# Final Report

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# Improving passive mine treatment through better understanding of biogeochemistry and mineralogy associated with Mn(II) oxidation

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William D. Burgos*[,1]
John M. Senko[1]
Colleen M. Hansel[2]

[1] The Pennsylvania State University,
Department of Civil and Environmental Engineering
[2] Harvard University,
School of Engineering and Applied Science

*Principal Investigator – address: Department of Civil and Environmental Engineering, The Pennsylvania State University, 212 Sackett Building, University Park, PA, 16802-1408; telephone: 814-863-0578; fax: 814-863-7304: e-mail: wdb3@psu.edu

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# IMPROVING PASSIVE MINE TREATMENT THROUGH BETTER UNDERSTANDING OF BIOGEOCHEMISTRY AND MINERALOGY ASSOCIATED WITH MN(II) OXIDATION

Bill Burgos, Associate Professor, Department of Civil and Environmental Engineering, The Pennsylvania State University, University Park, PA
John Senko, Assistant Professor, Department of Geology and Environmental Science, The University of Akron, Akron OH
Colleen Hansel, Assistant Professor, Division of Engineering and Applied Science, Harvard University, Cambridge, MA

## 1. Introduction

We have been studying biological Mn(II) oxidation in AMD limestone treatment systems since the summer of 2005, at the request of OSM and Pennsylvania Department of Environmental Protection (PA DEP) personnel. We have been funded by the OSM NTTT Applied Science Program since the summer of 2007 to characterize the microbial communities and mineral precipitates found in two Mn(II)-removal systems in western Pennsylvania. Personnel at OSM and PA DEP directed us to these treatment systems because of important differences among these treatment systems in influent Mn(II) concentrations and system construction. The treatment system at Site #1, constructed in Elk County in 2004, was filled with limestone gravel, was not inoculated with Mn(II)-oxidizing microorganisms, and treats an exceptionally high influent Mn(II) concentration of 130 – 150 mg/L. The treatment system at Site #2, constructed in Somerset County in 1999, was filled with limestone gravel, inoculated with a patented suspension of Mn(II)-oxidizing microorganisms (Pyrolusite Process®; Vail and Riley, 2000), and treats an influent Mn(II) concentration of 20 – 30 mg/L.

The purpose of this project was to improve our understanding and design capability of passive manganese(II)-removal systems for the treatment of acid mine drainage (AMD). The objectives of our project were to:

1) Characterize the microbial communities in these Mn(II)-removal systems using both culture-based and DNA-based techniques;
2) Measure Mn(II)-removal kinetics in controlled laboratory experiments; and
3) Characterize the $MnO_x$ precipitates formed in the two Mn(II)-removal systems.

## 2. Background

The removal of Mn(II) from AMD is a significant problem for both operating and abandoned mines in Appalachia and across the United States. Mine drainage containing high levels of Mn(II) exists in all Eastern coal producing states. According to OSM's mine drainage inventory, Mn(II) is being treated at more than 700 mine sites in Appalachia and most coal operators use caustic soda (NaOH) to remove Mn(II) from mine drainage (Brent Means, personal communication). This represents 60% of all regulated mine drainage in Appalachia. In Pennsylvania alone, the long-term water liability (the cost for perpetual treatment, should these companies go bankrupt) on these mine sites is ~$240 million dollars. The use of caustic soda in active treatment is both costly and presents a public safety issue. It has been shown that Mn(II) removal can double or triple treatment costs due to the chemical consumption needed to achieve high pH conditions (Means and Hilton, 2004). Vail and others have shown that Mn(II) can be removed passively from mine drainage through the use of biologically-active limestone beds (Vail and Riley, 2000; Johnson, 2003). Passive removal of Mn(II) is desirable as it eliminates the need for chemical reagent and the annual treatment costs can be a small fraction of those required for active treatment.

The success of passive Mn(II)-removal systems has been variable due to a lack of design criteria and a poor understanding of the mechanisms that govern Mn(II) oxidation at near-neutral pH. At circumneutral pH, Mn(II) may be oxidized by microbiological activity or by surface-catalyzed heterogeneous oxidation on Mn(III/IV) oxide surfaces (Davies and Morgan, 1989; Junta and Hochella, 1994). However, much of the Mn(II) oxidation observed in natural systems is believed to be microbiologically-mediated (Hungate et al., 1987; Nealson et al., 1988; Tebo, 1991; Tebo et al., 1997). Currently, the relative importance of biological Mn(II) oxidation versus abiotic Mn(II) oxidation in coal mine drainage treatment systems is uncertain (Means and Rose, 2005).

## 3. Results

We measured Mn(II) removal in the selected treatment systems at varied temperatures, seasons and flow conditions (Figure 1). Mn(II) is consistently removed below detection limit (0.02 mg/L) from AMD in the Site #2 treatment system, while Mn(II) removal is strongly dependent on the season (better removal in summer) in the Site #1 treatment system. The treatment system at Site #2 also employed a highly productive wetland upstream of the Mn-removal bed. We believe that higher concentrations of dissolved organic carbon and nutrients from this wetland stimulated greater microbial activity that, in turn, fortuitously promoted greater Mn(II) removal (on a %-removal basis).

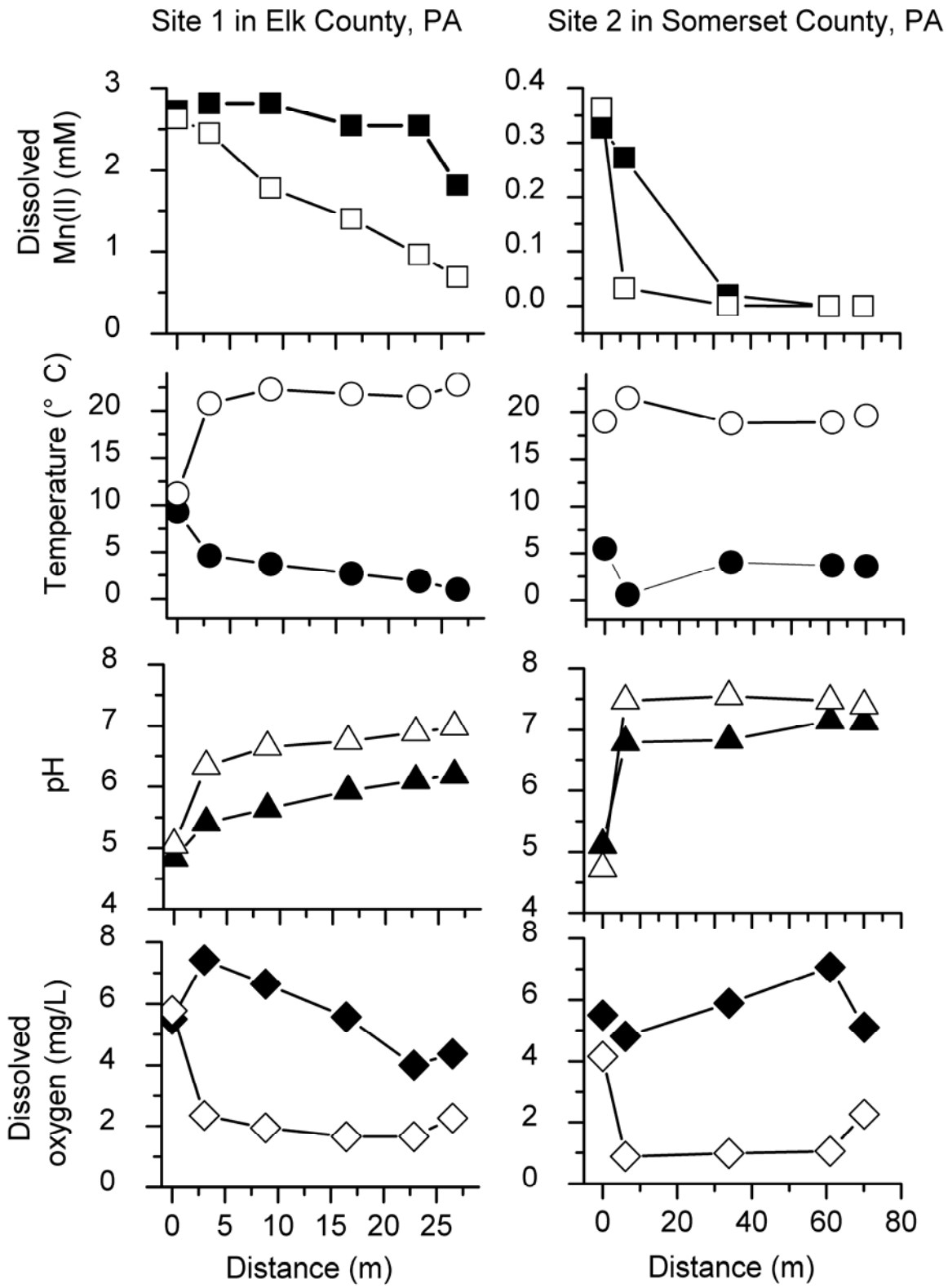


**Figure 1.** Seasonal water chemistry of Site #1 and Site #2. Open symbols represent warm season (July 2006) and filled symbols represent cold season (February 2006).

The first objective of our project was to characterize the microbial communities in these passive Mn(II)-removal systems using both culture-dependent (i.e. based on microbes in samples that grow on liquid or solid media) and culture-independent techniques (i.e. based on DNA extracted from microbes in samples). In an initial survey of the culturable bacterial population, we observed that Mn(II)-oxidizing bacteria, referred to hereafter as "Mn(II)OB", represented a large fraction (up to 22 %) of the total culturable bacteria in both the Site #1 and Site #2 treatment systems (Figure 2). Furthermore, culturable Mn(II)-oxidizing bacteria were present in considerably higher numbers in the treatment systems compared to adjacent "background" soil that did not receive Mn(II)-rich waters. It has been estimated, however, that far less than 1% of the total bacteria in the environment have been isolated, or may be cultured using current culturing approaches (Pace, 1997). Therefore, we characterized the phylogenetic diversity of microbial communities present in these two systems using a culture-independent approach that uses DNA sequencing of the 16S ribosomal RNA (rRNA) gene. Each colored wedge in Figure 3 represents a unique bacterial phylum, and the greater number of phyla (i.e. wedges) represents greater microbial diversity. Using this culture-independent approach, we were able to show that the conditions within the Mn(II)-removal systems give rise to microbial communities that are distinct, and less phylogenetically diverse, from those of the adjacent "background" soil. Combined with the culture-dependent approach which allows one to determine *the number of bacteria* in any particular environment (Figure 2), this culture-independent approach also allows one to specifically identify *what types of bacteria* (or fungi) are present in any particular environment (Figure 3).

An interesting and unexpected result of our culture-based examination of microbial communities was the abundance of Mn(II)-oxidizing fungi that were recovered from the Site #1 and Site #2 treatment systems. In these two Mn(II)-removal systems we found that **fungi constitute 88 % of the Mn(II)-oxidizing cultures** while bacteria constitute just 12 %. 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 these fungi are more tolerant to elevated Mn(II) concentrations as compared to well-studied Mn(II)OB. We have isolated several different Mn(II)-oxidizing fungi from these treatment systems to directly examine their nutritional requirements for optimal growth and Mn(II) oxidation. These experiments are carried out by

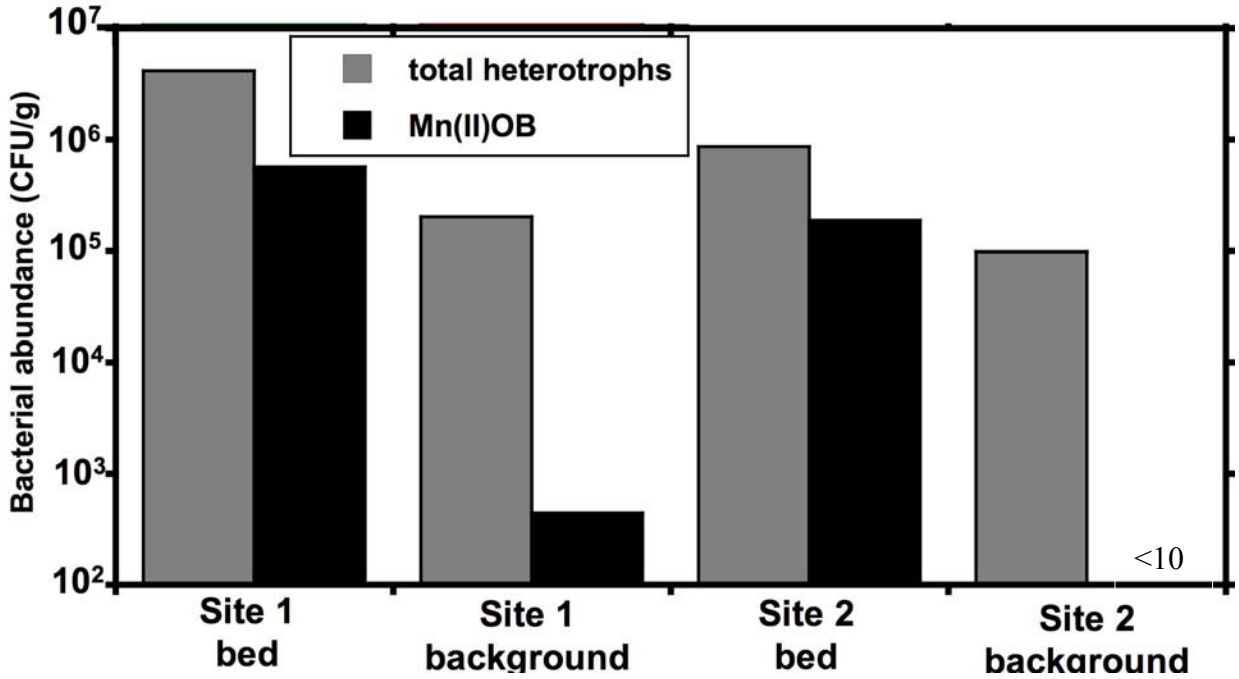


**Figure 2.** Abundance of culturable bacteria from the Mn(II)-removal systems at Site #1 and Site #2 and in adjacent "background" soils. Numbers of colony forming units (CFU) were determined for both total heterotrophic bacteria (gray bars) and Mn(II)-oxidizing bacteria (black bars).

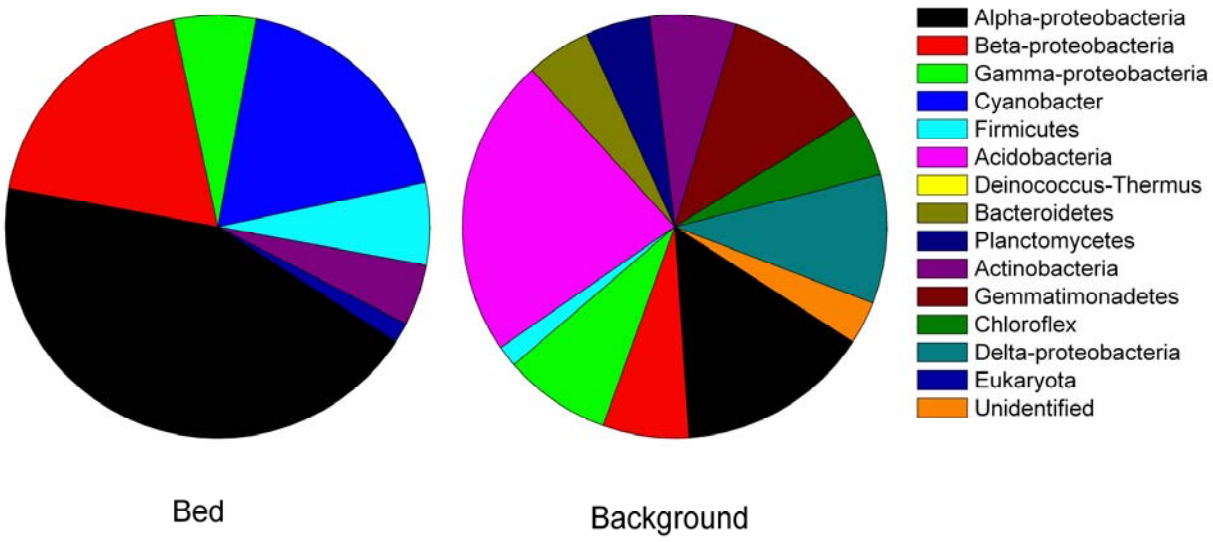


**Figure 3.** Culture-independent analysis of bacterial communities at Site #1. Distribution of 16S rDNA gene sequences from clone libraries of bacterial communities found in the Mn(II)-removal system at Site #1 and in adajacent "background" soil. At least 80 clones were sequenced from each 16S rDNA clone library.

inoculating fungi (using a "stab" technique) onto solid, agar-based media with varying concentrations of dissolved nutrients and/or Mn(II). As shown in Figure 4, two different fungal isolates from these Mn-removal limestone beds can tolerate astonishingly high levels of Mn(II) (e.g., 1,000 to >10,000 μM), while well-studied Mn(II)OB like *Leptothrix discophora* begin to show signs of metal toxicity at only ~100 μM Mn(II). 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.

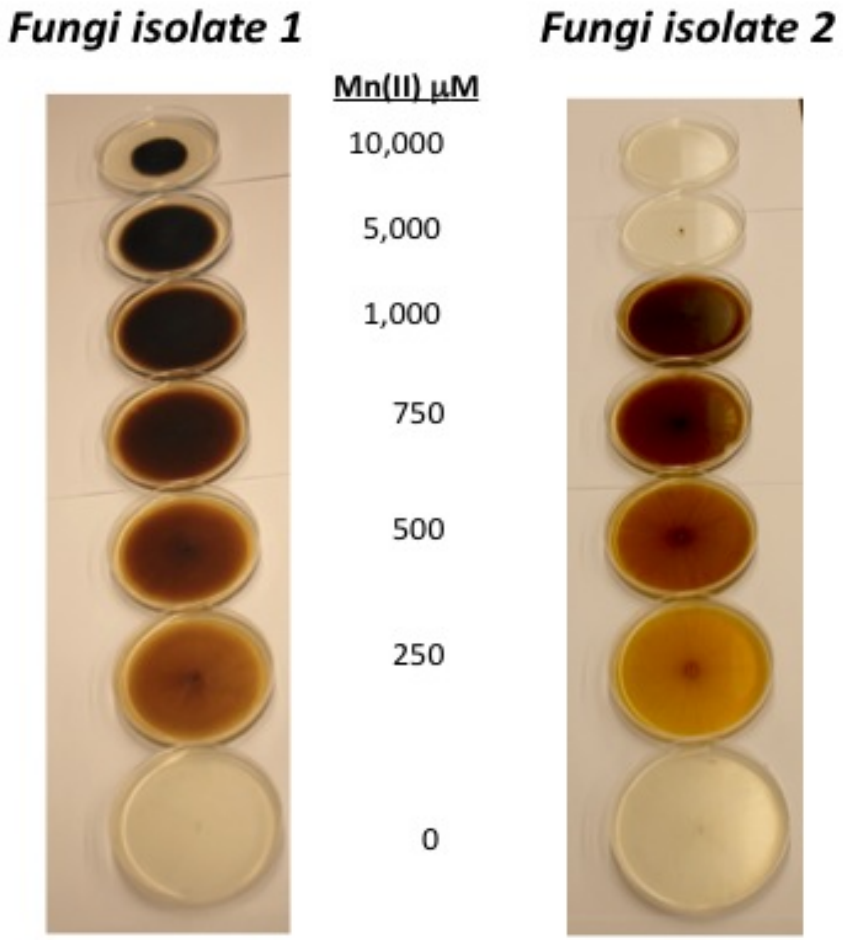


**Figure 4.** Tolerance of fungal isolates to elevated Mn(II) concentrations. Images show two different Mn(II)-oxidizing fungi isolates grown in petri dishes on agar-based media with increasing (0 to 10,000 μM) concentrations of dissolved Mn(II). Isolates were inoculated by "stabbing" the center of the 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.

The second objective of our project was to measure Mn(II)-removal kinetics in controlled laboratory experiments. Results from our laboratory experiments indirectly (but convincingly) reveal the great importance of fungi in Mn(II) oxidation in these systems. For these experiments we collected, sieved (<0.2-mm) and homogenized a large quantity of $MnO_x$-rich "crusts" from the treatment system at Site #1, and filter-sterilized (0.2-µm) large volumes of influent site water. Experiments were conducted by mixing $MnO_x$-rich crust (the microbial inoculum) and sterile site water, and maintaining them under a controlled atmosphere (e.g. variable $P_{O2}$). For control experiments, live sediments were maintained under a 100 % $N_2$ atmosphere or first sterilized by $^{60}Co$ gamma irradiation. We learned that we had to go through repeated additions of Mn(II) (e.g. to create "saw blade" concentration-vs-time profiles in Figures 5A and 5B) to better reveal the relative contributions of biotic versus abiotic removal processes. For example, the differences between all the reactors and controls were insignificant through the first Mn(II) removal cycle. However, the increasing difference in the Mn(II) concentrations between the "live under air" and "live under $N_2$" (black squares and white squares, respectively, in Figure 5A) reflect the relative importance of biological Mn(II) oxidation versus abiotic Mn(II) sorption/oxidation. The increasing lack of removal of Mn(II) from the "live under $N_2$" reactors reflect the gradual saturation of Mn(II) sorption sites.

To evaluate the relative importance of fungi on Mn(II) oxidation, identical experiments were conducted with or without the addition of the fungicide cycloheximide (0.2 g/L). Again, the repeated addition of Mn(II) helped reveal the relative contributions of biotic versus abiotic removal processes (e.g. differences between "live –fungicide" and "killed –fungicide"; black squares and white squares, respectively, in Figure 5B). In the case of +/– fungicide, the repeated addition of Mn(II) helped reveal the relative contributions of fungal versus bacterial Mn(II) oxidation. As this experiment proceeded, the increasing difference in the Mn(II) concentrations between the "live –fungicide" and "live +fungicide" (black squares and black circles, respectively, in Figure 5B) reflect the relative contribution of fungi-mediated Mn(II) oxidation (i.e. knocked out by fungicide). Based on these results, **fungi may account for over 80 % of Mn(II) oxidation in Mn-removal beds**. These results were not biased by bacteria being harmed by the fungicide, because cycloheximide did not decrease culture-based enumerations of bacteria (Figure 5C). (The increase in bacteria counts was likely caused by the absence of fungi colonizing a portion of the plates.).

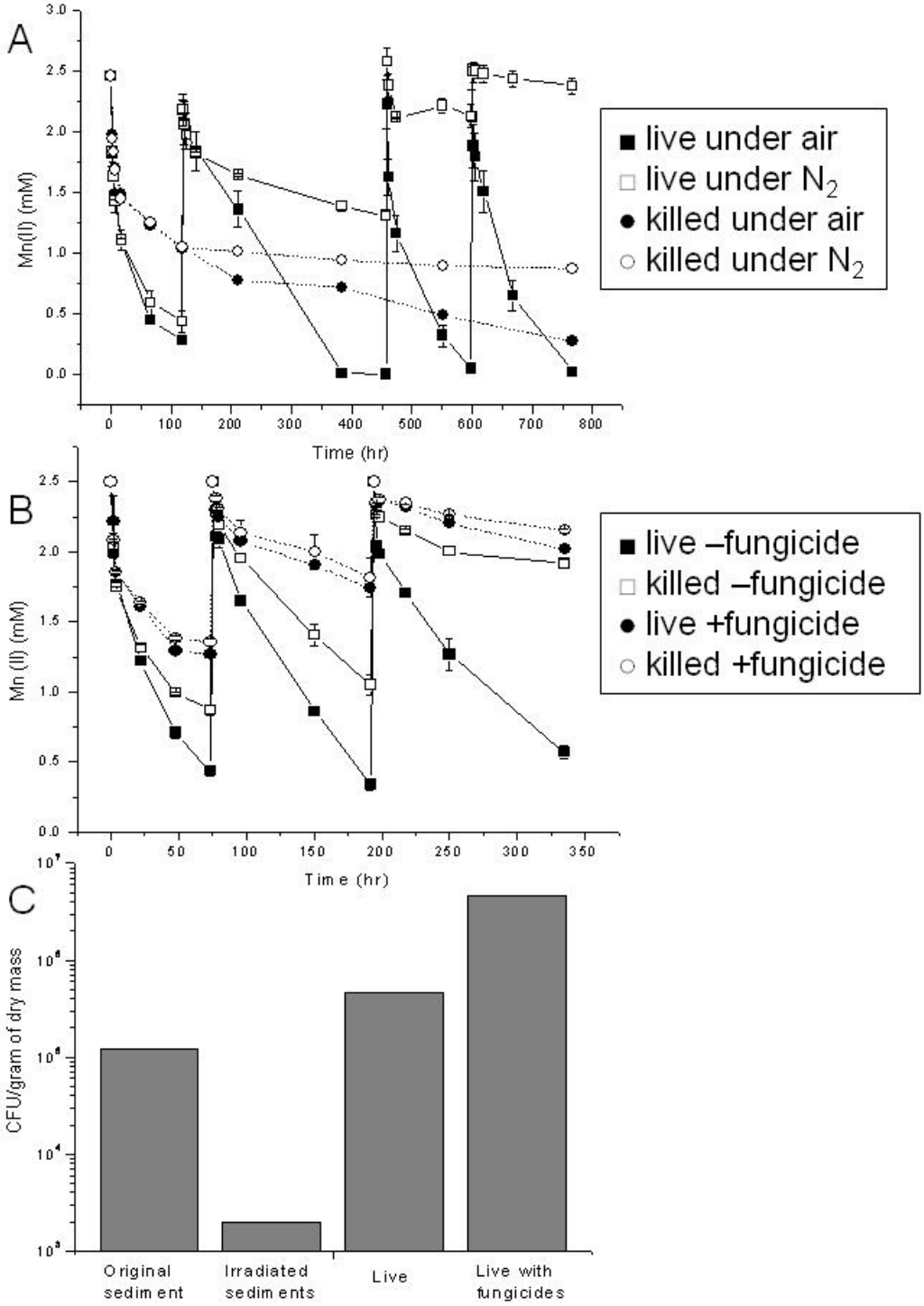


**Figure 5.** Laboratory sediment incubation experiments with $MnO_x$-rich "crusts" from Site #1 and sterile influent water. A) Effect of air and sterilization (γ-irradiation) on Mn(II) removal. Live reactors were re-spiked with Mn(II) (as $MnCl_2$) at ca. 120, 450 and 600 hours, while killed reactors were never re-spiked with Mn(II). B) Effect of the fungicide cycloheximide (0.2 g/L) on Mn(II) removal. C) Total heterotrophic plate counts for bacteria from sediments collected at the end of experiment shown in B.

The third objective of our project was to characterize the $MnO_x$ precipitates formed in the two Mn(II)-removal systems. For electron microscopy, $MnO_x$-rich crusts were "fixed" in the field with glutaraldehyde to preserve biological features within the samples. Scanning electron microscopy (SEM) showed that $MnO_x$ particles from the two different sites displayed similar morphological characteristics, and that bacterial cells and fungal mycelia were in close contact with the oxides (Figure 6). Transmission electron microscopy (TEM) coupled with energy dispersive spectroscopy (EDS) was used to better visualize the precipitates and confirm their elemental composition (Figure 7). For X-ray diffraction (XRD), $MnO_x$-rich crusts were analyzed in their natural moist state and then again after complete air-drying. We expected to find a combination of birnessite (layer structure) and todorokite (tunnel structure), common Mn(III/IV) oxides, in these systems. The drying of birnessite causes a collapse of its interlayer structure that can be observed by a shift in its XRD pattern, and can be used to distinguish it from todorokite (Webb et al., 2005) (Figure 8). We used the XRD patterns and Scherrer's equation to estimate the $MnO_x$ crystallite size and found that the smallest crystallites were located at the influent end of the Mn(II)-removal systems (ca. 5-nm). These small, high surface area $MnO_x$ particles were found to remove a significant fraction of Mn(II) and other trace metals (Co, Ni and Zn).

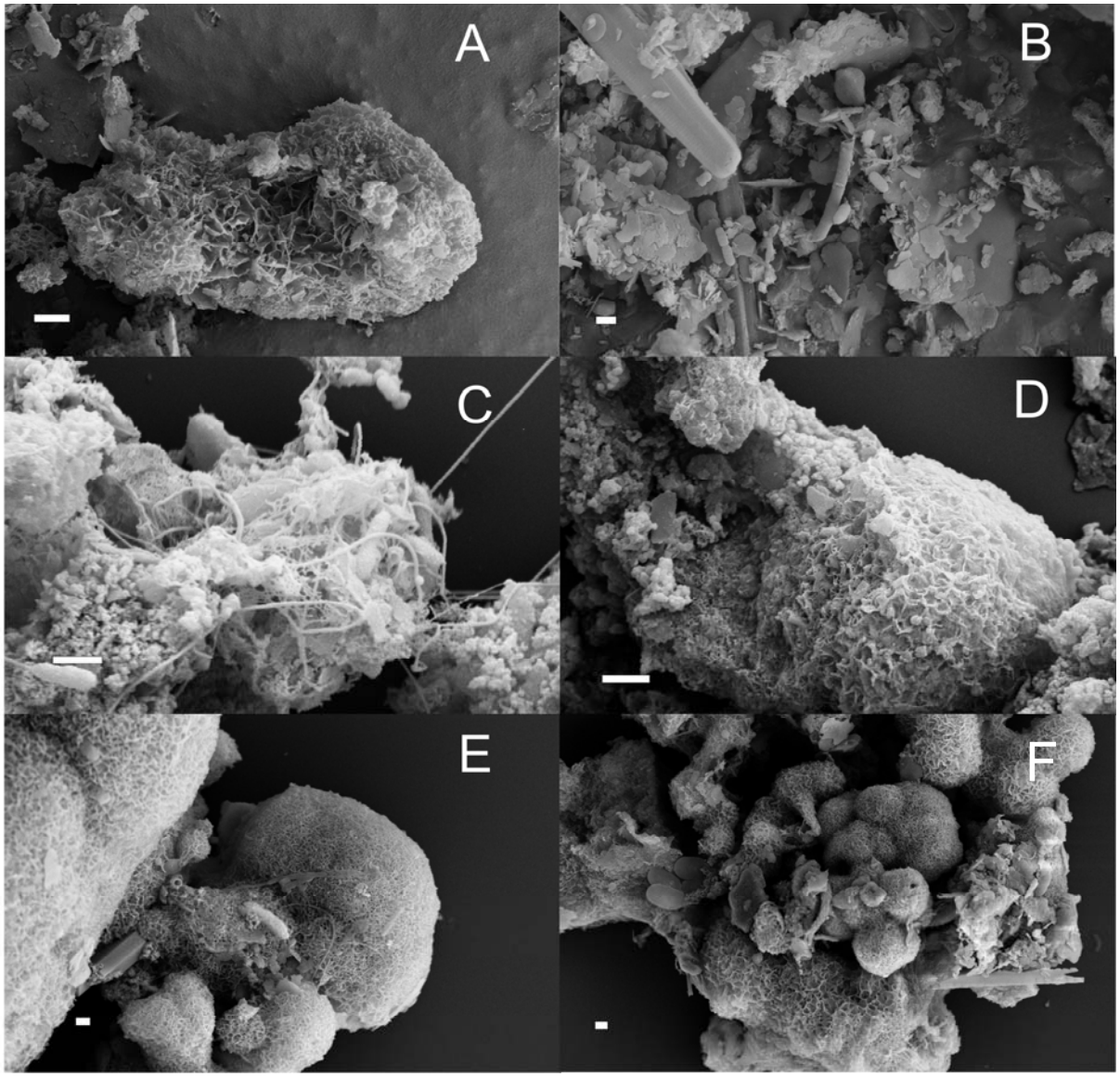


**Figure 6.** Scanning electron micrographs of MnOx-rich sediments collected from three Mn(II)-removal systems. A) Typical "sponge-like" morphology of $MnO_x$ minerals (Site 1). B) Bacterial cells of various shape and freshwater diatoms shown in close proximity to $MnO_x$ particles (Site 1). C) Fungal mycelium shown in close proximity to $MnO_x$ particles from Site 2. D) "Sponge-like" morphology of the $MnO_x$ crusts from Site 2. E) and F) $MnO_x$ crusts from third site where sandstone was used as a surface to precipitate Mn(II). Scale bar in all images represents 1 μm.

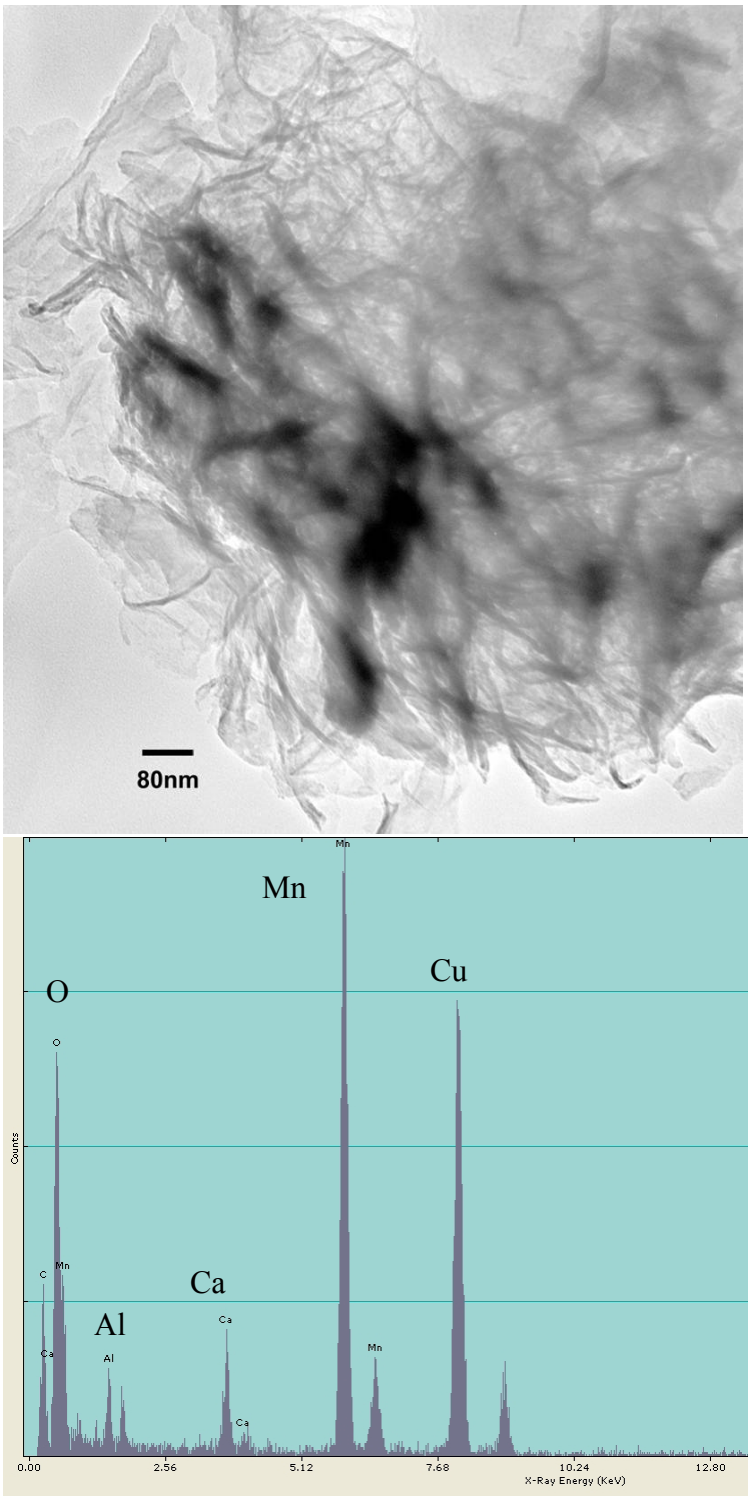


**Figure 7.** A) Transmission electron micrograph of $MnO_x$-rich crusts collected from Site #1 show thin layer morphology. B) Energy dispersive spectra from the most electron dense region in A. Cu signal comes from TEM Cu grid.

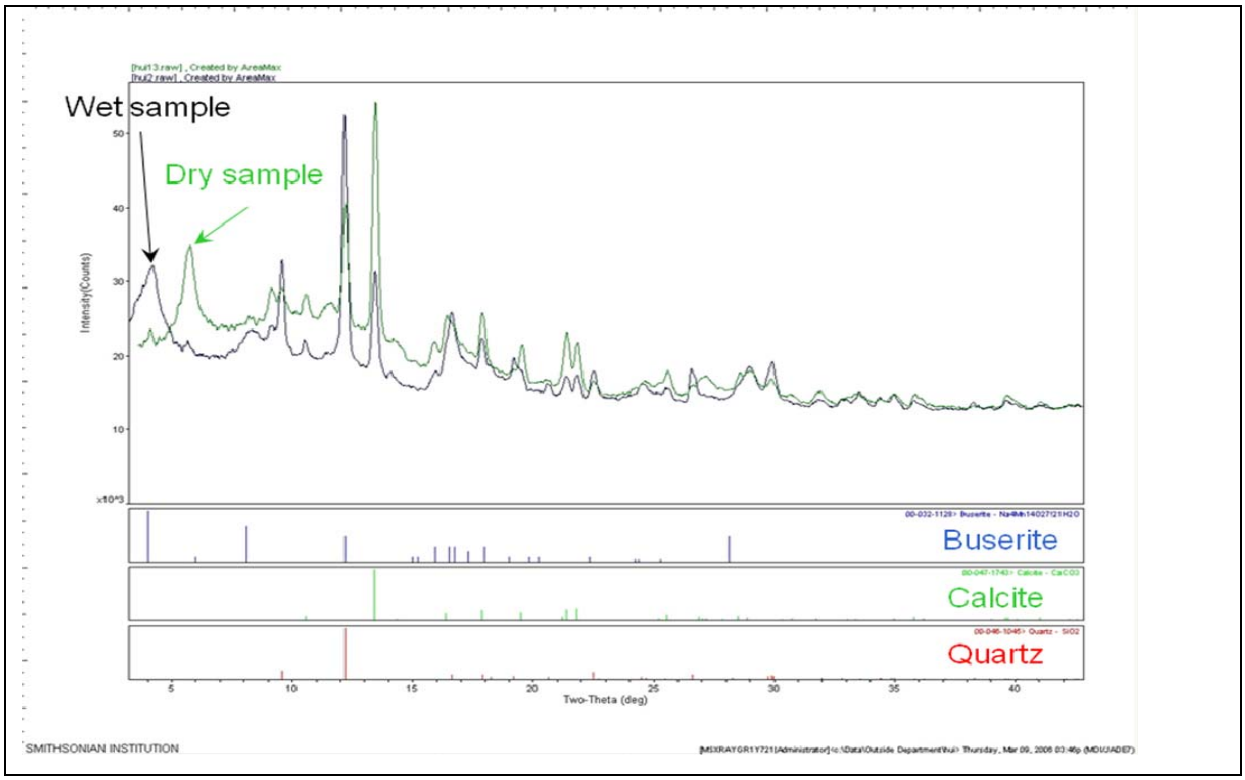


**Figure 8.** X-ray diffraction (XRD) patterns of wet and dry samples collected from Site #1. The shift of the 2-Θ peak from $4^o$ to $8^o$ upon drying suggests the presence of the layered manganese(III/IV) oxide buserite (unofficial mineral name, similar to birnessite).

## 4. Conclusions

Our microbial characterizations and laboratory experiments found that:

- In the two Mn(II)-removal systems we have studied, fungi constituted 88 % of the Mn(II)-oxidizing cultures while bacteria constituted just 12 %.
- Fungi isolated from these two Mn(II)-removal systems displayed an extremely high resistance to Mn(II) toxicity (e.g. up to 10,000 μM) as compared to well-studied Mn(II)-oxidizing bacteria (e.g. up 100 μM).
- In laboratory experiments using sediments collected from the Mn-removal beds (as the microbial inoculum) and sterilized site influent water, fungi accounted for over 80 % of Mn(II) oxidation activity in these Mn-removal beds.

Our mineralogical characterizations found that:

- The predominant Mn oxides at all sites were poorly crystalline birnessite and buserite with smaller amounts of todorokite.
- The surface morphology of the $MnO_x$ precipitates from all sites was coarse and "sponge-like" composed of nanometer-sized lathes and thin sheets.
- Trace metals such as Ni, Zn and Co were removed effectively, in most cases preferentially, into the $MnO_x$ precipitates

## 5. Future Research

- We need to expand our number of sampling sites (i.e. operational Mn(II)-removal systems) to better determine the composition, abundance, and frequency of detection of fungi in these systems.
- We need to conduct laboratory experiments with sediments from these Mn(II)-removal systems to quantify the relative importance of fungi versus bacteria with respect to Mn(II) oxidation.
- We need to conduct laboratory experiments with whole sediments and fungal isolates to determine optimal nutritional requirements for maximum Mn(II) oxidation.

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