

Report for 2003MT10B: Understanding and predicting changes in the microbial ecology of mine tailings in response to the addition of dissolved organic carbon

- Dissertations:
 - This work will contribute to the PhD dissertation of the principal investigator as well as the MS Thesis of Mr. Mark McBroom. Publication of this work in a peer reviewed journal is anticipated in calendar year 2005.
- Other Publications:
 - Two manuscripts are nearing submission to professional journals.

Report Follows

Abstract

Recent field- and laboratory-scale experimentation at MSU and elsewhere has indicated that microbial populations within acid-producing mine tailings can be influenced by the addition of dissolved organic carbon. Heterotrophic bacteria can be stimulated to consume dissolved oxygen from infiltrating water, thus decreasing the oxidation-reduction (redox) potential throughout the tailings pile and promoting the activity of anaerobic sulfate reducing bacteria (SRB). However, an unintended consequence of the addition of organic carbon may be the stimulation of heterotrophic populations within the mine tailings that are also capable of iron reduction. The stimulation of these populations via organic carbon addition is detrimental to remediation efforts. The research reported herein quantifies the specific response of SRB and iron-oxidizing bacteria (IOB) to commonly used organic carbon sources. Results indicate that both SRB and IOB populations may be stimulated by the addition of whey, molasses, or methanol. While IOB populations were much higher than SRB initially in untreated tailings from both highly weathered and fresh mine tailings, SRB were stimulated 3-5 orders of magnitude in some cases, whereas IOB were stimulated less than 100-fold in all cases. Bacterial population increases were not found to be well correlated with organic carbon concentration, but whey and molasses were clearly better substrates for SRB growth than methanol. Bacterial DNA examined using denaturing gradient gel electrophoresis (DGGE) indicated both that a high level of microbial diversity was present in tailings prior to treatment, and that this increased following treatment. DGGE also shows more microbial diversity in highly weathered tailings than in freshly placed tailings.

Background

Acid rock drainage (ARD) arises from waste rock and mine tailings containing sulfide minerals and lacking adequate acid-consuming carbonate minerals. Sulfide minerals, such as pyrite (FeS_2) are oxidized to form ferrous iron (Fe^{2+}), sulfate, and acidity when oxygenated water infiltrates tailings. The role of iron- and sulfur-oxidizing bacteria (IOB/SOB), such as *Acidithiobacillus (At.) ferrooxidans*, in accelerating the production of acidic drainage from sulfide-containing mine tailings has been known for decades (Silverman and Lundgren, 1959). IOB accelerate acid production by cycling ferrous iron to ferric iron (Fe^{3+}), which then reacts with pyrite to form more iron, sulfate, and acidity. The activity of IOB/SOB is estimated to result in increased acid production of up to six orders of magnitude over abiotic tailings oxidation (Brierley, 1978).

ARD from abandoned hard rock mine lands is a major environmental problem that impacts both ground- and surface water throughout the Western United States and is a major contributor to loss of habitat for fisheries. In Montana alone, there are estimated to be over 20,000 abandoned mines, many of which generate ARD which impacts over 1000 miles of streams. Many abandoned mines are located on public land (State, US Forest Service, or US Bureau of Land Management) or on patented parcels enclosed by public lands. It is therefore in the public interest to foster innovative, cost-effective solutions to ARD.

A remedial strategy that has received significant attention over the past 5 years is the addition of organic carbon directly to mine tailings either in solid form or dissolved in water applied to the tailings surface. Common solid additives include compost, manure, agricultural wastes and sewage sludge. Dissolved materials which have been studied include food processing wastes such as molasses and whey, as well relatively inexpensive non-waste products such as methanol and ethanol. These strategies seek to manipulate indigenous microbial populations in the mine tailings; specifically, stimulating sulfate-reducing bacteria.

Recent field- and laboratory-scale experimentation at MSU and elsewhere has indicated that microbial populations within acid producing mine tailings can be influenced by the addition of easily assimilable dissolved organic carbon. Results from this work have shown that heterotrophic bacteria can be stimulated to consume dissolved oxygen from infiltrating water, thus decreasing the oxidation-reduction (redox) potential throughout the tailings pile and promoting the activity of anaerobic sulfate reducing bacteria (SRB). This work has also shown that mine tailings mineralogy and pH can be favorably altered as a result of the activity of populations of general heterotrophic bacteria (GHB) and SRB. The control of microbial growth within tailings piles can potentially result in significant reductions in the rate and extent of mineral oxidation and subsequent acid production. However, an unintended consequence of the addition of organic carbon may be the stimulation of heterotrophic populations within the mine tailings that are also capable of iron and/or sulfur reduction. Other researchers (Johnson et al., 2001) have recently identified several phylogenetically distinct groups of Gram-positive bacteria which are capable of both heterotrophic growth and simultaneous iron oxidation. The stimulation of these populations via organic carbon addition may be detrimental to remediation efforts. In past work at the Center for Biofilm Engineering (partially funded by the Montana Water Center) stimulation of these populations following the addition of dissolved molasses and whey was periodically observed. Successful implementation of this technology at the field scale

requires a more thorough understanding of the presence, activity, and stimulation of these potentially detrimental populations, as well as beneficial populations (e.g. SRB). In particular, it is necessary to understand and predict the response of iron-oxidizing and sulfate-reducing populations to various organic carbon addition strategies. The research reported herein seeks to determine the specific response of these microbial populations to commonly used organic carbon sources. Mine tailings from the long-abandoned Mammoth Mine (Boulder River, MT) and recently deposited tailings from the Golden Sunlight Mine (Cardwell, MT) were fed varying concentrations of whey, molasses, and methanol in 6 week microcosm experiments. SRB and IOB populations were assessed before and after organic carbon treatment to determine the responses of these populations.

Methods and Materials

Mine tailings from both the Golden Sunlight Mine and the Mammoth Mine were collected from tailings piles in 5-gallon plastic containers and transported to the Center for Biofilm Engineering (CBE) at Montana State University. The Mammoth mine was operational during the early 20th century, and has been abandoned for approximately 70 years, while the Golden Sunlight Mine is in current operation. The tailings from these mines (which are approximately 15 miles apart) therefore represent highly weathered (Mammoth) and unweathered (GSM) conditions.

Microcosm Construction

Tailings were air dried for 1 day by spreading on clean plastic trays. 25 g aliquots of either Mammoth or GSM tailings were weighed and placed into 120 ml serum bottles. 100 ml growth media was then added. Microcosms that were to be incubated aerobically received sterile phosphate buffer solution (PSB), and microcosms that were to be incubated anaerobically received sterile modified Postgate C medium containing no added organic carbon. Organic carbon in the form of cheese whey, molasses, or methanol was then added to each of the microcosms to final concentrations of 100 mg/l, 1 g/l, or 5 g/l. Control microcosms received no added organic carbon. A full description of each microcosm, including tailings source, carbon source, and incubation condition is shown in Table 1. After the addition of organic carbon, aerobic microcosms were sealed with a gas-permeable foam stopper and anaerobic microcosms were nitrogen purged and crimp-sealed to maintain anaerobic conditions. All microcosms were then incubated in the dark at room temperature on a shaker table.

Microcosm Sampling and Analysis

Liquid phase samples were removed from the microcosms at days 1, 21, and 42 of incubation. The day 1 sample represents the pre-treatment condition of the tailings while the day 21 and 42 samples measure the progression of bacterial growth over the course of the experiment. During each sampling event, 1 ml of liquid solution was removed from each microcosm using a sterile syringe. From this sample, IOB and SRB were enumerated, and denaturing gradient gel electrophoresis (DGGE) was performed. IOB were enumerated using the most-probable number (MPN) technique (SM 9240 D.1F) and SRB were enumerated using API recommended practice 38/SRB MPN.

Table 1. Microcosm tailings source, carbon source, and incubation conditions.

#	Code	Tailings Source	Carbon Source	Carbon Conc.	Incubation conditions
1	MCO+	Mammoth	None	--	Aerobic
2	MCO-	Mammoth	None	--	Anaerobic
3	MW10+	Mammoth	Whey	100 gm/l	Aerobic
4	MW10-	Mammoth	Whey	100 gm/l	Anaerobic
5	MW20+	Mammoth	Whey	1 g/l	Aerobic
6	MW20-	Mammoth	Whey	1 g/l	Anaerobic
7	MW30+	Mammoth	Whey	5g/l	Aerobic
8	MW30-	Mammoth	Whey	5g/l	Anaerobic
9	MM10+	Mammoth	Molasses	100 gm/l	Aerobic
10	MM10-	Mammoth	Molasses	100 gm/l	Anaerobic
11	MM20+	Mammoth	Molasses	1 g/l	Aerobic
12	MM20-	Mammoth	Molasses	1 g/l	Anaerobic
13	MM30+	Mammoth	Molasses	5g/l	Aerobic
14	MM30-	Mammoth	Molasses	5g/l	Anaerobic
15	MA10+	Mammoth	Methanol	100 gm/l	Aerobic
16	MA10-	Mammoth	Methanol	100 gm/l	Anaerobic
17	MA20+	Mammoth	Methanol	1 g/l	Aerobic
18	MA20-	Mammoth	Methanol	1 g/l	Anaerobic
19	MA30+	Mammoth	Methanol	5g/l	Aerobic
20	MA30-	Mammoth	Methanol	5g/l	Anaerobic
21	GCO+	GSM	None	--	Aerobic
22	GCO-	GSM	None	--	Anaerobic
23	GW10+	GSM	Whey	100 gm/l	Aerobic
24	GW10-	GSM	Whey	100 gm/l	Anaerobic
25	GW20+	GSM	Whey	1 g/l	Aerobic
26	GW20-	GSM	Whey	1 g/l	Anaerobic
27	GW30+	GSM	Whey	5g/l	Aerobic
28	GW30-	GSM	Whey	5g/l	Anaerobic
29	GM10+	GSM	Molasses	100 gm/l	Aerobic
30	GM10-	GSM	Molasses	100 gm/l	Anaerobic
31	GM20+	GSM	Molasses	1 g/l	Aerobic
32	GM20-	GSM	Molasses	1 g/l	Anaerobic
33	GM30+	GSM	Molasses	5g/l	Aerobic
34	GM30-	GSM	Molasses	5g/l	Anaerobic
35	GA10+	GSM	Methanol	100 gm/l	Aerobic
36	GA10-	GSM	Methanol	100 gm/l	Anaerobic
37	GA20+	GSM	Methanol	1 g/l	Aerobic
38	GA20-	GSM	Methanol	1 g/l	Anaerobic
39	GA30+	GSM	Methanol	5g/l	Aerobic
40	GA30-	GSM	Methanol	5g/l	Anaerobic

DGGE Method

Microbial diversity was assessed using DGGE. Genetic material was extracted from liquid samples and amplified by polymerase chain reaction (PCR) using conserved, 16S rDNA-targeted primers. Nucleic acids were extracted and purified using a FastDNA Spin Kit and a FastPrep FP120 beadbeater (BIO101 Systems; Qbiogene, Inc, Carlsbad, CA). DNA was subsequently amplified using polymerase chain reaction (PCR). Complimentary regions of the 16S rDNA were amplified using 1 μ L each of 5' and 3' primer sets mixed with 25 μ L Accuprime SuperMix II, 23 μ L nuclease-free water (Sigma Chemical, St. Louis, MO) and 1 μ L sample. Reagents were mixed in a 0.2 mL Thermowell tube and transferred to a programmable Thermal Blok II thermocycler (Lab-Line, Melrose Park, IL) for replication. PCR products were separated using denaturing gradient gel electrophoresis (DGGE), which was performed using a Bio-Rad DCode universal mutation detection system and PowerPac 300, 100V power supply (Bio-Rad Laboratories, Hercules, CA). Acrylamide gradient gels (40%-70%) were poured using a Bio-Rad Model 485 Gradient Former and allowed to set up for 1 hour. Wells were loaded with PCR product (approximately 10 μ L) and run for 17 hr at 100V. The gel was then stained with SYBR Green for 30 minutes and gel images were recorded using a FluorChem 8800 imaging system (Alpha Innotech, San Leandro, CA).

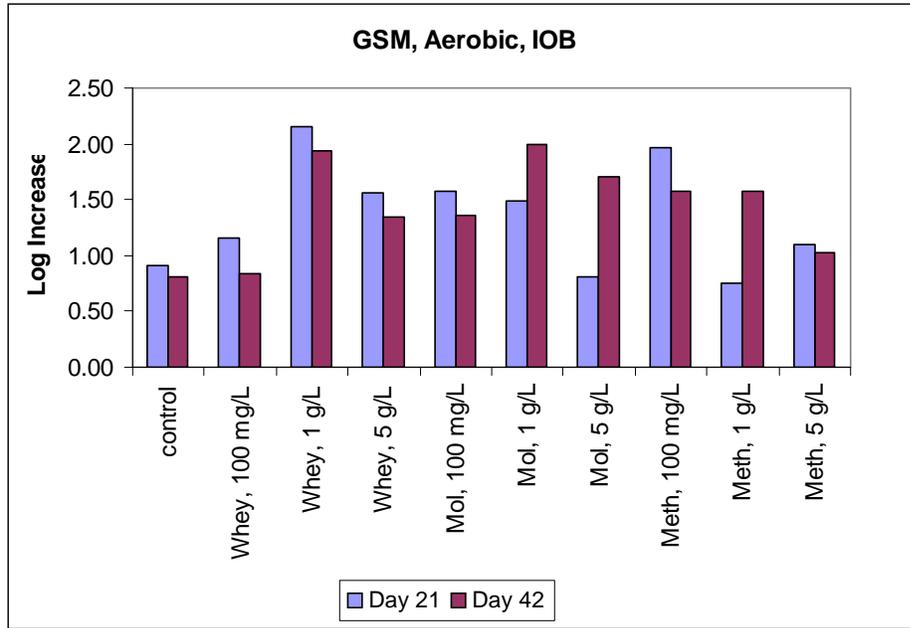
Results and Discussion

Iron Oxidizing Bacteria

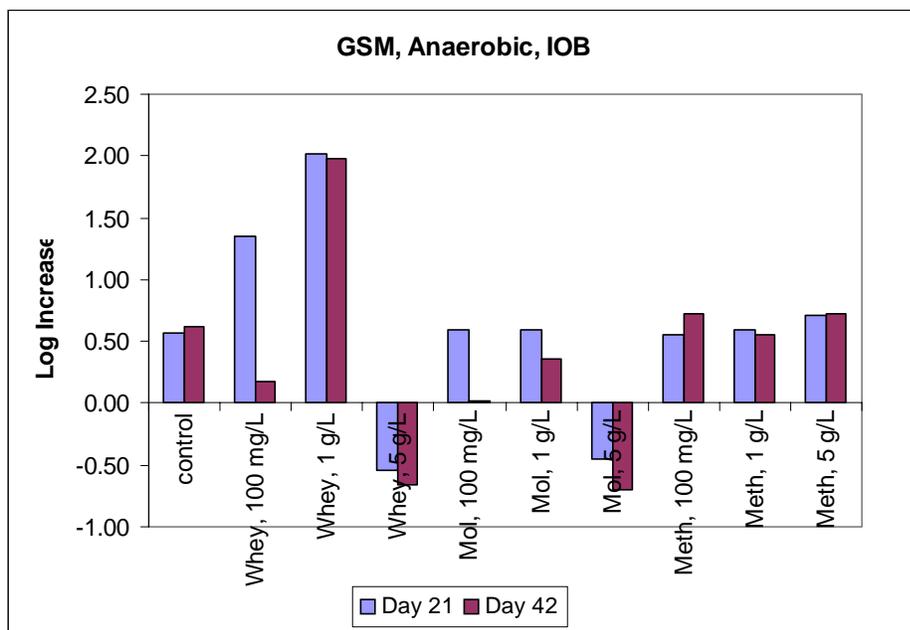
In Golden Sunlight Mine (GSM) tailings, IOB increased as a result of all organic carbon treatments when the microcosms were incubated aerobically. In contrast, anaerobic incubation conditions were more favorable than the control for IOB only in those GSM microcosms treated with 1 g/l whey (Figure 1). The day 42 IOB populations were typically very similar to the day 21 populations, with several notable exceptions where the populations fell between days 21 and 42 (e.g., 100 mg/l whey and 100 mg/l molasses). IOB population growth was not well correlated with organic carbon concentration for any of the three carbon sources tested.

In the Mammoth Mine tailings, aerobically incubated microcosms showed generally lower levels of IOB population increase compared to those observed with GSM tailings. In most cases, IOB population increases in carbon-amended microcosms were not significantly greater than the non-fed controls (Figure 2). In the Mammoth microcosms incubated anaerobically, several whey and molasses treatments resulted in 1.5-2 log increases in IOB populations. As with the GSM tailings, there was not apparent correlation between organic carbon loading and IOB population change.

Collectively, these data suggest that IOB populations can be expected to increase under aerobic conditions following organic carbon treatment, but that it is difficult to predict the response of IOB populations under anaerobic conditions. Although IOB have traditionally been considered chemolithotrophic (gaining energy from the transfer of electrons from ferrous iron to oxygen and utilizing CO₂ as their carbon source), recent research has shown that IOB are actually a metabolically diverse group that includes autotrophs, mixotrophs, and heterotrophs (Johnson, 1998). Because mine tailings are typically oligotrophic environments, with ferrous iron and

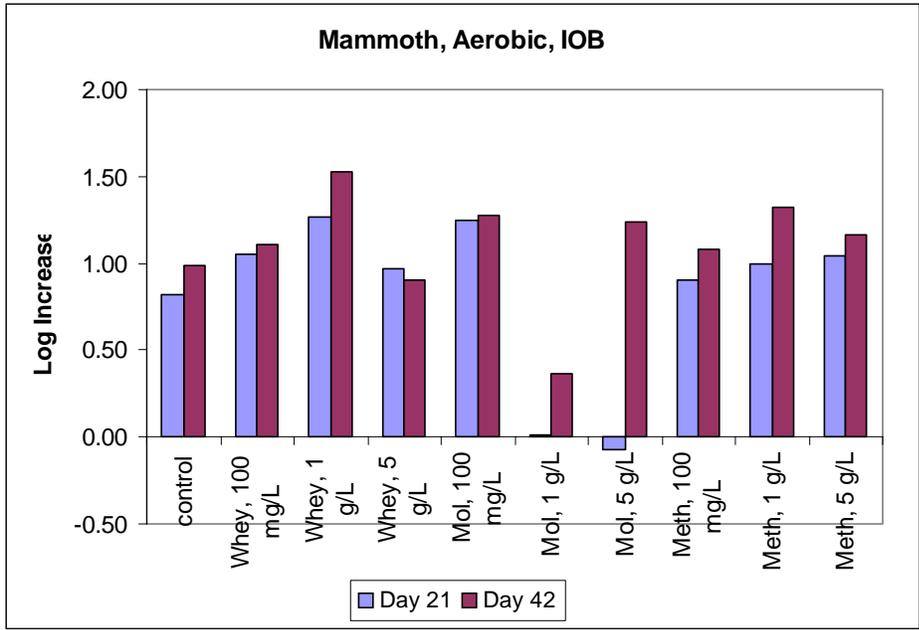


a)

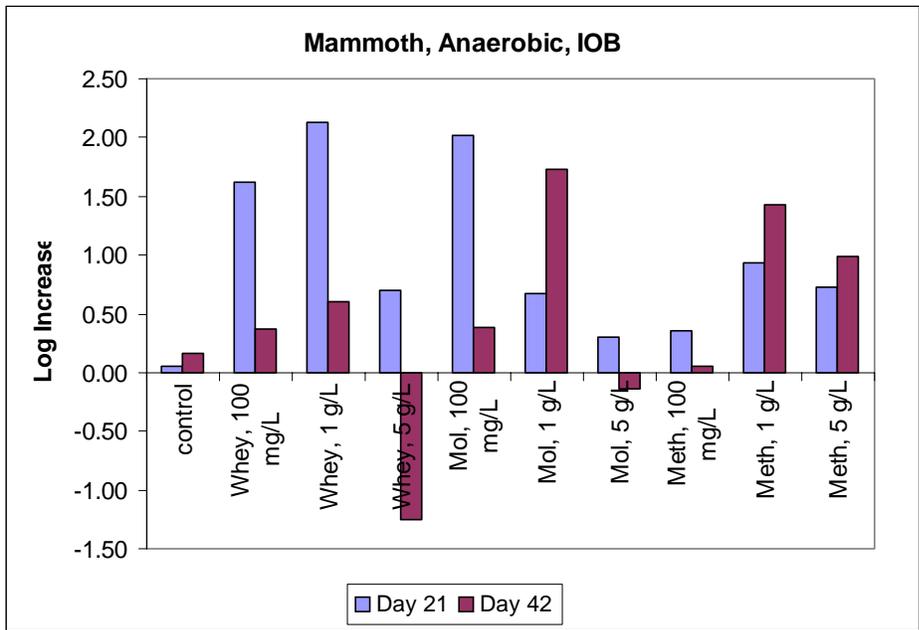


b)

Figure 1. Iron oxidizing bacteria (IOB) from Golden Sunlight Mine tailings 21 and 42 days following treatment with various organic carbon sources. Microcosms were incubated under a) aerobic and b) anaerobic conditions.



a)



b)

Figure 2. Iron oxidizing bacteria (IOB) from Mammoth Mine tailings 21 and 42 days following treatment with various organic carbon sources. Microcosms were incubated under a) aerobic and b) anaerobic conditions.

oxygen readily available, chemolithotrophy has distinct competitive advantages over heterotrophy under carbon-limited conditions. However, where organic carbon is available, some IOB have been observed to assimilate it for cell mass, while still oxidizing ferrous iron for energy (mixotrophy) (Pronk et al., 1991). These organisms do not require organic carbon for growth, but will utilize it if available. Members of this group include mesophiles such as *At. ferrooxidans* and moderate thermophiles such as the Gram-positive *Sulfobacillus acidophilus* and *S. thermosulfidooxidans* as well as members of *Acidimicrobium* (Johnson, 1998; Johnson, 2001). Some acidophilic iron oxidizers are obligate heterotrophs, being unable to fix CO₂. *Ferrimicrobium acidiphilum*, a Gram-positive IOB and *Sphaerotilus sp.* are representative of this group (Johnson et al., 1992; Johnson, 2001). Some obligately heterotrophic iron-oxidizers are relatively sensitive to high concentrations of organic carbon, and may be inhibited under carbon-rich conditions (Bacelar-Nicolau and Johnson, 1999).

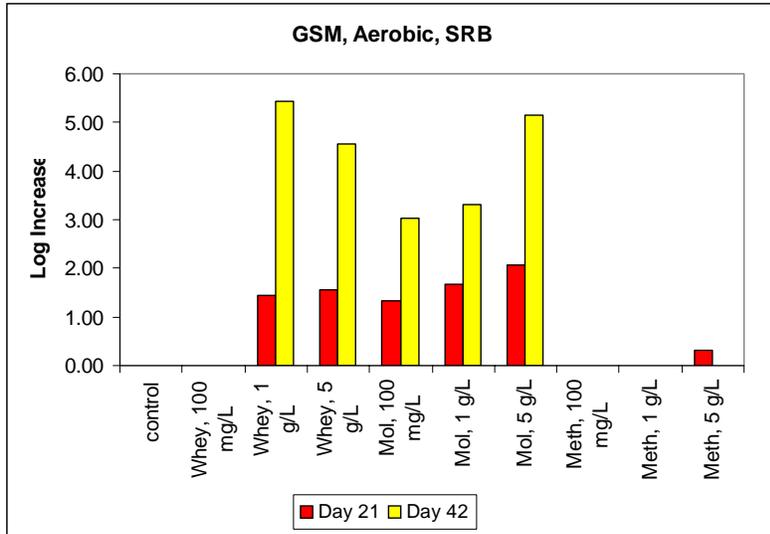
Clearly, the individual microbial ecology of a tailings environment is an important determining factor in the response of this community to organic carbon addition. In light of past research, it is not surprising that IOB populations were, under some conditions, stimulated by treatment. This situation is potentially problematic for application of organic carbon as a treatment method. IOB are principally responsible for acid and metals generation from tailings. Treatments that increase their population density are likely to increase ARD from tailings unless some alternative mechanism of control is present, as described below.

Sulfate Reducing Bacteria

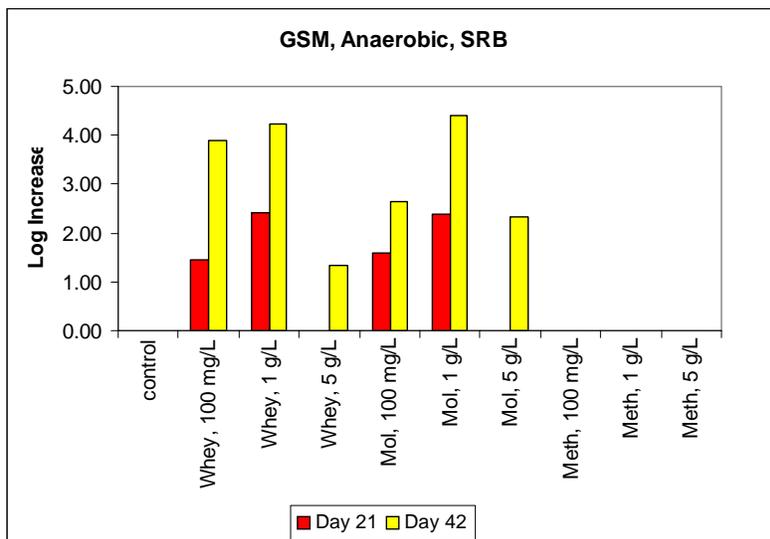
Through the generation of hydrogen sulfide, and subsequent precipitation of metals as metal sulfides, ARD is mitigated by the activity of SRB. SRB have been widely used for the treatment of ARD in engineered bioreactors, where influent organic carbon, pH, and oxidation-reduction (redox) potential can ostensibly be controlled to the benefit of SRB populations. The oxidized and acidic conditions present in most sulfidic mineral tailings would seem prohibitive to SRB, which are generally considered acid intolerant (Postgate, 1984). Nonetheless, SRB have been isolated from tailings piles (Fortin et al., 1995; Fortin et al., 1996; Fortin and Beveridge, 1997). In addition, SRB have been recovered from sediments deposited from mine waste effluent streams (Wielinga et al., 1999; Gyure et al., 1990; Herlihy and Mills, 1985) and their presence has been inferred from genetic sequencing of some of the most acidic drainage streams on Earth (Bond et al., 2000).

Results from GSM microcosms incubated both aerobically and anaerobically indicate the growth of SRB under all whey and molasses treatment conditions (Figure 3). Methanol, conversely, did not stimulate SRB in GSM microcosms under the conditions tested. In contrast to IOB, which in most cases did not continue to increase in number between days 21 and 42, SRB in most cases did, particularly when incubated aerobically. SRB have generally been considered obligately anaerobic bacteria, able to tolerate oxygen, but unable to grow in its presence. Recent evidence suggests that some SRB are capable of utilizing oxygen as an electron acceptor. Under aerobic conditions, no sulfate reduction would occur, yet the population would maintain the ability to reduce sulfate when conditions again become favorable. These data (Figure 3) indicate that SRB present in the GSM tailings increased in population when fed whey and molasses and incubated both aerobically and anaerobically (subsequent SRB enumeration was completely under anaerobic conditions). Results were similar with the Mammoth tailings, with the exception that

methanol also stimulated SRB populations, albeit generally not as well as whey and molasses (Figure 4). As with IOB, SRB population increases were not well correlated with organic carbon concentration. This suggests that SRB (and IOB growing heterotrophically) were not carbon limited during this experiment. It is likely that the levels of carbon used provided adequate carbon for the duration of the experiment. The lack of SRB growth under methanol addition in GSM tailings, and the lower SRB increases in Mammoth under methanol addition could result from the antimicrobial properties of methanol. At high concentrations, methanol (like ethanol) is toxic to bacteria. The threshold of this toxicity varies among populations, but could certainly account for the attenuated response to methanol observed here.

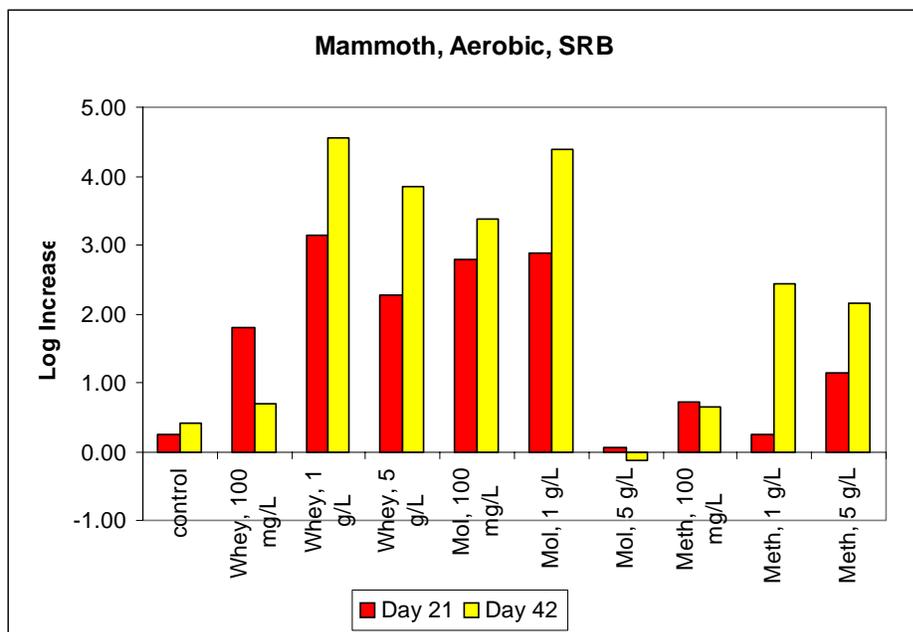


a)

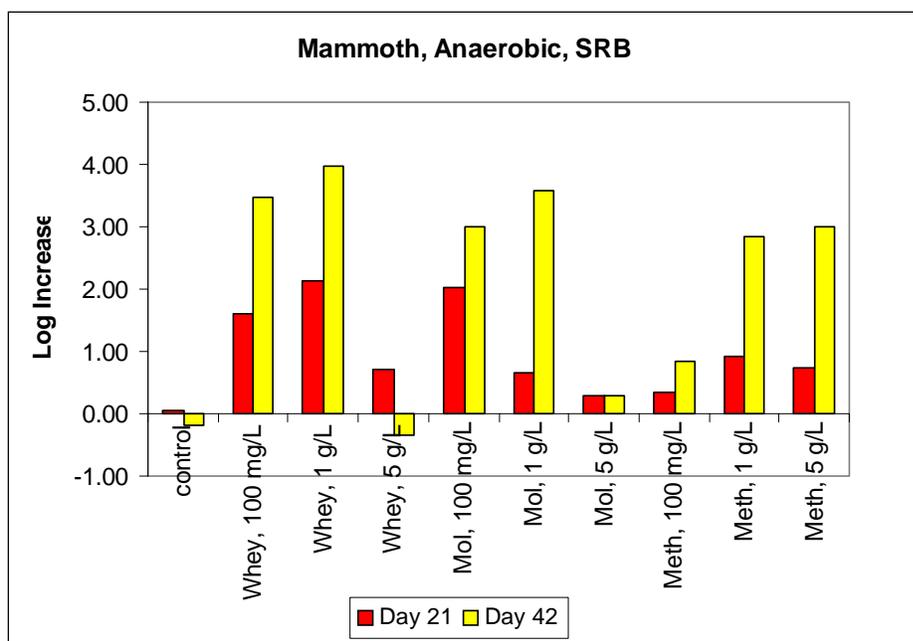


b)

Figure 3. Sulfate reducing bacteria (SRB) from Golden Sunlight Mine tailings 21 and 42 days following treatment with various organic carbon sources. Microcosms were incubated under a) aerobic and b) anaerobic conditions.



a)



b)

Figure 4. Sulfate reducing bacteria (SRB) from Mammoth Mine tailings 21 and 42 days following treatment with various organic carbon sources. Microcosms were incubated under a) aerobic and b) anaerobic conditions.

Denaturing Gradient Gel Electrophoresis Analyses

DGGE can be a powerful tool to determine the composition of microbial communities. In this study, it was used primarily as a means to qualitatively assess community differences between the various samples from both Mammoth and GSM. DGGE gels run from selected samples taken at day 42 illustrate distinct differences both between tailings types (GSM vs. Mammoth) and between organic carbon treatments. Each band in the various samples shown in Figure 5 represents an individual bacterial species. Time and resource constraints prevented the identification of each band; however, it is apparent that significant microbial diversity is present, particularly in the Mammoth samples. Comparison of the control samples from each tailings type (listed as “MCO+” and “GCO+” in Figure 5) suggests more diversity in unamended Mammoth samples. This is expected based on the length of time (> 70 yrs) the Mammoth tailings have been exposed to the open environment. GSM tailings, conversely, are relatively young (several months old). Mine tailings undergo a succession of bacterial colonization after placement. This progression may start in the meso-acidic range with filamentous IOB, such as *Metallogenium* (Walsh and Mitchell, 1972) and progress to the more acid-tolerant Gram-negative IOB, such as the *Thiobacilli* and *Acidithiobacilli* (Johnson, 2001), and progress to the archaea under highly acidic conditions. The rate of this progression is dependent on the mineral character and texture of the tailings, temperature, water infiltration, and site biota. Organic carbon addition clearly further promotes microbial diversity in samples, as indicated by the increasing number of bands in treated samples compared to the non-treated controls (Figure 5).

Close scrutiny of the banding patterns in Figure 5 reveals that, not surprisingly, different populations are stimulated when incubation occurs under aerobic vs. anaerobic conditions, particularly in Mammoth samples. Among GSM samples tested via DGGE, only those treated with molasses at 1 g/l (“GM2O+” and “GM2O-”) showed significantly different banding patterns between the aerobic and anaerobic samples. These data further suggest that the bacterial consortium present in both tailings samples is capable of either aerobic or anaerobic growth. The Mammoth DGGE gel suggests more population diversity, and a broader initial population from which individual populations (e.g., IOB or SRB) can grow. SRB populations were higher in Mammoth tailings than GSM at the beginning of the experiment, but not dramatically so, and IOB populations were of similar magnitude in both samples (Table 2). Given this parity of IOB populations, it is surprising that the GSM DGGE gel doesn’t show more diversity. This may be attributable to problems in the DGGE analysis, but this seems unlikely since the DGGE bands for GSM samples were very similar among samples.

Table 2. Initial Colonization of GSM and Mammoth Tailings at Day 0.

	SRB	IOB
GSM	2.89E+00	7.09E+05
Mammoth	1.14E+01	3.66E+05

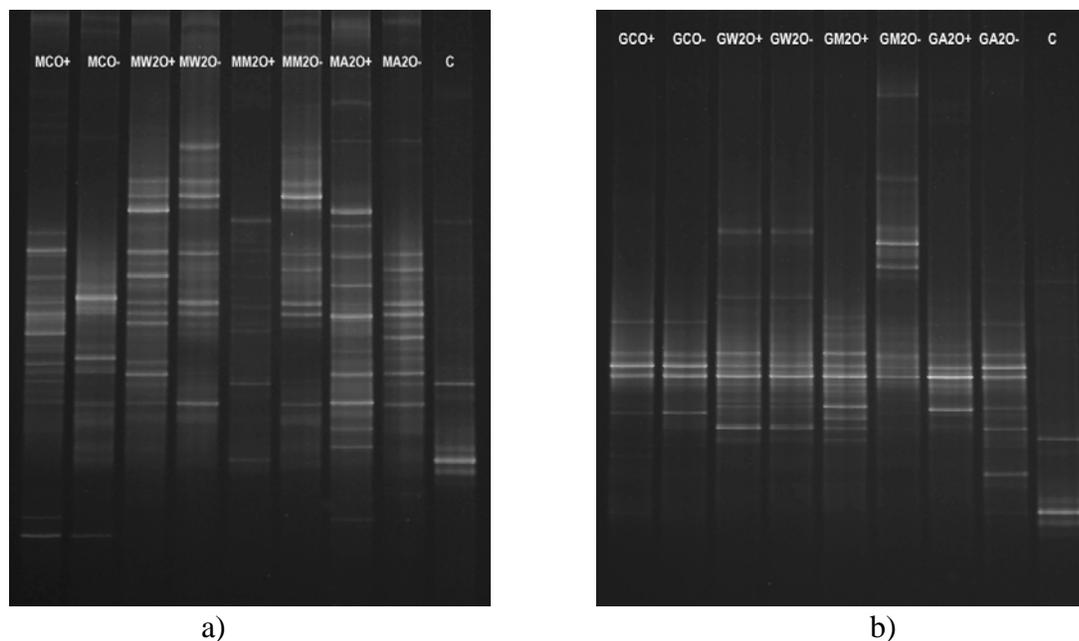


Figure 5. DGGE microbial community profile of treated a) Mammoth and b) GSM microcosms at day 42. Code names for treatments correspond to Table 1.

Conclusions

Treatment of mine tailings from two distinct sources, highly weathered tailings and freshly placed tailings, showed both similarities and differences in IOB and SRB response to organic carbon treatment. IOB, the activity of which is detrimental to ARD control efforts, were stimulated, albeit inconsistently, by organic carbon treatment. Under most organic carbon conditions, IOB populations in aerobic microcosms grew approximately 10-fold in Mammoth tailings and 10- to 100-fold in GSM tailings. Under anaerobic conditions, IOB population growth was much less consistent, with some treatments causing a drop in IOB numbers. Fortunately, SRB populations in both Mammoth and GSM tailings responded positively to both whey and molasses treatments. Methanol was not universally effective in SRB stimulation, however.

These experiments illustrate both the promise of using organic carbon to abate ARD through SRB stimulation and the potential to inhibit remedial efforts through the inadvertent stimulation of IOB. Although increases in bacterial populations were not well correlated with the concentration of organic carbon, it is apparent that even relatively low levels of whey and molasses addition can stimulate SRB populations over 1000-fold.

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