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ORIGINAL PAPER

Performance and microbial community dynamics of a sulfate-reducing bioreactor treating coal generated acid mine drainage

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Abstract The effectiveness of a passive flow sulfate-reducing bioreactor processing acid mine drainage (AMD) generated from an abandoned coal mine in Southern Illinois was evaluated using geochemical and microbial community analysis 10 months post bioreactor construction. The results indicated that the treatment system was successful in both raising the pH of the AMD from 3.09 to 6.56 and in lowering the total iron level by 95.9%. While sulfate levels did decrease by 67.4%, the level post treatment (1153 mg/l) remained above recommended drinking water levels. Stimulation of biological sulfate reduction was indicated by a +2.60‰ increase in $\delta^{34}S$ content of the remaining sulfate in the water post-treatment. Bacterial community analysis targeting 16S rRNA and *dsrAB* genes indicated that the pre-treated samples were dominated by bacteria related to iron-oxidizing *Betaproteobacteria*, while the post-treated water directly from the reactor outflow was dominated by sequences related to sulfur-oxidizing *Epsilonproteobacteria* and complex carbon degrading *Bacteroidetes* and *Firmicutes* phylums. Analysis of the post-treated water, prior to environmental release, revealed that the community shifted back to predominantly iron-oxidizing *Betaproteobacteria*. DsrA analysis implied limited diversity in the sulfate-reducing population present in both the bioreactor outflow and oxidation pond samples. These results support the use of passive flow bioreactors to lower the acidity, metal, and sulfate levels present in the AMD at the Tab-Simco mine, but suggest modifications of the system are necessary to both stimulate sulfate-reducing bacteria and inhibit sulfur-oxidizing bacteria.

Keywords Acid mine drainage · Sulfate reduction · Bioreactor · Iron oxidation · Sulfur oxidation

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Introduction

Acid mine drainage (AMD) is an environmental problem of major concern in areas where pyrite (FeS_2) -containing rocks are brought into contact with the oxygenated surface- or ground-waters via mining activities. Coal reservoirs in the Illinois Basin have been shown to have sulfur content ranging from 0.5 to

7% and pyritic sulfur with values as high as 5% (Korose and Elrick 2010). Exposure of these minerals due to mining of such coal leads to the oxidation of pyrite yielding waters with high levels of acidity, sulfate (SO_4^{2-}), ferric iron (Fe^{3+}), ferrous iron (Fe^{2+}) as well as other toxic metals. The presence of Fe^{3+} can act as a potent oxidant increasing the rate of pyrite oxidization while iron-oxidizing microbes can recycle the Fe^{2+} to Fe^{3+} creating a self-perpetuating process under low pH conditions. These iron-oxidizing bacteria, which thrive at a pH range of 2.0–4.0, can increase the rate of Fe^{2+} oxidation by factors greater than 10^6 (Brown et al. 2002). The resulting AMD from this process permeates throughout the mining region causing contamination to drinking water supplies, negative effects to plant and wild life, and corrosion to infrastructure (U.S. Environmental Protection Agency 2008).

While chemical additions can be used to raise the pH and promote metal precipitation within AMD, this augmentation does not address the high level of remaining dissolved sulfate. Currently, sulfate has a secondary maximum contaminant level of 250 mg/l in drinking water (U.S. Environmental Protection Agency 2006). Thus, sustainable methods for removal of acidity, metals, and sulfate are needed before waters from mining activities can be released into the environment.

Passive treatment methods that utilize naturally occurring materials, such as organic matter and limestone, have emerged as cost effective and low maintenance options for long-term acid mine drainage treatment. Specifically, sulfate-reducing bioreactors have been used to generate alkalinity, precipitate metals, and decrease sulfate levels from AMD waters (as reviewed by Neculita et al. 2007).

These bioreactors utilize sulfate-reducing bacteria (SRB)—anaerobic microbes that can couple growth to the reduction of SO_4^{2-} to H_2S using simple organic acids, alcohols, or H_2 as electron donors—to aid this process (Postgate 1984). Such electron donors are costly and would have to be continually added to the system but these bioreactors can be seeded with an inexpensive source like compost or wood chips that are converted by other heterotrophic bacteria into substrates for SRB growth (Neculita et al. 2007). This process is more sustainable and the degradation of the complex carbon source consumes oxygen providing an anaerobic system for the desired SRB.

A problem with sulfate-reducing bioreactors is that sulfate reduction is much slower under acidic conditions; sulfate reduction rates have been shown to be approximately 300-fold less at pH 3.5 compared to pH 6 with those cells showing limited longterm viability (Jong and Parry 2006). The SRB are able to produce alkalinity via the release of HCO_3^- during heterotrophic growth (Eq. 1) (Neculita et al. 2007), but the addition of limestone ($CaCO_3$) is beneficial to the system as it contributes alkalinity as well as providing a surface for biofilm formation to locally enhance sulfate reduction.

$$2CH_2O(aq) + SO_4^{2-} \rightarrow H_2S + 2HCO_3^- \quad (1)$$

While this metabolism removes SO_4^{2-} from the system, the by-product H_2S (Eq. 1) complexes with divalent metals, such as Fe^{2+}, to form FeS and FeS_2 precipitates. These iron sulfide precipitates are less soluble than iron hydroxides and are retained within the physical matrix of the limestone/organic material bioreactor (Eccles 1999). Once the AMD flows vertically through the bioreactor, under-draining pipes transport the water to an oxidation or settling pond in which continued iron hydroxide precipitation occurs (as reviewed by Neculita et al. 2007).

Studies indicate that passive flow bioreactors perform quite well the first few months post-construction, but long-term performance appears to be an issue. Reported problems include hydraulic retention time, additional flows, leakage, low pH, seasonal trends, substrate limitation, and precipitate/substrate clogging problems (as reviewed by Neculita et al. 2007). Maintaining sufficient complex carbon-degrading bacteria to break down the compost into simple by-products suitable for SRB is another critical factor affecting long-term performance of the bioreactor. Besides these factors, the presence of sulfur- and iron-oxidizing bacteria can reverse the desired product formation by oxidizing reduced sulfur species to sulfuric acid and Fe^{2+} to Fe^{3+}, thus recycling the oxidant and producing more acidity. A variety of studies have been done detailing the microbial communities including SRBs within AMD treatment systems, however these have focused on aspects such as the complexity of carbon source degraders within the reactor (Clarke et al. 2004), the bioreactor stimulated microbial community in response to different carbon sources (Schmidtova and Baldwin 2010), and the effect of different inocula sources on bench-top

bioreactors simulating the conditions of the field (Pereyra et al. 2008). These studies have yielded extremely valuable information, but more field scale analyses at a variety of different sites are necessary for a better understanding of how sulfate-reducing bioreactors work in situ.

The goal of this study was to evaluate the efficacy of a passive flow sulfate-reducing bioreactor on the AMD of a Southern Illinois coal mine. Here we report the geochemical and microbial profiles of the abandoned Tab-Simco coal mine site 10 months following bioreactor installation. This characterization included pH, dissolved oxygen content, iron, and sulfate measurements. To gauge the microbial community present in the AMD as well as how this community changed from exposure to the bioreactor, 16S rRNA and *dsrAB* (encoding the dissimilatory (bi) sulfite reductase involved in biological sulfate reduction) gene clone libraries were constructed and analyzed. Evaluating the performance of this treatment system by looking at geochemical data as well as present microbial communities is critical for improving the performance and longevity of sulfate-reducing bioreactors that are susceptible to constant dilution from area run-off.

Materials and methods

Characteristics of the study site

The Tab-Simco site is an abandoned coal mine located near Carbondale, IL, USA consisting of approximately 30 acres of underground mine works that were actively mined from 1890 to 1975. Subsequently, AMD pools were formed within the underground system that discharged through a series of seeps before exiting into a local creek (Smith 2002). Prior to reclamation, the acid pool within the underground works discharged approximately 132,500 l/day of AMD with a pH ranging from 2.6 to 3.0 (Lewis 2008). Initial reclamation included sealing the mine opening and any possible infiltration sites, construction of trenches to re-direct run off, backfilling of impoundments, and addition of lime and fertilizer to the site. In October 2007, a passive sulfate-reducing bioreactor was constructed on an area of approximately 3,000 m^2, which is subject to local run-off due to topology. Details regarding the site, construction of the limestone/compost (53% woodchips, 27% straw mulch, 11% seasoned compost, and 9% agricultural ground limestone) containing bioreactor, and geochemical monitoring of the site over a 3 year period have recently been described by Behum and coworkers (Behum et al. 2011).

Sampling and geochemical analyses

Water samples from the Tab-Simco site were collected in August 2008, 10 months after construction of the bioreactor. Sampling points included: 1) Well B-1 (WB1) (78 m down into the underground mine); 2) Acid Pond (AP) (water above the organic layer of the bioreactor); 3) Bioreactor Outflow (BRO) (water exiting the bioreactor); and 4) Oxidation Pond (OP) (non-agitated, open holding area before discharge into a local creek) (Fig. 1). Four samples corresponding to each site were collected in 125 ml bottles, stored on ice, and transported to the laboratory for analysis. Two of the samples were used for geochemical measurements while the other two samples were used

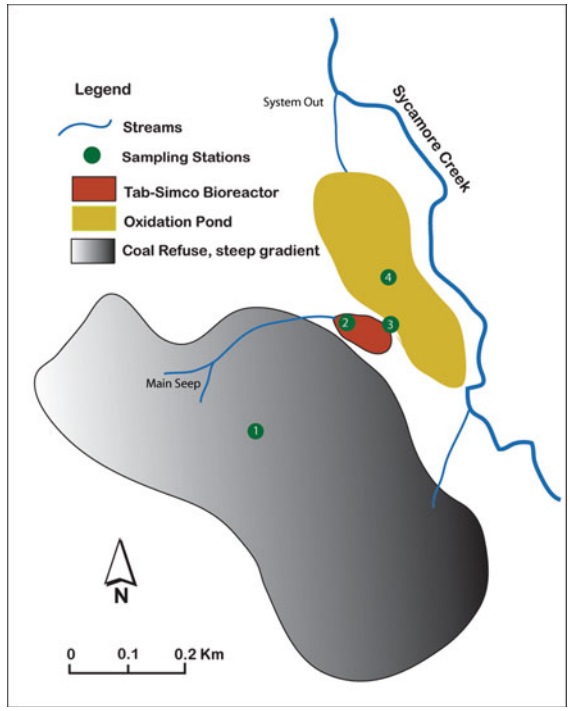


Fig. 1 Flow diagram of the Tab-Simco coalmine site with sample locations indicated. *1* Well B-1, *2* Acid Pond, *3* Bioreactor Outflow, *4* Oxidation Pond

for microbial analysis. At each sampling station the pH level and dissolved oxygen (DO) content was measured using a Hach (Loveland, CO) HQ40D pH/conductivity/DO meter. Water samples for chemical analysis were filtered using pre-baked and dried 0.22 μm pore-size quartz-fiber filters (Whatman, QM-A) immediately after transfer to the laboratory. Metal (Fe, Al, Mn, Ca, Mg, Na, and K) and sulfur isotope analyses were performed as previously described (Behum et al. 2011). Briefly, dissolved metals Fe^{2+} and total Fe concentrations were analyzed using colorimeter, Hach Ferrous (Method Number 8146) and FerroVer (Method Number 8008) methods, respectively. Other metal ions were analyzed using a Hitachi Z-2000 Polarized Zeeman Atomic Absorption Spectrometer (AAS) flame test method. For sulfur isotope quantification, barium chloride (BaCl) was added to precipitate the sulfate as $BaSO_4$. The dried $BaSO_4$ was mixed with vanadium pentoxide (V_2O_5) and sulfur isotope levels were measured using a Thermo Scientific (Waltham, MA) manufactured Finnigan/Mat Delta Plus® stable-isotope ratio mass spectrometer.

Molecular techniques

Microbial biomass was isolated from duplicate water samples from each site via 0.2 μm sterile analytical test filters. Total DNA was then isolated directly from the filters using the FastDNA SPIN Kit for Soil (MP Biomedicals). Both concentration and purity of the resulting DNAs were determined by Nanodrop spectrophotometry (Fisher Scientific). The duplicate DNAs were then pooled and PCR targeting the 16S rRNA and *dsrAB* genetic loci was performed. The PCR reactions were composed of 1× GoTaq buffer containing $MgCl_2$ (Promega), 0.2 mM each dNTP, 100 ng bovine serum albumen, 10 pmol each primer (including each primer in the respective DSR mixes), 1 U GoTaq Polymerase (Promega), and ~100 ng DNA in a total volume of 50 μl. The primers used to target the bacterial 16S rDNA were Bac8F (5′-AGAGTTTGATCCTGG CTCAG-3′) and Univ529R (5′-ACCGCGGCKGCTGGC-3′), while Arch21F (5′-TTCCGGTTGATCCTGCCGGA-3′) was used in conjunction with Univ529R to target the archaeal 16S rRNA gene (Baker et al. 2003). These primer sets have been used to create rRNA clone libraries in other environmental studies (Fierer et al. 2007). To amplify the 1.9 kb *dsrAB* fragment the following mixture of forward and reverse primers were used: DSR1F, DSR1Fa, DSR1Fb, DSR4R, DSR4a, DSR4b, DSR4c (Kjeldsen et al. 2007).

Cycling parameters for the 16S rRNA gene PCR consisted of a 94°C denaturation for 2 min; followed by 30 cycles consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and ending with a 5-min extension at 72°C. Touchdown parameters were used to amplify the *dsrAB* locus: 94°C denaturation for 2 min; followed by 20 cycles consisting of a 0.5°C decrease/cycle from 62°C to 52°C, and 72° for 90 s; then 15 cycles consisting of 94°C for 30 s, 52°C for 30 s, and 72°C for 90 s; and ending with a 5-min extension at 72°C. Controls for the PCR included *Desulfovibrio vulgaris* gDNA as a positive and no template DNA as a negative. The resulting PCR products were analyzed by gel electrophoresis and amplicons of the correct size were excised and purified using the QIAEX II Gel Extraction Kit (Qiagen). These purified DNAs were used to create clone libraries specific to each site using the TOPO TA cloning vector pCR2.1 and One Shot TOP10 *Escherichia coli* cells (Invitrogen). The resulting clones were screened for the presence of the desired insert and the corresponding plasmid DNAs were commercially sequenced using M13 vector primers (MCLAB, San Francisco, CA).

DNA sequence analysis

Partial 16S rRNA sequences were trimmed of the primer sequences and aligned using NAST on the Greengenes website (http://greengenes.lbl.gov) with sequences containing less than 75% identity to the database removed from the pool (DeSantis et al. 2006). The resulting 16S rRNA gene sequences from the clone libraries along with a subset of closely related pure culture 16S rRNA gene sequences identified from the BLAST analysis were analyzed for chimeras using Bellerophon (Huber et al. 2004). Operational taxonomic units (OTUs) were assigned based on 97% identity criterion using the mothur software package (Schloss 2009). Closest relatives for each OTU were determined by BLAST analysis. Rarefaction curves were generated in mothur to determine sampling of species diversity. Phylogenetic analyses of the 16S rRNA gene sequences were performed using treeing tools of the ARB software package (Ludwig et al. 2004). Inferred phylogeny was constructed by addition of clone sequences via

maximum parsimony into a pre-existing ARB database available on the Greengenes website. Consensus trees based on the final alignments were constructed using distance neighbor-joining method (Ludwig et al. 1998). Bootstrap re-sampling of the trees was performed using 1,000 replicates. To determine if the resulting 16S rDNA clone libraries from each site possessed significantly different communities, Unifrac analysis was performed (Lozupone et al. 2006).

Phylogenetic analysis of the *dsrAB* genes was performed similarly to the 16S rRNA gene with some modifications. A 150 inferred amino acid (AA) region corresponding to the N-terminus of DsrA was selected for further analysis. The inferred AA sequences were aligned along with pure culture and environmental clone sequences in the ClustalX program. The aligned sequences were inserted into a DsrAB ARB database maintained by the University of Vienna to infer phylogeny (Zverlov et al. 2005). Consensus phylogenetic trees were constructed using a maximum likelihood method with a DsrA filter for the first 150 amino acids using the ARB program. Partial DsrA sequences with ≥90% AA identity were grouped as OTUs (Kjeldsen et al. 2007).

Nucleotide sequence data

The clone sequences obtained from this data were deposited in GenBank under the accession numbers JN127415–JN127760.

Results

Bioreactor flow and geochemical analysis

Water chemistry values for the four sampling sites, including pre- and post-treated, are presented in Table 1. A comparison of the sample sites indicated that dissolved oxygen levels seem to correlate directly with sulfate concentrations. While the pretreated WB1 and AP samples possessed a similar acidic pH, the AP possessed the highest levels of sulfate (3538 mg/l; 36.83 mM), dissolved oxygen content (9.26 mg/l), and Fe^{2+} (432 mg/ml) amongst the samples (Fig. 2). To circumvent disturbing the bioreactor region at the bottom of the AP, samples from the water flowing directly out of the bioreactor were collected to monitor its effectiveness. Water directly from the BRO possessed a circumneutral pH of 6.53, a bicarbonate concentration of 500 mg/l, and a decreased dissolved oxygen content of 3.96 mg/l (Table 1; Fig. 2a). Total iron and sulfate concentrations in the treated water from the BRO decreased by 94.7 and 45.2%, respectively compared to the levels in the AP (Table 1). Concentrations of Al, Mg, Mn, Na, Ni, and Zn metals also decreased in the post-treated samples (Online resource 1).

Successful stimulation of sulfate reduction was demonstrated by the increased $\delta^{34}S$ content, 6.98–9.58‰, of the sulfate measured in the water from the BRO compared to the AP (Table 1). Due to the biological preference for lighter isotopes, biological reduction of sulfate can result in an increase of 2–46‰ in the $\delta^{34}S$ values of remaining dissolved sulfate (Bruchert et al. 2001). However, the OP still contained a sulfate concentration of 1153 mg/l, a level much higher than the recommended level of 250 mg/l for drinking water (U.S. Environmental Protection Agency 2006).

Water from the BRO also possessed a 36.8% increase in calcium concentration when compared to the AP (Table 1), suggesting dissolution of limestone ($CaCO_3$). This calcium concentration only accounted for 1.49 mmol of the 8.19 mmol of bicarbonate present, further supporting biological generation of

Table 1 Water chemistry of samples collected from the Tab-Simco site

Sample	pH	DO[a] (mg/l)	SO_4^{2-}(mg/l)	$\delta^{34}S$[b] (‰)	Fe^{2+} (mg/l)	Fe^{3+} (mg/l)	Ca (mg/l)	HCO_3^- (mg/l)
Well B-1	3.12	2.79	1981	7.40	175.50	159.50	247.00	0.00
Acid Pond	3.09	9.26	3538	6.98	432.00	176.00	161.49	0.00
Bioreactor Outflow	6.53	3.96	1939	9.58	29.25	2.95	220.93	500.03
Oxidation Pond	6.56	3.37	1153	9.96	9.50	15.60	156.81	123.91

[a] DO stands for dissolved oxygen content

[b] Sulfur isotope values of ^{34}S present in dissolved SO_4^{2-}

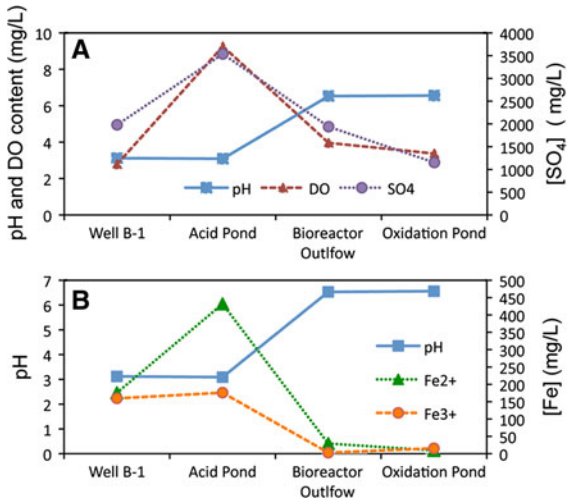


Fig. 2 Values measured in the pretreated Well B-1 and Acid Pond samples, as well as those measured in the post-treated Bioreactor Outflow and Oxidation Pond samples. **a** pH, dissolved O_2 (DO), and SO_4 content. **b** pH and iron species content

bicarbonate. Holding of the treated water in the OP resulted in further removal of sulfate, with an additional 40.5% decrease from the levels measured in the BRO. Fe^{3+} species also dominated over Fe^{2+} in the OP (Table 1).

Molecular analysis and community composition

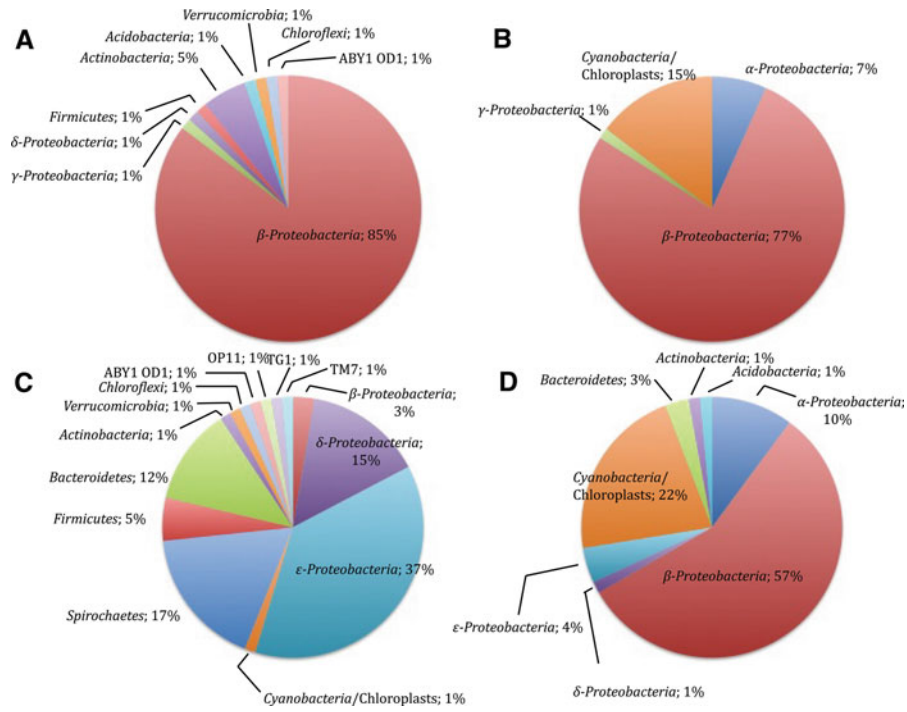
PCR targeting the archaeal 16S rRNA gene did not yield a product from any of the samples within the site, suggesting a limited archaeal population in the microbial consortia. *dsrAB* amplicons were only detected in the post-treated BRO and OP samples. Clone libraries were analyzed for chimeras and a total of 75 clones corresponding to a portion of the 16S rRNA gene were analyzed from each site, while 56 *dsrAB* clones were analyzed from the post-treated samples. Rarefaction analysis of the DNA sequencing data indicated that the level of microbial diversity could not be determined because sampling was not exhaustive (data not shown). Full descriptions of the 16S rRNA gene OTUs present for each site can be found in the Online resources.

Well B-1

Community analysis of the WB1 sample indicated the presence of 18 OTUs out of the 75 clones sequenced (Online resource 2). Members of the *Proteobacteria* dominated the library, (88% of total clones), with 85% of the sequences representing *Betaproteobacteria* phylotypes (Fig. 3a). Within these clones 58% were assigned to WB1-OTU10 and WB1-OTU11, which were most closely related to environmental clones from freshwater and acid-impacted lake samples (~97% identity) and the isolate *Gallionella capsiferriformans* (~95% identity). The remaining 36% of the *Betaproteobacteria* phylotypes corresponded to WB1-OTU12, which was most closely related to an environmental clone from AMD (~99% identity) and the isolate *Sideroxydans lithotrophicus* (~93% identity). At ≤95% sequence identity, these OTUs represent either a new species or possibly a new genus. Other phyla represented by the library included the *Actinobacteria*, *Acidobacteria*, *Firmicutes*, *Verrucomicrobia*, and *Chloroflexi*, but at very low abundance. Overall the community analysis indicated a low diversity of bacteria present in WB1 (Fig. 4), which may be attributed to the low pH and high iron and sulfate loads.

Acid pond

Sequence analysis of the AP library indicated the presence of 19 OTUs (Online resource 3). *Proteobacteria* still dominated the pre-treated AMD (85% of total clones), with 77% of the sequences representing *Betaproteobacteria* phylotypes most closely related to environmental clones isolated from other acid mine locations (Fig. 3b). Of these *Betaproteobacteria* phylotypes, five separate OTUs comprising 67% of the total clones were most similar to an environmental clone from AMD (DQ480476.1, ~88–99% identity) with AP-OTU12 specifically comprising 59% of the total clones (Online resource 3; Fig. 5). *Gallionella* and *Sideroxydans* type sequences that dominated WB1 were not detected. The *Alphaproteobacteria* phylotypes isolated (7% of total clones) separated into two groups with ~99% sequence identity to 1) the acidiphilic iron-reducing *Acidiphilum cryptum* (within AP-OTU6); and 2) *Rickettsiales*-type environmental clones (EF520417.1) from an acid impacted lake (within AP-OTU5). The remaining OTUs (15% of total clones) shared low sequence identity to cyanobacteria/chloroplast type sequences (≤90%). AP-OTU4 contained ~9% of the total clones from the library and was most similar to the chloroplast of the



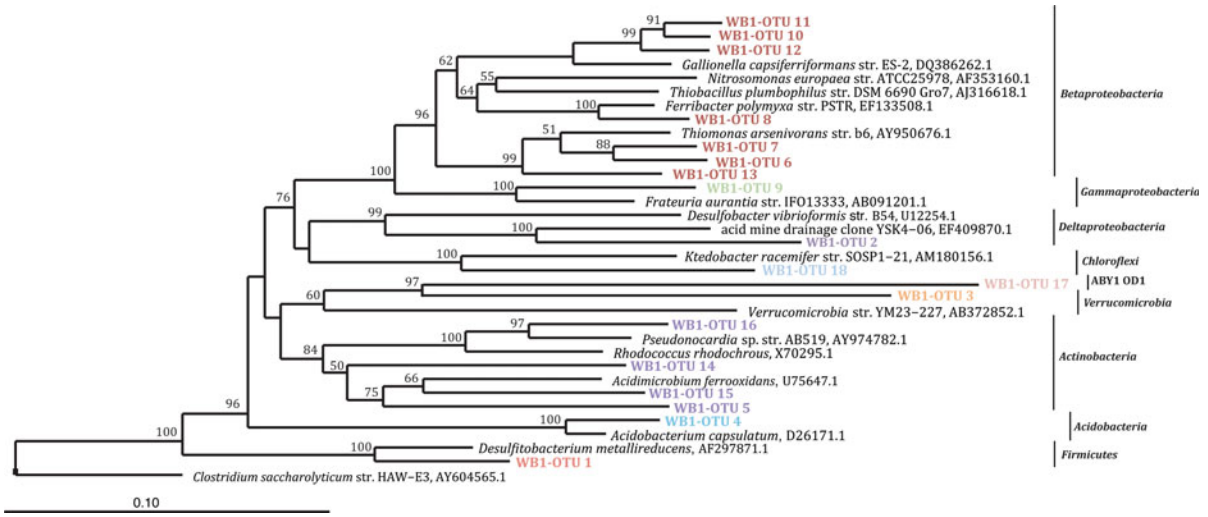


Fig. 3 Percentage distribution of sequences from the four sampled sites at the Tab-Simco mine. **a** Well B-1, **b** Acid Pond, **c** Bioreactor Outflow, **d** Oxidation Pond

Fig. 4 Neighbor joining phylogenetic tree based on partial 16S rRNA gene sequences showing the positions of Well B-1 clone sequences grouped into OTUs and close relatives. WB1 OTUs are represented in *bold* and color-coded to correspond to Fig. 3a. Bootstrap values (1,000 replicates) above 50% are represented at the nodes. The *scale bar* represents 0.10 changes

algae *Pedinomonas minor* (87% identity). Comparative phylogenetic analysis of these sequences indicated a new lineage and could possibly represent the chloroplasts of algae that were observed on the surface of the AP (Fig. 5).

Bioreactor outflow

Molecular analysis of the water directly exiting the BRO indicated the presence of 31 OTUs (Fig. 6; Online resource 4), illustrating an increase in diversity present

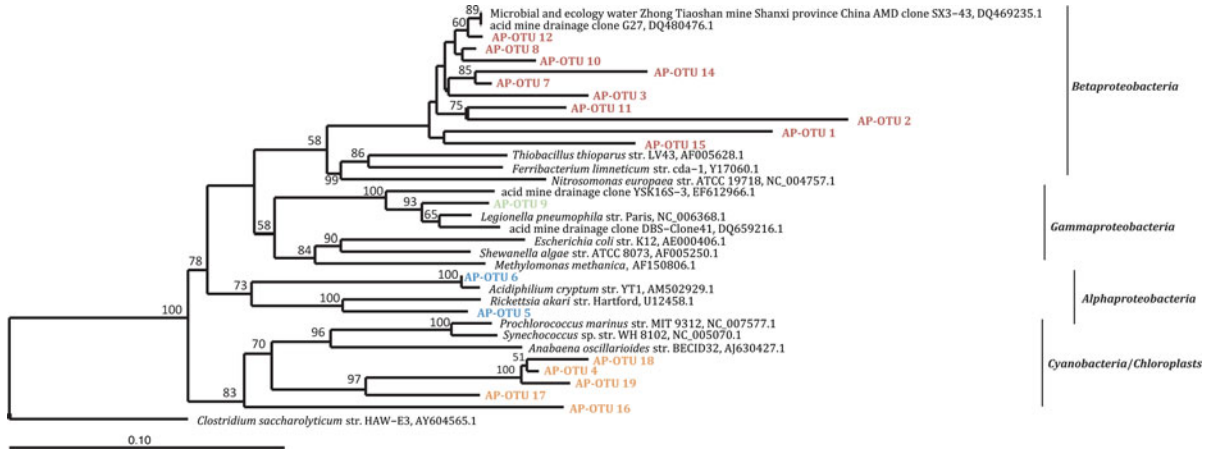


Fig. 5 Neighbor joining phylogenetic tree based on partial 16S rRNA gene sequences showing the positions of the Acid Pond clone sequences grouped into OTUs and close relatives. AP OTUs are represented in *bold* and color-coded to correspond to Fig. 3b. Bootstrap values (1,000 replicates) above 50% are represented at the nodes. The *scale bar* represents 0.10 changes

in the sample. This increase in community diversity was expected due to the increased pH and organic substrate present in the bioreactor. While *Proteobacteria* still dominated the sample (~56% of total clones), representatives from *the Epsilonproteobacteria* were most abundant (~37% of total clones) and separated into two OTUs (Fig. 3c). These two OTUs, BRO-OTU11 and BRO-OTU12, were most similar to environmental clones from a US Department of Energy well at the Oak Ridge Tennessee site (HM146443.1, ~99% identity), a mineral spring (AM167963.1, ~98% identity) and to *Sulfuricurvum kujiense* (AB080645.1, ~95% identity), which possesses sulfur-oxidizing capabilities. Only 7 of the 75 clones sequenced possessed similarity to phylotypes related to known sulfate-reducing bacteria, specifically the *Desulfobacteraceae* family and *Desufotomaculum* species (Fig. 6). OTUs most similar to the sacchrolytic and cellulolytic *Bacteroidales* and *Clostridiales* orders represented ~17% of the total clones while another ~17% of the sequences were designated as members of the *Spirochaetaceae* family and were most similar to environmental clones from a coal bed (EU073764.1), a petroleum contaminated aquifer (DQ664009.1), and technetium contaminated soil (EF508015.1) at 96–99% similarity.

Oxidation pond

Sequencing of 75 clones from the 16S rRNA gene clone library from the OP resulted in the assignment of 69 clones into 29 OTUs, with *Proteobacteria* dominating the community (~72% of total clones) (Online resource 5). The remaining 6 clones shared low sequence similarity (~79% identity) to the 18S rRNA gene of the algae *Prototheca wickerhamii* and were not included in the remaining analysis. While the epsilon class dominated the *Proteobacteria* detected directly in the BRO, the population shifted back to the beta class, with 11 OTUs and ~57% of the total clones (Fig. 3d). Of the *Betaproteobacteria*, nine separate OTUs (OP-OTU10-14, OP-OTU17-19, and OP-OUT-21) representing ~49% of the total clones shared the most sequence identity to environmental clones from samples analyzed from a landfill (AY381284.1), arsenic contaminated aquifer (GU183581.1), leaf material (EU542196.1), and *Sideroxydans lithotrophicus*, which was also detected with abundance in the WB1 sample. Phylogenetic analysis indicated that these OTUs formed a separate clade in the *Betaproteobacteria* (Fig. 7). Four other OTUs were assigned to the alpha class and were represented by members of the *Sphingomonadaceae* family (~10% of the total clones). Five OTUs that comprised ~22% of the total clones and were distinct from the *Proteobacteria*, were designated as cyanobacteria/chloroplast-like sequences (Fig. 3d). A similar number of these types of sequences were identified in the AP sample and possibly correlate to algal chloroplast sequences.

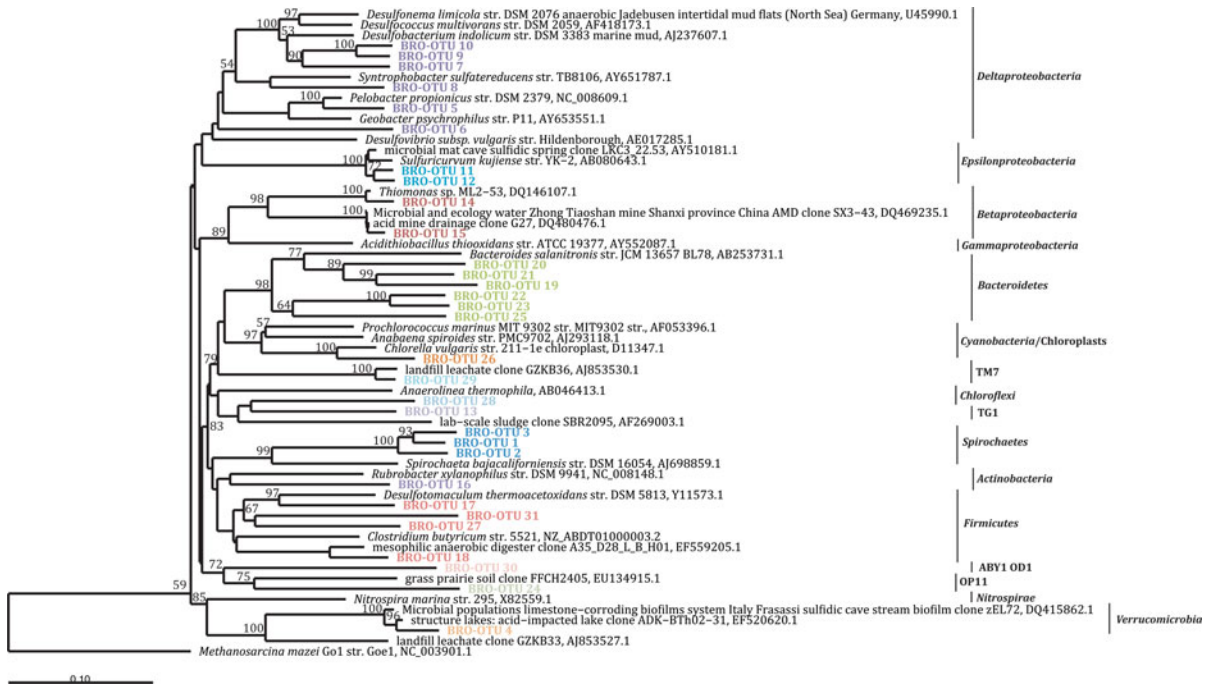


Fig. 6 Neighbor joining phylogenetic tree based on partial 16S rRNA gene sequences showing the positions of the Bioreactor Outflow clone sequences grouped into OTUs and close relatives. BRO OTUs are represented in *bold* and color-coded to correspond to Fig. 3c. Bootstrap values (1,000 replicates) above 50% are represented at the nodes. The *scale bar* represents 0.10 changes

Analysis of DsrA sequences from post-treatment samples

Sequences corresponding to the *dsrAB* locus were obtained from both the BRO and OP samples. The absence of amplicons from the pre-treated samples suggested a low abundance of DNA molecules containing the *dsrAB* locus. Due to the large size of the 1.9 kb *dsrAB* fragment and incomplete environmental sequences present in the databases, a 150 inferred amino acid region corresponding to the N-terminus of DsrA was selected for further analysis. This region has been targeted in previous environmental studies to determine SRB diversity (Giloteaux et al. 2010; Santillano et al. 2010). Analysis of the 56 DsrA sequences resulted in the assignment of 29 clones to 13 OTUs for the BRO and 27 total clones assigned to 7 OTUs for the OP (Online resources 6 and 7). No archaeal type DsrA sequences were detected, which is congruent to results obtained from the universal archaeal primers for the 16S rRNA gene. Of the bacterial DsrA OTUs, four were common to both samples, nine OTUs were exclusive to the BRO, and three were exclusive to the OP; the common OTUs are described in Online resource 8.

Phylogenetic analysis indicated that the DsrA sequences from the post-treated samples were affiliated with *Syntrophobacteraceae* (69.6% of total clones) *Desulfobacteraceae* (14%), *Nitrospiraceae* (7.1%) *Desulfovibrionaceae* (3.6%), and *Peptococcaceae* (5.6%) families (Fig. 8). For the BRO alone, DsrA analysis indicated that *Syntrophobacterales* and *Desulfobacterales* account for 59% and 14% of the total clones, respectively (Online resource 6). While these families were present in the OTUs for the 16S rRNA clones, they accounted for only ~1 and 8% of total clones indicating that total diversity for the site was not fully reached, specifically for the *Syntrophobacterales* (Online resource 4).

The four-shared OTUs indicated the presence of *Desulfobacca acetoxidans*, *Syntrophobacter fumaroxidans*, *Thermodesulfovibrio* sp., and *Desulfococcus multivorans*-like sequences. The predominant DsrA sequence detected in both samples (BRO-DSR-OTU-1 and OP-DSR-OTU2) formed one OTU comprising ~35 and ~78% of the total sequences from the BRO

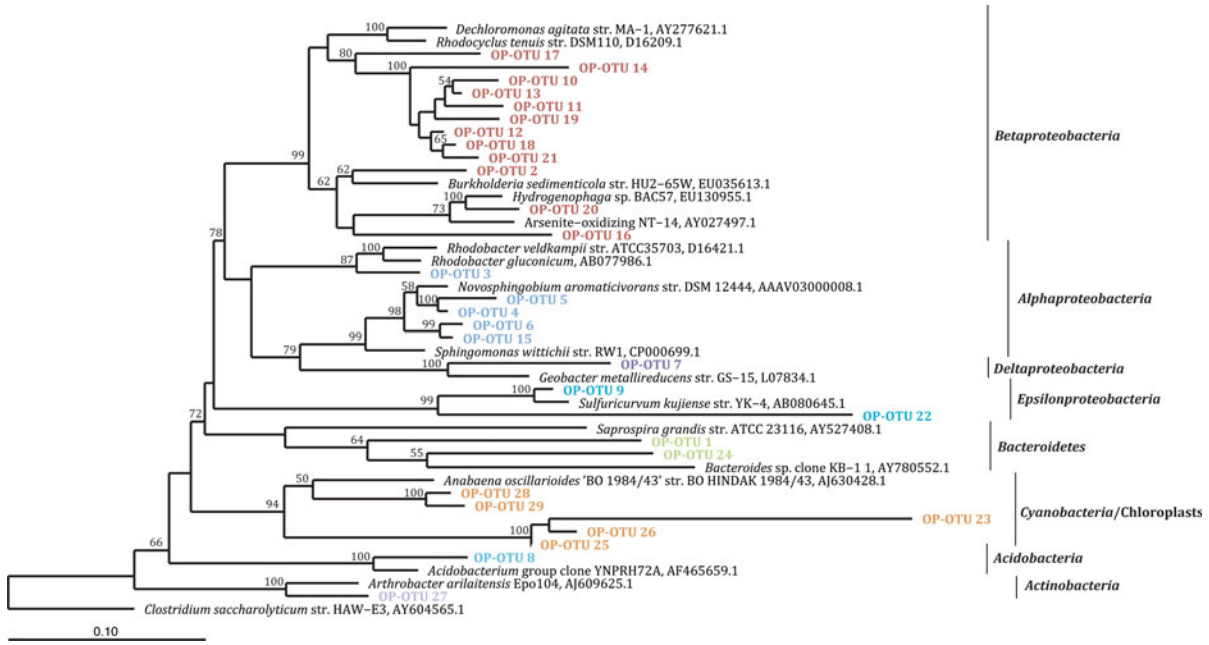


Fig. 7 Neighbor joining phylogenetic tree based on partial 16S rRNA gene sequences showing the positions of the Tab-Simco Oxidation Pond clone sequences grouped into OTUs and close relatives. OP OTUs are represented in *bold* and color-coded to correspond to Fig. 3d. Bootstrap values (1,000 replicates) above 50% are represented at the nodes. The *scale bar* represents 0.10 changes

and OP, respectively, and was most closely related to an environmental bay clone (Leloup et al. 2009) (CAQ77306.1) at 85% identity and to the isolate *Desulfobacca acetoxidans* at ~73% identity (Online resource 8). Thus indicating that microbes related to *Desulfobacca acetoxidans*, which has been shown to only use acetate as an organic carbon source (as reviewed by Dar et al. 2007), predominate in the acid mine drainage post treatment.

Discussion

Overall performance of the bioreactor

The aim of this study was to investigate the performance and microbial community dynamics of a passive flow sulfate-reducing bioreactor installed to treat acid mine drainage at the Tab-Simco abandoned coalmine. Geochemical data indicated that the bioreactor was successful in raising the pH, removing metals, and decreasing the sulfate content of the mine drainage. The Tab-Simco mine is not considered an extremely acidic environment with the AP having a pH of 3.09, but the sulfate-reducing bioreactor was still very adept at increasing the pH to circumneutral (Table 1). While this increase in pH can be partially attributed to the addition of limestone in the construction of the bioreactor, it is evident that there is SRB activity within the system. The stimulation of microbial sulfate reduction was indicated by the +2.60‰ increase in $\delta^{34}S$ content of the sulfate remaining in the treated water (Table 1; Fig. 2a). The BRO showed a reduction in sulfate levels by 45.2% and further sulfate reduction within the OP showed an overall reduction of sulfate levels by 67.4% (Table 1). Attempts to optimize sulfate-reducing bioreactors treating such waste with bench-scale systems and optimized inocula can reach overall 75% sulfate reduction (Schmidtova and Baldwin 2010). This rate of removal of nearly 68% lies in the upper range of long-term bioreactors treating similar sulfate levels even if the end levels of sulfate remain higher than the recommended drinking water levels (Hiibel et al. 2008; Steed et al. 2000; Boshoff et al. 2004). Additionally, the system showed an overall removal of approximately 95% of total iron with variable removal of other secondary metals (Table 1; Online resource 1).

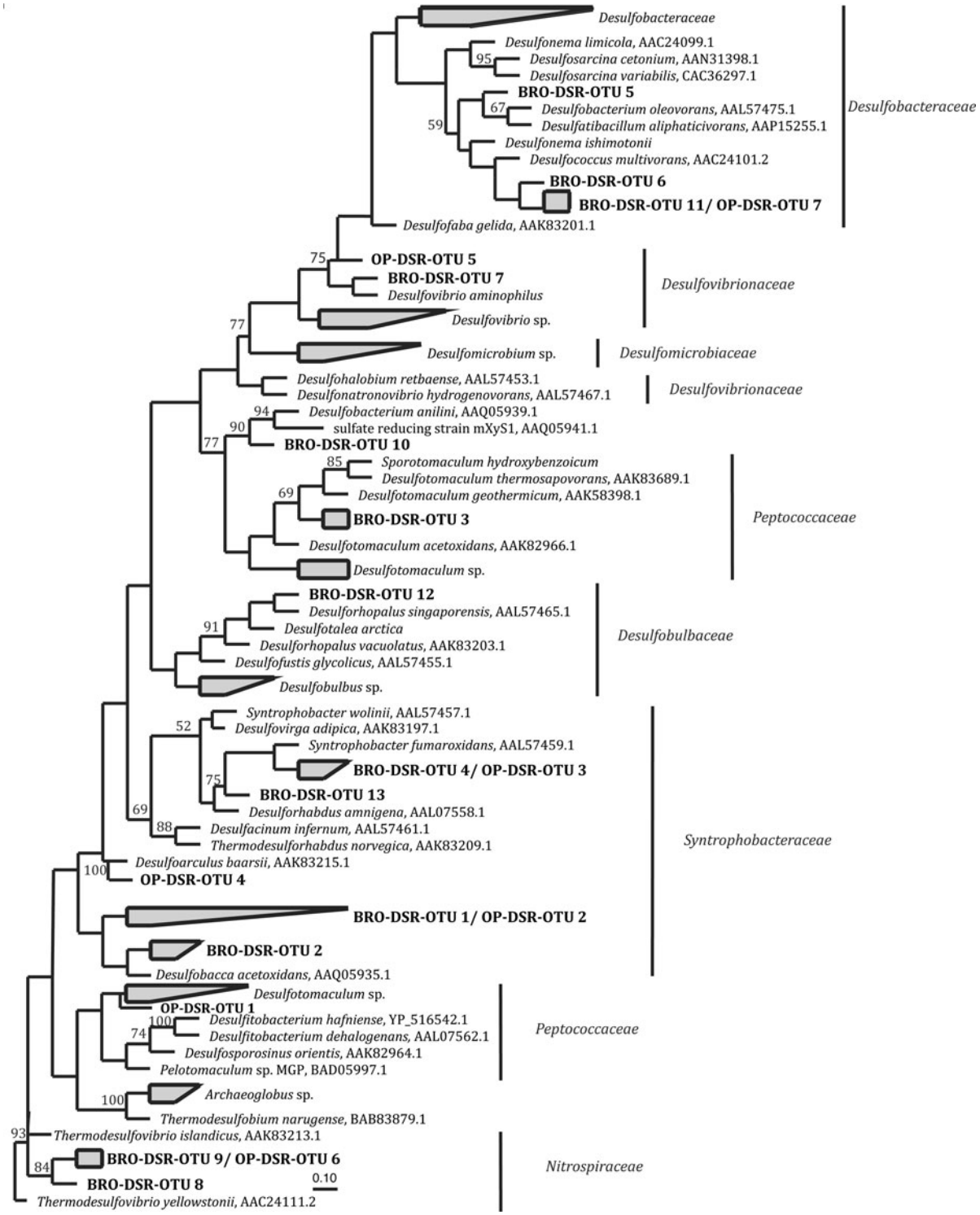


Fig. 8 Maximum likelihood tree based on the first 150 amino acids of the DsrA protein of Tab-Simco site sequences and close relatives. Bioreactor outflow and outlet pond OTUs are represented in *bold*. Bootstrap values (1,000 replicates) greater than 50% are represented at the nodes. The *scale bar* represents 0.10 changes

To monitor the effect the bioreactor had on the microbial community, 16S rRNA and *dsrAB* genes were targeted. The *dsrAB* genetic locus was selected to specifically target sulfate-reducing bacteria. Despite the intrinsic biases and limitations associated with molecular techniques, as well as the limited sampling performed here, a 'snapshot' of the microbial community present in each region was obtained. This preliminary data allows for hypotheses to be drawn regarding the ability of the bioreactor to stimulate specific types of bacteria.

Molecular analysis of the four sampling sites indicated that the microbial community shifted based on the geochemical parameters of the site. While archaeal-type sequences have been detected in extremely harsh AMD sites (Baker et al. 2003; Reysenbach and Pace 1995), no such sequences were detected in any of the four samples from the Tab-Simco mine, suggesting that the Tab-Simco site possesses moderately stressful environmental conditions. *dsrAB* sequences were also only obtained from the post-treated AMD samples, suggesting that the bioreactor was successful in stimulating SRB. The absence of *dsrAB* related sequences detected in the pre-treated samples is likely due to the acidic pH and high iron concentrations of the samples. While the *dsrAB* locus has been detected in sites affected by AMD, low pH and metal concentrations similar to those found in AMD have been shown to inhibit the activity of SRB (Moreau et al. 2010).

The limited diversity of bacterial 16S rRNA sequences and the predominance of *Betaproteobacteria* phylotypes related to Fe^{2+} oxidizers detected in both the WB1 and AP pre-treated samples were expected due to the acidic pH and high metal concentrations. Microbial recycling of Fe^{3+} would lead to further oxidation of pyrite, exacerbating the proton and sulfate loads. While WB1 was dominated by phylotypes similar to *Gallionella* and *Sideroxydans* species that are generally associated with circumneutral pH environments (Emerson and Moyer 1997), similar 16S rRNA sequences have been detected in acidic mine environments (Lüdecke et al. 2010; Heinzel et al. 2009b; He et al. 2007). *Gallionella* has also been shown to grow chemolithotrophically and dominate in microaerophilic waters where the total organic carbon ranges between <0.5 and 2.0 ppm and low concentrations of nitrogen and phosphorus exist (Cullimore 1999). Because of the underground nature of WB1, similar conditions would be expected to exist.

The shift to *Betaproteobacteria* phylotypes related to the uncharacterized iron oxidizer *Ferrovum myxofaciens* (Heinzel et al. 2009b) (Fig. 5) in the AP sample is likely a result of the increased oxygen and iron content of the AP water and/or increased nutrient availability due to run-off from the surrounding area. Thus it is tempting to speculate that *Ferrovum* species may have a higher O_2 and/or iron tolerance compared to the *Gallionella* and *Sideroxydans* phylotypes detected in the WB1 sample. *Ferrovum* (formally known as *Ferribacter*) like sequences have also been detected in other mining sites with a pH > 2 (Brown et al. 2011; Heinzel et al. 2009b; Heinzel et al. 2009a). Therefore this molecular analysis suggests that iron oxidization remains the predominant microbial metabolism prior to passage through the bioreactor.

Of particular interest was the finding that 15% of the total clones from the AP shared low sequence similarity with the 16S rRNA gene of cyanobacteria and chloroplasts (Fig. 3b). Since cyanobacteria are generally not present in acidic environments (Whitton 2000), it is likely that these sequences originate from algal chloroplasts. Universal bacterial primers in other culture independent community studies have also amplified sequences of small ribosomal subunits of plastids and chloroplasts (Lear et al. 2009; Ruckmani and Chakrabarti 2011). Because the percent similarity of the sequence is <97%, these OTUs potentially represent a new taxon (Stackenbrandt and Goebel 1994). Algae have been shown to tolerate extremely low pH (0.05) and have been found in acidic environments if adequate nutrients are available (Fryson et al. 2006; Rowe et al. 2007). Algae can influence AMD by complexing metals into the cell wall and through extrapolymeric substance (EPS) formation (Das et al. 2009). Algae have also been shown to contribute up to 60% of the biomass present in the Rio Tinto, an acidic river in Spain, which contains a high concentration of iron from pyrite dissolution (Amaral Zettler et al. 2001). Thus, the presence of these types of organisms will increase the oxygen load and carbon availability of the bioreactor pool.

Due to the compost seeded in the bioreactor, it was not surprising that the BRO sample possessed the greatest community diversity. This is evident by the number of OTUs present and their affiliation with

saccharolytic groups (Fig. 3c). Thus, the bioreactor was successful in stimulating cellulolytic bacteria to convert the inexpensive substrate to simpler carbon sources. However, the predominant phylotype from this sample was most closely related to the strictly autotrophic sulfur oxidizer *Sulfuricurvum kujiense* (Fig. 6). This member of the *Epsilonproteobacteria* is a facultative anaerobe capable of oxidizing reduced sulfur under microaerophilic and neutrophilic conditions. Unfortunately, sulfur-oxidizing bacteria create more H^+ through sulfuric acid production (Fliermans and Brock 1972) and thus reverse the desired bioreactor process.

Sequence analysis indicated that only ~11% of the 16S rRNA clones from the BRO possessed similarity to known sulfate-reducing bacteria and that the *dsrAB* sequences obtained were of low diversity. However, the sampling method was only able to monitor the planktonic community immediately out-flowing from the bioreactor. Therefore, it is possible that sulfate-reducing biofilms similar to those previously detected in a fixed-bed reactor treating AMD (Koschorreck et al. 2010) are present in the bioreactor channel. Further monitoring and sampling is ongoing to determine the attached community in the bioreactor. The majority of the DsrA clones detected were most similar to *Desulfobacca acetoxidans*, a sulfate reducer originally isolated from sludge that utilizes acetate as a carbon source and not propionate, butyrate, lactate, ethanol, propanol, hydrogen, formate, or glucose (Online resource 8) (Oude Elferink et al. 1999). The limitation of acetate utilization as a carbon source also suggests that other microbes present are efficient at metabolizing the compost supplied in the bioreactor to simple carbon sources and/or the presence of primary producers such as autotrophs. *D. acetoxidans* also requires circumneutral pH for growth and possesses a 1.7–2.2 day doubling time at 37°C. Similar *dsrA* and 16S rRNA gene sequences have also been detected in mining sites and a sulfidogenic bioreactor (Neculita et al. 2007). This suggests that this predominant DsrA OTU is resistant to metals and possesses a very slow growth rate. Therefore, this microbe is likely not responsible for the bulk of sulfate reduction taking place in the bioreactor. However, it is important to note that the *dsrAB* locus has been shown to be horizontally transferred (Klein et al. 2001) and that this OTU is only ~73% similar to *D. acetoxidans* and could possess different metabolic abilities.

While the geochemical analysis indicated that the OP remained at a circumneutral pH and possessed the lowest iron content of all the samples, (Table 1; Fig. 2) the predominance of *Sideroxydans*-like phylotypes suggests that the community in the treated AMD shifted back to an iron-oxidizing environment. This OTU was also identified as one of the two dominant iron oxidizers detected in the original WB1 sample. Thus, the activity of iron-oxidizing bacteria would assist in oxidizing the Fe^{2+} present in the circumneutral water to insoluble precipitates for removal from the water. Finally, sequences affiliated with cyanobacteria or chloroplasts were also present. As with the AP sample, we hypothesize that these sequences represent algae present in the OP and provide carbon for the in situ community as well as oxygen release in the pond to assist in precipitating iron minerals. The dominant DsrA OTU from the OP was also most similar to *D. acetoxidans*, further supporting that this phylotype is an abundant member of the planktonic sulfate-reducing community stimulated by the bioreactor (Online resource 8).

This study provides critical information regarding the performance of an in situ sulfate-reducing bioreactor treating acid mine drainage from an abandoned coal mine affected by local run-off. While geochemical profiling revealed that the bioreactor is successful in mitigating the pH, metal, and sulfate concentrations, further analysis is needed to determine that the bioreactor is able to continue this high level of functioning over a longer period of time. Our sampling of the microbial population revealed essential knowledge into the key microbes and metabolisms occurring within the system and more exhaustive sampling would provide further insight into the diversity of SRBs within the bioreactor. This community analysis and geochemical profile suggests the success of the current bioreactor design and the data set provides a base line for future studies to determine if variables such as longer retention times or additional substrates could further enhance the ability of the bioreactor to stimulate SRB and reduce the levels of sulfur oxidizers, thus allowing the system to further mitigate the environmental damages of AMD.

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