EFFECT OF ORGANIC SUBSTRATE COMPOSITION ON MICROBIAL COMMUNITY STRUCTURE OF PILOT-SCALE BIOCHEMICAL REACTORS TREATING MINING INFLUENCED WATER[1]

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Abstract: Mining-influenced water (MIW) is acidic, metal rich water formed when sulfide minerals react with oxygen and water. There are various options for the treatment of MIW; however, passive biological systems such as biochemical reactors (BCRs) have shown promise because of their low cost and maintenance requirements. The purpose of this study was to explore the effect of organic substrate on microbial communities present in pilot-scale BCRs treating MIW in order to understand how substrate-microbe interactions drive performance. Three organic substrates were evaluated: ethanol (ETOH); and two lignocellulose-based mixtures: hay and wood chips (HYWD), and corn stover and wood chips (CSWD). The microbial community compositions were characterized by cloning of 16S rRNA genes and *apsA* genes associated with sulfate reduction. Quantitative polymerase chain reaction (Q-PCR) was applied to quantify *Desulfovibrio-Desulfomicrobium* spp. and methanogens. Results revealed distinct differences in microbial compositions and relative quantities of total and sulfate-reducing bacteria (SRB) among the BCRs. In particular, the greatest proportion of SRBs were observed in the ETOH BCRs, but the total number of bacteria was low. The HYWD and CSWD BCRs had highly similar bacterial communities, which were complex in composition in comparison to the ETOH BCRs. Methanogens were found to be present in all BCRs at low levels and were the highest in the lignocellulose-based BCRs. This study demonstrates that substrate influences microbial community composition and diversity, which may play an important role in performance and reliability.

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Introduction

Mining-influenced water (MIW) is acidic; metal-rich water formed when sulfide minerals from abandoned or active mines are exposed to and react with oxygen and water. MIW represents a worldwide concern due to its potential to cause contamination of drinking water systems, disruption of growth and reproduction of aquatic life, and other problems related to its toxicity. Because most remediation sites are remote or associated with abandoned mines, treatment systems that are efficient and cost-effective are of great importance.

A variety of abiotic and biological remediation systems have been developed and used for the treatment of MIW. Abiotic methods usually involve the addition of neutralizing agents or other chemicals and thus can require an impractically high level of operation and maintenance. Alternatively, bioremediation has shown promise because of the low cost of maintenance and low external energy requirements. In particular, sulfate-reducing biochemical reactors (BCRs) have been applied with some success, but reports from the field are variable in terms of their overall performance and reliability (Benner et al. 1997; Barton and Karathanasis 1999; Johnson and Hallberg 2002). The basic principle of BCRs is the cultivation of sulfate-reducing bacteria (SRB) that reduce the SO_4^{2-} in the mine drainage to sulfide, which then reacts with heavy metals and precipitates them as metal sulfides. Metal sulfides are immobilized within the BCR and thus removed from the water (Johnson and Hallberg, 2005). Significant levels of alkalinity are also produced, which helps neutralize the acidity.

A significant aspect of BCR design is the choice of organic substrate. Relatively simple substrates, such as organic acids or alcohols, have the advantage in that they provide a carbon and energy source that SRB can utilize directly for growth. The disadvantage, however, is that organic acids and alcohols are used quickly and cannot be retained within the system, which increases the cost and maintenance requirements. Alternatively, complex lignocellulosic based organic material, such as wood chips, provides a slow-release source of carbon and energy that can be retained within the system. Because SRB cannot directly utilize cellulosic material, they must rely on the activities of anaerobic cellulose degraders and fermenters to break the complex material down into simpler components that they can utilize (Logan et al., 2005).

The purpose of this study was to explore the effect of organic substrate on the resulting microbial communities in parallel pilot-scale BCRs treating MIW. Recent studies applying molecular biological tools have revealed the complexity of microbial communities in

lignocellulose-based BCRs (Pruden et al., 2007; Johnson and Hallberg, 2005); however, a side-by-side comparison of the microbial communities resulting from complex versus simple carbon substrates has not been determined previously. Considering that microbes are the primary catalysts of BCR function, the composition of the microbial community potentially plays a highly important role in BCR performance. For example, complex versus simple substrate BCRs are likely to drive the development of distinct microbial communities. The relative levels of microorganisms that compete with SRB for hydrogen, acetate, and other available electron donors, such as methanogens, may have a negative overall impact (Daly et al., 2000). Distinct kinds of SRB may also result, which may then differ in their susceptibilities to toxins or their abilities to tolerate various stressors, such as low temperatures or shifts in pH or metal concentrations. Currently, the ability to recover from such stress events as well as the overall lifespan of performance are major challenges to BCR design and operation.

In this study, six pilot-scale BCRs were operated in Black Hawk, Colorado, and were fed with discharge from the National Tunnel, which is a major contributor of contaminants to this area (Buccambuso et al., 2007). Three organic substrate conditions were compared in duplicate: ethanol (ETOH 1, 2), hay and wood chips (HYWD 3, 4), and corn stover and wood chips (CSWD 5, 6). Microbial populations within these conditions were characterized by cloning of 16S genes, which are conserved in all bacteria, and the adenosine-5'-phosphosulfate reductase (*apsA*) gene, which is a functional gene present in SRB. In addition, quantitative polymerase chain reaction (Q-PCR) was performed targeting total bacteria, the *Desulfovibrio-Desulfomicrobium* genera of SRB, and methanogens in order to obtain a quantitative understanding of these groups within the community. The overall effect of substrate on BCR performance is described further elsewhere in the ASMR conference proceedings (Venot et al., 2008). Thus the broader goal of this project will be to understand better the role of substrate-microbe interactions in BCR performance. With this understanding of BCR microbial and organic substrate composition, better criteria may be developed for their design, operation, and overall success.

Materials and Methods

Sample Site and Collection

The bioreactors in this study are located in Black Hawk, Colorado, under the Mill Street Bridge, within the Central City Clear Creek Superfund site; and are fed with a water discharge

from the National Tunnel. The discharge contains elevated levels of sulfate and heavy metals (Zn, Cu, Mn, Cd, Pb, and As) resulting from historic gold mining. Four organic substrate conditions were studied in duplicate (further details are provided in Venot et al., 2008):

- Ethanol fed BCRs (ETOH 1, 2): Each of these vertical flow reactors consists of one non-metallic 55-gallon drum packed with limestone and a zero valence iron (ZVI) slag layer on the top. The reactors were inoculated with approximately 5 lb of horse manure layered on top of the limestone, and are constantly fed with ethanol and MIW from the National Tunnel.
- Solid substrate BCRs (HYWD 3, 4 and CSWD 5, 6): These 55-gal non-metallic drums contain mixed wood chips, limestone, horse manure, and hay or corn stover (Table 1), and are also fed with MIW from the National Tunnel in a vertical flow method.

Table1. Proportion of solid substrate components in the HYWD and CSWD BCRs.

	HYWD BCRs	**CSWD BCRs**
Component	**Percentage (%)**	
Wood Chips	50	35
Limestone	30	20
Horse Manure	10	15
Hay	10	0
Corn Stover	0	30

Five removable mesh bags were filled with the respective substrate and stacked in the center of each reactor within a vertically placed perforated PVC pipe, in order to facilitate sampling. For sample collection one mesh bag from each reactor (second from the topmost) was removed and placed on ice for immediate transport to the laboratory where they were then stored at -80 °C for subsequent analyses. Random grab samples were also collected from the main portion of the BCRs about 1 inch below the surface, in order to determine the efficacy of the mesh bags for sampling.

DNA Extraction

DNA was extracted from approximately 5 g of each of the collected samples using the PowerMax Soil DNA Isolation Kit (MO BIO, Carlsbad, CA) according to the manufacturer's protocol, and stored at -80 °C for subsequent studies. The exact mass of material used in each extraction was determined and recorded for downstream quantification purposes.

Real Time Quantitative Polymerase Chain Reaction (Q-PCR)

The 16S rDNA was targeted to quantify total bacteria and the SRB genera *Desulfovibrio-Desulfomicrobium*. Methanogens were quantified by targeting the gene that encodes the alpha subunit of the methyl coenzyme-M reductase (*mcrA*), an enzyme unique to methanogens.

All reactions were performed in a 7300 Real Time PCR cycler (Applied Biosystems, Foster City, CA). Reactions were set up in triplicate using 1X Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) and primers DSV230f and DSV838r (Daly et al., 2000) to target the 16S rDNA of *Desulfovibrio-Desulfomicrobium* species or the *mcrA*-targeted primers designed by Luton et al. (2002) to target methanogens. TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) and primers 1369f and 1492r (Suzuki et al., 2000) were used to target the 16S rDNA of total bacteria. A calibration curve was constructed for each set of primers using serial dilutions of PCR amplified 16S rDNA (for total bacteria and *Desulfovibrio-Desulfomicrobium* Q-PCR) previously purified and quantified (standards) or serial dilutions of genomic DNA (for the methanogens Q-PCR). An environmental sample consisting of DNA extracted from the CSWD6 sample was used as standard for the total bacteria Q-PCR, and a *Desulfovibrio salexigens* pure culture was used as standard for the *Desulfovibrio* Q-PCR. Genomic DNA from *Methanococcus maripaludis* (ATCC 43000D) served as standard for the methanogens Q-PCR.

16S rDNA and *apsA* PCR Amplification and Cloning

To characterize the overall composition of the bacterial community and to identify dominant bacteria, cloning of 16S rDNA was performed. In order to directly target SRB, *apsA* gene cloning was also performed. The 16S rRNA gene and *apsA* gene (Friedrich, 2002; Hallberg & Johnson, 2005) were PCR-amplified using primer sets 341F and 1492R (Primer 1 of Muyzer et al., (1993); Weisburg et al., 1991) and 7F and 8R (Friedrich (2002)), respectively. PCR products were cloned with the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Amplified rDNA restriction analysis (ARDRA) was performed

visually from MspI restriction enzyme (Promega, Madison, WI) digested PCR-amplified (using vector specific primers) inserts. 16S gene clones with ARDRA patterns appearing two or more times and all *apsA* gene clones with unique ARDRA patterns were sequenced. Because of the labor and cost associated with cloning of the 16S rDNA gene, and because it has already been applied in other studies (Johnson and Hallberg, 2005; Hiibel et al., in press), cloning of 16S rDNA was performed on only one of each of the duplicate BCRs.

Sequence analyses

The DNA sequences obtained from cloning were aligned to the sequences of the closest identified microorganisms by the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) and the Ribosomal Database Project II (RDP) (http://rdp.cme.msu.edu/). A literature review involving the microorganisms identified was used to assign a putative function to the identified bacteria. A phylogenetic tree was constructed using the 16S gene sequences of each of the unique clones identified, and 16S rDNA sequences of related organisms obtained from BLAST results.

Principal Coordinate Analysis (PCoA)

The phylogenetic trees representing the two genes were analyzed directly using PCoA and the UniFrac significance tests of the UniFrac algorithm (Lozupone and Knight, 2005; Lozupone et al., 2006; Lozupone et al., 2007). PCoA differs from principal component analysis (PCA) in that the input data are the phylogenetic distances between each pair of sampling locations rather than the number of times each clone sequence was observed at each location. The UniFrac significance test is used to determine whether two microbial communities are significantly different. Communities are considered different if the fraction of the phylogenetic tree unique to one is greater than would be expected by chance (Lozupone and Knight, 2005). For all analyses, the non-normalized abundance data, based on the ARDRA screening, were incorporated in the UniFrac significance test; reference strains used to construct the phylogenetic trees were not included. The raw p-values from the UniFrac significance tests are reported, with p-value ≤ 0.05 considered to be statistically significant.

Results

Microbial community diversity and composition

Rarefaction analysis of ARDRA patterns revealed that the overall bacterial diversity of a lignocellulose-based BCR (CSWD 5) was significantly higher than that of an ethanol-fed BCR (ETOH 2) (Fig. 1). The overall diversity of the HYWD 3 reactor was similar to that of the ETOH 2 reactor. However, it should be noted that the HYWD 3 reactor encountered significant operational problems in which the substrate material was inadvertently exposed to oxygen.

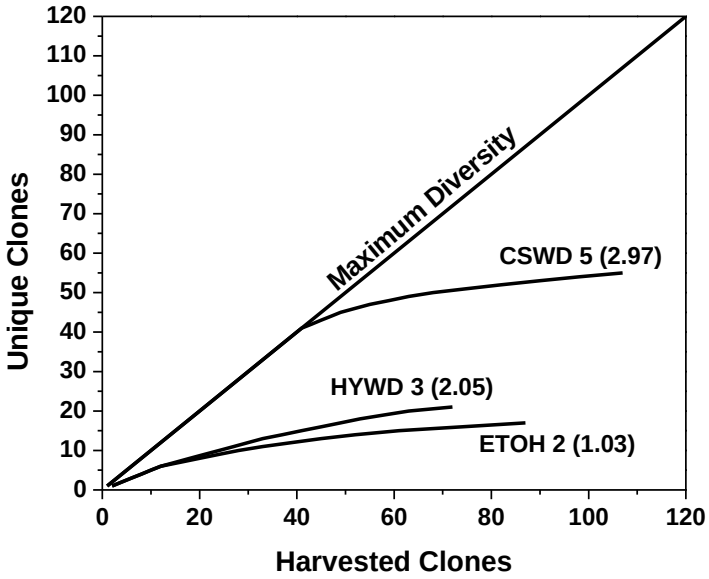

Figure 1. Relative diversity of the ETOH 2, HYWD 3, and CSWD 5 BCRs determined by cloning of the 16S gene. Shannon diversity indices for each BCR are indicated in parenthesis and were calculated based on the frequency of each clone in the clone library.

Sequencing the 16S rDNA of the dominant clones also revealed differences between the BCRs. Notably, the proportion of SRB in the ETOH 2 reactor was almost 70%, whereas SRB were only 2-5% in the HYWD 3 and CSWD 6 reactors (Fig. 2). Other putative functional groups were identified in the lignocellulose-based reactors that were not identified in the ETOH reactor, such as cellulose degraders. Fermenters were estimated at 25-30% of the lignocellulose-based communities and only 5% of the ETOH community.

ApsA gene profiling revealed some overlaps and some differences between the lignocellulose and ETOH BCRs (Fig. 3). An SRB most closely related to *Desulfovibrio* sp. JD160 and *Desulfovibrio aerotolerans* was found in all of the BCRs. A *Desulfomicrobium baculatum*

relative was only found in the two BCRs, ETOH 1 and HYWD 3. In the lignocellulose BCRs, some additional bacteria carrying the *apsA* gene were found: an uncultured bacterium that was present at about 10% in the three properly functioning BCRs (HYWD 4, CSWD 5, and CSWD 6), *Thiobacillus* spp. (only in HYWD 3), and *D. desulfuricans* and *D. burkinensis*. *Thiobacillus* spp. have been recognized in the literature as aerobic organisms that reverse the process of sulfate reduction.

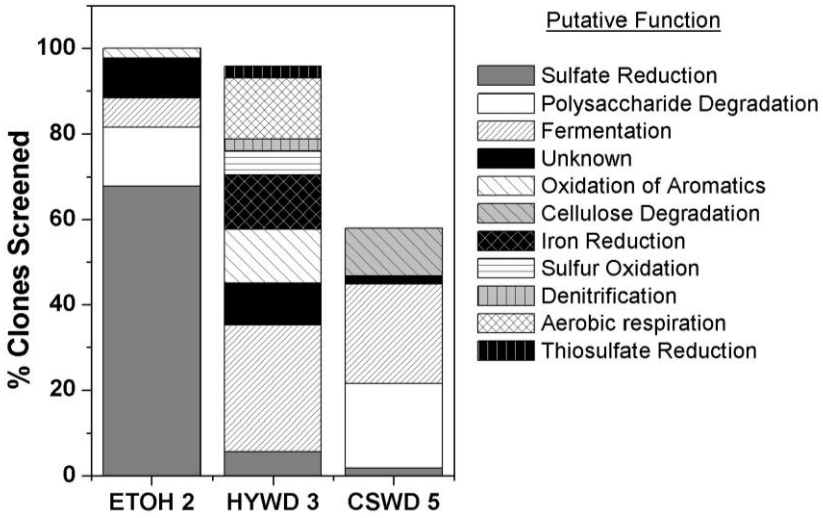

Figure 2. Distribution of microbial functions in each BCR based on the identity of the microorganisms identified through 16S gene cloning and DNA sequencing.

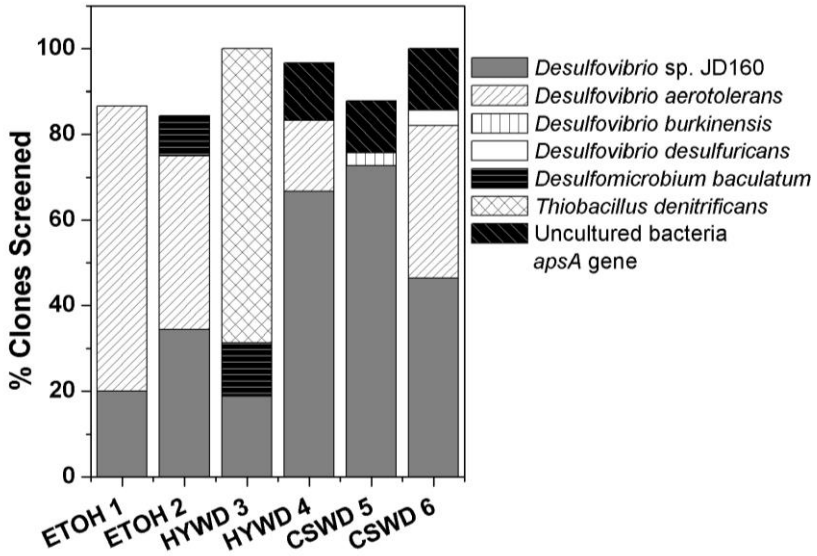

Figure 3. Identity and percentages of *apsA*-containing microorganisms in ETOH, HYWD, and CSWD BCRs.

PCoA provided a means to statistically compare the microbial communities and identify similarities and differences (Fig. 4). Based on PCoA analysis of 16S rDNA, it was found that the ETOH 2, HYWD 3, and CSWD 6 communities were distinct across both coordinates. PCoA analysis of *apsA* genes, however, demonstrated that all of the BCRs except HYWD 3 were relatively similar along the second coordinate, but distinct along the first coordinate, though the ETOH BCRs were near to each other along the first coordinate as were the lignocellulose-based BCRs.

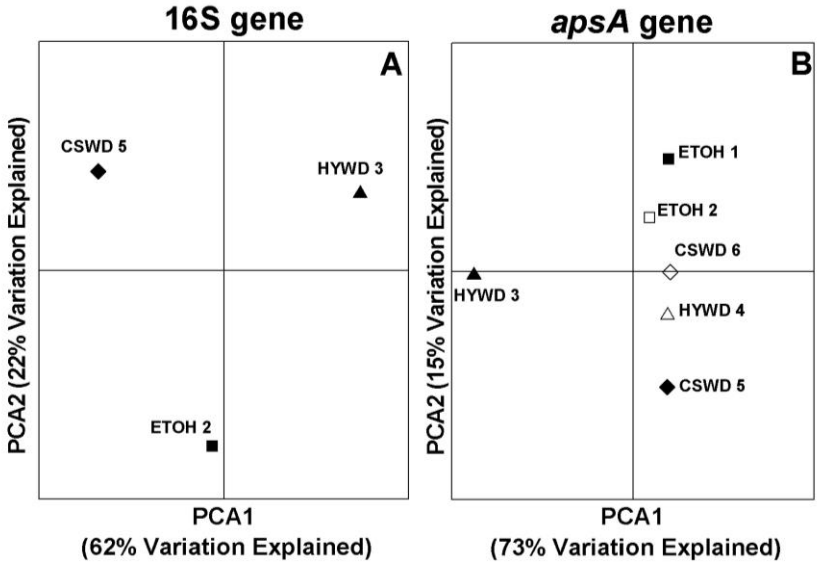

Figure 4. Principal coordinates analyses (PCoA) of the (A) total bacterial community based on 16S rRNA gene sequences and the (B) *apsA* gene-containing community based on *apsA* gene sequences.

Quantification of total bacteria, SRB, and methanogens

Q-PCR results targeting the 16S rDNA of total bacteria and the SRB genus *Desulfovibrio-Desulfomicrobium* provided a concentration of these genes per uL of DNA. These concentrations, related to the initial mass of the sample used for DNA extractions, yielded an estimate of the amount of 16S rDNA genes per gram of sample as well as the normalized ratios of 16S rDNA of *Desulfovibrio-Desulfomicrobium* species and methanogens with respect to total bacteria (Fig. 5). It was found that the ETOH 1 BCR contained a very high percentage of SRB (94±15%). However, the total number of bacteria was much lower in both ETOH BCRs than the lignocellulose reactors. Methanogens were found to be lower in the ETOH reactors than the

lignocellulose BCRs, especially in the ETOH BCR that was exposed to oxygen due to operational problems (ETOH 1).

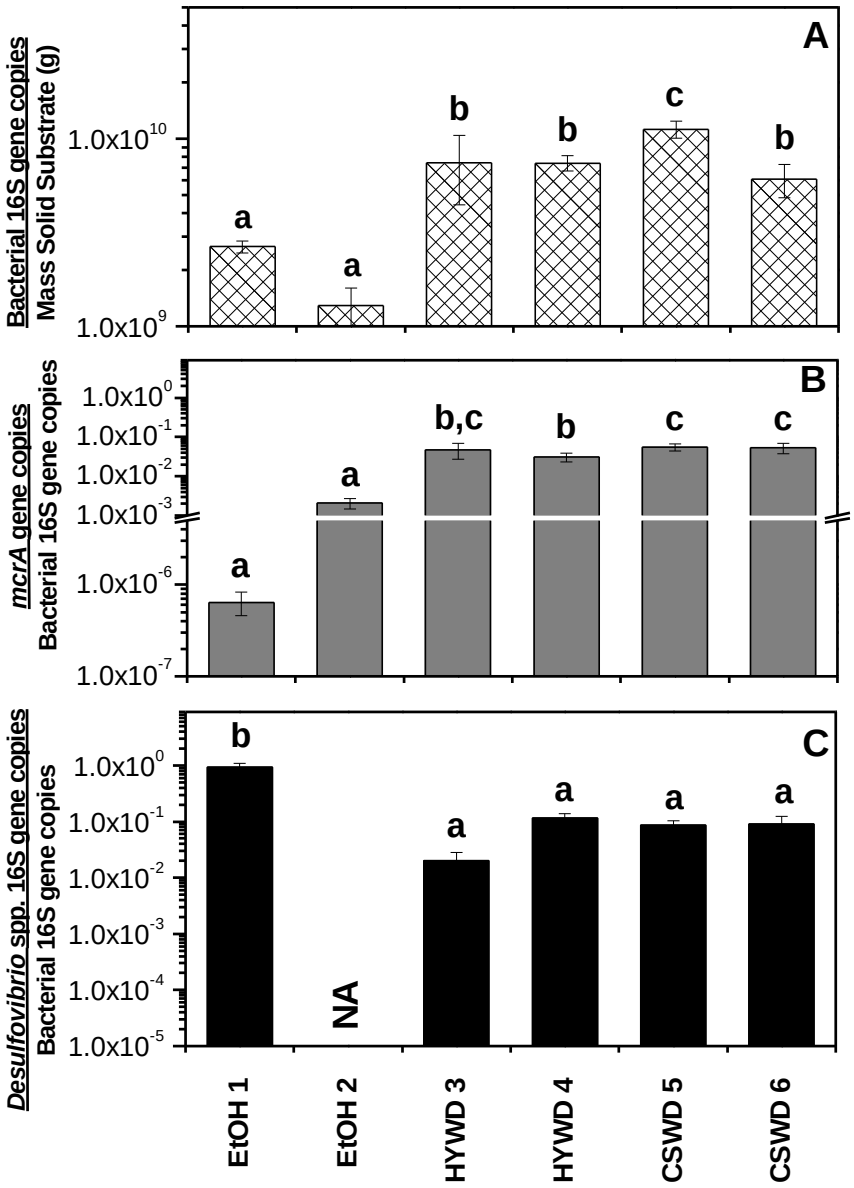

Figure 5. Quantification of total bacteria, the SRB of the genus *Desulfovibrio* and methanogenic bacteria for the ETOH, HYWD, and CSWD BCRs by Q-PCR. Total bacteria 16S rDNA copies normalized to mass of substrate (A), total *Desulfovibrio* spp. 16S rDNA copies normalized to total bacteria (B), and *mcrA* genes normalized to total bacteria (C). Bars with the same letter are not significantly different at the 0.05 level. NA – not analyzed.

Discussion and Conclusions

This study investigated the effect of organic substrate on microbial communities present in BCRs treating MIW. Considering that complex substrate conditions require complex communities of cellulolytic and fermentative microbes to break down the organic matter into simple substrates that SRB can utilize (Logan et al., 2005), it was hypothesized that different organic substrates would yield distinct communities. The results provided strong support for this hypothesis.

With respect to the overall community composition as determined by cloning of the 16S rDNA, SRB represented a much greater proportion of the community in the EtOH BCR than in the lignocellulose BCRs. The low levels of SRB in the lignocellulose-based BCRs were typical of what has been observed by others (Pruden et al., 2007; Hong et al., 2007; Johnson and Hallberg, 2005). These results were supported by the quantitative analysis of SRB based on Q-PCR. In terms of the diversity of the SRB found, there appeared to be a slight edge for the lignocellulose-based BCRs, in which 3-4 different kinds of SRB were identified, whereas 2-3 different kinds were identified in the ETOH BCRs. These SRB may vary from one another in terms of substrate utilization capabilities, kinetics and other characteristics such as tolerance to oxygen exposure and other stresses (Warren et al., 2005). This could have a significant impact on BCR performance. Though the proportion of SRB was higher in ETOH BCRs, the total bacterial community was smaller. This was likely an artifact of the reactor design in which the limestone provided a small surface area to mass ratio as compared to the lignocellulosic material.

Overall diversity of bacteria was found to be much higher in the lignocellulose-based BCRs. This was likely a result of the complexity of the substrate in the lignocellulose-based BCRs, which required a broader array of functional groups for its breakdown than ethanol. Possibly, this difference represented an important advantage to these systems in terms of resilience to stress. For example, redundancy within each functional group may have provided a competitive advantage because this increased the likelihood of individual species that tolerated the stress.. This factor would have important implications towards improving the design of BCRs for stress tolerance and thus this topic is now being explored further in a follow-up investigation.

Methanogens were found to be lowest in the ETOH BCRs, especially in ETOH 1, which was exposed briefly to oxygen. Assuming that methanogens had an overall negative impact on BCR performance due to their competitive relationship with SRB, this may have indicated an

advantage of ethanol based BCRs. However, methanogens may play a positive role as well. For example, some SRB have been inhibited in the presence of high concentrations of acetate, which has been removed easily by methanogens (Raskin et al., 1996).

It was also of interest that differences were found in BCRs that experienced operational problems resulting in exposure of the material to oxygen. For example, *Thiobacillus* spp. were only found in the HYWD 3 BCR, which was unintentionally exposed to oxygen due to operational difficulties. *Thiobacillus* spp. are aerobic or denitrifying organisms that convert sulfides to sulfates, thus reversing the process applied in remediation. This provided additional validation to the observed results and is consistent with results obtained in the field BCR Peerless Jenny King, where *Thiobacillus* spp. were found to be the dominant *apsA*-containing species (Hiibel et al., in press).

Interestingly, the performance of the six BCRs was comparable (Buccambuso et al., 2007), though the ETOH BCRs offered some advantage in terms of overall sulfate and metals removal. It can be concluded that ethanol bioreactors can be useful in situations where ethanol is readily available as a substrate and provided at low feed rates, because it directly supports the SRB performing the MIW treatment. Lignocellulose-based BCRs still represent a less expensive and lower maintenance option since the organic substrate is made up of waste material that can indirectly support the SRB via a complex microbial community, without having to be constantly replenished. The results of this study suggest that there may be advantages of lignocellulose-based BCRs in terms of the overall diversity of the microbial community, which may aid in providing resilience to stress. Future research exploring this hypothesis, as well as the effects of other substrates, could be helpful in improving design and performance of BCRs.

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