IMPORTANCE OF FUNGI IN BIOLOGICAL MN(II) OXIDATION IN LIMESTONE TREATMENT BEDS[1]

William D. Burgos[2], Hui Tan, Cara M. Santelli, and Colleen M. Hansel

Abstract. Coal mine drainage (CMD) is a serious threat to the environment of Appalachia in the United States, releasing large volumes of acid and metal contaminants (e.g., Mn, Fe). Passive limestone-based treatment systems are the most cost-effective method for manganese(II) (Mn(II)) removal from CMD. The success of passive Mn(II)-removal systems has been variable due to a poor understanding of the mechanisms of Mn removal and the microbial communities involved. We selected one Mn-removal system from northwestern Pennsylvania that treats an exceptionally high concentration of 150 mg/L Mn(II), and conducted laboratory experiments to evaluate the relative importance of abiotic versus biotic processes responsible for Mn removal, and to evaluate the relative importance of bacteria versus fungi on biological Mn(II) oxidation. We found that while abiotic processes such as Mn(II) sorption and heterogeneous oxidation contribute to Mn removal, biological Mn(II) oxidation is the most important process to ensure effective, long-term Mn removal. We also found, that fungal activity accounted for over 80% of Mn(II) oxidation in this Mn-removal bed. We also selected four additional Mn-removal systems from western Pennsylvania for an extensive culture enrichment survey of bacteria and fungi. From this survey, we found that Mn(II)-oxidizing fungi were isolated more readily than Mn(II)-oxidizing bacteria – fungal isolates outnumbered bacterial isolates 84:10 in 3 of the 4 systems, and that fungi were extremely tolerant to elevated concentrations of Mn(II).

Additional Key Words: acid mine drainage, metal oxidation, manganese removal

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Introduction

The removal of elevated concentrations of dissolved manganese, Mn(II), from mine drainage is a significant problem for many regions in the United States and throughout the world. In Appalachia, centuries of coal mining have left thousands of abandoned mines that are discharging metal-contaminated effluent, with Mn concentrations as high as 150 mg/L, and severely degrading water resources (Cravotta III, 2007 and references therein; Herlihy et al., 1990). Manganese, while not considered to be acutely toxic to humans (although this is poorly studied), can be damaging to ecosystems and water distribution networks. Furthermore, Mn(II) oxidation reactions produce protons, which perpetuates the generation of acid mine drainage (AMD). In the eastern United States, one of the most common methods to remediate high concentrations of dissolved Mn(II) in coal mine drainage (CMD) is the use of biologically-active limestone treatment beds, where oxidation and subsequent precipitation of Mn(III/IV) oxides is catalyzed by abiotic and biotic processes (Hallberg and Johnson, 2005; Johnson et al., 2005). In essence, Mn is immobilized via the precipitation of sparingly soluble minerals, which subsequently remove other metal contaminants (e.g., Cu, Co, Zn) through both co-precipitation and surface adsorption reactions. To date, the overall success of Mn removal within passive treatment systems is widely variable due to a poor understanding of the processes and mechanisms that govern Mn(II) oxidation at near-neutral pH in these systems.

Microorganisms accelerate Mn(II) oxidation rates up to several orders of magnitude faster than abiotic catalysis, thus it is believed that the precipitation of Mn(III/IV) oxide minerals in the environment is largely driven by microbiological activity (Nealson et al., 1988; Tebo, 1991; Tebo et al., 2004). Mn(II)-oxidizing bacteria are ubiquitously distributed in the environment and much research has recently been devoted to understanding the mechanisms, pathways, and products of Mn(II) oxidation by bacteria (Bargar et al., 2005; Dick et al., 2008b; Francis et al., 2001; Hansel and Francis, 2006; Johnson and Tebo, 2007; Ridge et al., 2007; Webb et al., 2005a; Webb et al., 2005b). Although much less studied, Mn(II)-oxidizing fungi have also been recovered from a wide variety of ecosystems, such as agricultural soil (Pedler et al., 1996), deep sea sediments (Shao and Sun, 2007), building stone, (de la Torre and Gomez-Alarcon, 1994), desert varnish (Grote and Krumbein, 1992; Krumbein and Jens, 1981), streambeds (Miyata et al., 2006a; Miyata et al., 2004; Takano et al., 2006), and an artificial wetland (Takano et al., 2006).

The contribution of microbial activity in the remediation of Mn-contaminated waters has frequently been observed (Bamforth et al., 2006; Haack and Warren, 2003; Hallberg and Johnson, 2005; Johnson et al., 2005; Johnson and Younger, 2005). Several different strains of Mn(II)-oxidizing bacteria have even been used in a patented bioremediation method, the "Pyrolusite Process", for treating manganiferous mine waters (Vail and Riley, 2000). A recent study by Mariner et al. (2008) identified Mn(II)-oxidizing fungi, in addition to bacteria, that successfully grow in a Mn(II) attenuation bioreactor for treating mine waters. To our knowledge, this is the first study to document the role of fungi in these bioremediation technologies. In general, however, the identities and growth characteristics of the Mn(II)-oxidizing community contributing to Mn remediation remains largely unresolved.

The purpose of this research was to improve our understanding and design capability of passive Mn(II)-removal systems for the treatment of coal mine drainage. The objectives of this research were to: 1) measure Mn(II)-removal kinetics in controlled laboratory experiments; and 2) characterize the microbial communities that promote Mn(II) oxidation in these Mn-removal systems using a culture-based approach.

Materials and Methods

Site description and sampling techniques

Five Mn-removal systems in western and northwestern Pennsylvania were examined in the current study (Table 1): Fairview (Elk County, PA), Saxman Run (Westmoreland County, PA), De Sale Phase 1, De Sale Phase II, and De Sale Phase III (Butler County, PA). The Fairview site has been described by Tan et al. (2010), and the four other sites have been described by Santelli et al. (2010). Sediments and water collected from the Fairview site were used to measure Mn(II) oxidation kinetics in controlled laboratory experiments. Sediments collected from all the sites were used to isolate, identify, and culture microorganisms that catalyze Mn(II) oxidation.

Water samples were filtered (0.2 μm) in the field and chemically preserved (dependent on analyte). Sediment "crust" samples were collected with sterile spatulas and placed in sterile endotoxin-free centrifuge tubes or whirl-pak bags. Sediments were scraped from the top 1-cm of black precipitates that armored the limestone cobbles. All samples were stored on ice for transport to the laboratory, except for samples intended for nucleic acid-based microbial

community analysis, which were transported on dry ice. Dissolved oxygen (DO) concentrations, temperature, pH and conductivity were determined in the field using portable meters.

Table 1. Geochemistry of influent waters to the five Mn-removal beds examined in the current study.

	Fairview	DeSale Phase 1	DeSale Phase 2	DeSale Phase 3	Saxman Run C1
pH	5.43	5.73	6.60	-[a]	6.62
Dissolved O_2 (mg/L)	5.5	5.4	7.1	-	11.3
Total Organic Carbon (mg/L)	2.2	5.5	2.9	-	3.5
Total Nitrogen (mg/L)	0.30	0.016	0.093	-	2.2
Al (mg/L)	5.1	0.55	0.01	-	0.06
Fe (mg/L)	<0.01	0.11	0.09	-	<0.01
Mn (mg/L)	150	54	46	119 [b]	28
Cd (mg/L)	0.024	<0.002	<0.002	-	0.002
Co (mg/L)	0.341	0.504	0.624	-	0.290
Cr (mg/L)	0.024	0.017	0.015	-	0.007
Cu (mg/L)	0.005	0.004	0.003	-	0.006
Ni (mg/L)	0.591	0.445	0.463	-	0.881
Pb (mg/L)	0.010	0.007	0.005	-	0.002
Zn (mg/L)	0.685	0.375	0.117	-	0.438
Post-treatment Mn (mg/L) [c]	70	33 [b]	24 [b]	67 [b]	<0.05
Mn removal efficiency (%)	53	39	48	44	100

[a] Not measured because water was not flowing into treatment pond on the day of sample collection.
[b] Data analyzed and contributed by the Slippery Rock Watershed Coalition (http://www.srwc.org/).
[c] Measurements of outflow waters from treatment system.

Sediment incubation experiments

Laboratory sediment incubation experiments were conducted to determine the relative importance of biological activity on Mn(II) loss from solution, the effect of dissolved oxygen on Mn(II) removal, the effect of supplemental organic carbon on Mn(II) removal, and the relative importance of fungi versus bacteria on Mn(II) removal (Table 2). Experiments were conducted with a single, large quantity of sediment collected from the Fairview Mn-removal bed. Sediment crusts were collected with sterile spatulas, homogenized, and wet sieved (<2-mm sieve fraction), using sterile tools in the field, into sterile mason jars. Experiments were conducted with 1.0 g moist "live" sediments (0.82 g dry mass) mixed with 50 mL of filter sterilized influent water in 120 mL serum bottles with air or 100% N_2 in the headspace and sealed with thin Teflon-coated stoppers and Al crimp tops. Abiotic controls were conducted with "killed" sediments that been exposed to 100 kilogray of ^{60}Co γ irradiation and maintained under a 100% N_2 headspace. No

loss of Mn(II) was measured in sediment-free controls under an air headspace. All reactors were kept in the dark and shaken at 100 rpm at room temperature (25 ^{o}C). Water and sediment suspension samples were periodically collected from the reactors using sterile needle and syringe to measure soluble Mn(II) and pH. Samples were centrifuged at 11,000 *g* for 10 min to separate liquid and sediments. Mn(II) concentration in the supernatants were measured using the PAN method (Goto et al., 1976). pH was measured using a combination electrode (Thermo Scientific, Waltham, MA). When the soluble Mn(II) concentration decreased to below detection limit (<0.1 mg/L) in the "live" reactors, $MnCl_2$ was re-spiked into the reactors to re-establish a Mn(II) concentration of ca. 150 mg/L; $MnCl_2$ was added to the reactors three times. This mode of reactor operation is referred to as "fed-batch", where batch refers to no-flow conditions, and fed refers to re-spiking Mn(II).

Table 2. Summary of conditions used in the Fairview sediment incubation experiments. All experiments were conducted with 1.0 g moist sediment (0.82 g dry) mixed with 50 mL filter sterilized influent water in 120 mL serum bottles (controlled headspace). All experiments included "killed" controls where the sediments were subjected to ^{60}Co γ irradiation.

Experimental variable	Objective of experiments	Results
"live" sediments vs. "killed" sediments	differentiate extent of Mn(II) removal due to biotic vs. abiotic processes	Figure 1
"live" sediments under 0.21 atm P_{O2} vs. "live" sediments under 0.0 atm P_{O2}	differentiate extent of Mn(II) removal due to oxidative precipitation vs. non-oxidative sorption	Figure 1
"live" sediments under 0.21 atm P_{O2} vs. "live" sediments under 0.10 atm P_{O2} vs. "live" sediments under 0.01 atm P_{O2}	determine the dependence of Mn(II) removal on the concentration of dissolved oxygen	Figure 2
"live" sediments with fungicides vs. "live" sediments	differentiate extent of Mn(II) removal due to fungi vs. bacteria	Figure 3
"live" sediments with 10 mg C/L glucose vs. "live" sediments with 10 mg C/L cellulose	determine if, and what type of, organic C source might stimulate Mn(II) removal	Figure 5

Culture enrichments and isolations

Rock and sediment samples were lightly crushed and homogenized using a mortar and pestle. All samples were diluted in sterile, artificial freshwater (AFW; 34.2 mM NaCl, 1.62 mM $MgSO_4$*$7H_20$, 1.36 mM $CaCl_2$*$2H_20$, 0.44 mM K_2HPO_4*$3H_20$, 20 mM HEPES buffer at pH 7) using serial dilutions to $1/10^4$. Dilutions were plated onto 7 different types of agar-solidified

media with 20 mM HEPES buffer (pH 7) and 200 µM $MnCl_2$. The culture media were described previously: HEPES-buffered AY medium (Miyata et al., 2004); K, Leptothrix, and M media with 0.2 µm filter-sterilized natural site water (Templeton et al., 2005); J and J+acetate media with AFW (Hansel and Francis, 2006); and Medium 3 (de la Torre and Gomez-Alarcon, 1994). Mn(II)-oxidizing bacteria and fungi were putatively identified by the presence of brown/black precipitates, Mn(III/IV) oxides, and transferred to fresh media a minimum of 5 times until cultures were deemed axenic. Mn(III/IV) was confirmed using the leucoberbelin blue (LBB) colorimetric assay – LBB reacts specifically with Mn(III) and Mn(IV) and turns deep blue (Krumbein and Altman, 1973).

Growth experiments

Fungal isolates were tested for their ability to grow and oxidize Mn(II) in the presence of varying metal concentrations. Fungi were grown in dark conditions on AY media supplemented with Mn^{2+} (added as $MnCl_2$) at the following concentrations: 0 µM, 250 µM, 500 µM, 750 µM, 1 mM, 5 mM, and 10 mM. Fungi were inoculated with a toothpick in the center of petri dishes and allowed to grow radially outward until the diameter of mycelia reached the plate edges (typically less than 10 days). Colony growth diameter was evaluated approximately every other day. The effects of light/dark and pH (5.5, 6.0, 6.5, 7.0, 7.6, and 8.0) on growth were also tested using the same protocol. MES buffer (20 mM) was used to maintain a solution pH of 5.5, 6.0, and 6.5, and HEPES buffer (20 mM) was used for pH values ≥7.0.

Results and Discussion

Mn(II) removal in the field

Limestone beds designed for Mn removal from CMD create a unique geochemical and physical environment for promoting biological Mn(II) oxidation. To function most effectively, Fe, Al, and some of the influent acidity are removed first through limestone-based systems before entering the Mn-removal system. In a survey of 140 abandoned coal mine discharges in Pennsylvania, the median Mn concentration was 2.35 mg/L with a range from 0.019 to 74.0 mg/L (Cravotta, 2008). Most of these discharges were from underground mines. For the five Mn-removal systems studied, all were bituminous surface coal mines, and influent Mn concentrations ranged from 28 to 150 mg/L (Table 1). For the Fairview site, the exceptionally high Mn concentration (130 – 150 mg/L) is believed to originate from reactions with the

overburden (e.g., $MnCO_3$ inclusions in limestone). Influent CMD to the Fairview Mn-removal system contains essentially no Fe; Ca and Mg are elevated from upstream neutralization. The elevated concentrations of Mn and Al make the water net acidic. Sulfate is the primary anion and nutrient concentrations (i.e., TOC, N) are relatively low (P < 0.1 mg/L). We have sampled this system on several occasions between December 2005 and May 2007 to capture seasonal effects and have found that Mn is never completely removed through the limestone bed (Tan et al., 2010). Greater removal of Mn(II) occurs in summer months (e.g., 35 mg/L effluent Mn), as compared to the winter sampling event (e.g., 70 mg/L). For the three DeSale systems, Mn removal efficiencies ranged from 39 to 48% (Table 1). For the Saxman Run system, 100% of the Mn was removed.

Mn(II) removal via biotic versus abiotic processes

Reactors containing wet-sieved (<2-mm), MnO_x-rich sediment crusts and their associated natural microbial communities were operated in a "fed-batch" mode with respect to dissolved Mn(II). $MnCl_2$ was periodically re-spiked into the reactors to re-establish the initial, influent Mn(II) concentration of ca. 2.5 mM. This fed-batch approach was required to overcome abiotic Mn(II) removal processes such as non-oxidative sorption and heterogeneous oxidation so that the contribution of biological Mn(II) oxidation on Mn removal could be better ascertained. Control reactors that had been sterilized by ^{60}Co γ-irradiation and/or maintained under a N_2 atmosphere were compared to "live" (i.e., non-sterile) reactors. Our simplified, operational interpretations were that Mn(II) could be removed only by non-reductive sorption in sterilized reactors maintained under N_2, while both heterogeneous oxidative precipitation of Mn(II) (as MnO_x) and non-reductive sorption could occur in sterilized reactors maintained under air.

As seen with the live reactors maintained under air or under N_2 (black and white squares, respectively, in Fig. 1), the difference between Mn(II) removal through each fed-batch cycle increased as the experiment proceeded. Our interpretation of these results is that the non-reductive Mn(II) sorption capacity of the sediments was essentially saturated after four cycles. The growing difference between the live reactors maintained under air versus the live reactors under N_2 was presumably caused by biological Mn(II) oxidation and the subsequent precipitation of MnO_x.

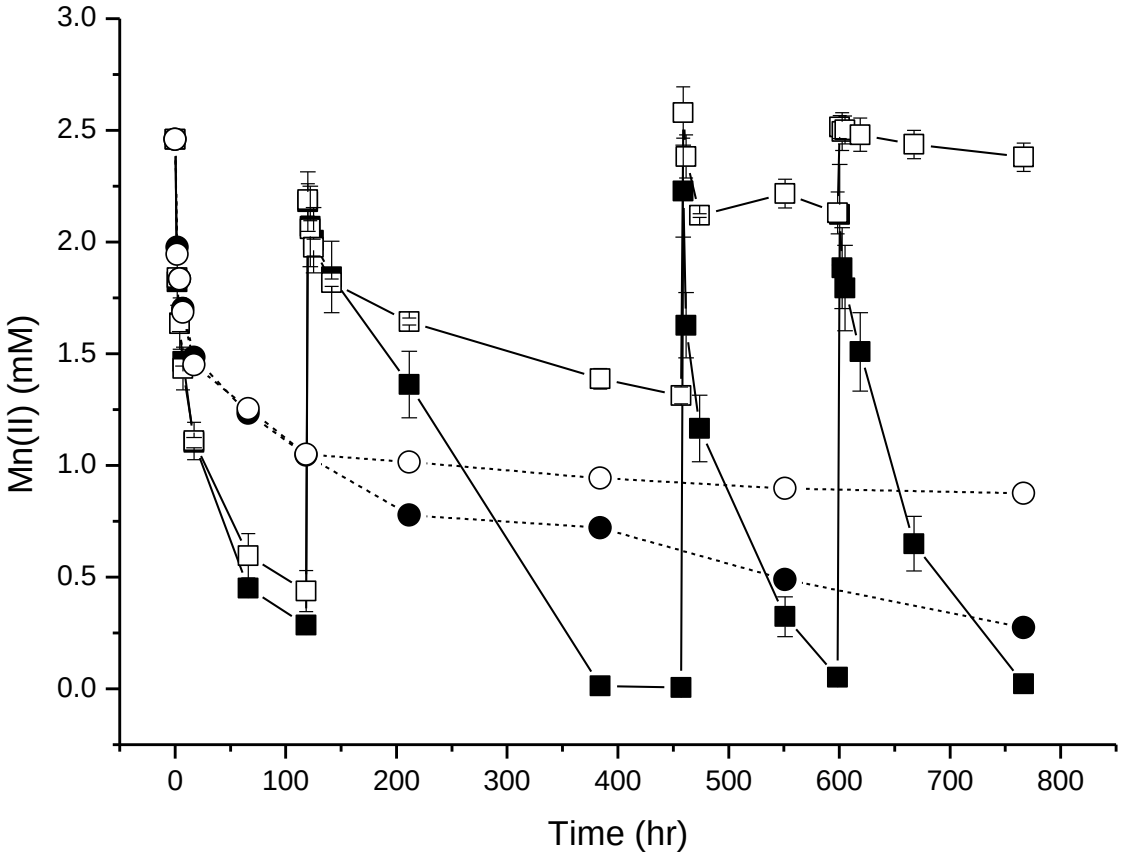

Figure 1. Mn(II) loss from solution in laboratory sediment incubation experiments. Black squares (■) – live sediments under air; white squares (□) – live sediments under N_2; black circles (●) – killed sediments (γ-irradiated) under air; and, white circles (○) – killed sediments under N_2. For live sediments Mn(II) was repeatedly added (as $MnCl_2$) at 120, 460 and 600 h, while killed reactors were never re-spiked with Mn(II). Experiments were conducted with 1.0 g moist sediment (0.82 g dry) and 50 mL filter sterilized influent site water. Figure from Tan et al. (2010).

A series of similar, fed-batch experiments were conducted to determine the effects of dissolved oxygen on Mn(II) removal (Fig. 2). Dissolved oxygen concentrations remained nearly constant throughout these experiments, and were found to be essentially saturated with respect to the corresponding P_{O2}. A significant difference in Mn(II) removal was observed when the headspace P_{O2} was maintained at 1% (v/v)-as compared to 10 and 21%. The difference became more pronounced after several cycles as the abiotic removal mechanisms contributed less to Mn(II) removal (e.g., due to saturation of Mn sorption). The rate of biological Mn(II) oxidation by *Leptothrix discophora* SS1 has been shown to be directly proportional to dissolved oxygen

(Zhang et al., 2002). Our results suggest a different response for the whole, natural microbial community in this Mn-removal bed as compared to *Leptothrix*. Specifically, our results suggest that dissolved oxygen had more of a saturation-type effect on Mn(II) removal where the rate and extent of Mn(II) removal were not different above a P_{O2} level of 10% (DO ca. 5 mg/L).

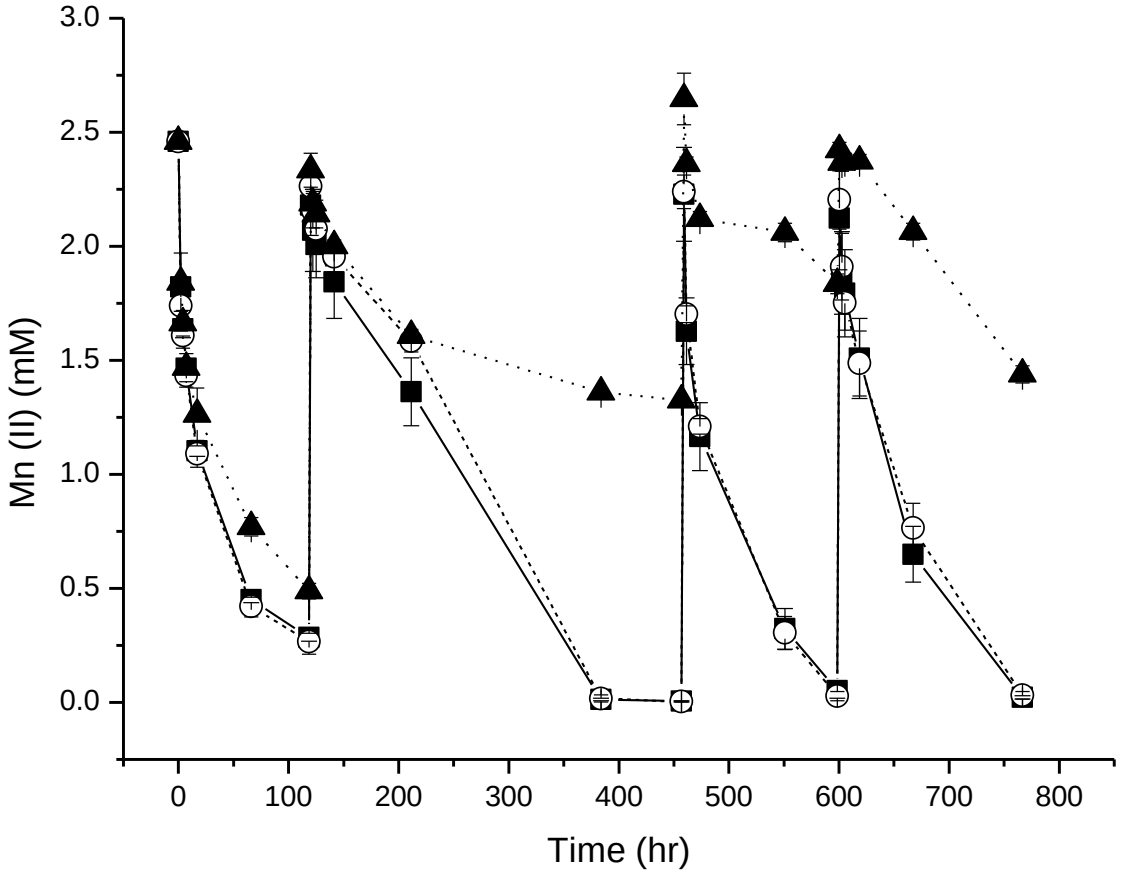

Figure 2. Mn(II) loss from solution under variable partial pressures of oxygen (P_{O2}). White circles (○) – incubated under 21% P_{O2} (i.e. air); black squares (■) – incubated under 10% P_{O2}: 90% N_2; and, black triangles (▲) – incubated under 1% P_{O2}: 99% N_2. Mn(II) was repeatedly added (as $MnCl_2$) at 120, 460 and 600 h. Experiments were conducted with 1.0 g moist sediment (0.82 g dry) and 50 mL filter sterilized influent site water. Corresponding controls using killed sediments (γ-irradiated) are not shown.

Mn(II) oxidation via fungal versus bacterial activity

A series of fed-batch experiments were conducted in the presence/absence of fungicides (0.2 g/L cyclohexamide plus 0.2 g/L pentachloronitrobenzene) to operationally asses the relative

importance of Mn(II) oxidation by fungi as compared to other microbial community members (Fig. 3). A somewhat surprising and important finding from these experiments was that the fungicides knocked out the majority of the sediment's ability to remove Mn(II). Reactors that contained sterile sediments and/or were maintained under N_2 all yielded results similar to those reactors that contained fungicides. Our interpretation of these results is that, e.g., the difference between the live reactors with fungicides (black circles in Fig. 3) and the live reactors without fungicides (black squares) was due to fungal activity. Furthermore, we believe that fungi were the most important bio-catalysts of Mn(II) oxidation in this treatment system.

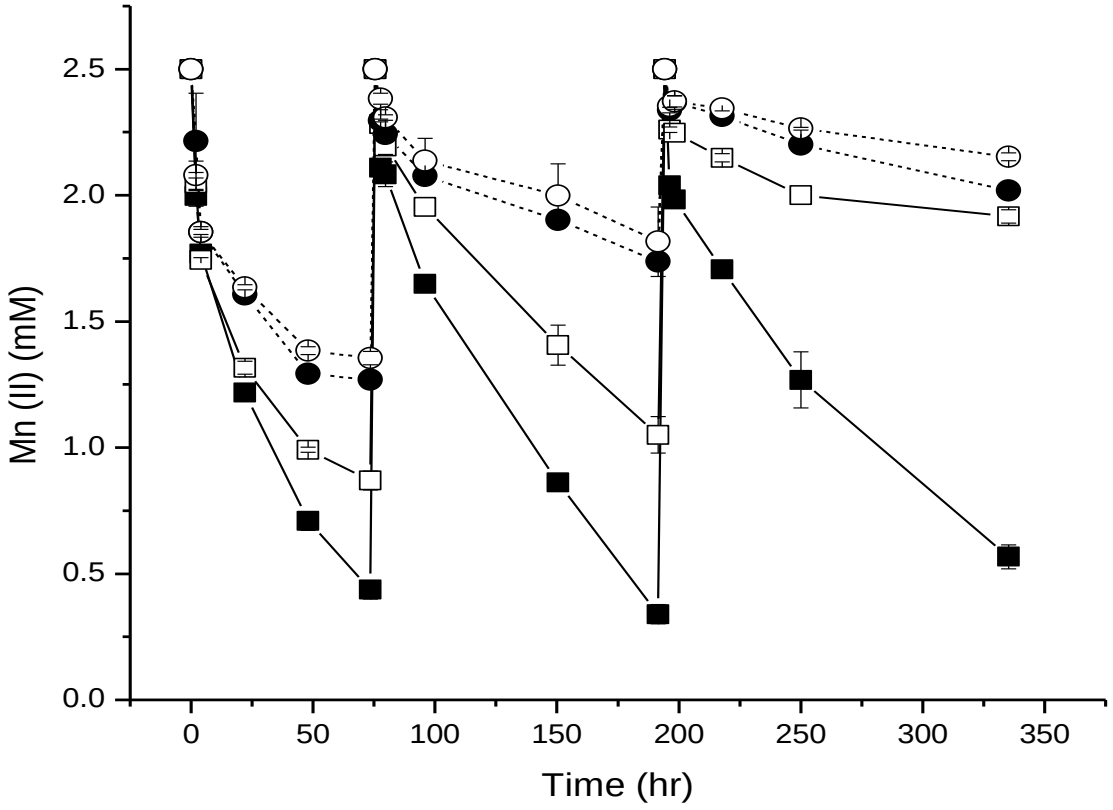

Figure 3. Mn(II) loss from solution in the presence and absence of fungicides. Black squares (■) – live sediments incubated with no fungicides; white squares (□) – killed sediments (γ-irradiated) incubated with no fungicides; black circles (●) – live sediments incubated with 0.2 g/L cyclohexamide and 0.2 g/L pentachloronitrobenzene; and, white circles (○) – killed sediments incubated with 0.2 g/L cyclohexamide and 0.2 g/L pentachloronitrobenzene. Mn(II) was repeatedly added as $MnCl_2$ at 75 and 190 h.

Based on enumerations of culturable heterotrophic bacteria measured at the end of these experiments, the fungicides did not non-specifically harm the bacterial population in the sediments (Fig. 4). We actually observed an increase in culturable bacterial numbers and speculate that this occurred because of the absence of fungi competing for space on the plates. Another possible explanation for increased numbers of culturable bacterial in the presence of fungicides is that fungi themselves may produce antibiotics to suppress bacterial activity.

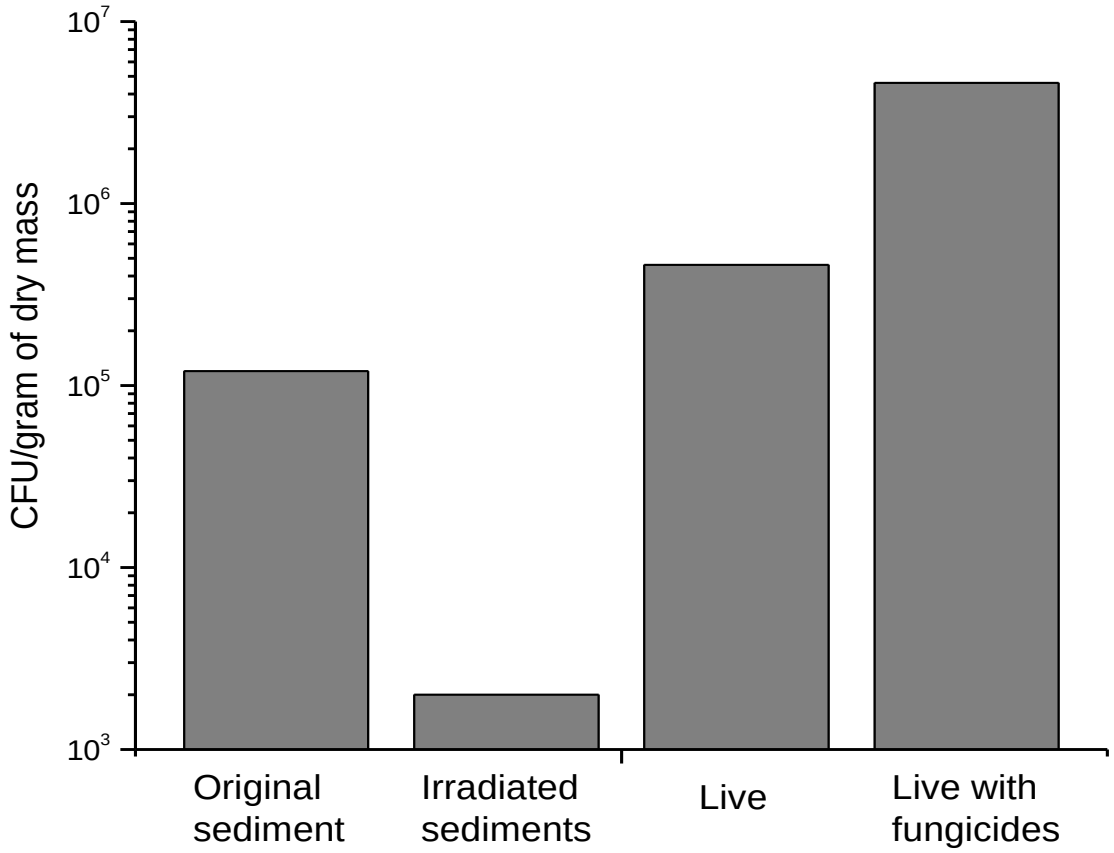

Figure 4. Culture-based bacterial enumerations at the end of select sediment incubations. Numbers of culturable heterotrophic bacteria were determined with K media. CFU refers to a colony forming unit of bacteria.

Effect of organic C addition on Mn(II) oxidation

Fed-batch experiments were also conducted to determine the effects of organic carbon addition on Mn(II) removal (Fig. 5). In practice, Mn-removal beds are often designed with an upstream wetland. Dissolved organic carbon (DOC) released via primary production in the

wetland into the Mn-removal bed presumably will increase microbial Mn(II) oxidation. Glucose and carboxymethylcellulose (CMC) were selected to represent labile and recalcitrant DOC, respectively. CMC was also selected to represent DOC that might be generated from wood dissolution with the thought that wetlands could possibly be replaced with a solid-phase carbon source emplaced within the bed. The addition of glucose was shown to slightly improve Mn(II) removal while the addition of CMC was shown to significantly inhibit Mn(II) removal.

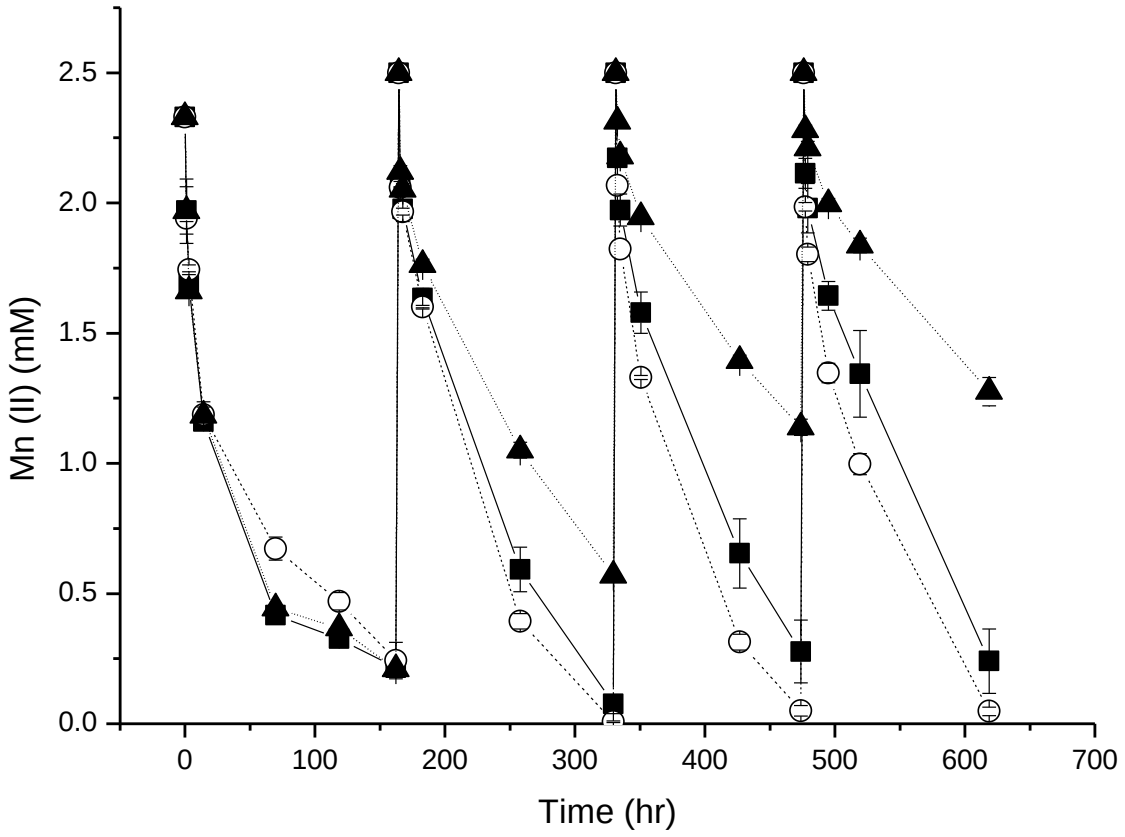

Figure 5. Mn(II) loss from solution with and without supplemental organic carbon source. White circles (○) – incubated with 10 mg C/L of glucose; black triangles (▲) – incubated with 10 mg C/L of carboxymethylcellulose; and, black squares (■) – incubated with no supplemental carbon. All incubations maintained under air. Mn(II) was repeatedly added (as $MnCl_2$) at 160, 330 and 480 h. Corresponding controls using killed sediments (γ-irradiated) are not shown.

Our interpretation is that glucose stimulated general heterotrophic activity that in turn fortuitously stimulated biological Mn(II) oxidation. We are unsure about the inhibitory effect of CMC, however, we speculate that CMC may complex Mn(II) to form soluble species that are

difficult to oxidize. Alternatively, the fungi in the Fairview system may not be able to utilize recalcitrant C such as cellulose or lignin. In fact, based on phylogenetic assignments for the fungi we isolated from these Mn-removal systems, we found the most common fungi were Ascomycetes (Santelli et al., 2010). In contrast, most "wood rot" fungi capable of oxidizing cellulose or lignin are typically classified as Basidiomycetes. Recently we found that the addition of organic C derived from white pine or red oak (10 mg C/L) inhibited Mn(II) removal in flow-through experiments using Fairview sediments and water (data not shown). Based on these observations, and considering the phylogeny of the predominant fungi, it is unlikely that wood chips would serve as a suitable solid-phase organic amendment for a Mn-removal bed.

Mn(II) oxidation by pure cultures of fungi

We conducted an extensive culture enrichment survey of several Mn(II)-treatment systems in western Pennsylvania. An interesting and unexpected result from this survey was that Mn(II)-oxidizing fungi were isolated more readily than Mn(II)-oxidizing bacteria, where fungal isolates outnumbered bacterial isolates 84 to 10. In other words, in these Mn(II)-treatment systems we found that fungi constituted 89% of the Mn(II)-oxidizing cultures while bacteria constituted just 11%. The most commonly recovered species, in terms of number of treatment systems and number of times obtained, was representative isolate DS2psM2a2 (Fig. 6, left). Based on phylogenetic analysis and morphological characterization, DS2psM2a2 was identified as *Plectosphaerella cucumerina* belonging to the class Sordariomycetes (Santelli et al., 2010).

We initially hypothesized that Mn(II) oxidation was predominantly mediated by bacteria; however, our results suggest that fungi are also important mediators of Mn(II) oxidation. Also of great practical importance, we found that most of these fungi were highly tolerant of elevated Mn(II) concentrations. As shown in Fig. 6, two representative fungal isolates from these Mn-removal limestone beds can tolerate astonishingly high levels of Mn(II) (e.g., 1,000 to >10,000 μM). It is generally believed that fungi are more tolerant than bacteria of high concentrations of heavy metals leading to a prevalence of fungi in heavy metal-contaminated soil (Chander et al., 2001a; Chander et al., 2001b; Kelly et al., 1999; Rajapaksha et al., 2004). Considering that Mn(II) concentrations in surface coal mine drainage in Appalachia often exceed 1,000 μM (= 55 mg/L) and can even exceed 3,000 μM, microbial catalysts in these Mn(II)-removal systems must be tolerant of high Mn(II) concentrations – further demonstrating that Mn(II)-oxidizing fungi likely play an important role in the treatment process. Consistent with

this conclusion, a recent study (Mariner et al., 2008) suggested that Mn(II)-oxidizing fungi are more abundant than bacteria in bioreactors treating mine water with elevated Mn(II) concentrations.

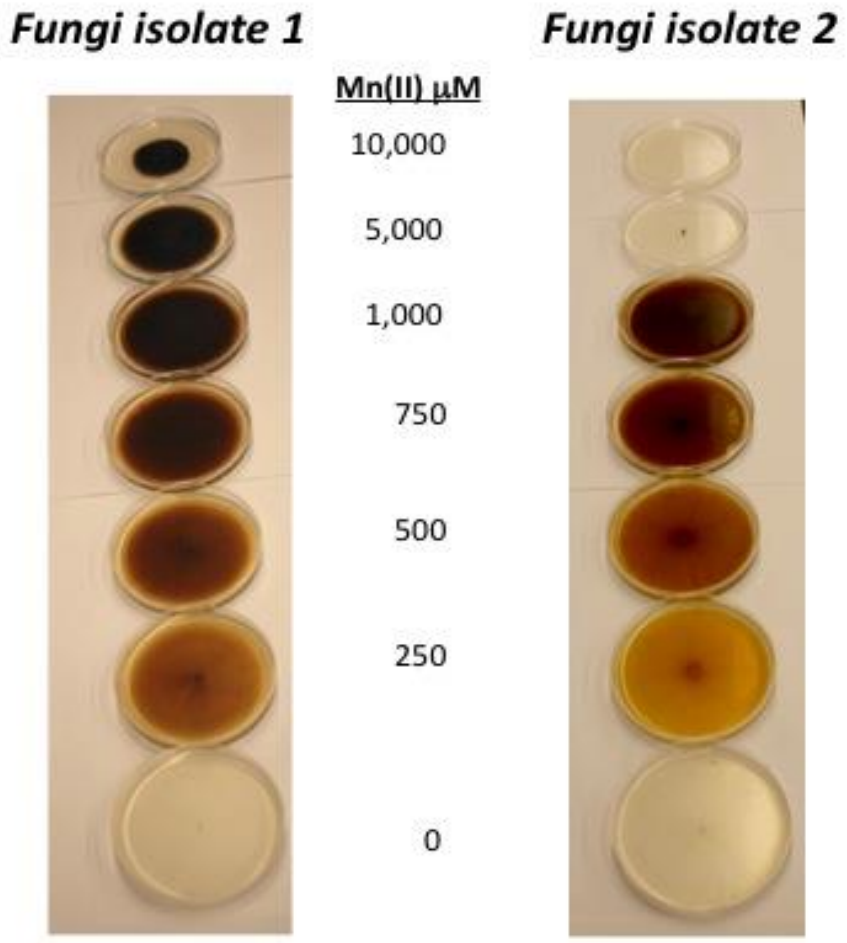

Figure 6. Tolerance of fungal isolates to elevated Mn(II) concentrations. Images show two different Mn(II)-oxidizing fungi isolates grown in petri dishes on agar-based media with increasing (0 to 10,000 μM) concentrations of dissolved Mn(II). Fungal isolate 1 is *Plectosphaerella cucumerina* and fungal isolate 2 is *Microdochium bolleyi*. Isolates were inoculated by "stabbing" the center of the petri dish, allowing the fungal hyphae to grow radially outwards. The brown color is due to the Mn oxide minerals precipitated on the fungi during growth.

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