MANGANESE OXIDE REDUCTION IN LABORATORY MICROCOSMS

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ABSTRACT

Manganese biogeochemistry holds special interest for the characterization of passive treatment systems designed to treat acidic mine waters while meeting enforceable effluent discharge limits set for manganese. In the present study, an initial anoxic enrichment culture was developed for use as an inoculum in experimental systems. Standard anoxic microcosms capable of reducing manganese from Mn^{4+} to Mn^{2+} were established from the initial enrichment and altered to study the effects of electron acceptor availability and inhibitors on manganese reduction. Manganese reduction was not significantly inhibited in aerobic and nitrate amended microcosms; however, systems amended with metabolic inhibitors (sodium azide or sodium molybdate) exhibited significant inhibition of manganese reduction relative to standard microcosms. The presence of iron was found to influence the partitioning of reduced manganese with adsorption becoming more important with increasing iron to manganese ratios.

INTRODUCTION

Manganese is a regulated constituent of acidic mine water; however, removal of manganese to compliance levels has generally proved difficult for passive treatment systems, e.g., constructed wetlands (Weider, 1989). A more thorough understanding of manganese biogeochemistry is necessary for improving manganese removal and retention in wetland treatment systems (Tarutis and Unz, 1996). Physical/chemical treatment processes for manganese removal typically require elevation of the pH of the mine water to facilitate an accelerated rate of manganese oxidation (Sung and Morgan, 1981), followed by gravity separation of the solid phase oxides. Manganese oxidation in passive treatment may be catalyzed by microbial action, often in conjunction with limestone systems (Thorton, 1995) or algae (Phillips et al., 1995). Stark et al. (1996) found that limestone-containing wetland mesocosms largely retained manganese as amorphous oxides that occurred predominately near the surface of the mesocosm. However, precipitation and migration of the manganic oxides to anoxic zones within the wetland sediments subjects the oxides to potential chemical and microbiological reduction, resulting in solubilization of the manganese and possible export of the metal from the treatment system. Tarutis et al. (1992) observed increased concentrations of interstitial manganese lower in the sediments relative to near surface values in a natural wetland receiving acidic mine drainage. Adsorption of manganese is more important as a removal mechanism than sulfide complexation or coprecipitation with iron sulfide (Arakaki and Morse, 1993; Wildeman et al., 1994). However, it is limited by the low affinity that manganese has for organic binding sites compared to other cations found in mine waters (Wieder

and Lang, 1986). Experiments conducted with fresh mushroom compost, a common substrate for constructed wetlands, revealed that manganese was poorly sorbed, especially when iron and copper were present (Machemer and Wildeman, 1992). The preferential adsorption of other metals, especially iron and aluminum, and hydrogen ion, hampers removal and retention of manganese (Wieder et al., 1990) providing for the export of manganese in the effluent. Manganese removal is further complicated by the instability of manganese oxides in the presence of sulfide. Manganese is rapidly reduced by sulfide (Burdige and Nealson, 1986; Aller and Rude, 1988). This places manganese removal at odds with sulfate reduction, which is an otherwise highly effective means of removing metals from solution and increasing pH (Christensen et al., 1996).

The direct bacterial reduction of manganese was also considered as a possible mechanism of manganese reduction in wetlands and in the test systems (Tarutis and Unz, 1996). Direct bacterial manganese reduction unconnected to respiratory needs has been demonstrated by several researchers (Ghiorse and Ehrlich, 1976; De Vrind et al., 1986; Ehrlich, 1987; Lovley, 1991; Nealson and Myers, 1992). The use of oxidized manganese as a terminal electron acceptor is thermodynamically more favorable than sulfate reduction (Zehnder and Stumm, 1988; Lovley, 1991; Nealson and Myers, 1992). Microbially mediated reduction of oxidized manganese may be important in manganese cycling with wetland sediments.

The present study has as its objective the examination of the fate of manganese oxide in anoxic enrichment cultures under various conditions of chemical treatment. Manganese oxide stability in anoxic environments is important in determining the retention of such oxides in wetland sediments designed to treat acidic mine waters.

METHODS

Inoculum Sources

The inoculum source for the standard enrichment culture produced in this study was a small scale, spent mushroom compost mesocosm treating synthetic acidic mine water (50 mg/l Fe and 25 mg/l Mn) at The Pennsylvania State University. Sediment was obtained in nitrogen-filled sterile sample bags (Whirl-Pak) and a 5% (weight:volume) (sediment:medium) suspension of the material was prepared (in a nitrogen atmosphere) to be used as the inoculum for the initial enrichment culture. All subsequent manipulations were also conducted in a nitrogen environment. The initial enrichment culture demonstrated manganese reduction and was then employed as the inocula source for the first standard enrichment cultures or microcosms, which in turn provided inocula for all standard and treatment microcosms. Triplicate standard microcosms were run parallel to all treatments and in each case were found to have at least 80% of the total manganese in the reduced form after 12 days, whereas uninoculated sterile control systems showed no discernible net manganese reduction. Testing standard microcosms parallel to all treatments allowed for verification that the culture's ability to reduce manganese was not changing over the course of the study. The previous series of standard microcosms was used to inoculate the next standard and treatment microcosms, ensuring that the inocula were capable of reducing manganese from the manganic to the manganous state.

Standard Microcosm Culture Preparation

Standard microcosms were developed in 125 ml serum bottles (containing 100 ml of media) sealed with butyl rubber septa, and purged with a nitrogen and carbon dioxide mixture (95:5) prior to inoculation. Standard incubation conditions for enrichment cultures were: 19°C, pH 7.3, and basic culture medium containing 2 mM manganese oxide suspension, which received a 1% (volume:volume) inoculum under a N_2:CO_2 (95:5) headspace. Standard microcosms were compared to all treatments to determine if the reduction of manganese was statistically different in the treatments relative to the standard anoxic microcosms.

Reagents and Culture Media

The culture medium used in microcosm studies was a modification of the medium of Myers and Nealson (1988). The basic culture medium contained (g/l): $(NH_4)_2SO_4$, 1.19; K_2HPO_4, $9.93x10^{-1}$; glucose, $5.40x10^{-1}$; KH_2PO_4, $4.49x10^{-1}$; $NaHCO_3$, $1.68x10^{-1}$; $MgSO_4$, $1.20x10^{-1}$; $CaCl_2$, $5.38x10^{-2}$; Na_2EDTA, $2.26x10^{-2}$; H_3BO_3, $3.50x10^{-3}$; $Ni(NH_4)_2(SO_4)_2$, $1.43x10^{-3}$; $FeSO_4$, $8.20x10^{-4}$; Na_2MoO_4, $7.97x10^{-4}$; $CoCl_2$, $6.49x10^{-4}$; NaCl, $5.84x10^{-4}$; Na_2SeO_4, $2.83x10^{-4}$; $MnCl_2$, $1.59x10^{-4}$; $ZnCl_2$, $1.42x10^{-4}$; DL-Serine, $3.99x10^{-5}$; $CuSO_4$, $3.19x10^{-5}$; L-Arginine, $1.66x10^{-5}$; DL-Glutamine, $1.65x10^{-5}$. The manganese oxide suspension was synthesized by the method of Balistrieri and Murray (1982) utilizing potassium permanganate to oxidize manganese chloride at pH 7. Sulfate reducing bacteria were enumerated by the most probable number (MPN, 5 tube) technique using medium E of Postgate (1984).

Treatments

The effect of iron on manganese dioxide reduction in enrichment cultures under standard conditions was evaluated at several iron to manganese ratios (reducing equivalents ratio and mass ratio as Mn and Fe per liter): 1:5 (26.5 mg Mn : 270 mg Fe), 1:1 (106 mg Mn: 216 mg Fe), and 5:1 (132.5 mg Mn : 54 mg Fe). Iron was added as a suspension of $Fe(OH)_3$ to the culture medium. Ratios are based on reducing equivalents of the metals (1 electron per iron and 2 for manganese). Experiments were performed in duplicate.

The effect of the two alternate electron acceptors and two respiratory inhibitors were investigated individually for their effect on manganese reduction: molybdate (20 mM Na_2MoO_4 final concentration), azide (3 mM NaN_3 final concentration), nitrate (4 mM KNO_3), and oxygen (aerobic incubation). Experiments were performed in triplicate including standard microcosms. Standard microcosm data were pooled for statistical analysis. All systems were incubated statically in sealed anoxic serum bottles except the aerobic microcosms, which were static but open to the atmosphere. Sterile controls were included in all experimental trials.

Manganese Analyses

Microcosm samples were analyzed for fractions of manganese: free soluble, adsorbed, acid extractable, and residual. All samples were taken from vigorously shaken reactors. Manganese determinations were made by atomic absorption spectroscopy (AAS). The definitions of the manganese fractions and the method used to obtain them are as follows:

Step 1: Free soluble: A sample of known volume (1 ml or 5 ml) was passed through a 0.2 µm polycarbonate membrane filter and analyzed for soluble manganese. The manganese concentration in the filtrate was defined as free soluble manganese.

Step 2: Adsorbed: The filter from step 1 (with the retentate) was soaked in 20 ml of 10 mM $CuSO_4$ for 24 hours. A fraction of the copper sulfate solution was used in determining the acid extractable and residual fractions of manganese. A 5 ml aliquot of the remaining solution was passed through a 0.2 µm polycarbonate membrane filter and analyzed for manganese. The manganese concentration was determined, and following appropriate correction for dilution, the value was reported as adsorbed manganese.

Step 3: Acid extractable: A 0.1 ml aliquot of the 24 hour old unfiltered copper sulfate solution produced in step 2 above was added to a scintillation vial containing 5 ml of 0.5 N HCl. The resulting solution was shaken and allowed to stand for 15 minutes. After 15 minutes, the mixture was filtered through a 0.2 µm polycarbonate membrane filter and analyzed for acid extractable manganese. Acid extractable manganese was defined as the concentration of manganese obtained in the step 3 filtrate corrected for dilution minus the adsorbed concentration.

Step 4: Residual: A 0.1 ml aliquot of the 24 hour old unfiltered copper sulfate solution from step 2 was added to a scintillation vial containing 5 ml of 0.25 N hydroxylamine hydrochloride in 0.25 N HCl. After 15 minutes, the resulting solution was filtered through a 0.2 µm polycarbonate membrane filter, analyzed for manganese, and the concentration corrected for dilution. Residual manganese was defined as the corrected concentration from the step 4 filtrate minus the acid extractable and adsorbed concentrations.

An abbreviated procedure to determine total manganese and total reduced manganese was only used to analyze samples taken during the inhibitor and alternate electron acceptor experiments. Total manganese was determined by adding 0.1 ml of sample to 5 ml of 0.25 N hydroxylamine hydrochloride in 0.25 N HCl, waiting 15 minutes, and filtering the resulting solution through a 0.2 µm polycarbonate membrane filter. The filtrate was analyzed by AAS and the value, corrected for dilution, was reported as total manganese. Total reduced manganese was determined by adding 0.1 ml of sample to 5 ml of 0.5 N HCl, waiting 15 minutes, and filtering the resulting solution through a 0.2 µm polycarbonate membrane filter. The filtrate was analyzed by AAS and the value, corrected for dilution, was reported as total reduced manganese.

Data Handling and Analysis

All treatments, excluding iron, were analyzed by Analysis of Variance (ANOVA) using a repeated measures design. The analysis indicated that all treatments were not identical ($p=0.006$). Subsequent analysis was done between standard (anoxic, inoculated, manganese oxide containing, no inhibitors) and treatment microcosms by using the 12 day values for percentage of reduced manganese and performing two tailed t-tests assuming unequal variances. An α value of 0.05 was used for the ANOVA and t-tests. Statistical analysis was performed using Minitab version 10.2 for Windows and Microsoft Excel version 7.0a for Windows 95.

RESULTS

Iron-Containing Microcosms

The fate of manganese after 22 days of incubation in microcosms containing iron and manganese was evaluated. Increased ratios of iron to manganese resulted in a greater proportion of manganese in the adsorbed fraction with lesser amounts of manganese in the acid extractable fraction (Figure 1). The relative distribution of total reduced manganese in the standard system (no iron present) was: acid-extractable > adsorbed phase >> soluble. Ferric hydroxide surfaces appeared to promote adsorption rather than precipitation of reduced manganese as an acid soluble solid such as manganese carbonate (Lovley and Phillips, 1988).

Alternate Electron Acceptor/Respiratory Inhibitor Effects on Manganese Reduction

Mean values of reduced manganese (Mn^{2+}) concentration, percentage of manganese in the reduced form, and t-test results are summarized in Table 1. The aerobic microcosms reduced some manganese oxide to the manganous state over the 16 days of testing and, although the mean value of the percentage of reduced manganese in the triplicate cultures was less than the standard anoxic microcosms, the difference was not statistically significant ($p=0.100$) (Figure 2). The nitrate-amended microcosms demonstrated increasing reduced manganese over the 16 days of incubation (Figure 3). The day 12 percentage of reduced manganese in the nitrate microcosms was not found to be significantly lower than the standard microcosms ($p=0.251$). Azide-amended microcosms (Figure 4) did not appear to reduce manganese, and the 12 day value for reduced manganese was significantly lower than that of the standard microcosms ($p=0.007$). Molybdate-amended microcosms behaved similarly to the azide systems, *i.e.,* no discernible manganese reduction (Figure 5). The t-test on 12 day values clearly demonstrated the difference between the molybdate and standard microcosms ($p=0.004$). The percentage of reduced manganese in the molybdate microcosm was also significantly lower than that in the aerobic and nitrate microcosms ($p= 0.031$ and 0.039 respectively). The pH of three of the standard microcosms (mean $\pm$ standard deviation) was 7.35 ± 0.04 and 7.03 ± 0.02 for zero and twelve days, respectively.

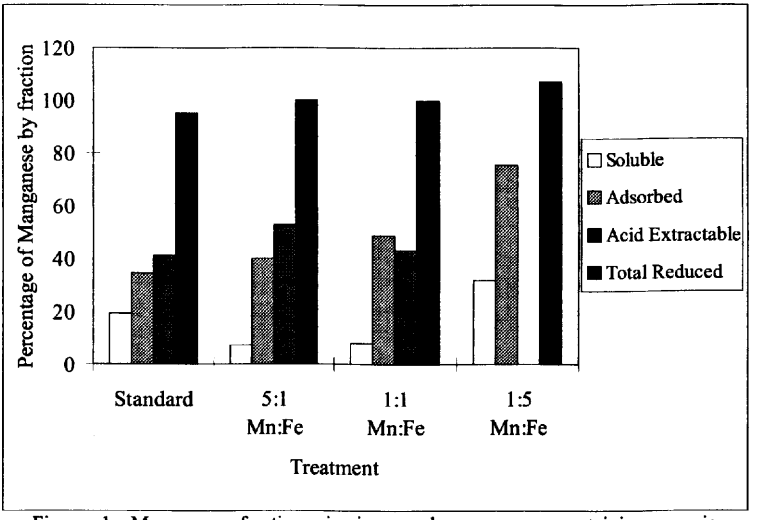

Figure 1. Manganese fractions in iron and manganese containing anoxic microcosms after 22 days (n=2). Acid extractable = Total Reduced - Soluble - Adsorbed.

Table 1. Summary of Reduced Manganese after 12 Days in Alternate Electron Acceptor/Inhibitor Containing Microcosms.

	Standard	Aerobic	Nitrate	Azide	Molybdate
Number of samples (n)	6	3	3	3	3
Mean Reduced Mn (mg/l) ± Standard Deviation	95.4±34.2	60.7±11.3	62.7±8.50	51.6±5.18	36.3±3.71
Mean % Reduced Mn* ± Standard Deviation	82.2±19.3	57.0±7.98	60.8±12.5	38.2±1.27	32.0±10.0
p-value for t-test using % of reduced Mn	----------	0.100	0.251	0.007	0.004

* Calculated as total reduced manganese divided by total manganese recovered; using this value accounts for recovery efficiency.

Sulfate Reducing Bacteria in the Standard Microcosms

The numbers of sulfate reducing bacteria increased to a maximum at day six and remained stable until the end of the sampling at day 11 (Figure 6). The increased density of sulfate reducers immediately prior to the increase in the rate of manganese reduction is consistent with sulfate reducers as critical to the reduction of the manganese oxide.

DISCUSSION

In the absence of iron, reduced manganese was predominately found in the acid extractable form. Acid extractable manganese may have been some form of manganese carbonate as reported by Lovley and Phillips (1988), who used X-ray diffraction to identify the precipitated reduced manganese in their experiments as manganese carbonate. Wildeman et al. (1994) have proposed the precipitation of manganese as rhodocrosite (manganese carbonate, $MnCO_3$) as a useful means of treatment. Stark et al. (1996) found manganese carbonate to be present but less important than oxidized manganese (16% vs. 72%) in a wetland mesocosm successfully removing manganese from synthetic acidic mine drainage.

The increased dominance of adsorbed manganese in high iron microcosms suggests that the exogenous $Fe(OH)_3$ provided additional surface area for adsorption of manganese thereby increasing the likelihood of adsorption versus solid phase precipitation of divalent manganese. However, adsorption of manganese generally proceeds weakly relative to many other metals leading to rapid displacement of adsorbed manganese when other metals are present (Wieder and Lang, 1986, Machemer and Wildeman, 1992).

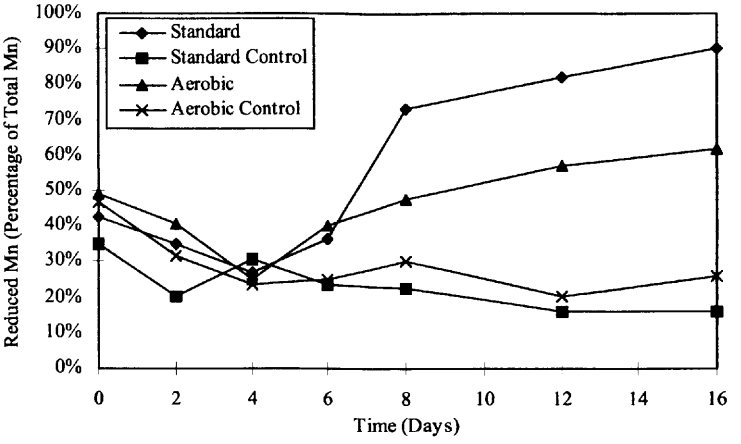

Figure 2. Percentage of reduced manganese (Mn^{2+}) in standard (anoxic; n=6), standard sterile control (anoxic; n=1), aerobic (n=3) and aerobic sterile control (n=1) microcosms. Statistical analysis of data presented in Table 1.

Manganese oxide reduction and dissolution was significantly inhibited by the addition of azide or molybdate to the standard systems. The inhibition of manganese oxide reduction by molybdate suggested that the most likely mechanism of manganese reduction was via reaction with sulfide, as molybdate is a specific inhibitor of sulfate reduction (Postgate, 1984). The enrichment of sulfate reducers in the standard microcosm over time tested provides additional support for sulfate reduction as a likely mechanism of manganese oxide reduction (Figure 6).

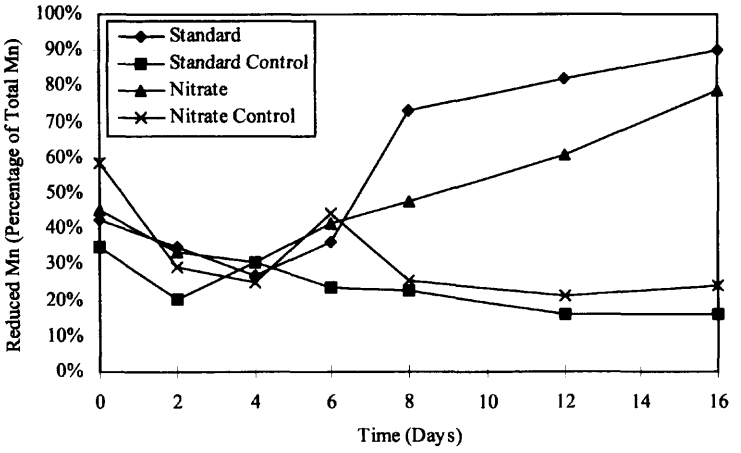

Figure 3. Percentage of reduced manganese (Mn^{2+}) in standard (anoxic; n=6), standard sterile control (anoxic; n=1), nitrate (anoxic; n=3) and nitrate sterile control (anoxic; n=1) microcosms. Statistical analysis of data presented in Table 1.

Direct respiratory reduction of manganese represents another possible anoxic mechanism of reduction. If direct reduction was occurring in the standard microcosms, then inhibition of such a reaction by molybdate, which is only known to inhibit sulfate reduction, would then be very unexpected. Indeed, in previous work with an organism capable of reducing manganese during respiration, Myers and Nealson (1988) and Burdige et al. (1992) found that 9 mM and 20 mM molybdate, respectively, did not inhibit manganese reduction. The inhibition of manganese reduction by azide in the present experiments is consistent with reduction of manganese via either sulfate reduction or direct respiratory reduction, as azide is a general inhibitor of respiration. As azide does not inhibit fermentative processes the inhibition of manganese oxide reduction in the azide microcosms demonstrates that fermentative processes alone could not significantly reduce manganese under the experimental conditions. The increase in density of sulfate reducing bacteria in the standard microcosm tested is consistent with the reduction of manganese oxide by biogenic sulfide. In light of our results and the results of others, it appears that manganese reduction via direct respiratory reduction did not contribute noticeably to the reduction of manganese in the microcosms described in this work.

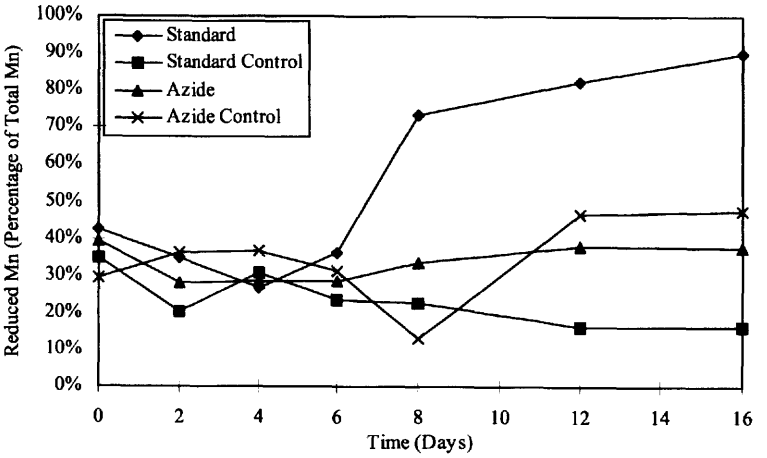

Figure 4. Percentage of reduced manganese (Mn^{2+}) in standard (anoxic; n=6), standard sterile control (anoxic; n=1), azide (anoxic; n=3) and azide sterile control (anoxic; n=1) microcosms. Statistical analysis of data presented in Table 1.

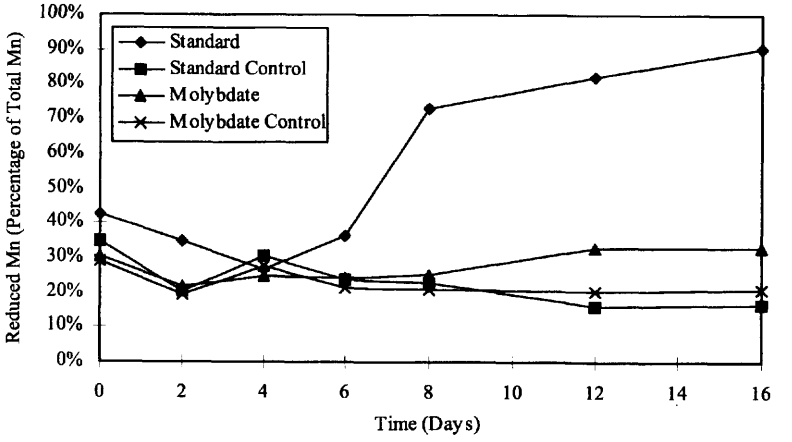

Figure 5. Percentage of reduced manganese (Mn^{2+}) in standard (anoxic; n=6), standard sterile control (anoxic; n=1), molybdate (anoxic; n=3) and molybdate sterile control (anoxic; n=1) microcosms. Statistical analysis of data presented in Table 1.

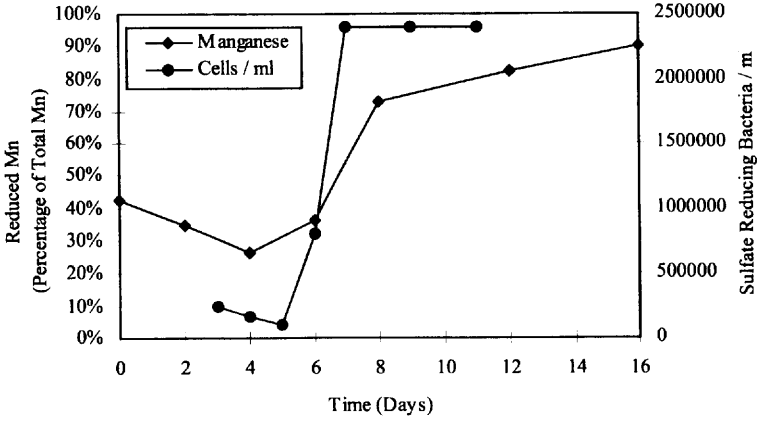

Figure 6. Percentage of reduced manganese (Mn^{2+}) (n=6) and sulfate reducing bacteria (cells/ml) (n=1) in standard anoxic microcosms.

The aerobic microcosms appeared to support less manganese reduction relative to sealed, static systems, but the difference was not statistically significant. The static incubation of the systems may have led to anoxic conditions due to slow mass transfer of oxygen into the bottom of the culture flask, making interpretation of the results difficult as the dissolved oxygen probably varied as a function of depth and time. Whether the reduction of manganese observed in the aerobic systems was due to sulfate reduction, a direct aerobic reduction mechanism (Ehrlich, 1996), or an indirect aerobic mechanism such as reaction with peroxide is unknown (Ghiorse, 1988; Lovley, 1991).

Nitrate-amended systems reduced manganese similarly to the standard system. The manganese reduction observed may be due to sulfate reduction as in the standard systems or some other mechanism such as reaction with nitrite (Lovley, 1991). The chemical reduction of oxidized manganese by nitrite is possible but requires nitrite to be released into the medium rather than be further reduced (Lovley, 1991). The depletion of nitrate and subsequent sulfate reduction is another explanation for the reduction of manganese in the nitrate-amended microcosms.

SUMMARY AND CONCLUSIONS

Manganese reduction in anoxic laboratory microcosms favored the distribution of reduced manganese in the adsorbed fraction relative to the acid-extractable forms with increased ratios of iron to manganese in the solid phase. Azide and molybdate significantly inhibited manganese

reduction while nitrate and oxygen did not. The most important mechanism of manganese reduction in the experimental microcosms appeared to be reduction via biogenic sulfide.

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