Microbial Communities Promoting Mn(II) Oxidation in Ashumet Pond, a Historically Polluted Freshwater Pond Undergoing Remediation

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An extensive culture-dependent and -independent study was conducted to identify microorganisms contributing to the biogeochemical cycling of manganese (Mn) in Ashumet Pond, a freshwater pond in Massachusetts currently undergoing remediation. A variety of bacteria (including Gamma-, Beta-, and Alpha-proteobacteria, Firmicutes, and Bacteroides) and Ascoymete fungi were isolated from the pond that promote Mn(II) oxidation and subsequent formation of Mn(III/IV) oxide minerals. Targeted-amplicon pyrosequencing of the bacterial and fungal communities associated with Mn oxide-encrusted samples show a highly diverse microbial community, of which the cultured phylotypes represent a minor proportion. This suggests a larger community, not identified through culturing, contributes to Mn oxide formation within the Pond.

Keywords: biogeochemical cycling, biomineralization, bioremediation, community structure, molecular ecology

Introduction

The biogeochemical cycling of manganese (Mn), which exists primarily in the +2, +3, or +4 oxidation states under environmentally relevant conditions, is largely driven by both the direct and indirect activity of microorganisms. In recent years, a wealth of studies have demonstrated that phylogenetically diverse bacteria (Carmichael et al. 2013; Dick et al. 2006; Hansel and Francis 2006; Maki et al. 1987; Stein et al. 2001; Tebo et al. 2005; Templeton et al. 2005) and fungi (Cahyani et al. 2009; de la Torre and Gomez-Alarcon 1994; Miyata et al. 2006; Santelli et al. 2010) promote the oxidation of Mn(II) compounds to sparingly soluble Mn(III/IV) oxide minerals.

These biogenic minerals (referred to hereafter as “Mn oxides”) are primarily nanoparticulate, highly disordered phyllogomanganates, similar to birnessite or vernadite (Bargar et al. 2005; Grangeon et al. 2010; Santelli et al. 2011; Toner et al. 2005; Villalobos et al. 2006; Webb et al. 2005a, 2005b). Due to their high reactivity and ubiquity in terrestrial and aquatic environment, biogenic Mn oxide minerals greatly impact the concentration and distribution of abundant nutrients, carbon sources, and organic and inorganic contaminants; thus, Mn(II)-oxidizing microorganisms and resulting biominerals play an important role in the remediation of polluted environments.

Distinct, black Mn oxide mineral coatings have long been observed (Lee and Bennett 1998; McCobb et al. 2003) on a portion of the shores of Ashumet Pond, a freshwater pond on Cape Cod in Massachusetts. Thick mineral coatings are particularly prevalent where a heavily contaminated groundwater plume emanating from a nearby decommissioned wastewater treatment facility at the Massachusetts Military Reservation intersects the pond. Plume chemistry, microbiology, and physical and hydrologic properties have been well studied by the United States Geological Society (USGS) Toxic Substances Hydrology Program and several others (Gorbushina 2007; Harvey et al. 1984; LeBlanc 1984; Lee and Bennett 1998; McCobb et al. 2003; McCobb et al. 2009; Stollenwerk 1996).

The sewage-contaminated plume contains high concentrations of dissolved phosphate (Stollenwerk 1996), boron, nitrate and ammonium (McCobb et al. 2003), and metals, particularly Mn and Fe (Lee and Bennett 1998). In an attempt to remediate the contaminant plume prior to discharging into Ashumet Pond, a zero-valent iron permeable reactive barrier (ZVI-PRB) was installed in the subsurface in 2004. Analyses of water quality in the pond several years after installation of the ZVI-PRB showed a 95% decrease in PO4 concentrations (McCobb and LeBlanc 2011). However, the continued observation of black Mn oxide coatings near...
the pond shore indicates that some dissolved Mn, and potentially other inorganic or organic compounds, are still being discharged into the pond.

The goal of this study was to identify the microbial communities contributing to the oxidation and precipitation of Mn, and thus potentially contributing to the remediation of metals and organics, in Ashumet Pond. An extensive culturing survey was employed to select for Mn(II)-oxidizing microorganisms contributing to the observed Mn oxide mineral formation in the pond. Because the genes, such as multicopper oxidases, involved in Mn oxidation are not specific to Mn (Anderson et al. 2009; Dick et al. 2008; Geszvain et al. 2013; Learman et al. 2011), a culturing approach is still considered de rigueur for identifying microbial communities specifically promoting the oxidation of Mn in an environment.

Further, the total bacterial and fungal microbial communities existing in Mn-oxide encrusted Ashumet Pond sediments were assessed using 454-pyrosequencing of the bacterial 16S rRNA gene and the fungal internal transcribed spacer (ITS) region to examine the environmental microbial community structure and determine if the cultured organisms represented a majority or minority of the overall community. These findings allow for a better understanding of the microbial communities that influence the biogeochemical cycling of Mn in human-impacted environments.

Materials and Methods

Site and Sample Descriptions

Samples were collected in June, 2007 and July, 2009 from the northwest shore of Ashumet Pond on Cape Cod, Massachusetts (Figure 1). The contaminant groundwater plume intersects the pond along its western and northeastern shores where samples were collected (location of star on map). Pond bottom water and near-shore pebbles and sands were collected for analyses in the vicinity of the ZVI-PRB. Thin, black Mn oxide mineral coatings were prevalent on the solid substrates, and reddish Fe-oxide staining was also observed in the surrounding area.

Prior to the installation of the barrier, Mn concentrations in the groundwater plume upstream of Ashumet Pond once measured as high as 32 mg/L (Savoie and LeBlanc 1998), and pond-bottom groundwater had reached concentrations of 10.3 mg/L in the north-central portion of the pond (McCobb et al. 2003) near where we focused our sampling efforts (see Figure 1). Bottom-water dissolved Mn concentrations were not measured during the time of this study, however measurements made October, 2011 in this area demonstrated Mn concentrations ranging from 0.4–0.7 mg/L.

Approximately one dozen samples were collected for microbial culture enrichments and total community DNA analysis. All samples were collected from the Mn oxide rich area of the pond and included Mn oxide coated pebbles, sands, and sediment (including samples AP2sed and AP3s9f used for community analysis) as well as freshwater directly above the pond bottom. The one exception was a sediment sample rich in Fe oxides that spanned the anoxic/oxic interface, as evidenced by the dark, sulfidic-smelling sediment just millimeters below the surface (this sample is denoted as “APFeOX” hereafter). Solid and liquid samples were stored on ice for culture enrichments or flash-frozen and stored on dry ice for molecular biological analyses.

Culture Enrichments

Culture enrichments were initiated using submerged Mn-oxide coated pebbles and sands and pond water from numerous samples taken along approximately 40 feet of shoreline. Rock and sediment samples were lightly crushed using a mortar and pestle. CaCl2*2H2O All samples were diluted in sterile, artificial freshwater (AFW; 34.22 mM NaCl, 1.62 mM MgSO4*7H2O, 1.36 mM CaCl2*2H2O, 0.44 mM K2HPO4*3H2O, 20 mM HEPES buffer at pH 7) using serial dilutions to 1/10^4. Dilutions were plated onto 7 types of agar-solidified media containing 20 mM HEPES buffer (pH 7) and 200 μM MnCl2 (11 mg L^-1 Mn^{2+}).

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 (Templetton et al. 2005); J and J+acetate (JAC) media with AFW (Hansel and Francis 2006); and Medium 3 (M3; 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).

Fig. 1. Map of the Ashumet Pond area on Cape Cod, Massachusetts (inset), showing the sampling area (white star) and location of the zero-valent iron permeable reactive barrier (gray box under star) designed to remediate the organic- and metal-rich groundwaters that intersect the pond.
Culture Identification

Fungal isolates were identified by phylogenetic analysis (using ITS as well as 18S and 28S rRNA genes). Genomic DNA of each culture was isolated using the FastDNA SPIN Kit (MP Biomedicals). DNA was amplified by polymerase chain reaction (PCR) using bacterial- and fungi-specific primers. Bacterial 16S rRNA was amplified with primer pair 8F/1492R using protocols described previously (Santelli et al. 2008). Fungal 18S rRNA was amplified using primer pairs NS1/NS302 and NS3/NS5 following protocols described by Takano et al. (2006). Fungal 28S rRNA was amplified with primer pair LR0R/LR5-F as described by Tedersoo et al. (2008).

Primers ITS1F and ITS4 (Gardes and Bruns 1993; White et al. 1990) were used to amplify the fungal ITS1-5.8S-ITS2 region using protocols from O’Brien et al. (2005). The PCR mixture for fungal DNA was similar to the bacterial mix except for the addition of Combinatorial Enhancer Solution (CES – 0.54 M betaine, 1.34 mM dithiothreitol, 1.34% dimethyl sulfoxide, 11 μg mL⁻¹ bovine serum albumin (Ralsier et al. 2006)). Amplifications were performed on a Dyad Thermal cycler (Bio-Rad Laboratories).

Amplified DNA was purified using the QIAquick nucleotide removal kit (QIAGEN) and sequenced at the Dana-Farber/Harvard Cancer Center DNA Resource Core. Chromatographs were visually edited with FinchTV v. 1.4.0 (Geospiza, Inc) and contigs were assembled using Sequencher v. 4.8 (Gene Codes Corporation). The BLAST nucleotide search program (Altschul et al. 1997) was used to search for close relatives in GenBank for all sequence fragments (16S, 18S, 28S, and ITS). ARB (Ludwig et al. 2004) was used to align sequences (isolates and related organisms) for phylogenetic analysis. Filters were constructed using the base frequency filter generator with 50% minimum homology for each data set (16S rRNA for all bacterial sequences analyzed, 18S rRNA for all fungi, and ITS for each specific species) and used for phylogenetic analysis.

Maximum likelihood trees for 16S and 18S rRNA genes were constructed with the PhyML package (Guindon and Gascuel 2003) in ARB using the generalized time reversible (GTR) nucleotide substitution model with 1000 bootstrap replicates. Tree-puzzle dendograms of the ITS region of fungal sequences were constructed in ARB using 10,000 puzzling steps with quartet puzzling, neighbor-joining parameter estimation, the Hasegawa, Kishino and Yano (HKY) nucleotide substitution model, and a uniform model of rate heterogeneity.

DNA sequences from cultured isolates were submitted to the GenBank database with the following accession numbers: bacterial 16S rRNA genes (KF561874-KF561880), fungal 18S and 28S rRNA genes (KF561881-KF561885 and KF561886-KF561890), and fungal ITS region (KF561891-KF561895).

Environmental DNA Sequencing

Total community DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals) following the manufacturer’s instructions. Environmental DNA for bacterial 16S rRNA gene analysis required no pre-sequencing PCR amplification, however the DNA for fungal ITS analysis required initial amplification prior to submission to the sequencing facility (the facility was unable to amplify the DNA without the addition of the CES mixture). Thus, fungal ITS was first amplified as above for cultured isolates and cleaned using the QIAquick PCR Purification Kit (QIAGEN). Bacterial 16S rRNA gene tag-encoded FLX ampiclon pyrosequencing (bTEFAP) was carried out directly from the environmental DNA as described by Dowd et al. (2008). Fungal ITS rTEFAP FLX ampiclon pyrosequencing was performed on cleaned PCR products with primers ITS1F and ITS4. All pyrosequencing was performed at the Research and Testing Laboratory (Lubbock, TX) as previously described (Dowd et al. 2008).

Bacterial 16S rRNA Sequence Analysis

For 16S rRNA genes, processing and analysis of the fasta and quality files were carried out in mothur 1.29.1 (Schloss et al. 2009), following the recommendations of Schloss et al. (2011) and the Schloss SOP (http://www.mothur.org/wiki/Schloss_SOP, accessed Jan–Feb 2013). In brief, low-quality sequences were removed, including sequences with ambiguous bases, homopolymers longer than 8 bp, or more than two mismatches to the sequencing primer. Sequences were trimmed when the average quality score of a 50-bp sliding window dropped below 25, and sequences shorter than 200 bp were removed. Remaining sequences were aligned using the SILVA bacterial reference alignment (http://www.mothur.org/wiki/Silva_reference_alignment); only the sequences spanning the targeted region of 16S rRNA were kept, and all sequences were trimmed to the same length (optimized alignment end and minimum length so as to keep 85% of sequences, which gave the best compromise between length and number of sequences).

Data were further denoised by clustering together sequences with 1 bp mismatch per 100 bp, and chimeras were removed using the mothur implementation ofuchime (Edgar et al. 2011), with the more abundant sequences as reference. Sequences were classified against the SILVA and RDP6 reference databases with 100 bootstrap iterations and a cutoff value of 80% for taxonomic assignment. Those identified as organelles, Archaea or Eukarya were removed. Finally, sequences were subsampled to give groups of equal size. Mothur was used to cluster sequences into operational taxonomic units (OTUs) at divergence levels ranging from 0.00 (identical sequences) to 0.25, and calculate coverage, Chaol richness estimates, inverse Simpson index and Shannon index. Log-transformed relative abundance, OTU richness and proportion unique curves were plotted in R (R Development Core Team, 2010) from the OTU by sample tables generated in mothur.

Fungal ITS Sequence Analysis

Processing and analysis of ITS sequences followed a pipeline similar to that of 16S rRNA, with the following differences.
The minimum sequence length was set to 150 bp instead of 200 bp. Following the initial clean-up in mothur, the ITS1 region was extracted using the ITS Extractor tool on the PlutoF Workbench (Abarenkov et al. 2010b) (http://unite.ut.ee/workbench.php). Chimeras were removed using the mothur implementations of uchime and perseus, and sequences were subsampled to the size of the smallest group. Multiple sequence alignments of ITS are problematic, so for OTU clustering, a distance matrix was constructed in mothur using pairwise distance values, with consecutive gaps treated as one and ignoring gaps at the ends of pairs. Further OTU-based analysis was carried out as described here.

The UNITE+INSDC database (UNITE_public_27.01.13.fasta, downloaded February 2013 from http://unite.ut.ee/repository.php) (Abarenkov et al., 2010a) was used as a reference for classification, with the following modifications. All entries without kingdom-level information were removed, as were entries with ambiguous bases. The modified database, split into separate fasta and taxonomy files and formatted for use with mothur, contained 16,279 entries. Classification against this database was carried out in mothur with 100 bootstrap iterations and a cutoff value of 55% for taxonomic assignment. Raw amplicon pyrosequencing data were entries with ambiguous bases. The modified database, split into separate fasta and taxonomy files and formatted for use with mothur, contained 16,279 entries. Classification against this database was carried out in mothur with 100 bootstrap iterations and a cutoff value of 55% for taxonomic assignment. Raw amplicon pyrosequencing data were uploaded to the NCBI Sequence Read Archive under BioProject number PRJNA212339.

### Results and Discussion

#### Culture Survey

A large number of microorganisms were obtained from the original culture enrichment survey, although only a handful of these oxidized dissolved Mn(II) compounds and were isolated to purity. Phylogenetic analysis of the Mn(II)-oxidizing microorganisms reveals a diversity of both bacteria and fungi, described below, that were all isolated from Mn oxide coated solid substrates (e.g., pebbles and surface sediment).

Not a single Mn(II)-oxidizing culture was obtained from the overlying pond water nor from sites lacking observable Mn oxide mineral coatings on the pond bottom. These findings suggest that Mn-oxidizing organisms are not evenly distributed throughout the water and across the solid surfaces of Ashumet Pond. Rather, they are found only in close proximity to where the anaerobic, metal-laden groundwater intersects the aerobic pond water at the sediment-water interface, the area with conspicuous Mn oxidation.

In total, 14 isolates of Mn(II)-oxidizing bacteria and 8 Mn (II)-oxidizing fungi were obtained from the culture survey (Table 1). Interestingly, 12 of the bacterial isolates (85%) were obtained using M medium, a nutrient lean medium containing trace amounts of yeast extract, and all fungal isolates

| Table 1. Mn(II)-oxidizing Isolates from Ashumet Pond |
|-----------------------------------------------|
| Representative isolate | Phylogenetic classification | Taxonomy (Phylum or Class) | Top BLAST hita (accession #) | Max identity (%) | Extra isolates | Mn oxidation capabilityb |
|-------------------------|-----------------------------|-----------------------------|------------------------------|-----------------|---------------|------------------------|
| **Bacteria**            |                             |                             |                              |                 |               |                        |
| AP5s2-K1a               | *Brevundimonas* sp.         | Alphaproteobacteria         | *B. bullata* strain 3P04AC (EU977700) | 99              | 1             | +/-                   |
| AP5s2-M1ld              | *Flavobacterium* sp.       | Bacteroidetes               | *Flavobacterium* sp. YO11 (DQ778310) | 99              | 0             | +/-                   |
| AP5s2-M2b               | *Comamonas* sp.            | Betaproteobacteria          | *C. testosteroni* I2gfp (EF421407) | 99              | 4             | +/-                   |
| AP5s2-M2lc              | *Pseudomonas* sp.          | Gammaproteobacteria         | *Pseudomonas* sp. CK8-10 (JN195810) | 99              | 1             | +/-                   |
| AP5s2-M5a               | *Delftia* sp.              | Betaproteobacteria          | *Delftia* sp. XYJ6 (EU707799) | 100             | 0             | +/-                   |
| AP5s2-M5c               | *Bacillus* sp.             | Firmicutes                  | *Bacillus* sp. SAP72_1 (JN872501) | 99              | 1             | +/+                   |
| AP5s2-3blb              | *Raoultella* sp.           | Gammaproteobacteria         | *R. ornithinolytica* ATCC 31898 (AF129441) | 99              | 0             | +/-                   |
| **Fungi**               |                             |                             |                              |                 |               |                        |
| AP5s2-J2a               | *Cladosporium* sp.         | Ascomycota                  | *C. cucumerinum* ATCC 26211 (AF393696) | 99              | 0             | ++                     |
| AP3s5-J2a               | *Paraconiothyrium* sporulosum | Ascomycota           | *P. sporulosum* Cs/6/1 (JN624891) | 100             | 3             | ++                     |
| AP3s5-J1a               | *Phoma* sp.                | Ascomycota                  | *P. macrostoma* WAC 7881 (DQ474117) | 97              | 1             | +/+                   |
| AP3s5-JAC2b             | Pleosporales sp.           | Ascomycota                  | Nonef                     | –               | 0             | ++                     |
| AP1s5-JAC1a             | *Pyrenochaeta* sp.         | Ascomycota                  | *Hyalodendriella* sp. FF39 (FJ379833) | 99              | 0             | ++                     |

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a The top BLAST hits are based on 16S rRNA gene analysis for bacteria and ITS1-2 for fungi.

b A capability of “+-” indicates Mn(II) oxidation is confirmed, but either slow or inconsistent, whereas “++” indicates robust and consistent oxidation.

cITS analysis yielded no closely related fungal organisms. Identification based on 18S and 28S rRNA gene analysis (see Figure 3).
were obtained using nutrient-poor J, JAC, or M media. Only a single bacterial isolate was obtained using a nutrient-rich (e.g., K) medium, which is commonly used to isolate Mn(II)-oxidizing bacteria from a variety of environments (Dick et al. 2006; Templeton et al. 2005). Prior to the installation of the ZVI-PRB, the influx of nutrients and organic compounds into the pond was considerable (McCobb et al. 2003; Savoie and LeBlanc 1998). With in situ remediation efforts underway, the amount of sewage and nutrients being discharged into the pond is substantially less than previous years (McCobb and LeBlanc 2011).

The culturing results imply that the microbial communities inhabiting the pond sediments have adapted to reflect a more oligotrophic environment. Alternatively, this could indicate that Mn(II) oxidation by these microorganisms is inhibited in high nutrient conditions, as was previously observed with Mn (II)-oxidizing microbes isolated from coal mine drainage passive remediation systems (Santelli et al. 2010).

**Bacterial Isolates**

Fourteen Mn(II)-oxidizing bacterial isolates represent 7 different phylotypes from 5 phyla or subphyla (based on 16S rRNA gene sequence similarities ≥99%; Table 1; Figure 2). Several of these isolates are identified as either *Pseudomonas*

![Phylogenetic tree](image.png)
sp. (AP5s2-M2lc and AP3s5-M1a) or Bacillus sp. (AP5s2-M5c and AP5s2-M2c), members of the Gammaproteobacteria and Firmicutes, respectively. This is not unexpected, as these genera represent two of the most commonly isolated microorganisms that promote Mn(II) oxidation in a variety of environments, including ocean sediments (Francis and Tebo 2002), hydrothermal vents and plumes (Dick et al. 2006; Templeton et al. 2005), limestone cave systems (Carmichael et al. 2013), and coal mine drainage passive remediation systems (Santelli et al. 2010). In fact, several Bacillus and Pseudomonas species have been rigorously studied to establish the mechanisms (e.g., multicomponent oxidase proteins) promoting Mn(II) oxidation (Dick et al. 2008; Francis and Tebo 2001; Francis and Tebo 2002; Geszvain et al. 2013; Geszvain and Tebo 2010). The mechanisms of Mn(II) oxidation for the phylotypes isolated from Ashumet Pond, however, have yet to be examined.

The only other Ashumet Pond bacterial isolate that is closely related to other known Mn(II)-oxidizing phylotypes is Flavobacterium sp. AP5s2-M1ld, a member of the phylum Bacteroidetes (Table 1, Figure 2). This isolate has 99% sequence similarity to the 16S rRNA gene of Flavobacterium sp. DS2psK4b from a passive remediation system treating Mn(II)-rich coal mine drainage in Pennsylvania (Santelli et al. 2010). All other isolates are less closely related to known Mn(II)-oxidizing microorganisms. However, several share high sequence similarity with organisms that contribute to the degradation of complex organic compounds and thus may be involved in the remediation of organic pollutants.

For example, the 16S rRNA gene sequence of the Betaproteobacterium Delftia sp. AP5s2M5a is 100% similar to Delftia acidovorans strain SPH-1, an isolate from a sewage plant that degrades sulfophenylcarboxylates (SPCs). Additionally, Comamonas sp. AP5s2-M2b shares 99% sequence similarity (16S rRNA) with Comamonas testosteroni strain 12g6p that degrades aniline compounds in activated sludge (Boon et al. 2000). The final two bacterial isolates (AP5s2-K1a and AP5s2-3blb) are identified as a Brevundimonas species and a Raoultella species, respectively (Figure 2). It is of interest that, in contrast to the fungal isolates which robustly continue to oxidize Mn(II) compounds with time, all of the bacterial isolates demonstrate poor Mn(II) oxidation capabilities with successive transfers (see Table 1). This loss in oxidation capacity was especially pronounced after reviving frozen cultures in the presence of high (>500 μM) concentrations of dissolved Mn(II) compounds did restore their oxidation activity (data not shown).

**Fungal Isolates**

Phylogenetic analysis of the eight Mn(II)-oxidizing fungi reveals 5 different phylotypes (based on sequence similarity ≥99% for ITS sequences), all belonging to the phylum Ascomycota. To date, only Ascomycota and Basidiomycota have been observed to promote Mn(II) oxidation, and many Ascomycetes are known for their tolerance to rapidly changing or extreme environmental conditions such as desiccation, UV radiation, and metal concentrations (Gorbushina 2007).

All isolates in this study are members of the class Dothideomycetes, and all but one belong to the order Pleosporales (Figure 3). The most commonly isolated Pleosporales organisms (3 of 8 strains; strain identifications AP3s5-J2a, AP3s5-JAC2a, and AP3s5-M1b1) are identified as Paraconiothyrium sporulosum, a common soil fungus belonging to the Montagnulaceae family, based on both phylogenetic analysis (Figure 3 and SI Figure 1A) and morphological identification of strain AP3s5-JAC2a. This strain produces pycnidia when grown on malt extract agar (MEA) with abundant 1-celled, brown, ellipsoid conidia with smooth walls.

Several previously identified Mn(II)-oxidizing fungi from Mn-rich environments are phylogenetically related to these 3 isolates (See Figure 3), such as another Paraconiothyrium sporulosum strain (99% ITS sequence similarity) from an artificial wetland in Japan (Takano et al. 2006), an isolate from Mn-oxide coated streambed pebbles (Miyata et al. 2006), and a Leptosphaerulina chartarum strain that contributes to the remediation of Mn-rich waters resulting from coal mine drainage (Santelli et al. 2010). The common physiology of this family indicates a shared Mn(II) oxidation mechanism, potentially resulting from the production of reactive oxygen species (ROS) by these organisms during asexual reproduction as was identified with several other ascomycete fungi (Hansel et al. 2012; Tang et al. 2012).

The remaining five isolates could not be identified to species level based on phylogenetic analysis of 18S rRNA or ITS sequences (Figure 3 and SI Figures 1 and 2). Additionally, these organisms largely grow through hyphal extension and rarely produce any identifiable asexual or sexual reproductive structures on defined media that can further assist with species-level identifications. Phylogenetic analysis reveals that the four remaining Pleosporales isolates represent three phylotypes, each from a different family (Figure 3).

Two of these Pleosporales phylotypes (AP1s5-IAC1a, identified as a Pyrenochaeta species; AP3s5-J1a and AP3s5-M1b2, identified as a single Phoma species) are related, albeit at ≤95% sequence similarity, to several Mn(II)-oxidizing fungal isolates (Figure 3 and SI Figures 1b and 2a) from Mn-rich freshwater environments (Takano et al. 2006), bioremediation systems (Santelli et al. 2010), soil Mn nodules (Cahyani et al. 2009), and building stone (de la Torre and Gomez-Alarcon 1994). The third Pleosporales phylotype, comprised of a single isolate (Pleosporales sp. AP3s5-JAC2b), is the only one not closely related to other known Mn(II)-oxidizing fungi, and the only isolate that could not be identified beyond the order level as BLAST analysis of the ITS sequence compares against few other identified organisms – 18S (Figure 3) and 28S rRNA gene sequence analysis, however, indicate that this isolate belongs to the Lindgomycetaceae family.

The single non-Pleosporales fungal isolate, identified as Cladosporium sp. AP5s1-J2a (Figure 3 and SI Figure 2b), is a member of the Capnodiales family. Interestingly, Cladosporium sp. AP5s1-J2a is related to a deep-sea sediment isolate, Cladosporium cladosporoides strain Psf-1 (Shao and Sun 2007), that sequesters Mn(II) intracellularly and forms unidentified mineral crystals, although this phenomenon is not observed in the Ashumet Pond strain. The relatedness of
of the Ashumet Pond fungal isolates to numerous previously identified Mn(II)-oxidizing species emphasizes the ubiquity of these organisms in metal-rich environments and further highlights the diversity of organisms contributing to the biogeochemical cycling of Mn and other metals in nature.

Environmental Community Survey

Of the twelve samples from Ashumet Pond, three representative samples were selected for targeted amplicon pyrosequencing of the bacterial and fungal communities – two Mn-oxide rich samples (AP2sed and AP3sed) are where the majority of Mn-oxidizing cultures were obtained and the third sample (APFeOX) represents a nearby (within 10 feet) Fe-rich environment that contains both Fe(II)- and Fe(III)-bearing minerals. In order to statistically compare the microbial communities, the number of DNA sequences was normalized to the sample containing the lowest number of highly quality sequences (APFeOX), resulting in 1061 sequences for bacterial 16S rRNA analysis (mean sequence length 376.9 bp) and 447 sequences for fungal ITS analysis (mean sequence length 145.7 bp) (SI Table 1).

A rank-abundance (RA) curve of bacterial 16S rRNA sequences (Figure 4a) shows that the community evenness differs among the three samples. The diversity of sequences (i.e., OTUs clustered at the 0.00 divergence level, meaning each OTU is comprised only of identical sequences) is most even in AP2sed, demonstrated by the shallowest slope of the RA curve (Figure 4a). In contrast, the steep initial slope in the RA curve for APFeOX shows that this sample is highly uneven, dominated by a single sequence with a relative abundance of 40% (an order of magnitude higher than the most abundant bacterial sequence in each of the other two samples). This steep RA pattern, indicating that the majority of the biosphere in this environment is “rare”, is typically observed in bacterial community surveys (Huse et al. 2010; Sogin et al. 2006).

Such differences in community evenness are not observed in the fungal data (Figure 4b), and all samples show greater
evenness in the distribution of sequences. Small differences are present, however, in the fungal communities. The most dominant sequences in AP2sed and APFeOX account for roughly 10% of the fungal diversity, as opposed to only 3% in AP3s9f. Furthermore, APFeOX has the highest Chao1 (Chao 1984) estimated fungal richness of the three samples (SI Table 1), whereas it has the lowest bacterial diversity and Chao1 richness (due to the dominance by a single organism).

Figure 5 shows the community richness (number of different OTUs) across a range of clustering levels. Although the general convention for bacterial 16S rRNA draws the species threshold at 0.03, the genus at 0.05, and the phylum at roughly 0.20, there is no biological justification for applying these same thresholds to fungal ITS. Including a wide range of clustering levels allows comparison of samples at increasingly coarse phylogenetic resolution without the need to choose arbitrary cut-offs. The richness profiles of the Ashumet Pond bacterial and fungal communities at these different levels (Figure 5a) show markedly different trends.

Bacterial OTU richness in all three samples declines steadily (over an order of magnitude) with increasing divergence, whereas fungal OTU richness changes little at divergence levels greater than 0.02 (i.e., there are approximately as many fungal OTUs clustered at 97% similarity as there are at 75% similarity). When clustered at low sequence divergence, bacterial richness surpasses fungal richness, but with OTUs clustered at higher levels of divergence, this is reversed. Additionally, large differences in richness among the three samples are observed in the bacterial community at clustering levels below 0.15 (driven in large part by the dominance of a single organism in APFeOX). In contrast, the fungal richness of the three samples is similar at all levels of OTU clustering.

Despite differences in bacterial OTU richness, the proportion of OTUs unique to each sample (Figure 5b) is similar in all three. At low clustering levels, few OTUs are shared between any samples (seen by the high proportion of unique OTUs). This proportion remains high (above 50%) even when OTUs are clustered at the bacterial phylum level (i.e., half of the phyla in each sample are not present in the other two samples). In contrast, most fungal OTUs in each sample
are also found in at least one of the other samples, even at the finest resolution (divergence of 0.00).

Further, differences in the fungal community between samples are more pronounced than for the bacterial community – in AP2sed, almost all OTUs at levels 0.02 and higher were also found in other samples, whereas roughly 25% of OTUs in AP3s9f were unique to that sample over the same range. All diversity and community structure indicators clearly reveal that the fungal community is far more homogeneous across the environment than the bacterial community, which suggests different dispersal mechanisms or less niche specialization in the fungal community.

**Microbial Community Taxonomy**

The distribution of sequences within the different bacterial phyla (and classes of Proteobacteria) is similar among the three samples (Figure 6), with the exception of the dominance of Deltaproteobacteria in APFeOX. Most of the Deltaproteobacteria sequences in this sample (541 out of 592) fall into the strictly anaerobic, sulfate-reducing family Desulfofabaetales, with 471 of those belonging to an unclassified member of the genus Desulfocapsa. Clearly, anaerobic conditions present in this sample are the greatest influence on the bacterial community structure. Generally, the Proteobacteria (Alpha-, Beta-, Delta-, and Gamma-) make up the largest proportion of sequences in all three samples.

These organisms have a cosmopolitan distribution and represent some of the greatest phylogenetic and physiological diversity among all organisms (Gupta 2000; Stackebrandt et al. 1988; Woese 1987). Furthermore, numerous representatives from these classes of Proteobacteria promote Mn(II) oxidation in a variety of environments (Carmichael et al. 2013; Dick et al. 2006; Tebo et al. 2005; Templeton et al. 2005), including several from Ashumet Pond (see Figure 2).

In addition to the Proteobacteria, there are also high proportions of Cyanobacteria (16.3% of all sequences), particularly in AP2sed and lesser in AP3s9f, and Acidobacteria sequences in AP3s9f (accounting for almost 10% of the total). Other bacterial phyla represented in the sequence database include Actinobacteria (2.6% of total), Bacteroidetes (3.2%), Chloroflexi (2.0%) and Verrucomicrobia (1.1%). Sixteen other phyla were present, nine of which had only one or two representatives out of the 3183 total sequences. Additionally, 8–12% of sequences in each sample (10.4% of the total) were unclassified at the phylum level (SI Table 1).

Although several Mn(II)-oxidizing Actinobacteria and Bacteroidetes have previously been isolated (Carmichael et al. 2013; Santelli et al. 2010), a role for Cyanobacteria in Mn(II) oxidation has not yet been directly observed. However, cyanobacteria have been shown to produce extracellular superoxide (Diaz et al. 2013; Latifi et al. 2009), which could indirectly promote Mn(II) oxidation reactions. We hypothesize that many of these uncultured organisms identified in our metagenomic analysis are contributing to the oxidation of Mn in Ashumet Pond.

The taxonomic breakdown of environmental fungi (Figure 7) shows that the phylum level community composition is, unexpectedly, most similar between AP2sed and APFeOX (samples from two highly different geochemical regimes). Both communities are heavily dominated by Basidiomycota (85% in AP2sed and 76% in APFeOX). Of these Basidiomycetes, the majority are Agaricomycete fungi with fewer Tremellomycetes. Sample AP3s9f, however, has a different taxonomic profile—although Agaricomycetes are still the most abundant Basidiomycete, accounting for 31.5% of sequences, the Ascomycota account for a larger proportion (34.0%) of sequences than in the other two samples (2.0% and 14.1%).

Very few Dothideomycetes, the class in which all of the Mn(II)-oxidizing isolates from Ashumet Pond belong, and Sordariomycetes, host to a large diversity of other previously identified Mn(II)-oxidizing fungi (Figure 3; Santelli et al. 2010), are present in the environmental sequences. AP3s9f is
the only sample in which additional phyla were found (one Glomeromycota and three Zygomyccota sequences), although a sizeable percentage (12.5% in AP2sed, 20.4% in AP3s9f and 9.6% in APFeOX) could not reliably be placed in any phylum.

Basidiomycota and Ascomycota are commonly observed growing in freshwater environments (Gareth Jones and Choeayklin 2008; Shearer 2001), so their presence in Ashumet Pond sediments is not unexpected. It is surprising, however, to find such a large number of sequences belonging to the Agaricomycetes in the Ashumet Pond environmental samples, largely because they are predominantly found in terrestrial environments with fewer known aquatic representatives (Hibbett 2007). These organisms, which include a variety of dry- and wood-rotting fungi, are known for their ability to degrade complex carbon compounds, like lignin and cellulose, from decaying leaf and wood litter (Gareth Jones and Choeayklin 2008; Highley 1980; Valaskova and Baldrian 2006) – thus, these fungi play an important role in the global carbon cycle.

Because the sampling sites are located near the heavily forested edge of the pond, it is possible that the large number of Basidiomycetes reflects the continual flux of decaying organic matter, an important nutrient source for these fungi, into the aquatic environment. These organisms could further be contributing to the degradation of residual organic contaminants in the groundwater plume, either input to the pond prior to the installation of the ZVI-PRB or bypassing the remediation system entirely.

The role of Basidiomycetes in the biogeochemical cycling of Mn in Ashumet Pond is uncertain, since no Mn(II)-oxidizing isolates from this phylum were obtained. However, it is known that several species of wood-rot Agaricomycetes can oxidize Mn(II) to Mn(III) compounds through the production of certain enzymes, such as Mn peroxidases, that ultimately promote lignin depolymerization (Ruttimann-Johnson et al. 1994; Scheel et al. 2000; Wariishi et al. 1992). Thus, we postulate that these organisms are contributing, either directly or indirectly, to the distribution and speciation of Mn in Ashumet Pond. A targeted culturing approach to select for Basidiomycetes in future studies may be valuable for establishing their physiological role in this and similar freshwater environments.

Cultured Representatives in Pyrosequencing Libraries

Although the culture assays to identify key Mn(II)-oxidizing microorganisms in Ashumet Pond were highly successful, the relative abundance of the resulting isolates is exceedingly low in most of the environmental pyrosequencing libraries (SI Table 2 and SI Table 3). Most bacterial isolates are not found in any of the sequence libraries, although several sequences with very high (>97%, approximately the same species or phylotype), or moderately high (95–96.9%, approximately the same genus) sequence similarity to Flavobacterium sp. AP5s2-M1ld are present in all three sample libraries. The recovery of cultured fungal isolate sequences from the pyrosequencing libraries is similarly low with the exception of Cladosporium sp. AP5s5-J2a (37 sequences with ≥98% similarity) and Pleosporales sp. AP5s5JAC2b (also 37 sequences with ≥98% similarity), although both of these isolate sequences are surprisingly abundant in APFeOX, the sample dominated by Fe oxides as opposed to Mn oxides.

There are several potential reasons for the low recovery of Mn(II)-oxidizing isolate DNA sequences in the targeted amplicon pyrosequencing surveys. First, the actual abundance of these isolates in the natural environment may be low. Second, the extraction or amplification procedure may be biasing the community analysis. And third, the organisms could be dormant and contain poor quality DNA for amplification and subsequent metagenomic analysis. These results, however, do not indicate that biologically enhanced Mn(II) oxidation is not important in Ashumet Pond. Given the abundance of Mn oxides observed at the sampling sites, it is possible that the activity of the isolates is disproportionate to their relative abundance in the total community DNA, which does not differentiate between active and dormant or dead cells.

Additionally, it is likely that the culturing survey is missing the majority of potential Mn(II)-oxidizing microorganisms, particularly the wood-rot Agaricomycetes which are prevalent in the pyrosequencing results. Culturing approaches, while necessary for confirming and examining Mn(II) oxidation processes, are usually only successful at obtaining a small fraction of the entire community, and it is possible that the culturing techniques used in this study are not adequate to enrich for Basidiomycete fungi or various other microorganisms identified through molecular techniques. Furthermore, a recent study by Diaz et al. (2013) demonstrates that a much greater diversity of bacteria than previously recognized have the potential to promote the production of superoxide, which is known oxidant of Mn(II) (Hansard et al. 2011; Learman et al. 2011).

This potential pathway is similarly recognized in several related Ascomycete (Hansel et al. 2012; Tang et al. 2012) and Basidiomycete fungi (discussed above). Consequently, a much larger and more diverse community of microorganisms is likely contributing to the in situ Mn biogeochemical cycle in Ashumet Pond and other Mn-rich environments. Further studies, using additional techniques (e.g., quantitative PCR targeting specific species and genes involved in ROS production) and in additional environments, will be necessary to evaluate this hypothesis. Regardless, this project reveals that both bacteria and fungi promote the biogeochemical cycling of Mn in Ashumet Pond.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

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