8.0 MICROCOSM EXPERIMENTS ON LAKE DEPUE SEDIMENTS

After characterizing the chemical speciation of zinc in the sediment, the interactions between metals and microbial communities under increased metal stress were investigated. The central hypothesis of this work is that resistant microorganisms will play an important role in controlling the speciation of metals in freshwater sediments and that their role will be seen through examination of the speciation. As shown as a result in the previous chapter, the chemical speciation of zinc in Lake DePue varies significantly between the metal loadings at the different collection sites and as a function of depth in the sediment. These changes in speciation may be driven in part by microbial processes. To investigate the metal-microbe interactions, several microcosms were prepared using sediment from Lake DePue. At various points in the evolution of the system, the microcosms were sampled to examine the chemical and biological properties. In these systems, the substrates added were observed to be consumed and the expected products of metabolism produced. In addition, major shifts in the particle zinc speciation were seen in the microcosms that received zinc amendments. This experiment allowed the characterization of the chemical and biological endpoints of the microcosm evolution under increased metal stress.

8.1 MICROCOSM SETUP

Sediments were collected from sites C2 and M1 in an aseptic manner. This was performed by scooping the upper 5 cm of the sediment at each site, storing the sediment in an autoclaved glass jar. The jars were filled to the top, sealed, and taped securely shut to prevent oxygen contamination of the anaerobic sediment. Once in the laboratory, the jars were stored in an anaerobic chamber until the inoculation of the microcosm was performed. For each site, two microcosms were created, one where zinc was added incrementally over the time span of the experiment, and a control that received no additional zinc amendments. In the following, each microcosm is identified by its site location (M1 or C2) and the presence (Z) or absence (NZ) of zinc amendments. Thus the C2 site control microcosm is labeled C2-NZ. The microcosms were designed in order to enrich for metal-reducing microbes, and were supplemented with acetate as the electron donor, and MnO_2 as the electron acceptor. Since metal-reducing bacteria may be more prone to metal exposure released from the surfaces of iron and manganese oxides, they may have more sophisticated mechanisms of resistance. For this reason, this group of bacteria was chosen to be the target for the enrichments.

Lake sediments were added at a 1:10 dilution by volume into an autoclave sterilized minimal media solution in a 2L bioreactor. The transfer of the sediments was performed using a sterile pipette in the anaerobic chamber. The reactors were sealed as soon as the sediments were added to the system. The total volume of the microcosm was approximately 1250 mL. The media contained major salts, trace metals, and required

metabolic vitamins. Table 8.1 gives the composition of the microcosm media. Sulfate was minimized in the media to limit the activity of sulfate reducing bacteria. The microcosms were incubated at a temperature of 30 °C and exposed to ambient room lighting.

Table 8.1: Composition of minimal media used in microcosm systems. Wolin vitamins are a solution of vitamins used in typical anaerobic enrichments, taken after a recipe from Wolin, *et. al.*, 1963.

Component	Concentation (g/L)
NaCl	0.8
NH_4Cl	1
KCl	0.1
KH_2PO_4	0.1
$MgCl_2 * 6H_2O$	0.165
$CaCl_2 * 2H_2O$	0.04
TES buffer	1.5
Wolin vitamins	10 mL / L

All microcosms were sampled twice weekly to measure the concentrations of: pH, acetate, metals (Mn and Zn), and headspace gasses (H_2, CH_4, and CO_2). Preservation of samples for 16S-rDNA phylogenetic analysis also was performed at these sampling periods. Samples were collected at more sparse intervals for DAPI counting and the determination of lipid content. These measurements were performed to give an idea of the amount of microbial biomass during the course of the experiment. Addition of substrates (acetate and MnO_2) and zinc in the appropriate microcosms was performed weekly. The volumes of the additions were designed to approximately equal the

volumes of the samples withdrawn from the system so that the total reactor volume changes were minimal. Typical additions of acetate were approximately 12 mL of 0.5 M total acetate solutions. This concentration was doubled to 1.0 M solutions at day 55 in the experiment, since it was observed that all of the 0.5 M acetate additions were being consumed within one week. Acetate was added as a balance of sodium acetate and acetic acid to so that the pH of the system did not depart significantly from 7.5. Since the acetate system is a weak buffer at this pH the proportion of acetate to acetic acid varied throughout the experiment depending on the pH trends of the system. The solutions used consisted of a pH 7.5 balanced acetate solution with a ratio of acetate to acetic acid of 55:1 and an equimolar solution of the acetate components at pH 4.75. Manganese oxides were added as thick slurries, with an approximate concentration of 1.0 M total Mn in each 30 mL addition. The MnO_2 was prepared by the oxidation of divalent Mn ($MnCl_2$) with permanganate ($NaMnO_4$) (Murray, 1974). This method results in the formation of δ-MnO_2. During the formation of the oxide, excess protons created during the reaction were titrated with NaOH. The resultant particles were rinsed in Milli-Q water until excess permanganate could no longer be detected. The slurry was added to the microcosms without sterilization due to the possibility that autoclaving might change the mineral form of the manganese present. Each of the additions to the microcosm resulted in an approximate increase in concentration of 5-10 mM acetate and 15-30 mM MnO_2. Figure 8.1 shows the input functions of the acetate, zinc, and MnO_2 amendments as a function of time during the experiment.

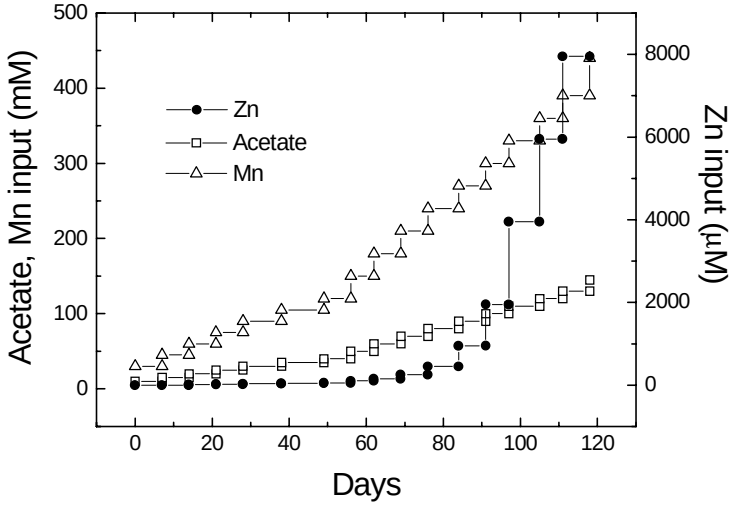

Figure 8.1: Input function of the various nutrient and metal amendments added to the microcosm systems. Zinc was not added to the "-NZ" systems.

With the microcosms setup in this particular configuration, we can describe many of the predicted chemical reactions that will occur. For example, the metal-reducing organisms that are selected for in this experiment can metabolize acetate using MnO_2 as the electron acceptor (Lovley, 1991).

$$CH_3COO^- + 4MnO_2 + 7H^+ \rightarrow 2HCO_3^- + 4Mn^{2+} + 4H_2O$$

Acetate may also be utilized as a substrate through methogenic pathways by archaean microorganisms (Madigan *et. al.*, 1997).

$$CH_3COO^- + H_2O \rightarrow CH_4 + HCO_3^-$$

Products of metabolism such as the bicarbonate ion will react with the metals in the solution, changing the chemical speciation of the metals as eluded to in Chapter 2. Metals present in the solution such as manganese and zinc react with bicarbonate to form solid carbonates as given by (Martell and Smith, 1974-1989):

$$Zn^{2+} + HCO_3^- \Leftrightarrow ZnCO_{3(s)} + H^+ \qquad \log K = 0.3$$
$$Mn^{2+} + HCO_3^- \Leftrightarrow MnCO_{3(s)} + H^+ \qquad \log K = 1.0$$

These reactions, as they release protons, have the tendency to release protons and lower the pH, leading to a release of carbon dioxide as well as the precipitation of the solid. However, since the microbial reactions that produce bicarbonate through manganese reduction consumes 3/2 protons per bicarbonate ion, the pH in the microcosms will increase. This is true as long as manganese reduction rather than methanogenesis is the dominant metabolic pathway that consumes acetate. Previous experiments performed on the DePue sediment system that lacked pH controls showed a significant increase in pH over the time.

Zinc introduced into the system may also have chemical interactions with the sediments that are present in the reactor slurry. These reactions include adsorption onto clay or metal oxide surfaces, as well as reactions with sulfides.

$$\equiv SOH + Zn^{2+} \Leftrightarrow \equiv SOZn^+ + H^+$$
$$FeS + Zn^{2+} \Leftrightarrow ZnS + Fe^{2+}$$

While some of these reactions, such as reactions utilizing acetate, can be monitored easily by measuring the concentrations of the species in solution, the zinc speciation in the particles once again poses a problem similar to that of measuring the speciation of zinc in the lake sediments. The issues of the metal speciation can thus be addressed through XAS.

8.2 MICROCOSM CHEMICAL OBSERVATIONS

A striking qualitative difference in the microcosms was the change in their visual appearances, as can be seen in the tubes that contain aliquots taken from the four microcosms, shown in Figure 8.2. First, sediment particles from the reactor slurries settled differently. In the two control microcosms, the sedimentation of the particles to yield a transparent solution after mixing was slow, on the order of many hours or days. In contrast to this, the rates of sedimentation of the slurries withdrawn from the metal amended systems were rapid, with The C2-Z microcosm settling almost immediately after resuspension (*i.e.* within a minute or two). This can be seen in Figure 8.2 where the liquid in the C2-Z suspension is clear just a few minutes after shaking. The M1-Z system, also settled quickly, but not as quickly as the C2-Z system, on the order of ten to

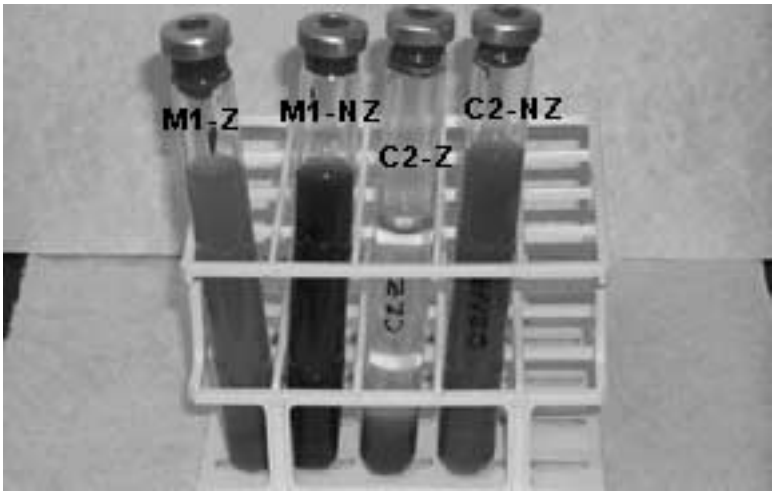

Figure 8.2: Visual appearances of particles removed from the microcosms. All tubes had been thoroughly mixed a few minutes previous to the taking of the picture.

fifteen minutes. In both of the control microcosms (NZ) shown in the figure, the particles remained in suspension for several hours (C2-NZ) to over a day (M1-NZ) after mixing. Additionally, the sediments from M1 evolved to become generally darker and blacker than the C2 sediments.

Figure 8.3 shows the rates of acetate consumption and the concentrations of acetate in the enrichments over the course of the first 100 days of the experiment. Rates were determined by taking the difference in acetate concentration between sampling points and dividing the utilization by the number of time elapsed. All enrichments consumed acetate after a brief lag period of approximately one week. However, the microcosms from M1, the low Zn contamination site, consumed acetate roughly twice as fast (1 mM

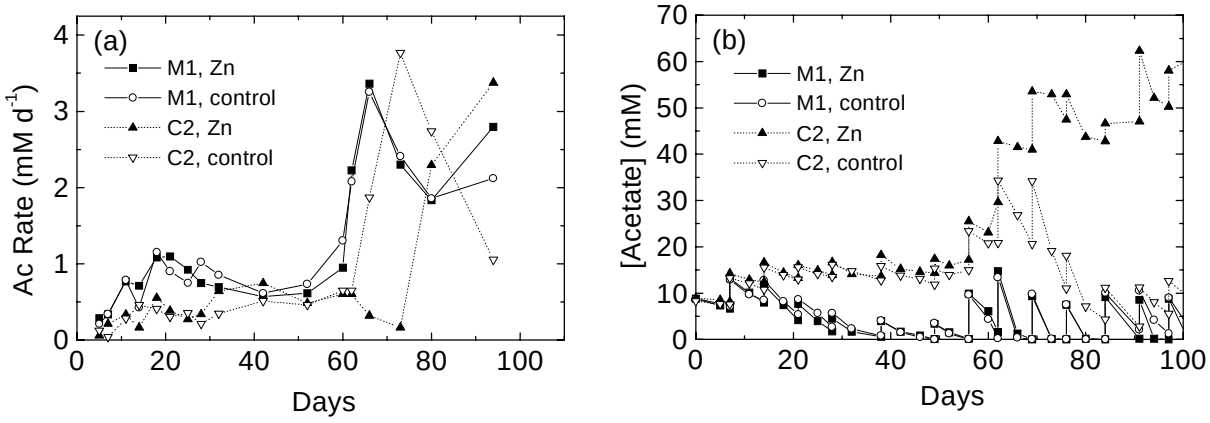

Figure 8.3: Acetate utilization in the microcosms over the first 100 days. **(a)** Rate of acetate consumption in the microcosm. **(b)** Concentration profiles of acetate.

d^{-1} vs. 0.4 mM d^{-1}) as the C2 microcosms until approximately day 35 when all enrichments consumed acetate at approximately the same rate (0.7 mM d^{-1}). It was noted that the M1 enrichments were consuming all of the acetate added by the end of the week, so acetate and manganese oxide additions were doubled starting at day 55 as shown in Figure 8.1. Again, the M1 systems adjusted to the increase and reached a new maximum faster than the C2 systems.

As acetate was consumed in these enrichments, the end products of metabolism, *i.e.* methane and carbon dioxide, were monitored in the headspace. Figure 8.4 shows the evolution of these gasses. The microcosms were observed to have bubbles that evolved from the sediment when shaken and also had significant overpressure when sampling. These plots show the progression of gas accumulation in the headspace before venting occurred to allow for the release of the products.

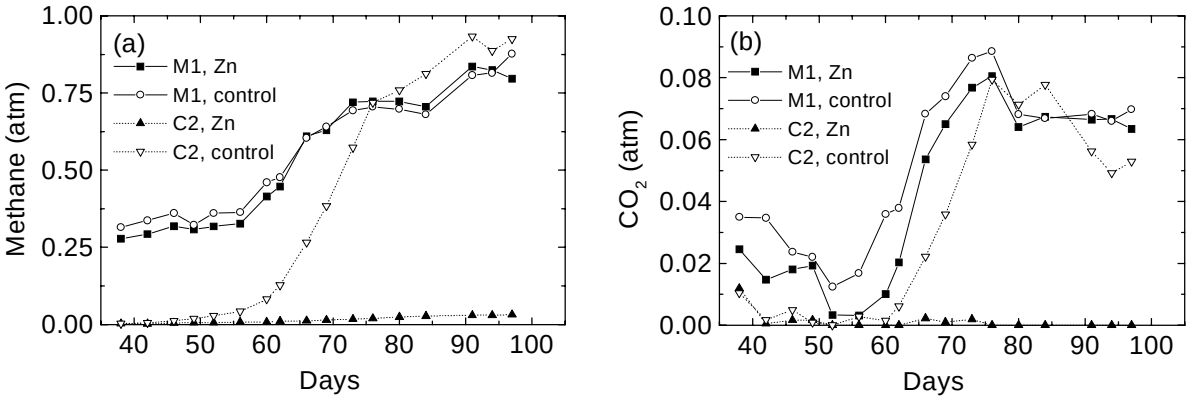

Figure 8.4: Concentration of headspace gasses: **(a)** methane **(b)** carbon dioxide, in microcosms. Gas sampling began at day 35.

As can be seen from these plots, all of the systems had some methanogenesis present, with the C2-Z microcosm producing the smallest amount of methane. Significant CO_2 was also produced in most of the enrichments. The most striking observation is that the C2-Z microcosm produced minimal gasses in comparison to the other systems. This occurred even though the C2-Z microcosm had similar rates of acetate utilization as the C2-NZ system. A possible explanation for this observation is that the zinc added to this system suppressed methanogenesis and the dissolved zinc and manganese acted as a net sink for CO_2 in the form of solid precipitates. The C2 control also showed similar low methane gas evolution until day 55 when the acetate additions were increased. After this point, when acetate in C2-NZ was as rapidly consumed as in the M1 microcosms, the headspace composition became more comparable to the M1 systems.

Dissolved zinc was measured in each system over the course of the experiment using GF-AAS as described in Chapter 4. The dissolved zinc concentration was operationally defined as the zinc passing through a 0.45 μM syringe filter. Figure 8.5 shows the concentration of zinc as a function of time in the enrichments. Initially, the profiles show that the enrichments from C2, the contaminated sediment, have much higher dissolved zinc concentrations than the M1 enrichments. The higher initial zinc is a result of the sediment for these systems originating from a significantly more contaminated environment. These concentrations rapidly drop to the concentrations that are typical of M1 microcosms by day 30. This is most likely due to the production of CO_2 in these systems through metabolic activity of the microorganisms leading to the precipitation of $ZnCO_3$. Additionally, it is important to note that in every case where Zn was added to

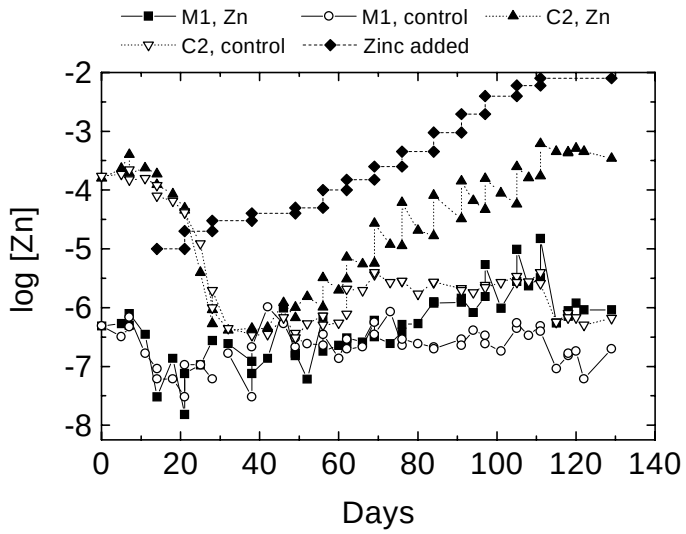

Figure 8.5: Dissolved zinc concentrations as a function of time in microcosm enrichments. Note that the concentrations are plotted on a log scale. The history of the zinc additions in the zinc added system is also plotted.

the systems, the concentration of dissolved Zn after the addition never was more than 20% of the total addition, *e.g.* an addition of 2 mM zinc at the end of the experiment resulted in an increase of only 0.4 mM dissolved zinc. A number of sediment particles from the zinc-amended microcosms were directly examined using scanning electron microscopy equipped with an energy dispersive X-ray ray detector to survey the zinc content of clay particles in the system. These results showed that little to no Zn adsorbed onto clay particles. Thus the loss of dissolved Zn is not due significantly to adsorption processes to particle surfaces within the microcosm. This again supports the notion that Zn was rapidly being removed from the system to a solid phase precipitate.

Several relations can be seen in the above datasets. Concurrent with the removal of dissolved zinc from the C2 systems was the increased utilization of acetate in these reactors. As mentioned above, the lower rates of acetate utilization in the C2 microcosms could be due to the higher concentrations of initial dissolved zinc present. This leads to a slightly longer lag time before the organisms are able to rapidly use the substrates provided. Once the carbonate begins to build in the system as a result of metabolic activity, zinc carbonate becomes oversaturated and is removed form the system. With CO_2 partial pressures around 0.03 atm and pH just above neutral, the maximum concentration of zinc in solution is approximately 2 μM as given by the equilibrium expression for $ZnCO_3$ (smithsonite) stated in Section 8.1. Since the microcosms are not shaken, carbon dioxide may build up in the sediment and it is likely that the partial pressures in of CO_2 in the sediment pores are even higher than in the headspace of the microcosm. The rates of acetate consumption in all four reactors were approximately equivalent during the next period of the experiment (day 30 through 50) when the dissolved zinc concentrations were all less than 1 μM. This is likely to be a result of the microcosms reaching equilibrium with the rate of carbon dioxide being produced from acetate consumption, and the limitation of the zinc concentration in the zinc amended systems at micromolar concentrations by continued carbonate precipitation.

It is also noteworthy that the apparent "death" (*i.e.*, significant build up of acetate and lack of CH_4 and CO_2 production) of the C2 zinc added enrichment occurred shortly after the increase in the acetate additions (day 55). This is also about the same time when the

concentrations of dissolved Zn in this microcosm began to rise again. Thus, there is a noticeable correlation between the dissolved zinc concentrations and the utilization of acetate over the history of these systems, although the cause and effect nature of this evidence is rather speculative at this time due to the complexity of the system.

8.3 XAS OF MICROCOSM PARTICLES

The microcosm sediments were characterized by X-ray absorption spectroscopy at the end of the experiment to determine the speciation and coordination of zinc and manganese. This was done in order to check the form of any metal precipitates that had formed and to verify that the MnO_2 added to the system had been utilized. The EXAFS signals obtained in these samples showed that the predominant zinc coordinative environment was either sulfide or carbonate. Table 8.2 gives the calculated proportions of ZnS and $ZnCO_3$ in the microcosms and Figure 8.6 shows the EXAFS spectra at the Zn K-edge for each of the samples and their associated fits. As noted previously in Chapter 4, the fits were verified by comparing the reconstructed XANES spectra to the XANES data that was collected.

Table 8.2: Calculated speciation of zinc in microcosm solids as determined by X-ray absorption spectroscopy at the end of the experiment.

	% ZnS	% $ZnCO_3$	Total conc. (ppm)	[ZnS] (ppm)	[$ZnCO_3$] (ppm)
M1-Zn	66 ± 6	34 ± 6	603 ± 18	398 ± 40	205 ± 40
M1-Control	71 ± 7	29 ± 7	172 ± 3	122 ± 13	50 ± 13
C2-Zn	11 ± 3	89 ± 4	1638 ± 16	180 ± 42	1458 ± 48
C2-Control	43 ± 5	57 ± 5	1245 ± 14	535 ± 60	710 ± 60

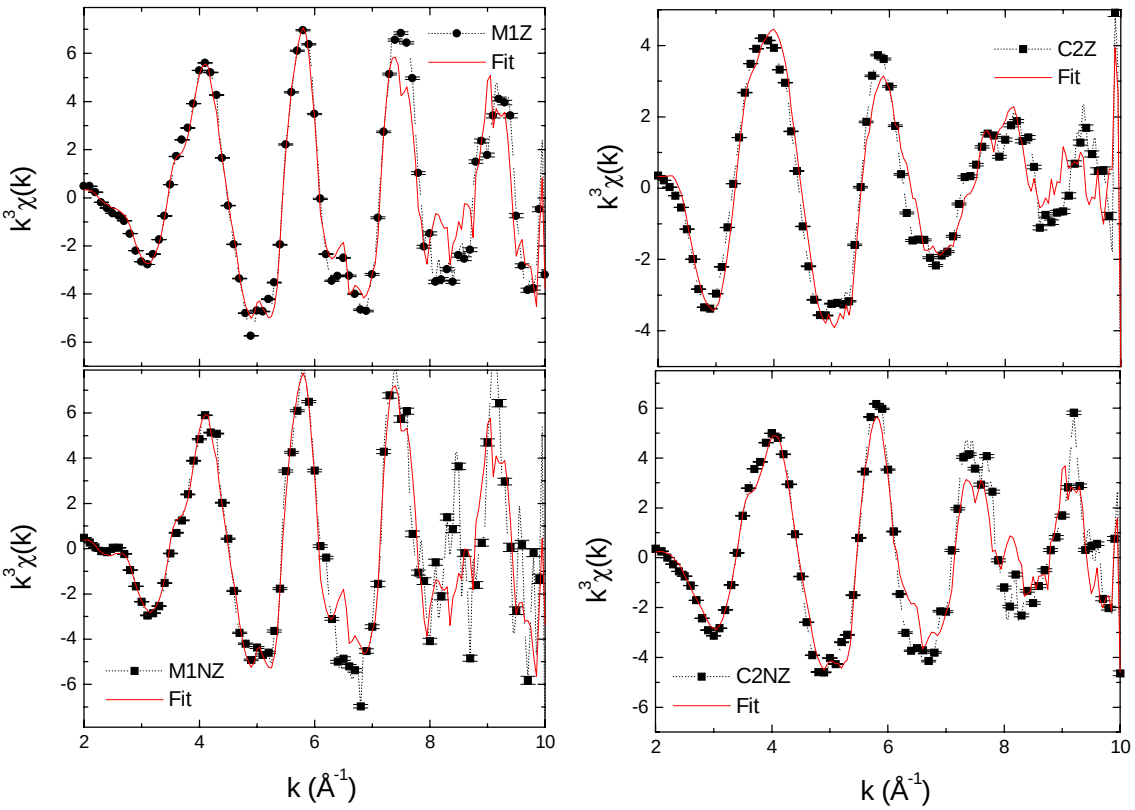

Figure 8.6: EXAFS spectra of the Zn-K edge for each of the samples taken from the microcosms at the end of the experiment. The spectral deconvolution fit is indicated in red. In each case, the fit is able to explain the major features of the spectrum. Fitting at higher k values is made difficult due to noisy EXAFS signals.

The microcosm sediments from M1 show very similar composition in both the control and the zinc added system. The composition of the solids is very similar to what is found originally in the sediments of Lake DePue at M1, with a small increase in the $ZnCO_3$ content. However, the zinc that was added to this system precipitated as a mixture of ZnS and $ZnCO_3$. Since there was very little initial sulfate present in the microcosm and no sulfate was added during the experiment to be reduced to sulfide, the concentrations present cannot lead to the magnitude of observed ZnS precipitation.

Thus the ZnS must have come from an alternative source other than as a result of dissimilatory sulfate reducing bacteria. The ZnS produced is likely to have originated from the conversion of available iron sulfides to zinc sulfides:

$$FeS + Zn^{2+} \Leftrightarrow ZnS + Fe^{2+}$$

The addition of dissolved zinc disturbed the normal sulfide equilibrium leading to the formation of ZnS over iron sulfides, as ZnS has a greater stability than the iron sulfides (Dyrssen and Kremling, 1990; Davison, 1991; Stumm and Morgan, 1996). The thermodynamic data for the solubility products of various iron, manganese, and zinc sulfides that supports this reasoning are given in Table 8.3. The data show that zinc has a much higher affinity for the sulfide ligand than either iron or manganese. A concentration excess of at least roughly six orders of magnitude of iron is required in order for FeS to be thermodynamically more stable.

Table 8.3: Thermodynamic data for metal sulfides at 25 °C and $I = 0$. The constants listed are for the reaction: $MeS_{(s)} + H^{+} = Me^{2+} + HS^{-}$. References: (a) Dryssen and Kremling, 1990; (b) Davison, 1991.

Sulfide	Log *Ks	Reference
FeS		
(amorphous)	-4.2	(a)
(amorphous)	-2.95	(b)
(troilite)	-5.25	(b)
(mackinawite)	-3.6	(b)
(pyrrhotite)	-5.1	(b)
MnS		
(green)	0.17	(a)
(pink)	3.34	(a)
ZnS		
(α-ZnS, sphalerite)	-10.93	(a)
(β-ZnS, wurtzite)	-8.95	(a)

The sediments from the C2-NZ enrichment at the end of the experiment showed a composition similar to the natural sediments present at this site in Lake DePue. However, the zinc-amended enrichment shows significantly more carbonate at the end compared to the control. In addition, the difference is more than the amount of zinc that was added to the system. This strongly supports the removal of zinc through extensive carbonate precipitation, as well as showing that ZnS in a sulfide limiting case can be dissolved and forms $ZnCO_3$. This is a possible explanation for the lack of CO_2 buildup in the C2-Z microcosm. The C2 site at DePue generally has less free sulfides in the system than the M1 site, and could be considered a sulfide limited sediment. Most sulfide that does exist in the surficial sediments is likely to be tied up as ZnS. The lack of excess sulfides in the system at C2 as compared to M1 then could lead to the dominance of zinc carbonate precipitation in the C2 microcosms.

Additionally, XANES spectra showed that all of the MnO_2 added to the enrichments had been converted to Mn(II) by the end of the experiment, as displayed in Figure 8.7. Mn(II) was present as a $MnCO_3$ precipitate. This supports microbiological reduction of the oxides for metabolic energy and the subsequent precipitation of Mn(II) by carbonates, also a product of metabolic activity. It should be noted that the C2Z microcosm did show a small fraction (5% ± 3) of MnO_2 present in the sediment at a level just above the possibility of detection. Because the deconvolution results in a small percentage of the oxide, the quantitative numerical result for its proportions should be scrutinized. However, qualitatively, it is still present in the system. Figure 8.8 shows the radial distribution functions for several of the manganese standards and samples

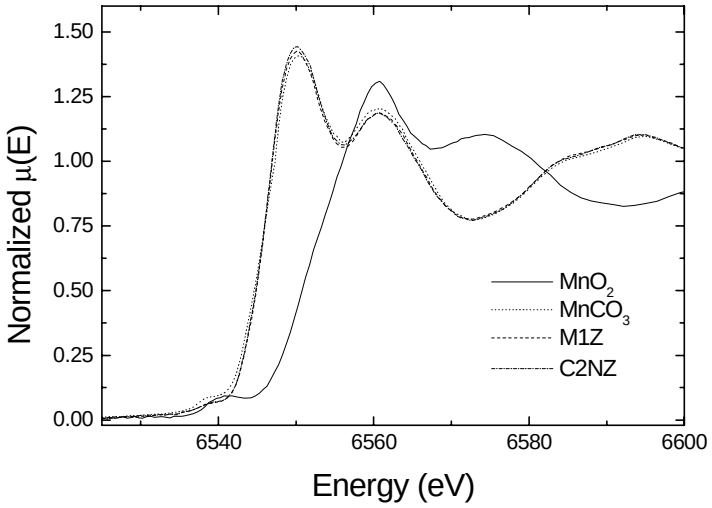

Figure 8.7: XANES spectra of standard manganese compounds and microcosm samples. Not all samples from the microcosms are displayed for clarity.

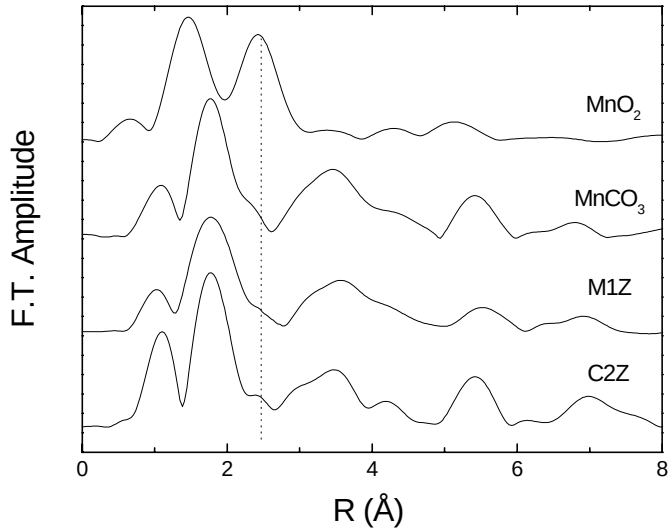

Figure 8.8: Radial distribution function of manganese oxide compounds and microcosm samples. No corrections for phase shift have made in this figure. Note that the C2Z sample has a small lobe at ~2.25 Å, indicating the presence of some MnO_2.

from the microcosms. The residual manganese oxide present in the C2Z system may be a result of the slowed acetate utilization at the end of the experiment in this microcosm. This is due to fact that the major pathway for the reduction of MnO_2 is through microbial

activity. Since the activity of the system was much smaller in the C2Z system than the other microcosms and the acetate added to the system was not fully utilized (Figure 8.3), the manganese oxides as a co-substrate can also be expected to build up in concentration in the system. Again, it is likely that this lack of substrate utilization was due to the build up of dissolved zinc in the microcosm.

8.4 MICROCOSM MICROBIAL POPULATIONS

Aliquots of microcosm solids were analyzed for bacterial populations by fingerprinting the 16S-rDNA genes. This work was performed in the Stahl lab and is the subject of the Ph.D. thesis of Heidi Gough. Thus, just a brief outline of the terminal restriction fragment length polymorphism (T-RFLP) technique and results will be presented here. This technique essentially differentiates organisms by the analysis of the cleavage patterns of their DNA. The DNA samples are digested with a particular restriction endonuclease enzyme that targets and cuts specific, usually palindromic, nucleotide sequences. Organisms that are genetically different will have different lengths of nucleotides between cleavage sites, and thus produce different fragment lengths after the digestion. The length patterns that result can be used to fingerprint the system.

Results show that similar bacterial species, as indicated by their base pair fragment lengths, existed in all of the microcosms. Ten signatures dominated the bulk of the DNA extracted from the systems. The populations of these bacteria went through some mild

fluctuations during the experiment, with the most drastic occurring at the very end when the reactors suffered a build up of metabolic end products. Although noticeable changes occurred chemically in the microcosms, changes in the bacterial makeup of the community were much less evident. These changes are likely due to a compounded series of variables, including, but not entirely consisting of the zinc concentration. One of the more interesting results with the T-RLFP was the fingerprinting of the archaea populations. These organisms showed greater variability, with over 30 fragment signatures observed. Additionally, the signatures fluctuated dramatically during the course of the experiment, however, not in any apparent related way to the chemical changes. The large diversity, and amount of archaean DNA extracted from the system may suggest that the archaea have an important role in the microbiological ecosystem in Lake DePue.

8.5 RESULTS OF THE MICROCOSM EXPERIMENTS

The above has shown that the amendments of zinc had little effect on the uncontaminated site, and the largest effect in the contaminated site. This shows that the microbial communities between sites behave differently through the chemical observations made. These are intriguing results, as one might expect that the microbial community in the contaminated section of the lake might be more likely to show increased resistance. However, the changes the zinc had on the microbial population is much less clear. The additions of zinc did lower the substrate utilization of both

methanogens and manganese-reducers in the C2Z microcosm. This was observed by the lack of significant methane production compared to the M1 and control systems, and the build up of the substrates added, both MnO_2 and acetate. However, the presence of carbonate precipitation leading to the removal of much of the amended zinc shows the problems involved in maintaining metal stress in a closed, anaerobic microcosm.

The large number of environmental and chemical variables in the microcosm system blurs many of the cause and effect relationships that might be hypothesized. With this system, it is difficult to determine which changes in speciation are due to direct microbial interactions, indirect involvement (*i.e.*, chemical interactions with metabolic end products), and those that are solely abiotic chemical reactions. In order to understand these relationships, a greater understanding of the microbial populations present and their resistance mechanisms must be achieved in a simpler system. To this end, the microcosms were also used as enrichment reactors to isolate some of the organisms in pure culture. The chemistry of the microbial interactions with zinc and was monitored with these organisms. The results of this study are discussed in the next chapter.