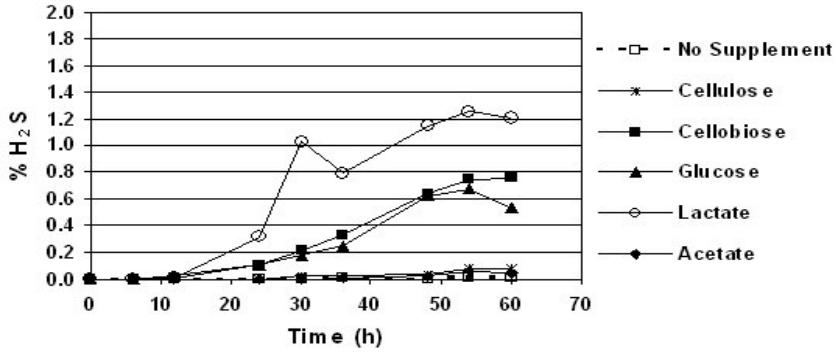

Figure 9. Headspace H_2S in batch study of Column Set 1 after 38 days. (Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 0.02%, cellulose ! 0.01%, cellobiose ! 0.24%, glucose ! 0.55%, lactate ! 0.40%, and acetate ! 0.02%.)

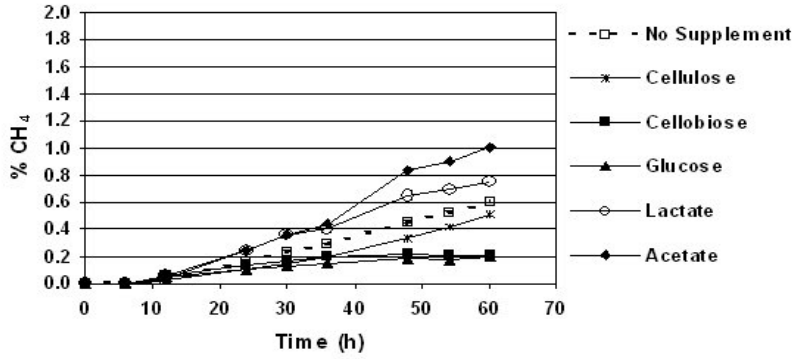

Figure 10. Headspace CH_4 in batch study of Column Set 1 after 38 days. (Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 0.11%, cellulose ! 0.07%, cellobiose ! 0.01%, glucose ! 0.04%, lactate ! 0.09%, and acetate ! 0.29%.)

Column Set 2. Data for Column Set 2 is presented below in Figure 11 through Figure 15. Cellobiose and glucose stimulated the greatest CO_2 production in the initial mixture for Column Set 2, again suggesting a rate-limiting step lies between cellulose and cellobiose in the degradation of organic material. Very little sulfate-reducing activity was exhibited in the initial mixture and no methane production was observed for the initial mixture under any of the conditions tested.

As observed with Column Set 1, CO_2 production in the reactive mixture from the sacrificed columns dropped substantially from the initial mixture, further supporting the hypothesis that labile organic carbon had diminished greatly. Again, activity was stimulated by cellobiose and glucose. The mixture from Column Set 2 produced 0.23% H_2S with no substrate supplement. Sulfate-reducing activity was greatly stimulated by cellobiose, glucose, and lactate. Lactate stimulated sulfate reducers directly, while cellobiose and glucose stimulated sulfate reducers indirectly. Methanogen activity was also observed after 27 days.

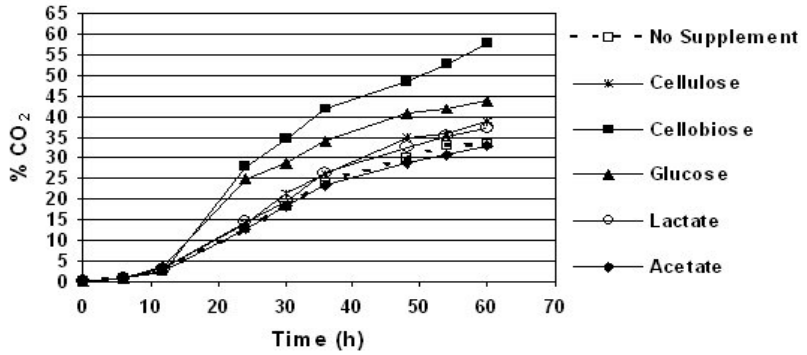

Figure 11. Headspace CO_2 in batch study of initial mixture from Column Set 2. (Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 1.37%, cellulose ! 2.24%, cellobiose ! 6.43%, glucose ! 2.26%, lactate ! 0.41%, and acetate ! 1.05%.)

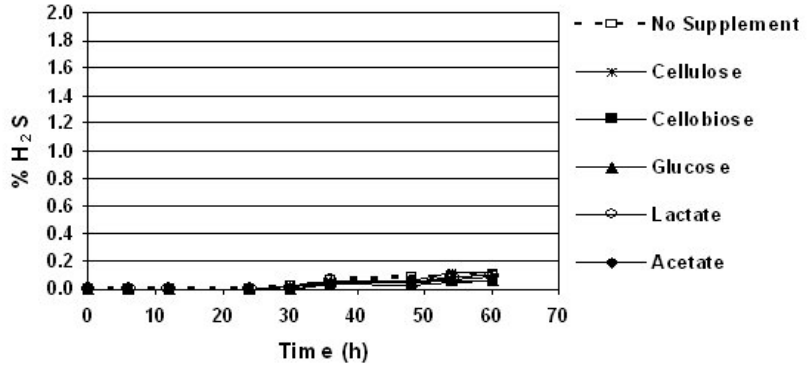

Figure 12. Headspace H_2S in batch study of initial mixture from Column Set 2.(Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 0.01%, cellulose ! 0.01%, cellobiose ! 0.01%, glucose ! 0.03%, lactate ! 0.00%, and acetate ! 0.01%.)

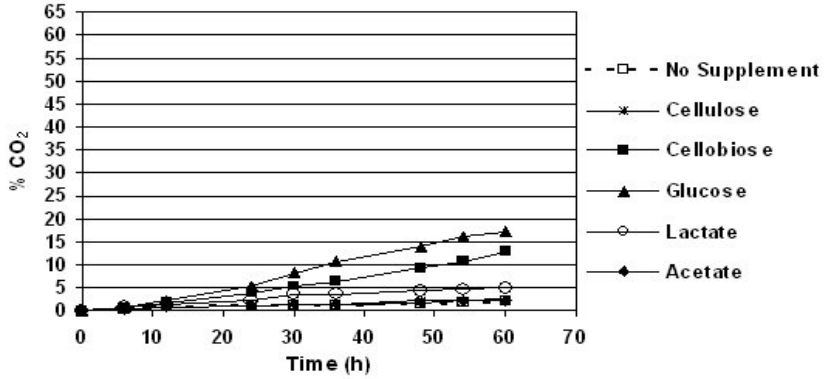

Figure 13. Headspace CO_2 in batch study of Column Set 2 after 27 days.(Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 0.06%, cellulose ! 0.22%, cellobiose ! 0.78%, glucose ! 0.58%, lactate ! 0.13%, and acetate ! 0.13%.)

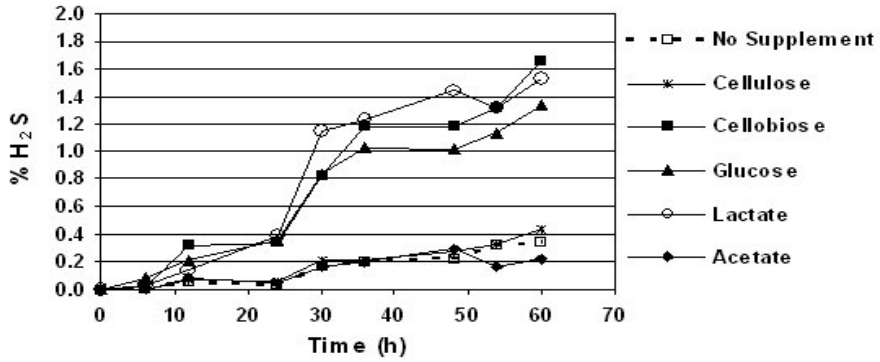

Figure 14. Headspace H_2S in batch study of Column Set 2 after 27 days. (Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 0.04%, cellulose ! 0.03%, cellobiose ! 0.30%, glucose ! 0.14%, lactate ! 0.17%, and acetate ! 0.15%.)

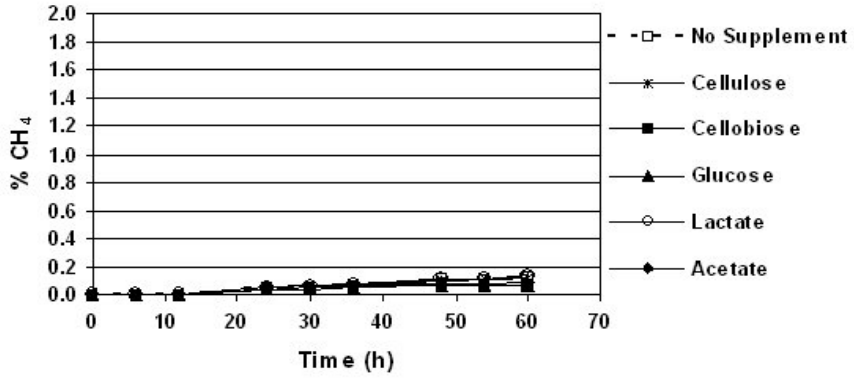

Figure 15. Headspace CH_4 in batch study of Column Set 2 after 27 days. (Data plotted represents an average of two replicates. Range of data is as follows: no supplement ! 0.01%, cellulose ! 0.01%, cellobiose ! 0.01%, glucose ! 0.01%, lactate ! 0.00%, and acetate ! 0.04%.)

Comparison of Initial Mixture Batch Studies. The batch study results at 48 h from initial mixtures for Column Sets 1 and 2 are presented below in Table 1 and Table 3. A comparison of the initial mixtures gives insight into the importance of reactive mixture composition for system performance. The mixture used in Column Set 2 exhibited higher overall microbial activity, as measured by the total CO_2 produced (nearly twice as high) when no supplement was added, compared to the mixture used in Column Set 1. The higher activity was likely due to the use of fresh manure in Column Set 2, which may have contained a higher concentration of microorganisms and/or a higher percentage of easily degraded soluble organic material.

A comparison of H_2S produced suggests the initial mixtures were comparable in terms of sulfate-reducing activity, as both were able to produce close to the same amount of H_2S with no supplement (0.17% in Column Set 1 compared to 0.08% in Column Set 2). However, solution phase sulfate and total sulfur concentration data are needed to support this observation. The initial mixture from Column Set 1 exhibited the presence of a competing methanogen population, while no methane production was observed for the initial mixture from Column Set 2. It is likely that a methanogen population was established in the manure used for Column Set 1, which had been stored for nearly one year before its use in the reactive mixture.

Table 3. Headspace gas at 48 h in batch study of initial mixture for Column Set 1.

Supplement	% CO_2	% H_2S	% CH_4
No Supplement	16.60 ! 1.47	0.17 ! 0.01	0.34 ! 0.00
10 mM Cellulose	17.12 ! 1.20	0.10 ! 0.02	0.09 ! 0.01
10 mM Cellobiose	30.16 ! 0.14	0.04 ! 0.01	0.04 ! 0.04
10 mM Glucose	22.80 ! 0.81	0.08 ! 0.04	0.13 ! 0.06
10 mM Lactate	18.76 ! 0.78	0.11 ! 0.04	0.13 ! 0.07
10 mM Acetate	11.53 ! 3.54	0.47 ! 0.28	0.85 ! 0.49

Table 4. Headspace gas at 48 h in batch study of day 38 sacrifice of Column Set 1.

Supplement	% CO_2	% H_2S	% CH_4
No Supplement	2.00 ! 0.28	0.00	0.45 ! 0.08
10 mM Cellulose	1.85 ! 0.07	0.03 ! 0.01	0.33 ! 0.04
10 mM Cellobiose	24.20 ! 1.94	0.64 ! 0.18	0.22 ! 0.00
10 mM Glucose	26.39 ! 1.25	0.62 ! 0.10	0.18 ! 0.04
10 mM Lactate	5.54 ! 0.43	1.15 ! 0.21	0.65 ! 0.08
10 mM Acetate	2.04 ! 0.09	0.03 ! 0.00	0.83 ! 0.29

Table 5. Headspace gas at 48 h in batch study of initial mixture for Column Set 2.

Supplement	% CO_2	% H_2S	% CH_4
No Supplement	29.95 ! 0.69	0.08 ! 0.00	0.00
10 mM Cellulose	36.64 ! 1.38	0.06 ! 0.00	0.00
10 mM Cellobiose	48.48 ! 6.43	0.03 ! 0.00	0.00
10 mM Glucose	40.48 ! 0.56	0.05 ! 0.03	0.00
10 mM Lactate	32.63 ! 0.09	0.04 ! 0.00	0.00
10 mM Acetate	28.73 ! 0.44	0.06 ! 0.01	0.00

Table 6. Headspace gas at 48 h in batch study of day 27 sacrifice of Column Set 2.

Supplement	% CO_2	% H_2S	% CH_4
No Supplement	1.60 ! 0.02	0.23 ! 0.04	0.11 ! 0.01
10 mM Cellulose	2.20 ! 0.22	0.27 ! 0.03	0.10 ! 0.01
10 mM Cellobiose	9.24 ! 0.78	1.19 ! 0.30	0.06 ! 0.01
10 mM Glucose	13.79 ! 0.34	1.01 ! 0.14	0.06 ! 0.01
10 mM Lactate	4.21 ! 0.08	1.44 ! 0.17	0.11 ! 0.00
10 mM Acetate	1.71 ! 0.11	0.30 ! 0.15	0.07 ! 0.04

Comparison of Sacrificed Column Batch Studies. The batch study results at 48 h from sacrificed columns in Column Sets 1 and 2 are presented above in Table 4 and Table 6. CO_2 production, and thus overall activity, was significantly reduced in both mixtures after 27 to 38 days in a column for all conditions tested. Headspace CO_2 dropped from 16.60% initially to 2.00% for Column Set 1, and from 29.95% initially to 1.60% for Column Set 2. Again, cellobiose and glucose always stimulated the greatest CO_2 production, suggesting a rate-limiting step lies between cellulose and cellobiose in the degradation of organic material.

Despite the failure of Column Set 1 to reduce sulfate, SRB activity was present at a comparable level to Column Set 2 when stimulated with lactate. Thus, given the right carbon source, the sulfate reducers in Column Set 1 were as productive as the sulfate reducers in Column Set 2. This suggests that either the pathway leading to the production of lactate and other carbon sources needed by sulfate reducers in Column Set 1 was inhibited, or a different population was somehow interfering with the carbon flow to the sulfate reducers.

With sulfate-reducing activity on the same order as methanogenesis in both mixtures, it is clear that methanogens represented a significant competitive group to sulfate reducers within the column system. CH_4 production in Column Set 1 was greater with no supplement than was sulfate reduction, suggesting methanogens may have out-competed sulfate reducers for simple substrates, contributing to the poor performance of these columns in terms of sulfate reduction. In Column Set 2, however, sulfate-reducing activity with no supplement was two to three times higher than methanogenesis, which is consistent with the active sulfate reduction observed in these columns.

Another possibility for the lack of sulfate-reducing activity in Column Set 1 was an inhibitory effect due to the metals in the influent solution. Zinc and manganese have been used at these concentrations during several previous experiments with no inhibitory effects observed (data not shown). However, this was the first experiment in which nickel was included in the influent composition. The addition of nickel may have caused inhibition of sulfate-reducing activity directly, or inhibited a group upstream of sulfate reducers that function to degrade complex compounds to simple ones used by sulfate reducers.

Discussion of Method. The major objectives of these experiments have been to (1) develop a method for assessing the activities of major microbial functions in anaerobic passive treatment

systems, and (2) apply the method to a column system for the purpose of discerning the rate-limiting step(s) in the degradation of organic material. The method appears to be useful in quantifying the activities of sulfate reducers and methanogens, and providing information about rate-limiting steps in the degradation of organic material within the column system. However, many considerations can be addressed in refining the method.

The batch experiments were carried out to 60 h. A period of 36 to 48 h was sufficient to observe the trends in gas production within the serum bottles. Less than 36 h was not long enough, as the samples required a certain amount of start-up time before differences could be assessed. A 48-h sampling period seemed to yield more representative data than 60 h, and thus was the length of time used for the data presented in the tables above.

Liquid samples have not yet been analyzed in these experiments. They can be used to provide information about sulfur speciation in the serum bottles, and information about the amount of each substrate supplement that was actually degraded. Throughout these batch studies, both the headspace and liquid in each serum bottle were sampled nine times in a period of 60 h. Each time the serum bottles were sampled 3 mL of liquid was removed to ensure enough would be available for IC, HPLC, and ICP analyses after filtering through a 0.2 μm filter. This led to a 27 mL increase in the headspace volume of each serum bottle over the 60-h sampling period. It was not necessary to sample the liquid as often as the headspace to provide the above information and that portion of the method should be modified for future use, as it causes significant increases in headspace volumes over the 60-h sampling period.

Although not presented here, future analysis of the data should take into account the changes in headspace volume and changes in pressure as gas production increases over time within the serum bottles. It should also take into account the carbon added in each 10 mM substrate supplement. Normalizing the data based on the amount of carbon added to the serum bottles in the form of each substrate supplement may make trends already observed in the data even more apparent. Subtracting the control from the other supplements appears to be a useful way to look at the data, as well. A large positive difference between the supplement sample and the control could indicate the supplement stimulated activity, while a large negative difference could indicate possible inhibition caused by the supplement, as observed with acetate in a batch experiment for Column Set 1.

Gas analyses have proven to be a useful tool with which to measure overall activity in the system and quantify the activities of sulfate reducers and methanogens as headspace gas composition in the serum bottles changes over time. Quantifying the activities associated with important functions provides information about the flow of carbon in the system, and the efficiency with which it is proceeding in a direction that supports the desired activity, sulfate reduction. Results of the gas analyses in batch experiments can be correlated to performance of the column system in terms of sulfate-reducing activity.

Gas analyses have also been useful in identifying the rate-limiting step(s) in the degradation of organic material in the system as it relates to sulfate reduction. Cellobiose and glucose have been found to consistently stimulate increased production of CO_2 and H_2S, while cellulose has not. This suggests cellobiose and glucose are present in limited amounts in the system. Thus, processes that produce these components, through the degradation of more complex components, represent the rate-limiting step(s) in the system.

Future Work

Future work for this research will include three more batch studies to be performed using the remaining six columns currently running as part of Column Set 2. The next pair of columns will be sacrificed soon, as metals have been added to the influent feed. The last pair of columns will be sacrificed when they begin to fail in terms of sulfate-reducing activity. Future work also includes additional analyses to be performed on liquid samples collected during the batch studies, as well as column effluents. These analyses will include IC for sulfate, ICP-AES for sulfur and metals, and HPLC to measure degradation of substrate supplements. Data from the batch studies will be normalized to carbon and analyzed with changes in headspace volume and pressure taken into account.

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