COMPARISON OF INOCULA APPLIED IN THE REMEDIATION OF ACID MINE DRAINAGE BY SULFATE REDUCTION[1]

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Abstract: Sulfate-reducing permeable reactive zones (PRZs), such as anaerobic wetlands, sulfate-reducing bioreactors, and permeable reactive barriers, are an attractive means of passively treating mining influenced waters contaminated with heavy metals. While the low cost and maintenance requirements are significant advantages of PRZs, the lack of clear design criteria is a disadvantage. It is not known why some systems will function for long periods of time without need for intervention, while others fail or do not recover well when exposed to stresses such as winter weather or other changes in conditions. This study explores the role of microorganisms in PRZs and the potential to use selected inocula to improve performance with respect to start-up time, sulfate-reducing activity level, and activity retention time. We have compared these attributes using various inocula, including: dairy manure, anaerobic digester sludge, acclimated column inoculum, and inoculum collected from two sulfate-reducing bioreactors operated in the field (Luttrell and Peerless Jenny King). Our results demonstrate that there are clear differences between the inocula and that the Luttrell bioreactor inoculum performs the best in terms of start-up time and overall activity. Sulfate concentrations, metal concentrations, and pH were measured in the aqueous phase to evaluate the ability of the different inocula to remediate acid mine drainage (AMD). In subsequent studies, DNA-based methods that profile the microbial community will be used to determine what kinds of microorganisms are present and to quantify key functional groups, including sulfate reducers, methanogens, and cellulose degraders. The ultimate goal will be to transfer these results to the field by developing the capability to intelligently design inocula for site-specific concerns.

Additional Key Words: microbiology, acid mine drainage, sulfate-reduction, permeable reactive zones, bioremediation

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Introduction

Acid mine drainage (AMD) is a major environmental issue in the United States and other countries with estimated costs of treatment in the tens of billions of dollars (Benner et al., 1999). AMD is associated with mining activities that expose sulfide minerals such as pyrite (FeS_2) to air and water. The oxidation of sulfide minerals releases acidity, sulfates and Fe(II) which contaminate the water. The generated acidity also mobilizes toxic trace metals.

Treatment of AMD is a major concern because of its deleterious effects on the environment. Treatment systems fall into two categories: active and passive. Active treatments usually neutralize AMD with an alkaline reagent. These systems are reliable but expensive and require access and maintenance to function properly. This makes them impractical for remote or abandoned mines. Sulfate-reducing permeable reactive zones (PRZs), as applied in anaerobic wetlands, sulfate-reducing bioreactors, or permeable reactive barriers, are an attractive passive means for treating AMD given their low cost and low required maintenance. Additionally, PRZs produce minimal hazardous waste requiring disposal compared to active treatment methods. These barriers are installed in the flow path of the contaminated water and consist of organic matter and a consortium of microorganisms which are responsible for the remediation of the water. The principle mode of function of PRZs is metal sulfide precipitation due to bacterial sulfate reduction. Sulfate-reducing bacteria (SRB) reduce sulfates to hydrogen sulfide using simple organic substrates or H_2 as a source of energy (Eq. 1). Hydrogen sulfide reacts with divalent dissolved metals to form insoluble metal sulfides that are retained within the barrier (Eq. 2).

$$2\,CH_2O + SO_4^{-2} \rightarrow \quad 2HCO_3^- + H_2S \qquad\qquad Eq.1$$

$$Me^{+2} + S^{-2} \quad \rightarrow \quad MeS \qquad (Me^{+2} = metal\ cation) \qquad Eq.2$$
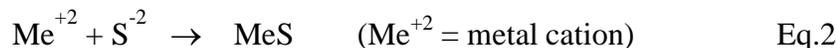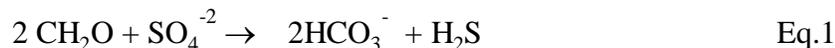
PRZs have been implemented in the field and at laboratory scale with mixed success. Many perform as designed in the first months after establishment but in the long term their performance declines with respect to what is expected in theory (Blowes et al., 2000). In other cases, PRZs effectively treat AMD and perform as designed (Waybrant et al., 1995; Benner et al. 1997, 1999). It is evident that additional work is needed to refine the design of these barriers and improve their performance and reliability.

Since the operation of PRZs relies on microbial activity, a better understanding of the role of the microbial community in these systems will help improve their design and performance. In our model, a complex community is responsible for AMD remediation in PRZs (Logan et al., 2003). SRB are obligate anaerobes that use short chain organic compounds or H_2 as electron donors. Thus, they depend on microorganisms that have the enzymes to degrade cellulose, lignin and other polymers to monomers and on fermenters that transform these simple sugars into short chain compounds. According to our model, methanogens compete with sulfate reducers for fermentation products and might reduce the overall rates of sulfate reduction.

Our past research has demonstrated the controlling influence of microbial inoculum on the performance of sulfate-reducing columns simulating PRZs (Messner et al., 2004). Columns were inoculated with an acclimated inoculum, dairy manure and no inoculum and their performance was evaluated according to their capacity to remove metals, sulfates and to neutralize the pH. It was found that the columns inoculated with the acclimated inoculum gave the best overall performance and demonstrated the highest metal removal. There was no

noticeable difference between the columns inoculated with dairy manure and those with no inoculum.

Improvement of microbial inocula either by developing them from strains of known function or by enrichment of inocula known to have the desired microorganisms is a promising low cost means of improving the performance of PRZs. In this study the ability of five different inocula to remediate AMD was compared in a batch experiment. Sulfate and metal removal and the ability to neutralize pH were evaluated. The objective of the study was to identify microbial inocula with key functions in order to optimize PRZs performance and reliability for AMD remediation.

Materials and Methods

Candidate inocula

Five different inocula were used for this study: twice-acclimated inoculum from a sulfate-reducing column simulating a PRZ (Messner et al., 2004), dairy manure, anaerobic digester sludge, and inocula taken from two sulfate-reducing bioreactors operated in the field (Luttrell and Peerless Jenny King, near Helena, MT, kindly provided by Jim Gusek of Golder Associates, Inc.). Anaerobic digester sludge samples were obtained from the Drake Water Reclamation Facility of the city of Fort Collins, CO. Fresh dairy manure was obtained from La Luna dairy, Wellington, CO. The dairy manure was included in this study for comparison purposes as it is the typical inoculum used in field PRZs.

Batch experiment set up

125 mL serum bottles filled with inoculum, simulated mine water, and organic substrate were used for the experiment. The bottles were prepared in an anaerobic hood under a nitrogen atmosphere. The simulated mine water was prepared by adding 1.3204 g/L $MgSO_4$; 0.0268 g/L NH_4Cl; 0.088 g/L $ZnSO_4.7H_2O$; 0.0623 g/L $FeSO_4.7H_2O$ and 0.0088 g/L $CdCl_2$ to deionized water purged with nitrogen (Messner et al., 2004). Because this study focused on the microbiology, the ammonium was added to ensure that there were no limiting factors for microbial growth. The final pH of the water was between 5.5 and 6. To each bottle 7.27 g of beech wood chips; 0.66 g of pulverized alfalfa; 3.64 g of pine shavings and 1.65 g of limestone was added. The amount of inoculum added was such that all the bottles would initially have the same number of cells. Bottles inoculated with twice-acclimated inoculum received 80% less cells than the other bottles because a limited amount of this inoculum was available. Total phospholipids were used to determine viable cells in each inoculum using the procedure described by Findlay et al., 1989. The control bottles received no inoculum. The final amount of material in each bottle was 33 g. Silica sand was used to reach the target weight, accounting for differences in the phospholipids to mass ratio in the inocula. After the addition of the organic substrate and inoculum, 90 mL of simulated mine drainage water were added and the bottles were capped with ¼ inch butyl rubber septa (Bellco Glass Inc., Vineland, NJ) and sealed with aluminum crimp seals. The bottles were maintained upside down in the dark to keep the septa moist and inhibit the growth of photolithotrophic bacteria (Waybrant et al., 2002). Bottles were named according to the inoculum each received: ACC for the twice-acclimated inoculum, DM for the dairy manure, ADS for the anaerobic digester sludge, LUTR for the Luttrell site, PJK for the Peerless Jenny King site, and BL for the control bottles. An additional set of bottles were prepared with LUTR and DM inocula and placed upside down in a shaker rotating at 60 rpm in order to determine the effect of shaking versus maintaining the bottles static. For each type of inoculum and condition, static or agitated, 13 bottles were prepared. At 4 different times

corresponding to week 0, 2, 4 and 9 of the experiment, 2 bottles of each type were sacrificed. The liquid from the sacrificed bottles was collected in 60 mL Nalgene bottles and were stored at 4 ^{o}C for sulfate and metal determination. The solid material was stored in 50 mL sterile centrifuge tubes at –80 ^{o}C.

Monitoring gas production

   Bottles were vented periodically to measure gas production and prevent pressure build-up. Excess gas was removed with a 10 mL hypodermic syringe conditioned with a luer lock valve and the total volume of gas removed was recorded. The headspace of three randomly chosen bottles of each type of inoculum and condition were sampled to analyze for CO_2, CH_4 and H_2S. Sampling was done twice a day for the first two weeks of the experiment and subsequently once per day. Gas samples of 300 µL were taken with a gas-tight SampleLock syringe (Hamilton, Reno, NV) and analyzed on a Hewlett Packard 5890 series II gas chromatograph with a thermal conductivity detector (TCD).

Sulfate concentration

   Sulfate measurements were carried out as an indicator of desired sulfate reduction. Therefore by determining the sulfate concentrations in the liquid phase an estimate of the extent of reaction is possible. Sulfates were measured with the SulfaVer 4 procedure (Hach Company, Loveland, CO) using a DR/3000 spectrometer (Hach Company). This procedure was based on turbidity of sulfates present following the addition of reagent. 30 mL of the liquid from the sacrificed bottles were filtered through a 0.45 µm filter membrane (Whatman, Clifton, NJ). 0.625 mL of filtrate was diluted in 24 mL of deionized water and the absorbance at 450 nm was measured before (as a blank reading) and after adding the contents of the SulfaVer4 packets and waiting for 5 minutes. To ensure that the sample matrix did not interfere with the spectrophotometric readings of sulfate, a spiked sample matrix study was performed. Comparison of the concentration of sulfates measured in the spiked and the original sample confirmed that there were no apparent matrix effects in our study.

Metals concentration

   Zinc, cadmium, iron and sulfur concentrations in the filtered liquid phase were determined with a Thermo Jarrell Ash IRIS Advantage ICP (Thermo Electron Corp., Milford, MA) by inductively coupled plasma atomic emission spectroscopy (ICP-AES). A digestion step was performed in order to acquire an accurate determination of metals remaining in solution. 10 mL of sample were digested in a CEM microwave digester model MS-2000 (Matthews, NC) using EPA method SW-3015.

Results and Discussion

   Total gas production was used an indicator of overall microbial activity. The bottles that were agitated showed higher gas production than those that were static (Figure 1). Control bottles, which were not inoculated (but were not sterilized either), produced less gas than those inoculated with acclimated inoculum, anaerobic digester sludge, dairy manure and material from the Luttrell site. PJK bottles, however, produced less gas than the control bottles until day 40 of the experiment. This was of interest considering that the inoculum was field-collected and thus expected to yield good performance, yet for the first 40 days it showed less overall activity than no inoculum at all. The highest rates of gas production were observed on day one of the experiment in uninoculated bottles, bottles inoculated with dairy manure, Luttrell and Peerless

Jenny King sites material. Peak rates for the bottles with acclimated inoculum and ADS were observed at day three.

Analysis of CO_2, H_2S and CH_4 produced in the headspace of the bottles provided a means to monitor the activity with time of microorganisms such as sulfate reducers that produce H_2S and methanogens that produce CH_4. Chromatograms were used to estimate the sulfate-reduction start-up time for the bottles (Table 1). A distinct H_2S odor at the time of sampling supported the observations. An increase in pH was observed in all of the bottles with time, except for the ADS bottles which experienced a drop in pH at week 4 (Fig. 2). The pH of the LUTR bottles (both agitated and static) was consistently higher than that of the other bottles. The differences in pH response among the bottles would be expected to be associated with bicarbonate buffering due to the generation of bicarbonate during sulfate reduction considering that all bottles received an equivalent dose of limestone.

Table 1. Start-up time determined as the time of detection of H_2S in the headspace of the bottles.

Inoculum –condition	Start-up time (hs)	Inoculum –condition	Start-up time (hs)
Luttrell-static	28	Anaerobic digester sludge-static	40
Luttrell-agitated	6	Twice-acclimated inoculum-static	40
Dairy manure-static	40	Control-static	40
Dairy manure-agitated	6	Control-agitated	40
Peerless Jenny King-static	28		

Monitoring of the sulfate concentration provided a direct means to compare the relative sulfate-reducing activity of the bottles. Initial sulfate concentration in the bottles was 1,103 mg/L. Sulfate concentration in the ACC, ADS and BL bottles stayed fairly constant throughout the experiment with a slight decrease at week 9 (Fig. 3). In the PJK, LUTR and DM bottles, sulfate concentration decreased steadily over the course of the experiment. In the first two weeks, the sulfate concentration decreased 34% and 32% for the static LUTR and DM bottles respectively and 62% and 50% for the agitated LUTR and DM bottles respectively. By week 9, static LUTR and DM bottles removed 68% and 45% respectively of the original sulfate concentration and the corresponding agitated bottles removed almost 100% of sulfates. These results indicate that agitating the bottles clearly increases the rate of sulfate reduction. Of the static bottles, the LUTR-inoculated bottles demonstrated the highest sulfate removal. The material used to inoculate the ACC bottles originated from a column with significant sulfate-reducing activity. The fact that the ACC bottles did not manifest significant sulfate removal might be due to the extensive manipulation and storage of this inoculum before being used in this batch experiment. ACC bottles were inoculated with 80% less cells than the other bottles and this might have also influenced the low sulfate removal observed. Even though ACC bottles initially had 80% less cells than the other bottles sulfate removal in these bottles reduced by

more than 80%. This suggests that if all the bottles had been inoculated with the same number of cells, the ACC bottles could have been among the bottles with higher sulfate removal. PJK bottles did not remove as much sulfate as LUTR and DM bottles. Sulfate reducers from the bioreactor from which the PJK material was taken were reported to have been exposed to relatively low sulfate concentrations (influent concentrations of approximately 100 mg/L, Jim Gusek, personal communication). One possibility for the inferior performance of the PJK inoculum is that the low concentration of sulfate had a detrimental effect on sulfate reducers from which they were not able to recover. However, in this case it would be expected that when exposed to excess sulfate, as they were in this study, recovery would occur rapidly. Our hypothesis is that the inoculum to PJK may have been inadequate and therefore excessive time was required for the microbial community to acclimate.
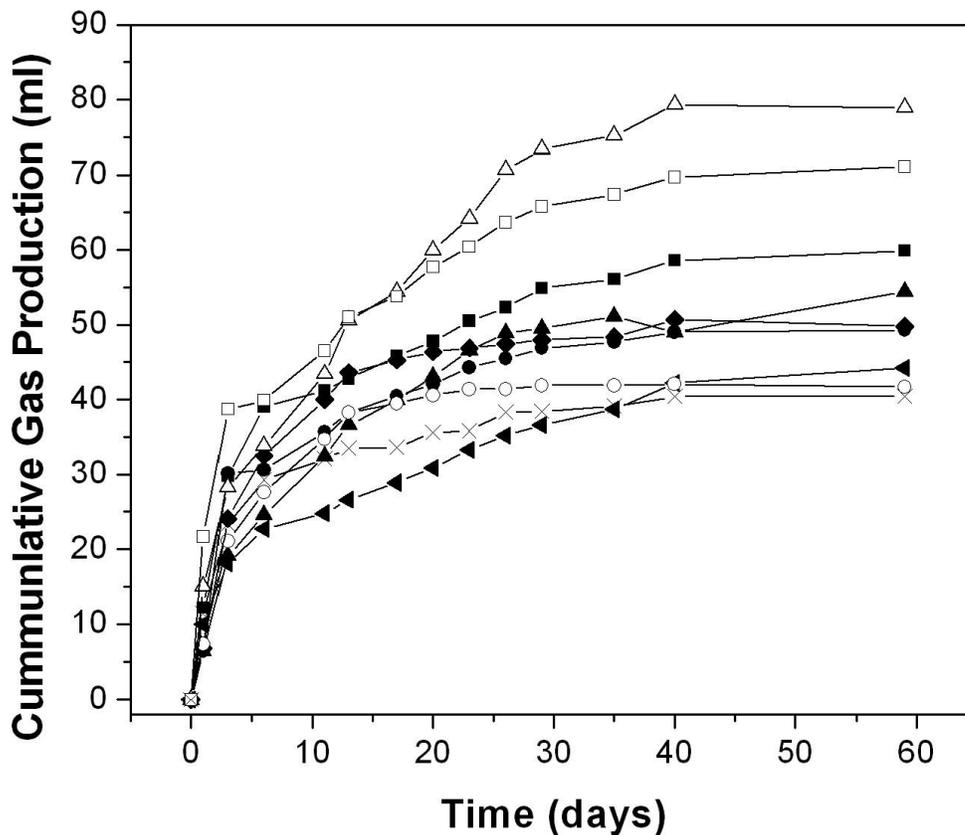

Figure 1: Cumulative gas production during the first 60 days of the experiment for (○) static control bottles; (●) static bottles inoculated with twice-acclimated inoculum, (▲) dairy manure, (■) Luttrell PRZ material, (◄) Peerless Jenny King PRZ material, (◆) anaerobic digester sludge; (✗) agitated control bottles; and agitated bottles inoculated with (△) dairy manure and (□) Luttrell PRZ material. The values are the average of five to eleven independent measurements, depending on the number of unsacrificed bottles remaining at the time of sampling.
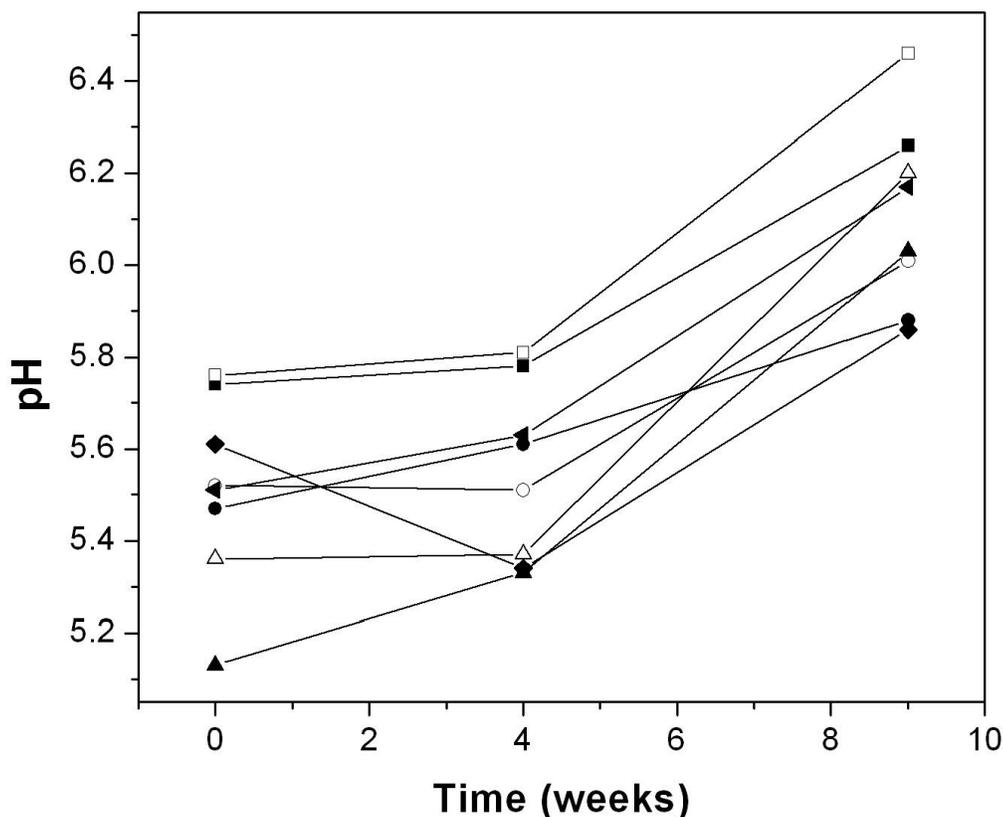
Figure 2: pH in the liquid phase at the beginning, week 4 and week 9 of the experiment for (○) static control bottles; (●) static bottles inoculated with twice-acclimated inoculum, (▲) dairy manure, (■) Luttrell PRZ material, (◄) Peerless Jenny King PRZ material, (◆) anaerobic digester sludge; and agitated bottles inoculated with (Δ) dairy manure and (□) Luttrell PRZ material.

The physical appearance of the bottles also provides some indication of the level of sulfate-reducing activity. The color of the bottles inoculated with LUTR material changed from beige to black in the first 2 weeks. This indicates the precipitation of metals sulfides and is consistent with the high metal removal observed in these bottles. A slight darkening was observed in the DM bottles. ACC, ADS and control bottles did not show significant color change over time. This is consistent with the low sulfate removal in these bottles. PJK inoculum was originally dark possibly due to the presence of metal sulfides. As a consequence, the contents of these bottles were dark from the beginning of the experiment and it was not possible to notice changes in color. Darkening of the bottles was accompanied by the distinct odor of hydrogen sulfide.
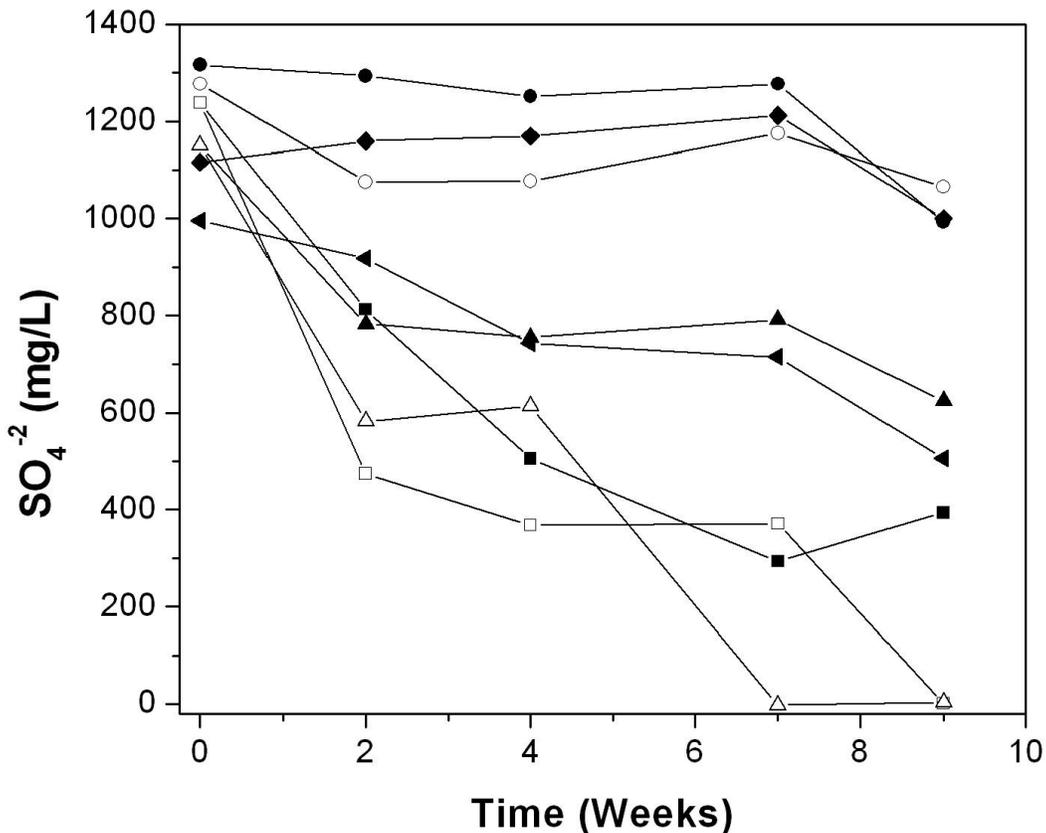
Figure 3: Sulfate concentration in the filtered liquid phase for (○) static control bottles; (●) static bottles inoculated with twice-acclimated inoculum, (▲) dairy manure, (■) Luttrell PRZ material, (◄) Peerless Jenny King PRZ material, (◆) anaerobic digester sludge; and for agitated bottles inoculated with (Δ) dairy manure and (□) Luttrell PRZ material. Each data point is the average of six independent measurements.

Metal removal occurs due to a combination of physical (i.e.: adsorption) and biological processes (i.e.: sulfate reduction). The effect of the physical processes is expected to be the same in all the bottles considering that they all received the same substrate. Thus, differences in metal removal among bottles with different inocula can be attributed to different sulfate-reducing activity levels as a result of the inoculum used. Zinc concentrations in the liquid phase were observed to decrease significantly in agitated and static LUTR and DM bottles (Figure 4). Removal of zinc by ACC and ADS bottles, however, was about equal with that of the control bottles. The removal of metals in the controls would be expected to be due mainly to adsorption processes (Jong, et al., 2003), although microbial processes were also taking place at low levels. Fe and S removal among the bottles showed a similar removal pattern to zinc.
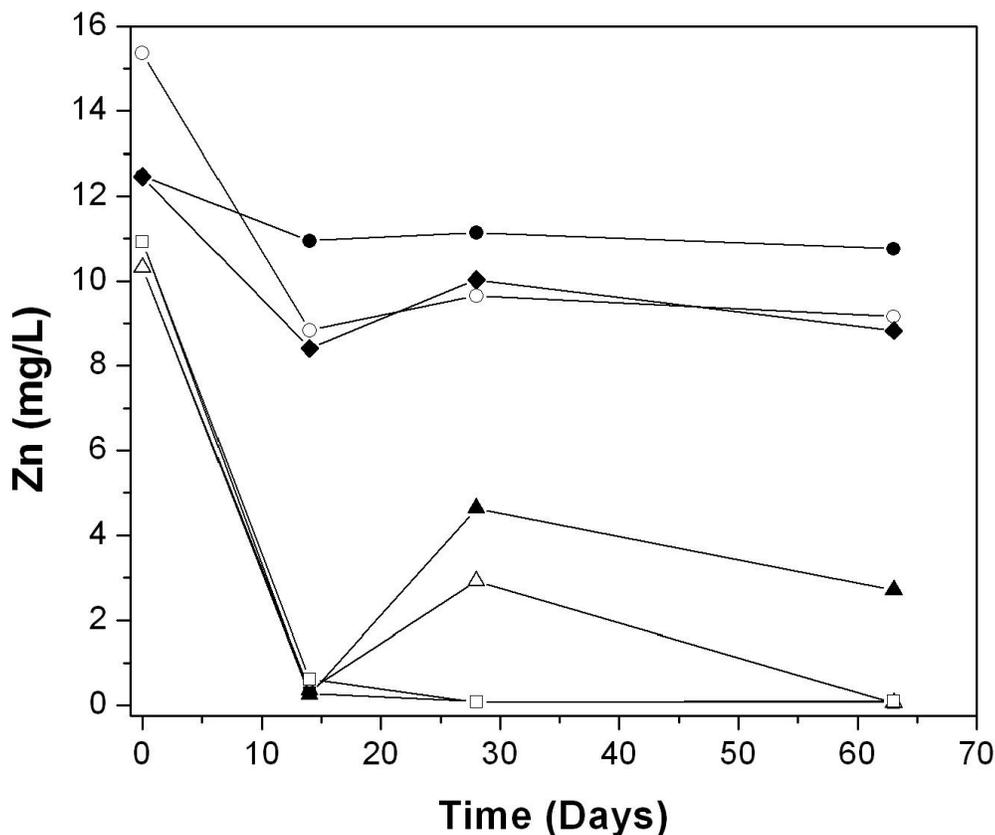
Figure 4. Zn concentration in the filtered liquid phase for (○) static control bottles; static bottles inoculated with (•) twice-acclimated inoculum, (▲) dairy manure, (■) Luttrell PRZ material, (◆) anaerobic digester sludge; and for agitated bottles inoculated with (Δ) dairy manure and (□) Luttrell PRZ material. Each data point is the average of two independent measurements.

The results of this study indicate that bottles inoculated with LUTR show higher sulfate and metal removal than bottles inoculated with the other four inocula or no inoculum. Agitated bottles were also observed to perform better than static ones. It is hypothesized that agitation helps solubilize carbon substrate in the system so that it is more bioavailable. Another possible explanation could be that the agitation allowed for the liberation of any hydrogen sulfide microbubbles trapped by the substrate. These released bubbles would have direct exposure with the dissolved metals in the liquid phase. However, during the extent of this research bottles were manipulated on almost a daily basis for sampling and no obvious bubbles were observed. It must also be cautioned that the agitation is not representative of the static conditions in the PRZs. The results support our previous research which demonstrates the importance of substrate availability for sulfate reduction and metal removal. Nonetheless, inocula that performed well under agitated conditions also showed superior performance under static conditions, therefore it is clear that inocula had an impact in this study. Current work is focusing on molecular analysis of microbial

communities present in the bottles to help identify microorganisms that play a key role in AMD remediation so that they may be better implemented.

Conclusions

This study demonstrates the influence of microbial inoculum in the removal of sulfates and metals from AMD and supports our previous findings in column experiments (Messner, 2004). Bottles inoculated with LUTR inoculum collected from a field PRZ showed the best overall performance and demonstrated the highest metal removal. Future work will focus on further characterization and refinement of inocula for optimized PRZ performance.

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