RESPONSE AND RECOVERY OF SULFATE-REDUCING BIOCHEMICAL REACTORS FROM AEROBIC STRESS EVENTS[1]

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Abstract. Microbially-mediated treatment of mining-influenced water (MIW) through the implementation of sulfate-reducing biochemical reactors (BCRs) is an attractive option for passive, in situ remediation with low operating costs and reduced maintenance requirements. However, BCRs can be unpredictable in terms of how they recover from environmental stresses such as oxygen exposure. Previous studies have demonstrated that the inoculum can impact performance positively, suggesting that engineered control of the microbial community structure could improve the resilience of BCRs to stress. The purpose of this study was to determine the effect of bioaugmentation and biostimulation on performance and recovery from oxygen stress. Twelve columns (six conditions in duplicate) were packed with a complex organic substrate (wood chips, etc.), inoculated with dairy manure, and fed simulated acid mine drainage containing Fe, Cd, and Zn at pH 5.5. The conditions tested were: 1.) bioaugmentation with cellulose degraders (CD); 2.) bioaugmentation with sulfate reducers (SRB); 3.) biostimulation with ethanol (EtOH); 4.) biostimulation with carboxymethyl cellulose (CMC); 5.) dairy manure only control (DM); 6.) un-inoculated control (CR). Once the columns reached steady state anaerobic performance, they were exposed to oxygen, allowed to reach steady state again, and then oxygen-exposed for a second, longer time. The results indicate that all columns performed well in terms of pH neutralization and removal of sulfate and heavy metals. However, the cellulose and ethanol biostimulated columns appeared to be the most resilient to oxygen exposure and performed best by the end of the experiment (171 days), while the bioaugmented columns performed similarly to the controls. The microbial communities were evaluated at each steady state using a suite of biomolecular tools, including active community profiling (ACP) and quantitative real-time PCR (Q-PCR) targeting key functional groups of sulfate-reducers, cellulose-degraders, fermenters, and methanogens. It was found that the active microbial community structure was distinct among the six column conditions and that bioaugmentation significantly impacted the kinds and diversity of bacteria present following stress events. Functional gene analysis supported these observations, but provided finer resolution regarding the response of each functional group to stress.

Additional Key Words: sulfate-reducing biochemical reactors, oxygen stress, functional genes, column studies

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Introduction

Sulfate-reducing BCRs treat mining-influenced water (MIW) by precipitating the metals with biogenic sulfide. The two main components of a BCR are the substrate and the microbial community. The substrate provides hydraulic conductivity, physical support and energy sources for the microbial community. Typically, the substrate is a lignocellulose-based inexpensive material such as wood chips or compost (Johnson and Hallberg 2005). This provides a slow-release, long-term source of carbon and also has a significant influence on the microbial community. Sulfate-reducing bacteria (SRB) are of key importance because they produce sulfide through the reduction of sulfate. Additionally, cellulose-degrading and fermentative bacteria are important members of the BCR microbial community as they transform the lignocellulosic material into energy sources for the SRB through hydrolytic and fermentative reactions, respectively. Methanogenic archaea are also of interest because they can compete with SRB for energy sources.

Although BCRs have many positive attributes, their performance can be unpredictable. Some systems do not remove metals to the expected levels and others have a short lifetime. On the other hand, there are some successful systems such as Nickel Rim in Ontario Canada (Benner et al. 1999; Benner et al. 1997). A significant body of prior work has focused on the role of substrate in BCR performance (Bechard et al. 1994; Gibert et al. 2004; Waybrant et al. 1998; Zagury et al. 2006). However, the characteristics of the microbial community as well as environmental conditions are also important to consider. BCRs are subject to a variety of environmental stresses such as below-freezing temperatures during winter and exposure to air during periods of low flow. In is not clear exactly how these stresses affect performance. Tsukamoto and others (2004) investigated the effect of low temperatures and low pH on MIW remediation. Interestingly, they did not observe an effect of temperature on treatment efficiency, but a pH below 3.0 resulted in loss of sulfate reduction.

Recent 16S rRNA gene-based characterization of the microbial communities in laboratory and pilot-scale lignocellulose-based BCRs has provided new insight into the inner workings of these reactors. Particularly notable is the high degree of bacterial diversity that has been observed in these systems using 16S rRNA gene-based methods (Hiibel et al. 2008; Pereyra et al. 2008; Pruden et al. 2007). SRB have been discovered to represent only a small fraction of the microbial community, which makes their detection implementing 16S rRNA gene-targeted

fingerprinting methods, such as denaturing gradient gel electrophoresis (DGGE), extremely difficult.

In this study, we explored the effect of oxygen exposure on duplicate lab-scale lignocellulosic BCR columns operated under varying microbial community conditions: 1.) bioaugmentation with cellulose degraders (CD); 2.) bioaugmentation with sulfate reducing bacteria (SRB); 3.) biostimulation with ethanol (EtOH); 4.) biostimulation with carboxymethyl cellulose (CMC); 5.) dairy manure (DM); and 6.) uninoculated control (CR). New and innovative biomolecular tools were developed and applied in order to profile and quantify key functional groups of cellulose degraders, fermenters, SRB and methanogens. Active community profiling (ACP), which compares relative 16S RNA:DNA ratios of dominant phylogenetic groups via capillary electrophoresis single stranded conformation polymorphism (CE-SSCP), was also applied. This study provides key biomolecular insight into response and recovery of BCRs from environmental stress.

Materials and Methods

Column Set-Up

Twelve PVC columns (10.2 cm ID) with seven, 0.635 cm (¼") sampling ports distributed alongside 40.5 cm of operating height were operated vertically at an average upward flow of 250 mL/day. The influent port was located 10 cm from the bottom of the columns and the effluent port, 2 cm from the top. The remaining five sampling ports (spaced 5 cm apart) were used for collection of substrate samples during column operation. The bottom (12 cm) and top (4 cm) layers of the columns were packed with gravel (particle size 0.3 - 0.5 cm) to ensure homogeneous flow distribution and were separated from the substrate by a Nylon mesh to prevent loss of substrate. Once filled, the columns were sealed with a monitoring well plug. The hydraulic residence time (HRT), determined in a tracer test with 10 g/L NaBr in a test column, was 4.4 days.

Each column received 866 g (dry wt) of substrate, which consisted of 232 g beechwood (3.33 – 4.75 mm), 112 g pine shavings (3.33 – 4.75 mm), 17 g ground alfalfa, 51 g of limestone sand, and 454 g of washed playground sand. Fresh manure was collected from a dairy in Wellington, CO, slurried with 2032.5g:1098mL sterile DI water and used to inoculate all columns except the uninoculated CR columns. The same mass of DM slurry was added to each column, with a target biomass concentration of 10^9 cells/g substrate (dry weight) as determined by 16S rRNA

gene Q-PCR. The CD and SRB columns received an additional 10^9 cells/g substrate of a cellulose-degrading and a sulfate-reducing enrichment culture, respectively. The cellulose degrading enrichment was obtained from an inoculum of sheep manure and creek sediment that was incubated anaerobically with lignocellulosic material as the sole carbon substrate. Sodium molybdate was added to inhibit sulfate reduction. The sulfate reducing enrichment was obtained from an inoculum collected from an ethanol-fed reactor in operation at the National Tunnel site in Blackhawk, CO. Ethanol, a fermentation by-product (and thus unattractive to fermenters), was provided as a sole carbon substrate. Daily serial transfers were necessary in order to propagate the culture and prevent product inhibition.

Influent Composition

Columns were fed simulated mining influenced water consisting of 1.32 g/L Na_2SO_4, 0.03 g/L NH_4Cl, 0.089 g/L $ZnSO_4 \cdot 7H_2O$, 0.01 g/L $CdCl_2$, and 9.15 x 10^{-4} g/L $FeSO_4 \cdot 7H_2O$ in deionized water, modeled after Logan et al. (2003). This formulation was chosen to represent moderate water that had gone through pre-treatment to remove the majority of the Fe. The feed for the CMC columns also contained 0.507 g/L of CMC. Feed solutions were adjusted to pH 5.5 and delivered to the columns via peristaltic pumps (Isamatec, Glattbrugg, Switzerland). The feed solutions were continuously bubbled with nitrogen in the feed tank to exclude oxygen. In addition to the simulated MIW, the EtOH columns were fed 200 proof ethanol at 2.8 μL/h using a syringe pump (kd Scientific, Holliston, MA) connected into the feed line between the peristaltic pump and the columns. Because CMC is a solid substance, it could not be injected into the feed line. Therefore, while the other columns received feed from the same bottle, the CMC feed was prepared in the same manner, but in a separate feed bottle containing 0.507 g/L of CMC. The amount of CMC and ethanol in the feed corresponded to the equivalents required to reduce 50% of the sulfate based on the stoichiometry of the reactions and the yields described by Badger (2002).

Aerobic Stress Events

When the effluent SO_4^{2-} concentration in all the columns reached a pseudo steady-state (defined as changes in SO_4^{2-} concentration of 10% or less over 3 HRTs), the substrate of the columns was sampled for biomolecular analyses. The columns were disconnected from the influent line, opened from the top, and allowed to drain from the influent port. After they

drained, the columns were subjected to stress by exposing them to the atmosphere for exactly one hour while the substrate was sampled and immediately frozen in liquid nitrogen. The packing material removed from each column was replaced with sterile gravel in order to preserve flow conditions. After one hour of being exposed to air, the columns were re-sealed, filled with simulated MIW, and the continuous flow was resumed. Once the columns reached a second pseudo steady-state on Day 105, they were sampled again and exposed to the atmosphere for exactly 8 hours. The columns were sacrificed on Day 171 upon achieving a third steady-state.

Analytical Methods

Sulfates were quantified by ion chromatography (IC) using a Metrohm 861 Advanced Compact IC with MSM and CO_2 Suppressor and a 250 mm Metrospec A Supp 5 High Resolution Anion Column (Houston, TX). The IC NET Chromatography Control and Data Acquisition System was used to calibrate the instrument and to collect and analyze chromatograms. Metals were analyzed using inductively coupled plasma absorbance emission spectroscopy (ICP-AES) (Thermo Jarrell Ash IRIS Advantage) following U.S. EPA Method 3015 (1994). Samples were filtered through 0.45-µm filters and 10 mL were digested according to EPA Method 3015 (1994) prior to analysis. Detection limits were 0.01 mg/L for Zn and 0.005 mg/L for Cd. The pH was measured with an Accumet® AB15 Basic pH meter (Fisher Scientific, Pittsburgh, PA) immediately upon opening the anaerobically stored effluent samples. Mixed linear regressions were fit to the data sets using the PROC MIXED function of SAS 9.1 (SAS Institute Inc., Cary NC). Significance was defined by a pair-wise comparison p-value $\leq$ 0.05.

DNA and RNA Extraction

DNA was extracted from approximately 5 g of each of the column substrate samples collected prior to the aerobic stress events 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. Exact masses of material used for this procedure were determined and recorded for quantification purposes. Genomic DNA extractions were also performed on the cellulose-degrading and sulfate-reducing enrichment cultures and the dairy manure slurry using the UltraClean™ Soil DNA Isolation Kit (MoBio, Carlsebad, CA) according to the manufacturer's protocol. Genomic DNA from the three pure cultures: *Clostridium cellulovorans* (ATCC #35296), *Ruminococcus flavefaciens* (ATCC #4994), and *Fibrobacter succinogenes* (ATCC

#51214) was also extracted using the UltraClean™ Microbial DNA Isolation Kit (MoBio, Carlsebad, CA) according to the manufacturer's protocol. A small portion of the extracted genomic DNA was treated for RNA contamination with RNase ONE Ribonuclease (Promega, Madison, WI). Total RNA was extracted from the column substrate material, inoculum, and enrichment cultures using the RNA PowerSoil™ Total RNA Isolation Kit (MoBIO, Carlsebad, CA) according to the manufacturer's protocol with the addition of two freeze-thaw steps added to the mechanical lysis. RNA extracts were stored at -80°C for subsequent studies and exact masses of material used for this procedure were determined and recorded for quantification purposes. A small portion of the extracted total RNA was treated with RQ1 RNase-Free DNase (Promega, Madison, WI) for DNA contamination. The DNase-treated RNA was reverse transcribed using AMV Reverse Transcriptase (Promega, Madison, WI).

Real Time Quantitative Polymerase Chain Reaction (Q-PCR)

Primer sets were designed targeting *cel5* and *cel48*, *hydA*, *dsrA*, and *mcrA* genes to quantify cellulose-degrading bacteria, fermenters, SO_4^{2-} reducing bacteria, and methanogens, respectively. These functional genes were chosen because they produce enzymes that directly catalyze functions of interest. This allowed the corresponding functional groups to be quantified directly, rather than relying on indirect inference of function based on the 16S gene. The selection of *dsrA* and *mcrA* genes as targets was relatively straightforward because these genes are well conserved within sulfate reducers and methanogens, respectively (Luton et al., 2002: Ben-Dov et al., 2007). In the case of fermenters, the *hydA* gene was chosen because it encodes an Fe hydrogenase that catalyzes H_2 evolution (Meyer, 2007). This is in contrast to Ni hydrogenases that catalyze H_2 uptake. Unfortunately, there was no ideal gene that captured all known cellulose degraders. Nine families of cellulases encoding cellulsomal components have been described to date. Family 5 and family 48 were chosen because together they cover about 50% of known cellulose degraders and include the majority of anaerobic and ruminal species that have been detected in previous studies (Pruden et al., 2007; Hong et al., 2007). To our knowledge, this study represents the first to design and demonstrate methods for Q-PCR quantification of uncultured environmental bacteria possessing cellulase and fermentation genes. The sequences of the primers designed in this study are presented in Table 1.

Table 1: Sequences of degenerate primers targeting functional genes applied in Q-PCR

Target	Primer	'5-3' sequence	Approximate Amplicon Size (bp)
Family 48 glycoside hydrolases	*cel48*_490F	TNATGGTTGAAGCTCCDGAYTAYGG	430
	*cel48*_880F	CAYTGG HTNNTG GAYGTTGAY AACTGGTA	
	*cel48*_920R	CCAAANCCRTACCAGTTRTCAACRTC	100
	*cel48*_980R	CCTGTTCACCTCTYTGRWARGTRTT	
Family 5 glycoside hydrolases	*cel5*_392F	GAGCATGGGCTGGAAYHTNGGNAA	133
	*cel5*_525R	GAAAGGAATACGGACGGYNTTRAAHCC	
	*cel5*_754R	CATCATAATCTTTGAAGTGGTTTGCAATYTGDKTCCA	362
Alpha subunit iron hydrogenases	*hydA*_1290F	GGTGGAGTTATGGAAGCWGCHHT	248
	*hydA*_1538R	CATCCACCWGGRCAHGCCAT	
Alpha subunit dissimilatory sulfite reductase	dsrA_290F	CGGCGTTGCGCATTTYCAYACVVT	140
	RH3-dsr-R'	GTGGMGCCGTGCATGTT	
	dsrA_660R	GCCGGACGATGCAGHTCRTCCTGRWA	370
Alpha subunit of methyl coenzyme M reductase	*mcrA*_1035f	GGTGGTGTMGGATTCACACARTAYGCWACAGC	495
	*mcrA*_1530r	TTCATTGCRTAGTTWGGRTAGTT	
	*mcrA*_1450r	TTTGAAGCWCCRCAYTGGTCYT	415
	*mcrA*_1430f	TTCTATGGTTACGACTTVCAGGACCARTGYGG	100

[a]The primers *mcrA*_1035F/1530R were designed by Luton and others (2002) and the primer RH3-dsr_R' is a modified version of the primer RH3-dsr-R designed by Ben-Dov and others (2007).

Q-PCR targeting functional genes was performed on in the purified DNA extract from the samples collected at the beginning and end of the experiment and during the two pseudo steady-states. In addition, total bacterial 16S rRNA genes were quantified using the conditions, universal BACT1369F and PROK1492R primers and the TM1389F probe described by Suzuki et al. (2000). Q-PCR was performed in a 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). All samples were analyzed in triplicate.

Active Community Profiling (ACP)

The treated DNA and reverse transcription products were PCR amplified targeting the highly variable V3 region of the 16S rRNA gene using primers W49f (5'- ACG GTC CAG ACT CCT ACG GG) (Brosius, Dull et al. 1981) and W104r. For each capillary electrophoresis-single strand conformation polymorphism reaction, 1 μL of PCR product was added to a mixture of 18.8 μL of HiDi formamide (Applied Biosystems) and 0.2 μL ROXTM 400 (Applied Biosystems). The formamide aids in denaturation and the ROX was used as an internal standard

for data alignment and normalization. The reaction mixture was heat denatured at 95°C for 5 min in a thermal cycler, then immediately placed in an ice bath for 15 min to form single-stranded conformations. Capillary electrophoresis was performed with a Genetic Analyzer 310 with a 47 cm x 50 um ID capillary and 5.6% GeneScan polymer (Applied Biosystems, Foster City,CA). Electrophoresis run conditions were performed at 12 kV for 32 min at 32°C (Pruden et al. 2007). GeneMapper 3.2 software (Applied Biosystems) (Chackhiani et al. 2004) was used to align the electropherograms from all samples and for peak integrations. Peak areas were normalized to the ROX 220 bp peak of each run to correct for injection variation.

Results

Column Performance, Response, and Recovery from Stress

In general, columns performed similarly during start-up, but SO_4^{2-} removal rates began to diverge following each of the two aerobic stress events. A total of three pseudo steady-states were achieved, the first just prior to the first stress event, the second just prior to the second, more intense, stress event, and the third at the end of the experiment prior to column sacrifice.

After 15 d (approximately 3.4 pore volumes), the effluent SO_4^{2-} concentrations decreased in all columns to values between 304 and 505 mg/L and remained at pseudo steady-state for approximately 20 d (Fig. 1). At this first steady-state, the SO_4^{2-} concentration in the effluent of all columns was significantly lower than that of the feed and there were no significant differences among the different types of columns. Cadmium and Zn effluent concentrations (Fig. 2) were also similar for all columns and remained significantly below the feed concentrations throughout the experiment, except those biostimulated with CMC. The effluent concentrations of the CMC-stimulated columns were similar to those from the other five columns for the first 15 d of operation, at which point they began to increase and fluctuate. The spike in effluent metal concentrations for the CMC columns did not correlate with a decrease in the other performance-determining metrics (pH or sulfate removal). For the first 10 d of operation, effluent pH was between 5.5 and 6.0 (Fig. 3). After this time, pH increased in all columns to between 6.5 and 7.5, which was significantly higher than the feed. Throughout the experiment, there were no significant differences among the different columns in terms of pH.

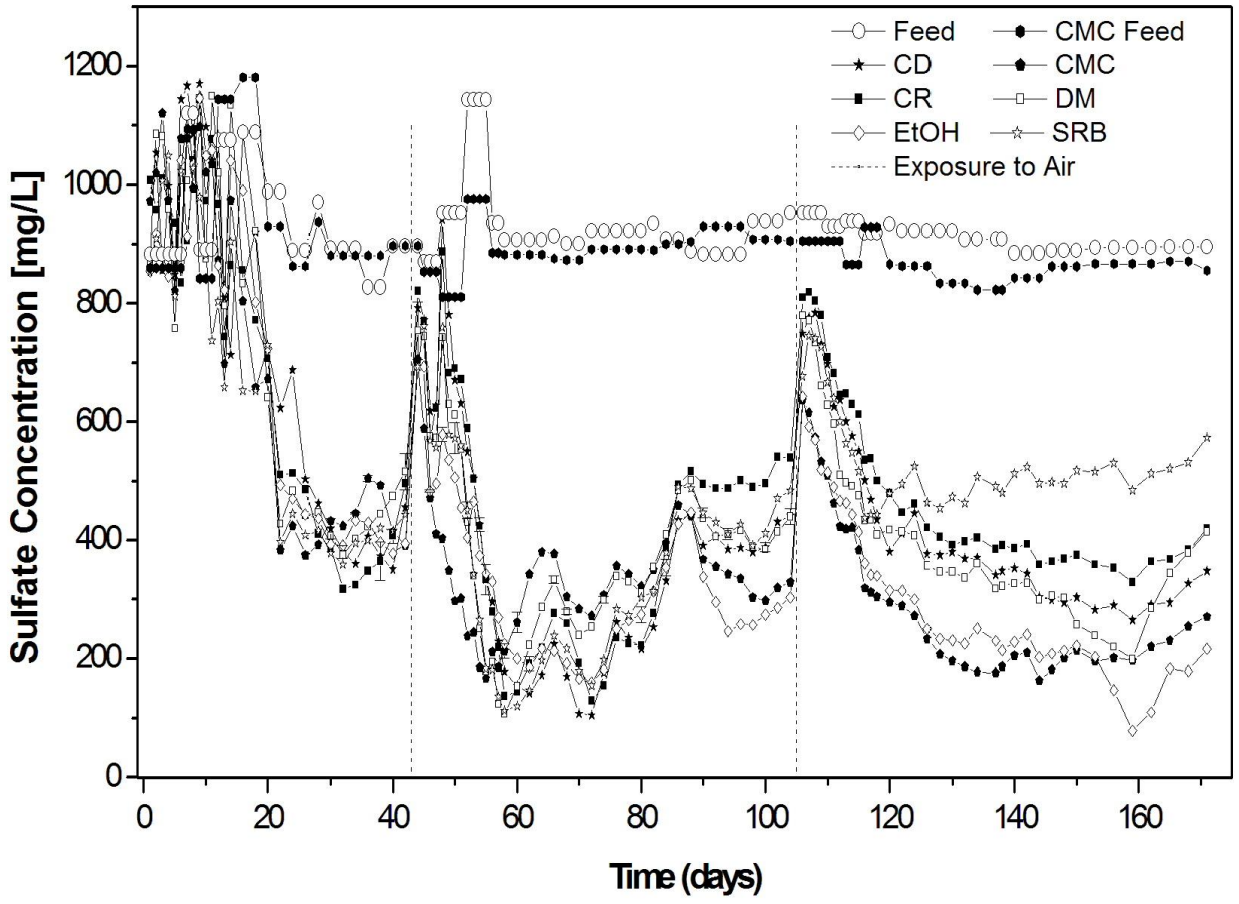

Figure 1. Averaged influent and effluent sulfate data from the six duplicate column conditions. DM=dairy manure only inoculum, SRB=DM and sulfate reducing bacterial enrichment inoculum, CD=DM and cellulose-degrading bacterial enrichment inoculum, EtOH=DM and biostimulated with ethanol, CMC=DM and biostimulated with carboxy methyl cellulose, and CR=uninoculated columns.

After the first air exposure stress event on Day 43, there was a significant increase in the effluent SO_4^{2-} concentrations in the CD, CR, DM, EtOH, and SRB columns ($p < 0.0001$ in all cases). In the CMC columns, the increase in effluent concentration was not significant (p-values of 0.0541 and 0.2219 for the comparison between Days 32 and 38 with Day 45). Following the stress, the effluent sulfate concentration from all columns decreased over the next 10 d (~2.5 HRTs) to levels below those observed before the stress was applied (between 60 and 213 mg/L). At Day 80, the effluent sulfate concentrations of all columns increased and then finally achieved a second pseudo steady-state from about Day 86 to Day 106. Generally, the effluent sulfate concentrations at the second pseudo steady-state were similar to those observed at the first

steady-state before the stress was applied. However, performance differences among the columns began to emerge at the second pseudo steady-state. As expected, the uninoculated CR columns had the slowest SO_4^{2-}e reduction rate. The EtOH and CMC biostimulated columns were observed to have the highest sulfate reduction rates, while the SRB and CD bioaugmented columns had moderate SO_4^{2-} reduction rates that were not significantly different from the DM control. Effluent metal concentrations in the CD, CR, DM, EtOH, and SRB columns remained similar before and after the first stress event. The CMC effluent metals concentration remained significantly higher than the other columns (p-value < 0.0001 for Cd and Zn). Just prior to the first stress event, the effluent pH of all columns trended sharply upward. Immediately following the stress event pH in all columns began to trend gradually downward before stabilizing around 7.0. There was no significant difference among the columns in terms of pH.

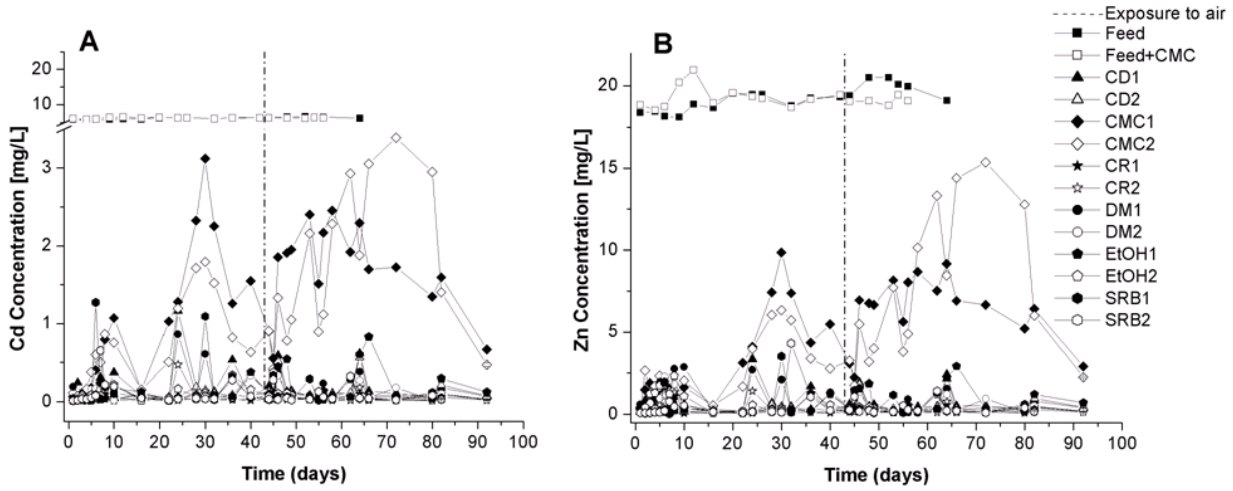

Figure 2. Feed and effluent concentrations of (A) cadmium and (B) zinc as determined by ICP-AES.

The columns were subjected to a second, more intense, stress event consisting of 8 hours of air exposure on Day 105. The SO_4^{2-} concentrations increased in the effluent of all the columns to levels comparable to the feed and then gradually decreased over the next 25 d (~6 HRTs). Performance differences among the columns were further enhanced by the second stress event. Following the second stress event, the SRB columns were found to perform the poorest in terms of SO_4^{2-} reduction rate, followed by the uninoculated CR columns. On Day 116, the effluent SO_4^{2-} concentrations in the SRB columns began to increase steadily from 437 to 574 mg/L by Day 171 when the columns were sacrificed. Effluent SO_4^{2-} concentrations in the EtOH, CMC,

CD, DM, and CR columns continued to decrease and eventually reached a third pseudo steady-state. The EtOH and CMC column SO_4^{2-} effluent decreased more rapidly, resulting in the lowest concentrations, 216 and 270 mg/L, respectively, by Day 171. The CD columns did not perform significantly different from the DM controls at the third pseudo steady-state. Clogging in one of the DM columns at Day 150 reduced the average effluent concentration for these columns.

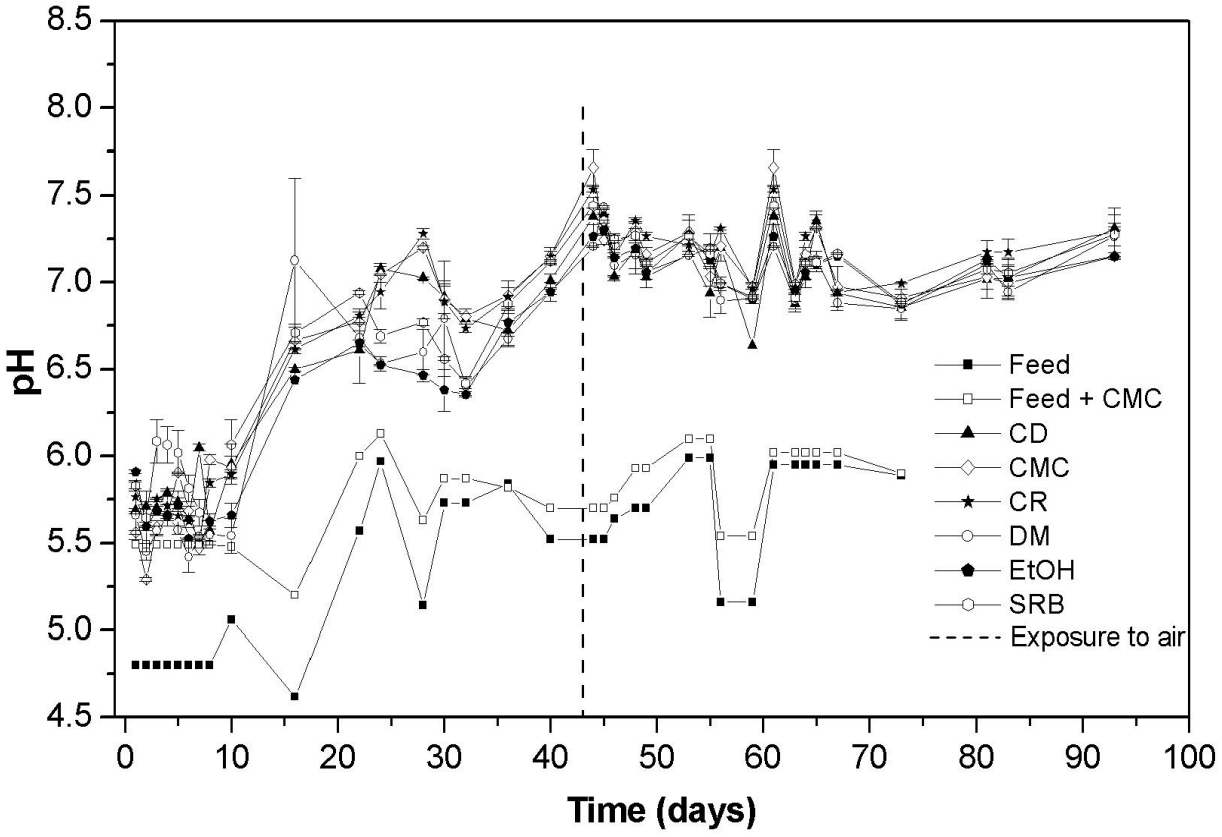

Figure 3. Feed and effluent pH in the columns. Data points represent the average of biological replicates and the error bars represent the average deviation of the biological replicates.

Response of Functional Groups to Stress Events

Q-PCR provided quantitative information about total bacterial biomass and key BCR functional groups from the initial time point through the second pseudo steady-state following the first stress event. Data is not yet available for the third pseudo steady-state, which followed the second stress event, but is expected to be added prior to presentation at the annual conference.

The 16S rRNA gene was used as an indicator of overall bacterial biomass. Initially, there were significantly lower numbers of bacterial 16S rRNA genes in the CD and SRB columns, indicating that bioaugmentation did not take place to the extent intended. In all the other columns (including the uninoculated CR columns), the initial number of copies of the 16S rRNA gene was comparable. In all the columns, there were more 16S rRNA genes at the first two pseudo steady-states than in the initial material. At the first pseudo steady-state, numbers in the CR columns were significantly lower than in the inoculated columns, which were not different from each other. In the second pseudo steady-state, no clear trends were observed.

The number of the genetic markers for cellulose-degrading bacteria (*cel5* and *cel48*) increased in all columns at the first pseudo steady-state with respect to the initial inoculated substrate (Fig. 4B & 4C). At the first pseudo steady-state, the SRB columns contained higher numbers of *cel5* and *cel48* genes than the other columns. At the second pseudo steady-state, the numbers of these genes decreased in all columns except the CR and CD columns, where they remained the same as the first steady-state.

In all the columns, the number of *hydA* genes (fermenters) increased with time (Fig. 4D). At the first pseudo steady-state, all the inoculated columns contained comparable numbers of *hydA* genes while the CR columns had significantly lower numbers. In each column, the numbers in the second pseudo steady-state were significantly higher than the first pseudo steady-state and all the columns contained comparable numbers except for the CD #1 and DM #1 columns, which contained more *hydA* genes.

The number of *dsrA* genes (SRB) in the initial substrate was significantly lower than at the two pseudo steady-states for all columns (Fig. 4E). At the first pseudo steady-state, the highest numbers were observed in the SRB columns and in columns DM #2 and CMC #1. At the second pseudo steady-state, after exposure to air, the highest number of *dsrA* genes was observed in the SRB columns followed by the CMC and EtOH #2 columns.

In general, more *mcrA* genes (methanogens) were detected in the CMC, DM, and SRB than in all the other columns (Fig. 4F). In the CD columns, the number of *mcrA* genes increased significantly after exposure to air. In the EtOH columns the number of *mcrA* genes did not change between the two pseudo steady-states and was generally lower than that of the other inoculated columns. In the CR columns, the number of *mcrA* genes was significantly lower than in the other columns at all times.

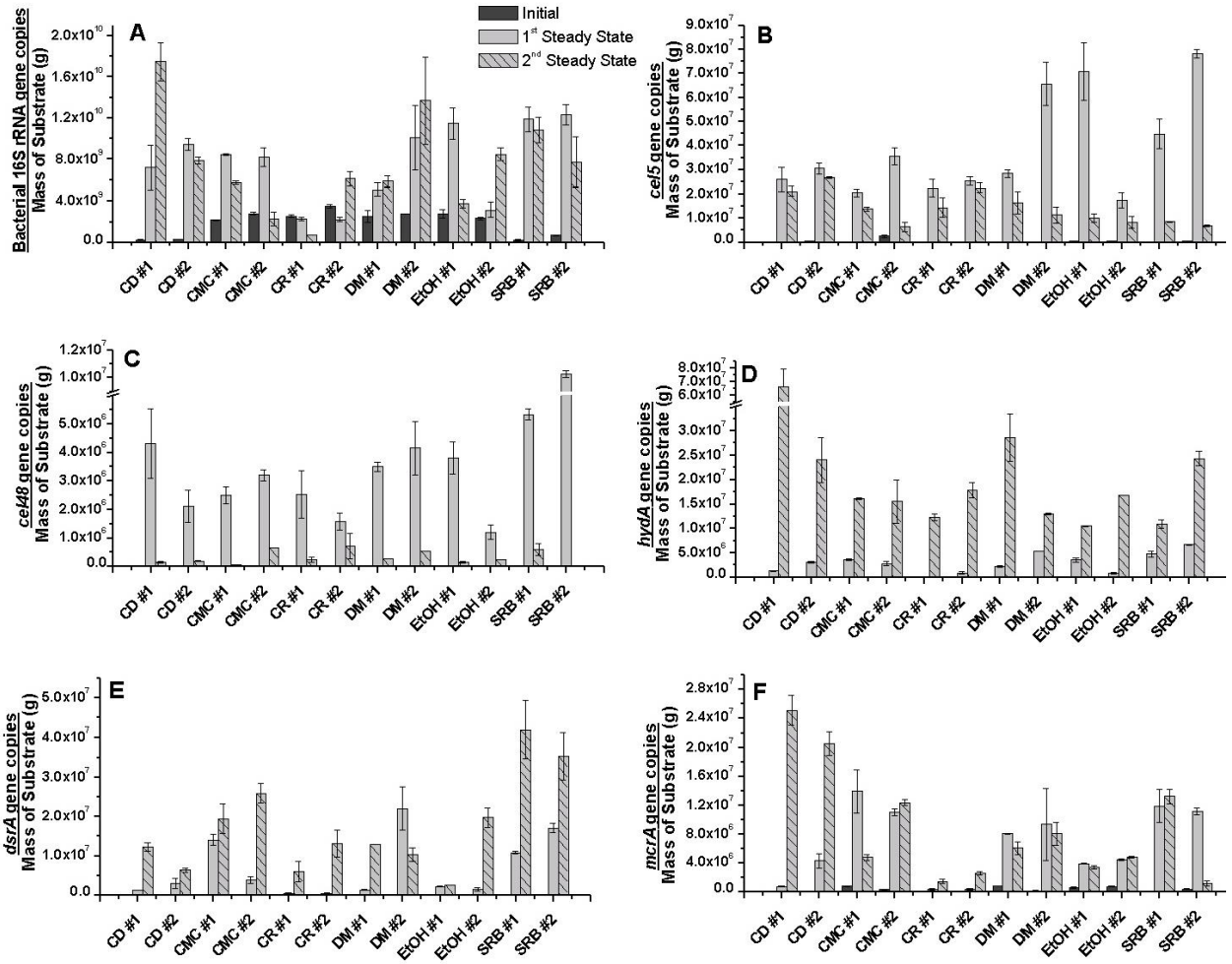

Figure 4. Number of (A) 16S rRNA genes, (B) *cel5* genes, (C) *cel48* genes, (D) *hydA* genes, (E) *dsrA* genes, and (F) *mcrA* genes representing family 5 cellulose-degraders, family 48 cellulose degraders, fermenters, sulfate reducers, and methanogens, respectively, in the columns in the initial inoculated substrate and during the two pseudo steady-states normalized to the mass of substrate as, determined by Q-PCR.

Active Community Profiling (ACP)

Representative initial and pseudo steady-state ACP profiles from control, biostimulated, and bioaugmented columns are presented in Fig. 5. ACP profiles provide a snapshot of active bacteria in the form of a fingerprint of the 16S rRNA:16S rDNA ratio. In general, the ACP results provide insight into the relative diversity and overall structure of the active bacterial community at the initial time point and at pseudo steady-state. ACP profiles are not available for the post-stress sampling events, but several key observations can be made at this point in the study.

As expected, it was observed that the initial time point of the uninoculated control column, CR 1, had lower species diversity than the other columns (Fig. 5). All of the dominant peaks in the initial CR profile persisted through the first six weeks of the experiment, aligning well with dominant peaks at the first steady-state profile. Interestingly, the diversity at the first steady-state increased from that of the initial condition. The initial 16S rRNA:16S rDNA profiles of the six columns inoculated only with DM were highly similar to each other, as expected, providing evidence of the repeatability of the ACP approach.

The initial 16S rRNA:16S rDNA profiles of columns bioaugmented with SRB aligned very well with the enrichment culture profile. As expected, the dominant peak in the profile of both columns aligned with the dominant peak of the enrichment culture. The initial 16S rRNA:16S rDNA ratio profiles of the columns bioaugmented with cellulose degraders (CD1 and CD2) were considerably less complex than the first steady-state profile (Fig. 5). The three dominant peaks of the initial profile persisted throughout the experiment and were present at pseudo steady-state. None of the dominant peaks of the cellulose degrader enrichment profile aligned with peaks in the initial ratio profile, although several of the enrichment peaks aligned with the steady-state dominant peaks. Even though the enrichment may not have survived well during inoculation, it appeared to flourish later at pseudo steady-state

The active species in the columns biostimulated with carboxymethyl cellulose remained consistent for CMC 2, based on the CMC columns' rRNA:16S rDNA profiles (Fig. 5). Unlike the CMC-stimulated columns, the EtOH active microbial community appeared to decrease in diversity over the course of the experiment. The steady-state EtOH profile had fewer total and dominant peaks than the initial profiles. Thus, EtOH seems to have had a stronger enrichment effect (i.e. decreasing overall diversity) than CMC.

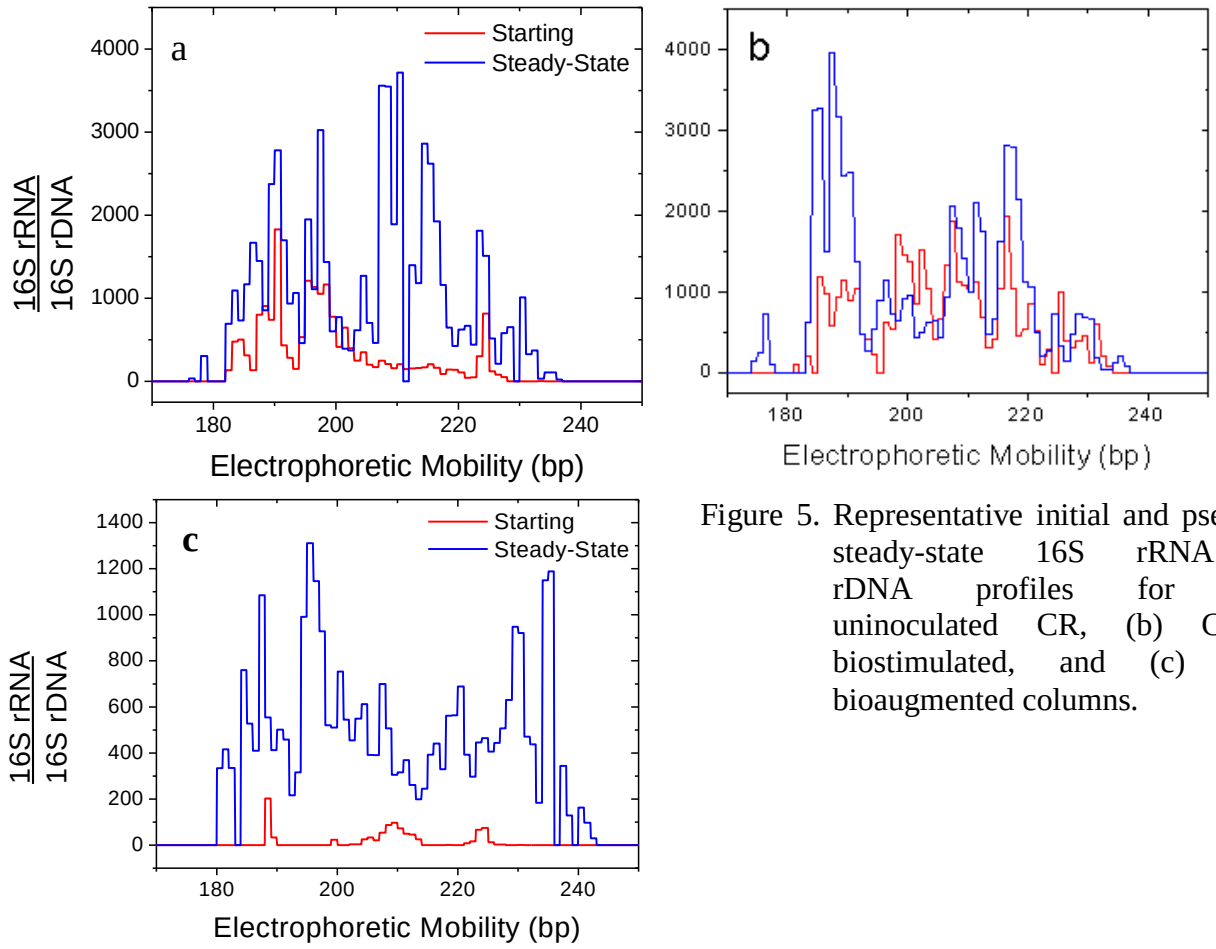

Figure 5. Representative initial and pseudo steady-state 16S rRNA:16S rDNA profiles for (a) uninoculated CR, (b) CMC biostimulated, and (c) CD bioaugmented columns.

Discussion and Conclusions

In this study, sulfate removal was used as the primary indicator of column performance. Available metals data also provided insight, but do not necessarily capture short-term changes in biological phenomena because of adsorption processes. During the first pseudo steady-state and the two following exposure to air, the effluent concentrations of SO_4^{2-}, Cd, and Zn in all columns were significantly lower than those of the feed and the effluent pH was significantly higher. This indicates that all the columns achieved sufficient anaerobic conditions for sulfate reduction to proceed and were successfully remediating the simulated Zn and Cd MIW. The performance of the bioaugmented and biostimulated columns began to diverge from the uninoculated and dairy manure controls following the stress events. Considering the relatively short time scale of this experiment (~6 months) compared to actual field operation (years), such distinctions are likely to be amplified with time.

Immediately after exposure to air, there was an initial increase in the sulfate concentration in the effluent of all columns, which suggests that a stress response was successfully induced. Since BCRs are anaerobic systems, the exposure to air was expected to reduce the microbial activity initially in the columns and, thus, cause a decrease in performance denoted by increased sulfate in the effluent. Once the microbial community recovered, the concentration of sulfate and metals in the effluent was expected to return to the previous or higher levels (if some key members of the community could not recover from the stress). Interestingly, the sulfate levels initially decreased to levels that were lower than before the first oxygen stress event before increasing to levels similar to those at the first pseudo steady-state. After the second exposure to air, sulfate levels decreased at a slower rate to first pseudo steady-state levels. By day 121, there were obvious differences in column performance. The biostimulated columns achieved the highest SO_4^{2-} reduction rates, followed by the CD columns, which performed similarly to the DM controls. Interestingly, the SRB bioaugmented columns performed the worst, followed by the uninoculated control. There is evidence that some sulfate-reducing bacteria and *Clostridium* spp., which are cellulose degraders and fermenters commonly found in BCRs, are more tolerant to oxygen than others that perform the same function (Kawasaki et al., 2005; Krekeler et al. 1998; LeGall and Xavier 1996). If such microorganisms that are key for MIW remediation tolerated the oxygen exposure better and recovered more rapidly than other microorganisms that compete with them, they would be in an advantageous position immediately after the stress.

In all the columns, the numbers of *cel5*, *cel48*, *hydA*, *dsrA*, and *mcrA* genes were higher during column operation than in the initial inoculated substrate, indicating that members of the microbial groups represented by these genes were able to survive and grow in the columns. Quantification of 16S rRNA genes also indicated an increase in the number of total bacteria for the inoculated columns. Although surprisingly the uninoculated columns initially contained similar numbers of bacteria as the DM columns, the numbers did not increase during column operation except for the CR #2 column at the steady state following exposure to air. Although the CR columns performed similarly during start-up and the first pseudo steady-state, their performance deteriorated following the stress events. This result suggested that initial microbial community diversity may be critical to long-term performance and survival of stress events. The ACP profiles confirmed that the initial microbial community diversity was lower in the CR (control) columns, providing support for this conclusion. At this point, the study evidence points

to the importance of *initial* diversity (that of the inoculum) in column performance, since the addition of EtOH decreased diversity at the first pseudo steady-state by selecting for specific bacteria, and these columns eventually achieved the best overall performance. However, this needs to be examined in more detail. In addition, in this study the ethanol addition was combined with the complex lignocellulosic substrate, which may have enhanced complexity and buffered its resistance to stress as compared to a field system that receives ethanol only. Also, the attachment medium may also be important. For example, biomass attached to rocks may slough off in response to stress, thus devastating the system.

Interestingly, bioaugmentation with CD and SRB bacteria did not improve performance as expected. Q-PCR quantification of total bacteria revealed that the initial number of 16S rRNA genes in the CD and SRB columns was much lower than the other inoculated columns. This was intriguing given that all of the inoculated columns received the same mass of dairy manure inoculum as the other columns in addition to CD and SRB enrichments. Q-PCR quantification of specific functional groups further revealed that there was no indication of higher numbers of cellulose degraders or sulfate reducers in the initial material of the CD or SRB columns; though the SRB columns did have the highest levels of SO_4^{2-} reducers at the first and second pseudo steady-states. These results strongly suggest that bioaugmentation did not occur as intended. A likely possibility is that inhibitory metabolic byproducts may have been present in the enrichment culture used for bioaugmentation, which killed a significant portion of the initial microbial community. At least in the case of the SRB bioaugmented columns, the relative proportion of SRB was likely still initially higher as indicated by their dominance at the first and second pseudo steady-states. Interestingly, higher levels of SRB did not translate into improved performance and the SRB columns had low sulfate reduction rates at the second pseudo steady-state and the lowest at the third pseudo steady-state. These results further supported prior work that had suggested that upstream microbial groups, such as cellulose degraders and fermenters, may be more critical to BCR performance than SO_4^{2-} reducers (Logan et al., 2003). This study showed that enhancing SRB populations alone may not be a fruitful approach to improving BCR performance, or that the enhancements that were used were of limited, if any, value. The results were an indicator of the potential long-term importance of the initial microbial community. Though the performance of the SRB columns was initially indistinguishable from the others, by

the end of the experiment these, columns exhibited the worst performance in terms of SO_4^{2-} reduction rate.

Supplementation with CMC was expected to stimulate cellulose-degrading bacteria and increase their numbers. However, there was no obvious effect of CMC on the *cel5* and *cel48* gene numbers, which were comparable to those in the DM columns. The different types of cellulose (crystalline vs amorphous, soluble vs unsoluble) required the use of different enzymes for its hydrolysis and microorganisms differ in their affinities for the different types of cellulose. Therefore, cellulose degraders from these two families may not have been enriched. However, CMC had a notable positive effect on the sulfate reduction rate by the second and third steady-state, but in contrast, the effluent levels of Zn and Cd were higher in the CMC columns. The carboxymethyl and hydroxyl groups in CMC have chelating properties (Hosny et al., 1995) and might have formed complexes with the metals, preventing their precipitation as metal sulfides. Therefore, use of CMC as a biostimulant may not be advisable for remediation of some types of MIW in the field.

The EtOH columns achieved the best long-term performance in terms of sulfate reduction, likely because of decreasing availability of the lignocellulosic substrate and the consistent presence of a substrate that could be directly utilized by SRB. Supplementation with ethanol was expected to enrich SO_4^{2-} reducers, but the EtOH columns did not contain significantly more *dsrA* genes. Genes involved in cellulose degradation were the lowest among the columns, as would be expected, in the EtOH 2 column, but not in the EtOH 1 replicate. Compared to the other inoculated columns, the EtOH columns contained the fewest *mcrA* genes (methanogens). This result supports a previous report by Prieto et al. (2008), in which methanogens were lowest in concentration in ethanol-fed as compared to lignocellulose-fed BCRs. Suppressed methanogens may be advantageous considering that they compete with SRB for resources.

Exposure to air caused an increase in *dsrA* and *hydA* genes and a decrease in *cel5* and *cel48* genes in the majority of the columns, suggesting that the sulfate-reducing and fermentative bacteria in the columns were more resilient to oxygen stress than the cellulose-degrading bacteria. This could be a potentially important finding considering that conventional wisdom of BCR operation is typically concerned with the negative impact of oxygen exposure on SRB, not cellulose degraders. This result may be especially key considering the growing body of evidence supporting the critical role that cellulose degraders play in solid substrate BCRs. The cellulose

degraders present in the CR and CD columns survived better following the first stress, suggesting that aerotolerant cellulose degraders exist and may have applications for improving BCR performance. Future MIW microbial research should concentrate on the role of cellulose degraders in solid substrate BCRs, since it appeared in this study that they played a significant role in BCR performance.

ACP confirmed that the overall structure and the active species of their microbial community were significantly different among the columns at the first steady-state. The diversity and number of active species in the CD bioaugmented and the CMC stimulated columns were much greater after six weeks of operation than in the starting communities. The opposite trend was observed in columns stimulated or bioaugmented with sulfate reducers, where the diversity of active species in the starting community was much greater than at the end of the experiment. These results suggest that targeting the top level of the ecological carbon chain (i.e., cellulose degradation) via either biostimulation or bioaugmentation can increase the activity of a larger portion of the microbial community than can be achieved by targeting the lower levels of the carbon chain (i.e., sulfate reducers). The increase in diversity may offer redundancy of functional groups within the BCR, and thus improved resilience to system perturbations and stresses.

This work is the first systematic examination of the relationships between BCR remediation performance, the active microbial community, key functional groups, and the parallel use of bioaugmentation and biostimulation in MIW bioremediation systems. This is an initial work in the study of the effects of stress on the microbial community and BCR performance. The applied research approach may be beneficial to advancing the understanding of other microbially-driven systems, thereby progressing towards the integration of microbial community dynamics with bioreactor design.

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