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TECHNICAL ARTICLE

Biological Source Treatment of Acid Mine Drainage Using Microbial and Substrate Amendments: Microcosm Studies

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Abstract Microcosm studies in the laboratory demonstrate that sufficient dosages of wastewater effluent (microbial inoculum) and returned milk (substrate) can effectively raise the pH of pyrite-amended acid mine drainage water to circumneutral levels under aerobic conditions in as little as 7 days, and the pH remains at these levels for >19 months. Microbial analysis indicates that a complex biofilm (>70 species) forms over the pyrite. The biofilm dominantly consists of facultative anaerobes, which potentially interact with obligate anaerobes, such as sulfate-reducing *Desulfosporosinus* sp., to maintain an oxygen-free micro-environment surrounding the pyrite, even though the overlying water remains aerobic. The biofilm became established in water samples with an initial pH as low as 2, and subsequently caused the water pH to increase to circumneutral levels. Concurrently, concentrations of Al, As, Cu, Fe, Pb, Ni, and Zn all decreased substantially compared to baseline concentrations in the control microcosms.

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Introduction

Acid mine drainage (AMD) poses a serious threat to ecosystems (Levings et al. 2004) as well as human health (Batten and Scow 2003; Carlson et al. 2002). Well-characterized metal sulfide minerals such as pyrite (FeS_2), when exposed to dissolved oxygen (DO) in water, can oxidize and dissociate into sulfate and protons (acidity), lowering the pH of the surrounding environment. Under these conditions, acidophilic *Acidithiobacillus ferrooxidans* can catalyze the oxidation of metal sulfide minerals and generate AMD (Johnson et al. 2002). Treatment strategies for AMD have ranged from biotic to abiotic and passive to source treatment technologies. Biotic treatment strategies that use microorganisms to reduce sulfate into sulfide, which results in metal-sulfide precipitates and proton consumption, were first envisioned decades ago (Tuttle et al. 1969).

Considerable research has been conducted investigating the use of bacteria, dominantly sulfate reducing bacteria (SRB), to remediate AMD effluent (Chang et al. 2000; Dvorak et al. 1992; Elliot et al. 1998; Johnson et al. 2002; Jong and Parry 2003; Lyew et al. 1994; Machemer and Wildeman 1992; Tabak et al. 2003; Van Houten et al. 1994; Webb et al. 1998). These techniques, generically defined as "passive treatments", treat AMD effluent away from the source; as long as oxygenated water flows through the source material, the AMD load on these systems continues indefinitely. In cases where remediation work is underway, most treatments focus on treating the effluent by (1) raising the pH to circumneutral or alkaline levels using additives that range from limestone to sodium hydroxide (Coulton et al. 2003; Doye and Duchesne 2005; Johnson and Hallberg 2005; Kleinmann et al. 1998; Skousen et al. 1998), (2) aerating effluent to form mineral precipitates

(Skousen et al. 1998), (3) adding coagulants to form mineral precipitates (Skousen et al. 1998), and (4) running the effluent through wetlands and other biological systems (Johnson and Hallberg 2005; Skousen et al. 1998; Webb et al. 1998). Depending on the situation, any combination of these treatments (and others) may be employed to raise the effluent pH and remove high concentrations of potentially toxic metals including Cd, Cu, Fe, Pb, Ni, and Zn. These methods usually produce large amounts of waste sludge (some toxic with high metal concentrations) that must be disposed of, which can create secondary disposal problems and increased cost. Additionally, because these treatments occur on the surface, they are subject to high flows during spring runoff and fluctuating temperatures that can negatively affect the performance of biological and passive effluent treatments.

Comparatively, limited work has focused on using bacterial consortia such as SRB to treat and prevent AMD at its source (Adams et al. 1995; Canty 1998; Fallgren and Jin 2005; Kim et al. 1999). In order to bioremediate AMD at its source, the mechanisms behind source control of AMD must be understood. These mechanisms include proton consumption and bicarbonate production through sulfate reduction and the normal metabolism of SRB and other associated microbes, as well as the potential formation of biofilm and biofilm-like structures on AMD source material, which creates a robust barrier to oxygen diffusion, pyrite oxidation, and consequent AMD generation (Characklis 1990; Christensen and Characklis 1990; Elsetinow et al. 2003; Zhang et al. 2003a, b, 2006).

The purpose of this research was to determine if suitable microbial inoculum and sufficient substrates could treat and prevent AMD for an extended period of time in AMD water and pyrite. Several microcosm studies were conducted in the laboratory to (1) determine if a robust consortium of SRB and other bacterial communities exist and function actively in our inoculum source, (2) identify the optimum inoculum and substrate dosages, (3) evaluate if the microbial consortia could survive and be effective at low pH, and (4) analyze the microbial community in the biofilm growing on pyrite. These experiments were conducted in support of developing a biological source treatment (BST) technique for identifying and treating sources of AMD in the field.

Materials and Methods

Ground Water and SRB Inoculum

Ground water was collected from a backfilled area impacted with AMD at the Sequatchie Valley Coal Mine, near Dunlap, Tennessee. A bladder pump was used to collect ground water from a 10.6-cm diameter well with a water column depth of 3.2 m. The pump was allowed to purge three full well volumes of ground water before water collection. Ground water was collected in 19-L plastic containers with zero headspace, sealed, and shipped to the laboratory where it was stored at 4°C and used in microcosm studies. Effluent/solids (ES) were collected from a nearby wastewater treatment plant in Dunlap, Tennessee, and used as the SRB-containing inoculum. Our previous work and the work of others have demonstrated that high concentrations of SRB are present in ES (Fallgren and Jin 2005; Ingvorsen et al. 2003; Kjeldsen et al. 2004; Lens et al. 1995; Manz et al. 1998; Schramm et al. 1999). The presence of SRB in this specific source was verified with biological activity reaction test (BART) tubes (HACH, Loveland, CO, USA). The BART test semi-quantitatively enumerates the population of SRB based on sulfide produced from sulfate reduction. Dairy waste (returned milk), obtained from a dairy farm in Southern Tennessee, was used as the substrate and nutrient source based on favorable results from a previous study we conducted using this substrate source (Fallgren and Jin 2005). The AMD ground water and returned milk were analyzed for dissolved (0.45 μm filter) organic carbon (DOC), ammonium nitrogen (NH_4^+–N), chloride (Cl^-), nitrate nitrogen (NO_3^-N), sulfate (SO_4^{2-}), Al, As, Ca, Cu, Fe, Mg, Ni, K, Na, and Zn, as described in Chemical Analyses below.

Microcosm Experiments

In all of the following experiments, microcosms were established in triplicate in 125-mL serum bottles under aerobic conditions. Pyrite obtained from a mineral vendor (Laramie, Wyoming) served as AMD source material. Pyrite was crushed to particle size of <0.850 mm. Each microcosm contained 100 mL of ground water collected from the field site and 3 g of crushed pyrite. Pyrite was washed with deionized water before being added to the microcosms. All microcosms were incubated at room temperature (22–25°C) in the dark and left unsealed and exposed to air to maintain aerobic conditions. Dissolved oxygen was measured at the beginning and ending of the test and intermittently during the incubation period to verify aerobic condition in the microcosms. A Thermo probe was used for DO measurement in aliquots immediately sampled from the microcosms. The sample was taken upon slight agitation of the microcosms to ensure the measured DO reflected the actual redox condition.

ES Inoculum Dosage Experiment

The purpose of this experiment was to determine the optimal amount of ES needed to successfully inoculate the

microcosms with a bacterial consortium; therefore, different concentrations of ES were added to microcosms containing lactate as a substrate. The amount of lactate added was based on the stoichiometric concentration of carbon required by SRB to deplete sulfate in the microcosms through sulfate reduction. The concentrations of ES inoculum added to each microcosm were 0.1, 1.0, and 3.0 weight % (wt%) of the volume of water in each bottle. These concentrations were chosen based on results from a pilot test we conducted using ES concentrations ranging from 0.01 to 10 wt% (data not shown). Sodium L-lactate was added at a final concentration of 1.0 wt% ($\approx$89 mM) to all microcosms. Microcosms containing no substrate or inoculum served as controls. All microcosms were set up at an initial pH between 5.3 and 5.7 and we determined whether sulfate-reduction was occurring based on sulfate depletion and the presence of black precipitates (possibly metal sulfide) in the microcosms. Dissolved oxygen was measured at the end of the experiment to verify that the overlying water in each microcosm remained aerobic; pH was monitored periodically throughout the experiment.

Substrate Dosage Experiment

The purpose of this experiment was to determine the optimal substrate and ES dosage combinations required to prevent acid generation in each treatment. Microcosms were established with six different treatment combinations of substrate (0, 0.05, 1, and 5× the stoichiometrically required amount of C) and ES inoculum (0 or 3 wt%; Table 1). The stoichiometrically required amount of C (returned milk) is the amount needed to completely deplete the sulfate concentration in each microcosm, assuming all sulfate is used as a terminal electron acceptor by SRB utilizing returned milk as a sole electron donor. For example, 2 mol of C are consumed for every mole of sulfate reduced; therefore, we added approximately 40 mM of C to corresponding microcosms that contained approximately 20 mM of sulfate (Table 2) or 100% of the required stoichiometric concentration of C. To calculate this, we corrected the DOC concentration in the returned milk (1,458 mM of C) by subtracting the 14 mM of the C that would be consumed upon reduction of the 7 mM of sulfate already in the returned milk, which left us with 1,444 mM of C. Therefore, we added 2.8 mL of returned milk to the 100 mL of water in each microcosm that was to receive 100% of the required stoichiometric concentration of C. Microcosms containing substrate only, ES inoculum only, or no amendments served as controls. This experiment ran for 571 days. To offset water loss due to evaporation from these unsealed vials and maintain a relatively constant volume in the microcosms, 50 mL of deionized water was added to each microcosm on day 70. Microcosms containing returned milk received an additional milk amendment (equivalent to 0.05× the stoichiometric requirement of C given the sulfate concentrations at the beginning of the test) on day 153 due to decreasing trends in pH (see Results section). The pH of each microcosm was monitored periodically throughout the experiment. At the end of the 571-day incubation period, pyrite was collected for microbial characterization of biofilm, and water samples from one microcosm from each treatment were collected for analysis of Al, As, Cu, Ni, Pb, and Zn on day 571.

Table 1 Treatments for substrate dosage study

Treatment	Proportion of required C	wt% ES
1	0	0
2	1.0	0
3	0	3
4	0.05	3
5	1.0	3
6	5.0	3

Proportion of the stoichiometric requirement of C (added as returned milk) needed to completely deplete sulfate in each microcosm, and the weight % of effluent solids (ES; 88% moisture) added to each treatment

Threshold pH Experiment

An additional experiment was conducted to determine the threshold pH at which the BST technique could

Table 2 Baseline chemical parameters of acid mine drainage (AMD) water and returned waste milk used in microcosm experiments

Parameter	AMD water	Returned milk
PH	4.78	4.51
DOC (mg L^{-1})	BDL	17,510
Cl^- (mg L^{-1})	3.6	1,000
SO_4^{2-} (mg L^{-1})	1,917	674
NH_4–N (mg L^{-1})	15.1	7.1
Ca (mg L^{-1})	151	10.4
Fe (mg L^{-1})	230	BDL
K (mg L^{-1})	16	13.7
Mg (mg L^{-1})	150	0.97
Na (mg L^{-1})	12	4.8
Al (μg L^{-1})	120	8.5
As (μg L^{-1})	9.4	BDL
Cu (μg L^{-1})	1.7	3.0
Ni (μg L^{-1})	993	BDL
Zn (μg L^{-1})	601	60.7

BDL below detection limit

successfully begin to raise and maintain the pH in an oxidizing environment similar to that in an AMD situation. Microcosms used in this experiment were established in triplicate with 3 g of pyrite (as in the other experiments), 3 wt% ES, and 1.4× the required stoichiometric concentration of C (as returned milk) at nominal pH values of 2 to 4. Control treatments did not receive an inoculum or substrate amendments and pH values were adjusted with sulfuric acid (H_2SO_4). Microcosms were incubated for 522 days, sampled periodically for changes in pH, and samples from one microcosm from each treatment were collected for analysis of Al, As, Cu, Ni, Pb, and Zn on day 522.

Microbial Characterization of Biofilm

Pyrite was collected from a microcosm from the substrate dosage experiment that received 3 wt% ES and 5× the required stoichiometric C concentration (as returned milk) and divided into two subsamples. One subsample was mounted to a glass slide, coated with carbon, and photographed with a JEOL model 5800LV scanning electron microscope (JEOL USA Inc., Peabody, MA, USA) at an accelerating voltage of 10 kV.

The other subsample was processed with the MO BIO Soil DNA kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) to extract DNA from the sample. Following extraction, 16S rRNA genes from the total bacterial community were amplified using polymerase chain reaction (PCR) with universal primers 341F (Muyzer et al. 1993) and 1492R (Weisburg et al. 1991) to amplify ≈1,100 bp portions of these genes. The PCR product was then cloned into *Escherichia coli* using the Invitrogen TOPO TA cloning kit (Invitrogen Corporation, Carlsbad, CA, USA) and the presence of inserts was verified using M13 PCR specific to the cloning vector. A total of 136 clone inserts were randomly chosen and an *MSP1* restriction enzyme analysis was performed in order to compare inserts and lump them into categories of "same" or "unique". Clone inserts that occurred more than once and the majority of inserts occurring only once were sequenced to determine their identity (a total of 90 inserts were sequenced). DNA sequences were compared with the National Center for Biotechnology Information (NCBI) Blast database (Altschul et al. 1997) to determine the identities of the microorganisms in the sample.

Chemical Analyses

All chemicals used for this study were reagent grade and purchased from Sigma-Aldrich (St Louis, MO, USA), unless otherwise indicated. The pH was measured with an Orion® Thermo model 720A+ pH meter equipped with an Orion® Ag/AgCl combination electrode. Chloride, NO_3^-N, and SO_4^{2-} were analyzed by ion chromatography on a DIONEX DX-100 ion chromatograph (Sunnyvale, CA, USA). Ammonium–nitrogen was measured by the indophenol blue colorimetric method (Keeney and Nelson 1982) on a Shimadzu UV-VIS spectrophotometer (Columbia, MD, USA). Dissolved organic carbon was analyzed on a Shimadzu total organic carbon analyzer (Columbia, MD, USA). Cations including Ca, K, Mg, and Na were analyzed by atomic absorption spectrophotometry. Metalloids and metals including Al, As, Cu, Fe, Ni, Pb, and Zn were analyzed by inductively coupled plasma-mass spectrometry (ICP-MS). All water samples were filtered (0.45 μm) and cation samples were acidified (pH ≤ 2) with trace-metal grade nitric acid (HNO_3) and DOC samples were acidified (pH 2–3) with HCl. BART tubes (HACH, Loveland, CO, USA) were used to detect and quantify SRB populations in the samples.

Results

Baseline Characterizations

The pH of the AMD water and the returned milk was slightly acidic (4.8 and 4.5, respectively; Table 2). The DOC concentration in the AMD water was below detection limit (<0.08 mg/L); however, the DOC concentration in the returned milk was high (17,510 mg/L; Table 2). Ammonium was detected at concentrations of 15.1 and 7.1 mg/L, respectively, in the AMD water and returned milk. The ammonium concentration is within the normal stoichiometric concentration range that supports microbial activity (based on a molar C:N:P ratio of 100:10:1). Concentrations of NO_3^-N were below detection limit (0.1 mg/L) in the AMD water and returned milk. Concentrations of all other baseline parameters are listed in Table 2.

ES Inoculum Dosage Experiment

The ES inoculum contained an SRB population of approximately 700,000 cfu/mL based on the BART test. The moisture content of the ES inoculum was 88%. The ES inoculum dosage experiment indicated that adding 1 wt% lactate and 1 to 3 wt% ES inoculum was required to raise the pH initially, but that only a 3 wt% ES inoculation could maintain a circumneutral pH for up to 35 days in the laboratory (Fig. 1a). The pH of non-amended microcosms, microcosms amended with 1 wt% lactate only, and microcosms amended with 1 wt% lactate and 0.1 wt% ES

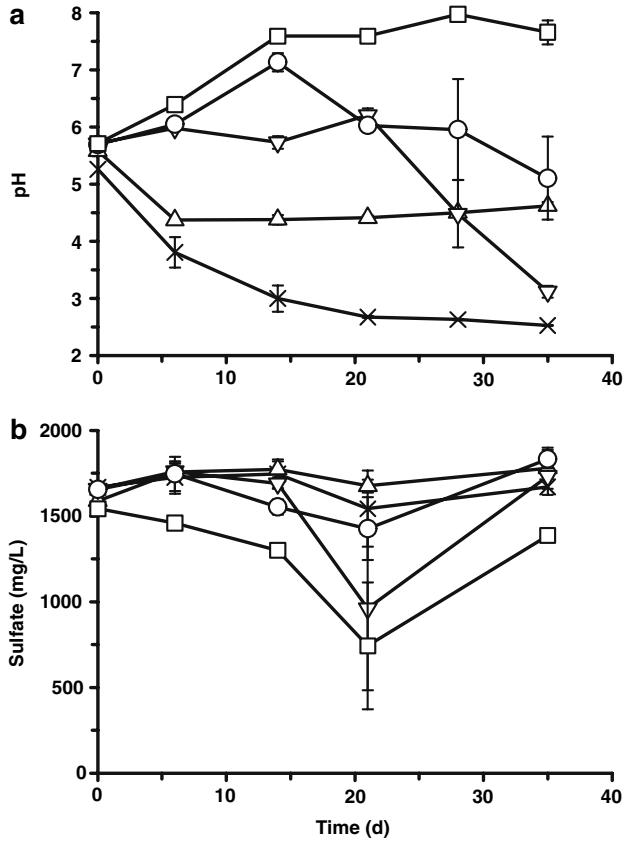

Fig. 1 ES inoculum dosage experiment: changes in pH (**a**) and sulfate (**b**) in microcosms filled with acid mine drainage water, pyrite, and amendments of effluent solids (*ES*) and (or) carbon (*C*). The treatments receiving C received 1% of the required stoichiometric concentration of C (as sodium lactate) that bacteria would consume while reducing all the SO_4^{2-} in each microcosm. The treatments included a control with no ES or C amendments (X), C only (*open triangle*), C and 0.1 wt% ES (*inverted open triangle*), C and 1 wt% ES (*open circle*), and C and 3 wt% ES (*open square*). *Error bars* are mean ± standard error (n = 3)

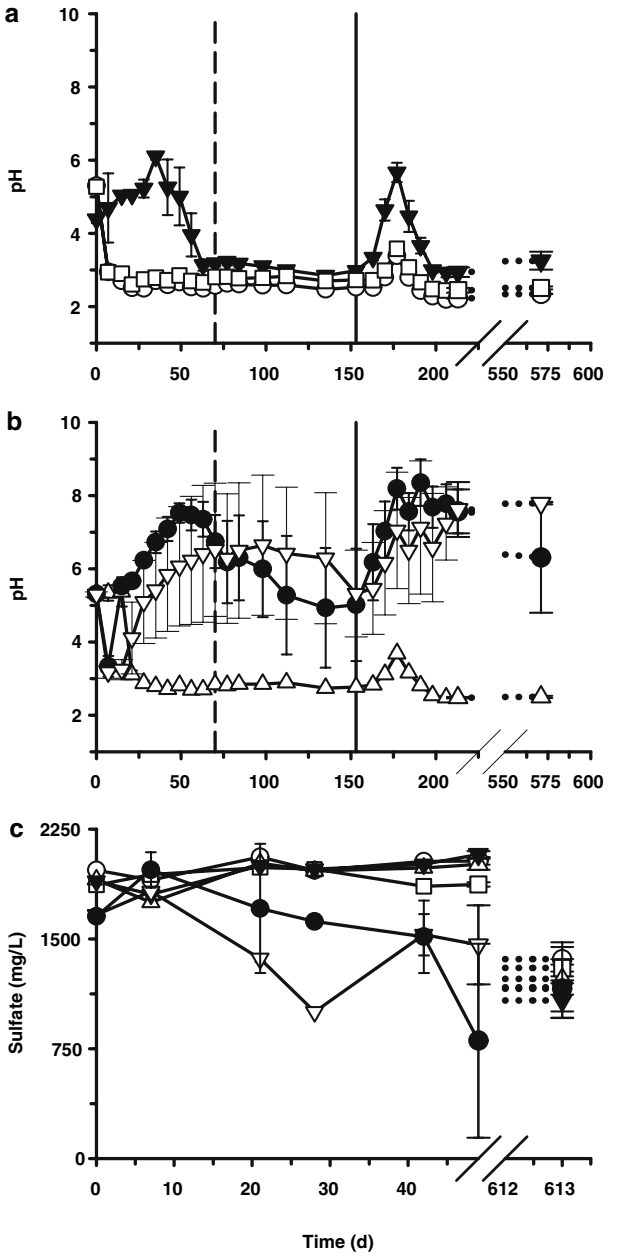

Fig. 2 Substrate dosage experiment: changes in pH (**a**, **b**) and sulfate (**c**) in microcosms filled with ground water impacted with acid mine drainage, pyrite, and amendments of effluent solids (*ES*) and (or) carbon (*C*). The treatments amended with C received 0.05, 1, or 5× the required stoichiometric concentration of C (as returned milk) that bacteria would consume if they reduced all the SO_4^{2-} in each microcosm. All treatments that contained ES received 3 wt% (moisture content was 88%). The treatments included a control with no ES or C amendments (*open circle*), ES (*open square*), 1× C (*inverted filled triangle*), ES and 0.05× C (*open triangle*), ES and 1× C (*filled circle*), and ES and 5× C (*inverted open triangle*). All microcosms received 50 mL of distilled water on day 70 (*dashed vertical line*) and microcosms containing returned milk received an additional 0.05× the stoichiometrically required C concentration on day 153 (*solid vertical line*). No samples were taken for pH analysis between days 213 and 571 in **a** and **b** and no samples were taken for sulfate analysis between days 70 and 613 in **c** (indicated with *dotted lines*). *Error bars* are mean ± standard error (n = 3)

inoculum declined from ≈5.7 to 2.5, 4.6, and 3.1, respectively, within 35 days (Fig. 1a). Sulfate concentrations in treatments that received C and an ES inoculum appeared to decrease initially but then returned to beginning values, suggesting little SRB activity occurred throughout the experiment (Fig. 1b).

The overlying water in all treatments remained aerobic and by 23 months of incubation, the DO concentration in the control treatment (no amendments) was 6.5 (±0.1) mg/L. The DO in treatments receiving 1 wt% lactate and 3 wt% ES was 6.3 (±0.2) mg/L. Interestingly, the pH in the control continued to drop, and by 23 months was 1.8 (±0.04) while the pH in the 1 wt% lactate and 3 wt% ES treatment had continued to increase to 9.7 (±0.01). It was not necessary to measure redox potential in all microcosms due to the high DO concentrations.

Substrate Dosage Experiment

The initial pH in the microcosms ranged from 4.3 to 5.4 (Fig. 2a, b). The pH in the treatments with no amendments, C only, and ES only decreased to 2.3, 3.3, and 2.5, respectively, during this 571-day experiment (Fig. 2a). The pH in the treatments amended with ES and 1 and 5× the required stoichiometric concentration of C increased after day 7 to nearly 8, then decreased until day 153 when additional C was added to the microcosms, after which the pH increased again and remained in the cricumneutral range for the remainder of this experiment (Fig. 2b). The pH did not increase in the treatment containing 3 wt% ES and 0.05× the required C concentration (Fig. 2b).

Sulfate concentrations steadily decreased in treatments containing 3 wt% ES and 1 and 5× the required C concentration, suggesting sulfate reducing activity could have occurred in these treatments. The sulfate concentration did not decrease in the remaining treatments. Although the final sulfate concentration measured on day 613 appeared to decrease substantially in these treatments, this was due to dilution after the addition of 50 mL of de-ionized water to the microcosms on day 70 (Fig. 2c).

Concentrations of Al, Cu, and Fe decreased to well below baseline in treatments that received ES and 1 or 5× the stoichiometrically required C concentration (Fig. 3a, c, d, respectively). Similarly, concentrations of Ni and Zn decreased substantially in treatments that received 5× the

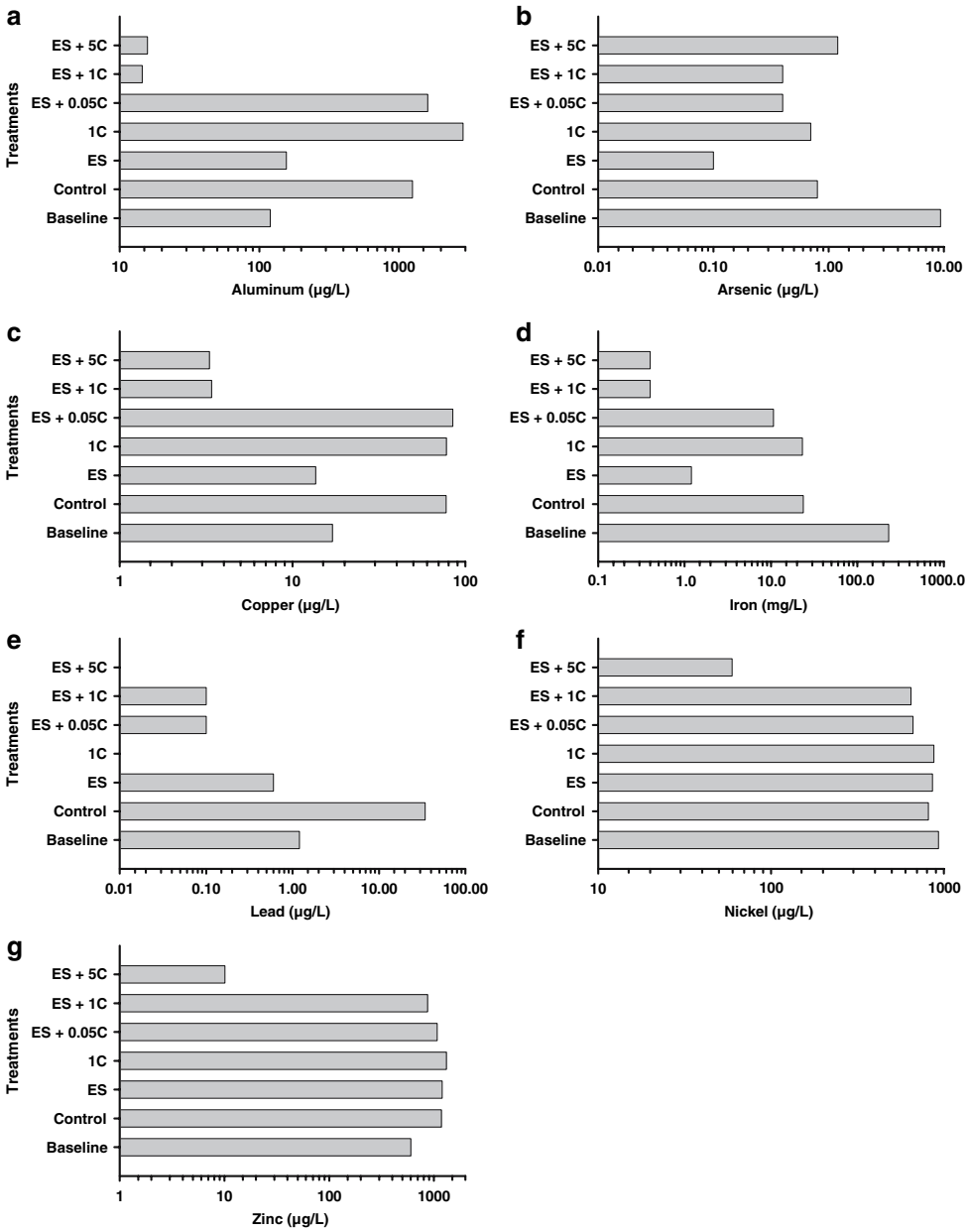

Fig. 3 Substrate dosage experiment: concentrations of aluminum (**a**), arsenic (**b**), copper (**c**), iron (**d**), lead (**e**), nickel (**f**), and zinc (**g**) in microcosms filled with ground water and AMD, pyrite, and amendments of effluent solids (*ES*) and (or) carbon (*C*). The treatments amended with C received 0.05, 1, or 5× the required stoichiometric concentration of C (as returned milk) that bacteria would consume if they reduced all the SO_4^{2-} in each microcosm. All treatments that contained ES received 3 wt%. The treatments included a control with no ES or C amendments (control), ES (ES), 1× C (1C), ES and 0.05× C (ES + 0.05C), ES and 1× C (ES + 1C), and ES and 5× C (ES + 5C). Baseline concentrations of metals (baseline) were measured in AMD water 2 days prior to addition to microcosms and samples were collected for final metal analysis on day 571

stoichiometrically required C concentration (Fig. 3f, g, respectively). Lead concentrations decreased in all treatments except the control, which increased (Fig. 3e). Concentrations of As decreased the most in the treatment that received ES only (Fig. 3b) and the least in the treatment that received 5× the stoichiometrically required C concentration, which may be due to the lower pH in the ES treatment, which would not cause dissolution of As-containing minerals and would promote sorption of negatively charged dissolved As to surfaces in the microcosms.

Threshold pH Experiment

The pH in microcosms initially adjusted to pH 2 surpassed pH 6 within 100 days and gradually increased to >7.5 during the remaining 452 days (Fig. 4a). Additionally, the pH in the microcosms that had initially been adjusted to pH 3 and 4 surpassed pH 6 within 40 days, surpassed pH 7.5 within 75 days, and remained relatively constant during the remaining 477 days of this 552-day experiment (Fig. 4b, c, respectively).

Concentrations of Al, Cu, Ni, Pb, and Zn increased over baseline values in controls and decreased in treatments that received ES and C (Fig. 5a, c, e–g, respectively), except for the Al concentration in the treatment initially adjusted to pH 4 that received ES and C, which increased slightly over baseline (Fig. 5a). Iron concentrations decreased in all controls and treatments; however, concentrations were lower in treatments that received ES and C than in controls (Fig. 5d). Overall, As concentrations decreased in all treatments with no obvious trends in the data (Fig. 5b).

Microbial Characterization of Biofilm

Scanning electron microscope images show a biofilm-like structure covering the majority of the pyrite surfaces in the subsample taken from a microcosm containing ES and a high concentration of returned milk (Fig. 6). The diversity of the microbial community in this biofilm was unexpectedly high (>70 different species). The majority of the microbes identified were associated with anaerobic environments and most of the aerobic microbes identified were facultative denitrifiers. One confirmed sulfate-reducer was identified in the sample (*Desulfosporosinus* sp.), which only accounted for about 1% of the total community. This particular species is noted to be particularly acid tolerant and has been successfully applied in other studies for reducing AMD near the source (Kusel et al. 2001). The organism with the highest frequency (≈19%) in this sample matched "uncultured bacterium clone B-42" in the NCBI Blast database. This organism appears to be related

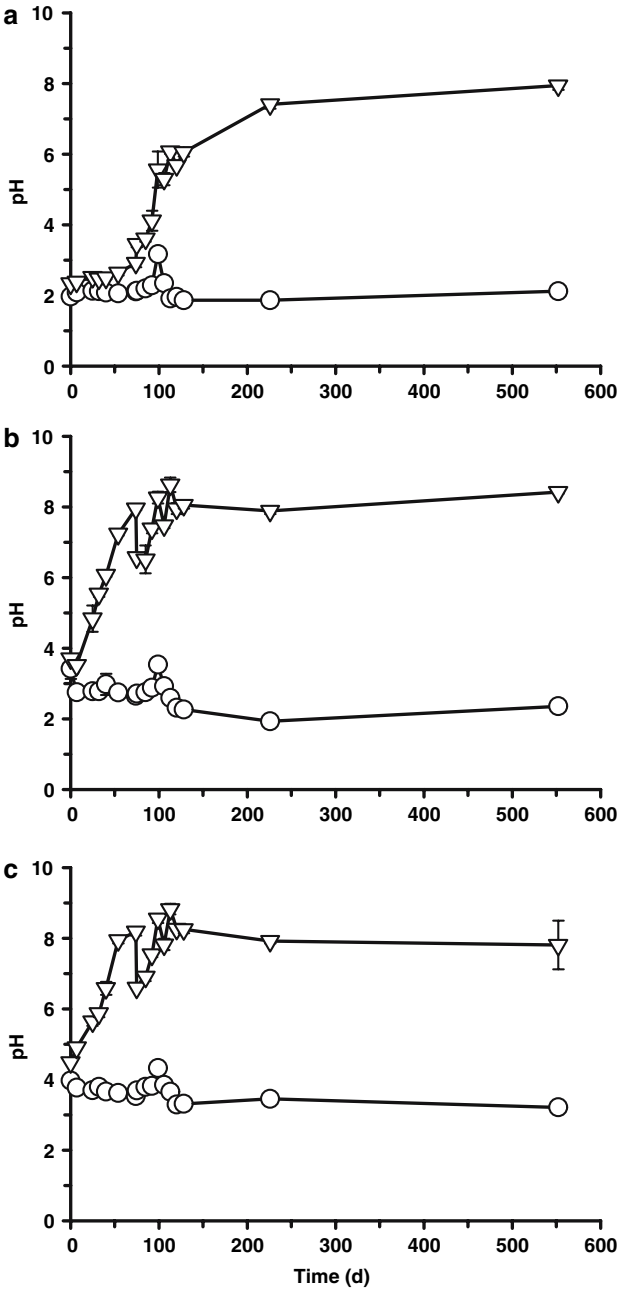

Fig. 4 Threshold pH experiment: changes in pH in microcosms filled with ground water impacted with acid mine drainage and pyrite. One treatment received no additional amendments (*open circle*) and the second treatment received 3 wt% effluent solids (*ES*) and 1.4× the required stoichiometric concentration of C (as returned milk) that bacteria would consume while reducing all the SO_4^{2-} in each microcosm (*inverted open triangle*). The pH of each treatment was adjusted with H_2SO_4 on day 0 to nominal values of 2 (**a**), 3 (**b**), or 4 (**c**). *Error bars* are mean ± standard error (n = 3)

to cow teat canals and up-flow anaerobic sludge blanket reactors (Gill et al. 2006; Purohit and Kapley 2005). Other species of note included an anaerobic bacterium (clone 159) associated with whey degradation (≈1% of total

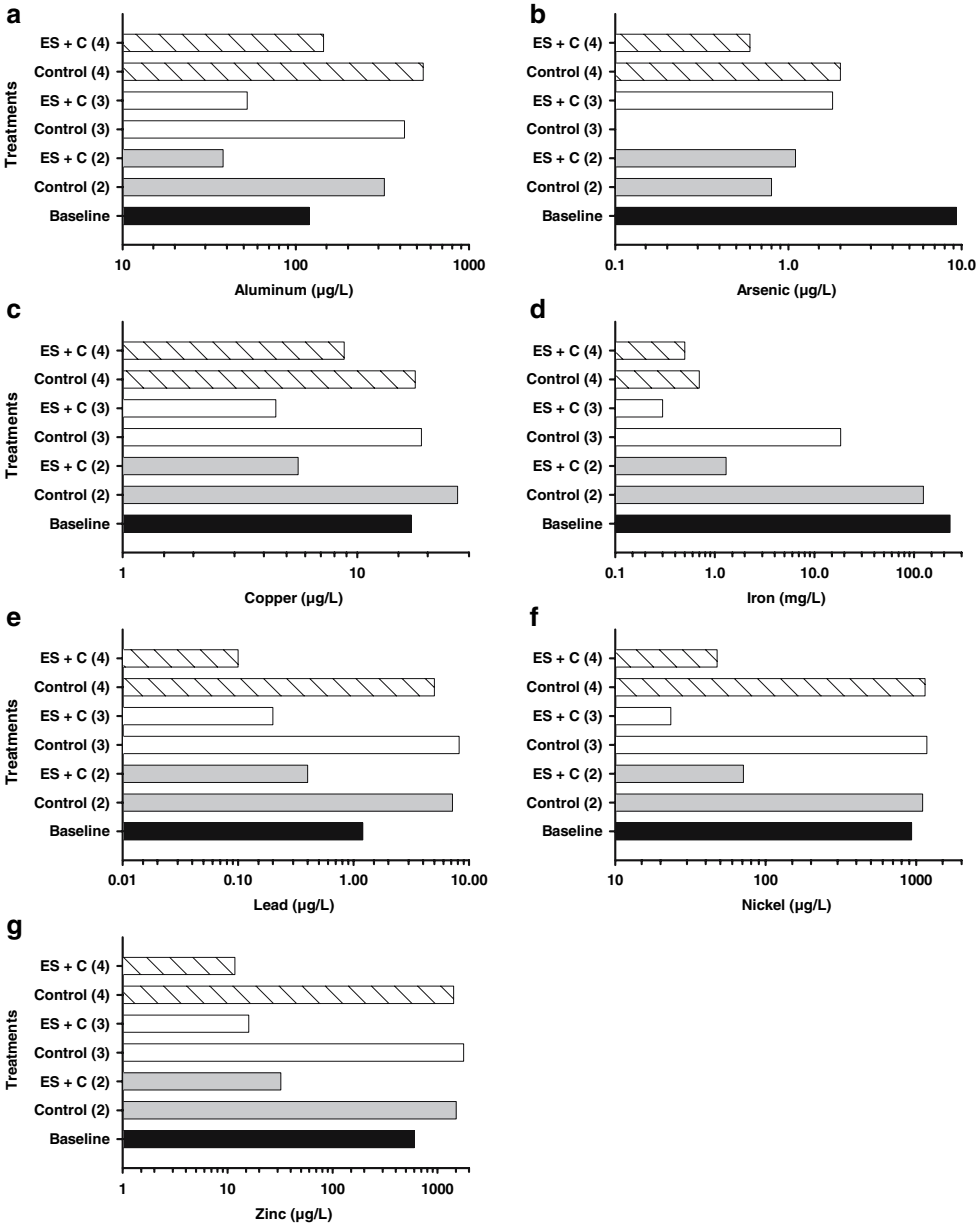

Fig. 5 Threshold pH experiment: concentrations of aluminum (**a**), arsenic (**b**), copper (**c**), iron (**d**), lead (**e**), nickel (**f**), and zinc (**g**) in microcosms filled with ground water impacted with acid mine drainage and pyrite. One treatment received no amendments (control) and the second treatment received 3 wt% effluent solids (*ES*) and 1.4× the required stoichiometric concentration of C (as returned milk) that bacteria would consume while reducing all the SO_4^{2-} in each microcosm (ES + C). The pH of each treatment was adjusted with H_2SO_4 on day 0 of this 226-day experiment to nominal values of 2 (*gray bars*), 3 (*open bars*), or 4 (*hatched bars*) the pH of each treatment is also indicated in *parentheses* following each treatment description. Baseline concentrations of metals (baseline; *dark bars*) were measured in AMD water 2 days prior to addition to microcosms and samples were collected for final metal analysis on day 226

community) and *Acidovorax avenae* (7% of total community), which is a nickel tolerant denitrifier (Abou-Shanab et al. 2003; Table 3).

Discussion

Ammonium was detected at a concentration of 15.1 mg/L in AMD water. This concentration is relatively high when compared to water samples collected from other mining sources. We presume that organic matter in the backfilled materials, such as degraded plant tissues, might have contributed to the ammonium. The results from the threshold pH experiments demonstrate that the BST system is effective in low pH water (as low as 2) and can raise the pH to circumneutral levels after a brief lag time (<100 days; Fig. 4). This is very important as some AMD situations experience extremely low pH (≤2). Furthermore, the protective biofilm layer that grew on the AMD source material in our study prevented AMD generation for >19 months. In comparison, pH in the non-treatment controls dropped and stayed in the acid range, apparently due to the oxidation and subsequent hydrolysis of ferrous iron, as attested by the formation of orange-colored precipitation in the control microcosms. Although a small amount of orange-colored precipitates were also observed in the treatment microcosms, they were probably caused by the oxidation of remaining ferrous ions in the overlying water column, which stayed aerobic throughout the testing period.

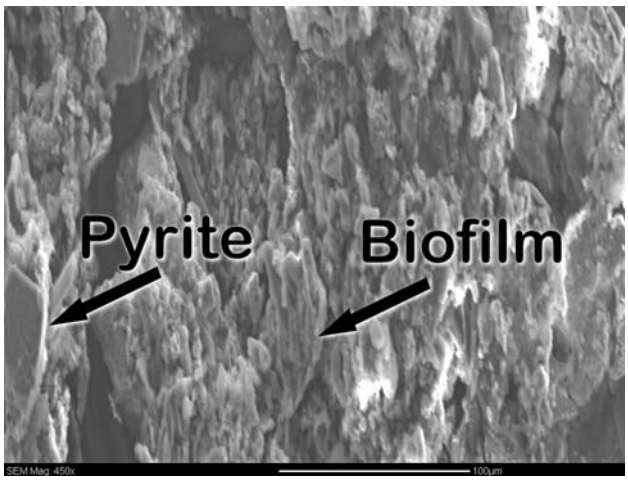

Fig. 6 Substrate dosage experiment: biofilm growing on pyrite after 213 days in a microcosm filled with ground water impacted with acid mine drainage, pyrite, 3 wt% effluent solids (*ES*) and 5× the required stoichiometric concentration of C (as returned milk) that bacteria would consume while reducing all the SO_4^{2-} in the microcosm. This image was taken at ×450 magnification with a scanning electron microscope

Table 3 Species frequency of bacteria composing ≥2% of the biofilm community growing on pyrite in a microcosm containing 3 wt% effluent solids and 5× the required stoichiometric concentration of C (as returned milk) that bacteria would consume while reducing all the sulfate in the microcosm

Sequence identification	Percentage of total community
Uncultured bacterium clone B-42	19
Acidovorax avenae	5
Uncultured bacterium/petrimonas sulfuriphila	4
Uncultured bacterium clone PL-36B1	4
Uncultured bacterium clone AKAU4168	3
Uncultured alpha proteobacterium	2
Uncultured actinobacterium clone: KY204	2
Acidovorax sp. R-24607	2
Nitrosomonas sp. NM 41	2
Uncultured bacterium clone 75-ORF02	2
Uncultured beta proteobacterium clone: HAuD-MB34	2

Species were identified using polymerase chain reaction and 16S rRNA techniques. In all, this biofilm contained at least 70 different species

Additionally, the ES inoculum dosage and substrate dosage experiments demonstrated that inexpensive materials such as ES from wastewater treatment facilities and returned milk are suitable sources of inoculum and substrate for establishing the BST system on AMD source material. Returned milk is composed of lactose, triglycerides, and proteins. The microbial consortium in the ES and maybe the milk itself can break down these organic compounds into smaller molecules such as acetate, fatty acids, and amino acids, to be used by SRB and other bacteria of narrow substrate ranges. Treatments of ES and returned milk in these experiments resulted in increased pH and decreased metal concentrations. Due to the fact that the pH actually decreased in the controls, we believe that the increase in pH in the treated microcosms was caused by initial proton consumption by SRB and other bacteria, and that subsequent shielding of the pyrite by the biofilm prevented fresh AMD generation, as suggested by Elsetinow et al. (2003) and Zhang et al. (2003a, b). The decrease in metal concentrations in these treatments could have been due to a number of processes including: (1) precipitation of metal-containing minerals and (or) adsorption to biotic and abiotic surfaces in the microcosms when the pH increased to circumneutral levels, (2) absorption into bacterial cells once the biofilm began to grow, and (3) precipitation as metal sulfides with sulfide generated through sulfate reduction by SRB. Because we recognize potential problems that may arise if wastewater effluent is introduced into potentially sensitive ground water environments linked to delicate ecological areas or municipal water sources, we are investigating the feasibility of isolating and culturing microbial consortia in non-toxic media enrichments for use as inoculum in the field.

Our initial hypothesis was that SRB play a crucial role in our BST system, similar to the role they play in a typical SRB reactor, which is to consume protons and produce sulfide through metabolic activity, thus increasing pH and decreasing metal concentrations by forming metal-sulfide precipitates, respectively. However, microbial analysis of the biofilm that formed on AMD source material in our microcosms indicates that facultative anaerobic bacteria, other than SRB, are abundant and, therefore, we attribute the observed increase and stabilization in pH to the group function of a bacterial consortium. Based on the populations identified, we contend that this biofilm likely consists of a multilayered community with aerobes and facultative anaerobes on the outermost layers and obligate anaerobes (such as SRB) on the inner layers (Fig. 7). The interactions among all the species in this population are unknown at this time; therefore, we do not fully understand how this biofilm functions as a whole. For example, sulfide-oxidizing bacteria were detected (particularly *Thiobacillus denitrificans*) and may exist in some of the inner layers where abundant sulfide would be generated by SRB in adjacent layers closer to the source material.

Data suggests that the mechanism preventing acid generation in our experiments is the oxygen-reducing capacity of the complex biofilm that covers the source material, such that a reductive microbial barrier consumes oxygen before it can reach the source material and cause

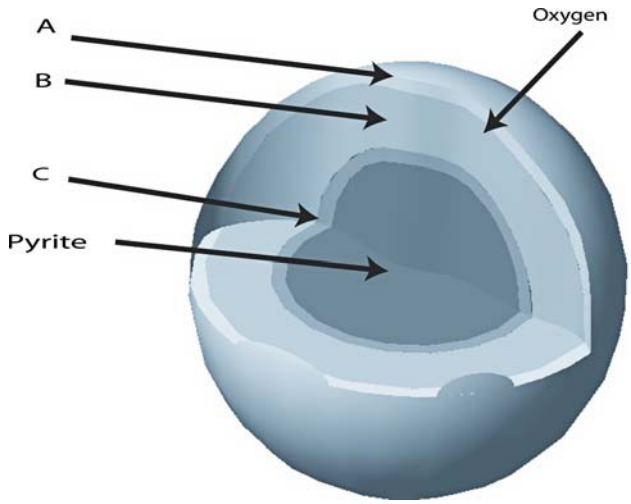

Fig. 7 Conceptual model of the community structure of biofilm growing on pyrite in microcosms. Layers A and B of the biofilm are composed of aerobic and facultatively anaerobic bacteria that consume oxygen (O_2) diffusing through the biofilm from overlying water. Layer C is an anaerobe-dominant layer containing sulfate reducing bacteria and other facultative anaerobes; therefore, oxygen diffusion to the pyrite and generation of acid mine drainage is prevented

acid generation. Additionally, the biofilm may also form a hydrophobic physical barrier, due to the exopolymer secretions in the biofilm, which would also shield the pyrite from exposure to oxygen by eliminating contact between pyrite and the oxygenated water (e.g. Zhang et al. 2003a). The source of the bacteria could be from both inoculum and the amended substrates; however, the biofilm that eventually established on the pyrite surface during the extensive incubation period is obviously an effective system to raise the pH of the water and prevent fresh acid generation.

Conclusions

Applying this BST technique in the laboratory prevents AMD generation for >19 months. We concluded that a complex biofilm that formed on the pyrite effectively scavenged all O_2 near the source material and prevented oxidation and acid generation. We anticipate that the main technical challenges associated with implementing this technique in the field in some cases will be locating source material and delivering sufficient amounts of inoculum and substrate to the source material. If these challenges are met, we believe that this BST system has the potential to prevent AMD generation at many field sites with surface or subsurface source material.

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