Profiling microbial communities in manganese remediation systems treating coal mine drainage

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Abstract

Water discharging from abandoned coal mines can contain extremely high manganese levels. Removing this metal is an ongoing challenge. Passive Mn(II) removal beds (MRBs) contain microorganisms that oxidize soluble Mn(II) to insoluble Mn(III/IV) minerals, but system performance is unpredictable. Using amplicon pyrosequencing, we profiled the bacterial, fungal, algal and archaeal communities in four variably-performing MRBs in Pennsylvania to determine whether they differed among MRBs and from surrounding soil, and to establish the relative abundance of known Mn(II)-oxidizers. Archaea were not detected; PCRs with archaeal primers returned only non-target bacterial sequences. Fungal taxonomic profiles differed starkly between sites that remove the majority of influent Mn and those that do not, with the former dominated by *Ascomycota* (mostly *Dothideomycetes*) and the latter by *Basidiomycota* (almost entirely *Agaricomycetes*). Taxonomic profiles for the other groups did not differ significantly between MRBs, but OTU-based analyses showed significant clustering by MRB with all four groups ($p<0.05$). Soil samples clustered separately from MRBs in all groups except fungi, whose soil samples clustered loosely with their respective MRB. Known Mn(II) oxidizers accounted for a minor proportion of bacterial sequences (up to 0.20%) but a greater proportion of fungal sequences (up to 14.78%). MRB communities are more diverse than previously thought, and more organisms may be capable of Mn(II) oxidation than are currently known.

Introduction

Coal mine drainage (CMD) from operating and abandoned mines often contains elevated concentrations of dissolved manganese (Mn). Removing this metal from contaminated water is a significant challenge in mining regions across the world, including the Appalachian Coal Basin in the eastern USA, where centuries of coal mining have left thousands of abandoned mines with effluent dissolved Mn concentrations as high as 150 mg L^{-1} (1, 2). At such levels, Mn can damage ecosystems and water distribution systems. One of the most common remediation methods in this region involves biologically-active limestone treatment beds (3). These beds raise the pH of the CMD and promote the oxidation of soluble Mn(II) to sparingly soluble Mn(III/IV) minerals (4), which are retained in the beds and can be periodically removed. Unfortunately, the performance of these Mn(II) removal beds (MRBs) is highly variable due to insufficient knowledge of the processes (biotic and abiotic) involved (2, 5). A better understanding of the primary mechanisms contributing to Mn(II) oxidation will aid technological advances and removal efficiencies.

Recent studies have demonstrated that, although abiotic Mn(II) oxidation is thermodynamically inhibited below pH 9 when oxygen is the oxidant (6), Mn oxide surfaces (7) and reactive oxygen species (8, 9) catalyse oxidation of Mn(II) at near-neutral pH. Mineral surface-catalysed Mn(II) oxidation was shown to occur in simulated CMD treatment bioreactors, though microbial activity dominated the oxidation of Mn(II) to Mn(III/IV) oxides under certain treatment conditions (2). A diversity of bacteria (10–15) and fungi (12, 15–18), isolated from a range of aquatic and terrestrial environments, are known to oxidize Mn(II) when grown in pure culture, though not as an energy-

conserving process but rather as a side reaction of unknown physiological basis. The remediation of Mn-contaminated waters is thought to rely largely on such organisms. Indeed, culture-based studies of Mn(II) removal systems in Wales, UK (19) and across Pennsylvania, USA (5, 20) have identified numerous resident bacteria and fungi that oxidize Mn(II), though the abundance and activity of these isolates relative to the total microbial communities in the treatment systems is unknown. Since Mn(II) oxidation is not an energy conservation process (i.e. respiration) in any known Mn(II)-oxidizing microorganism (21, 22), it is possible that it does not correlate positively with abundance. For example, superoxide-mediated Mn(II) oxidation would be negatively correlated to abundance if an organism used superoxide as a signal at low cell densities but not at higher densities.

While culture-based studies are essential for elucidating the mechanisms promoting biological Mn(II) oxidation (22–28), these isolated organisms exist in complex communities whose interactions could be modulating their Mn(II) oxidation activity in the environment. Other community members, notably phototrophs and diazotrophs, could be providing essential carbon and nitrogen compounds. Conversely, heterotrophs could be competing for limiting resources. Finally, other unidentified community members could be oxidizing Mn(II) but have resisted laboratory cultivation. A culture-independent, community-level approach that encompasses groups known to contribute to biological Mn(II) oxidation (bacteria and fungi), as well as other potentially relevant community members (algae and archaea) has, to our knowledge, not been performed on these treatment systems but would greatly improve our understanding of key organisms and community interactions promoting Mn(II) oxidation *in situ*.

Using an amplicon pyrosequencing approach, we characterized four microbial target groups (bacteria, archaea, fungi, and algae) existing in four variably-performing MRBs in Pennsylvania that have previously been described (2, 5, 20) and from which Mn(II)-oxidizing bacteria and fungi were isolated (20): De Sale 1 and 2 (DS1 and DS2), PBS, and Saxman Run bed C1 (SRC1), also known as Derry Ridge (2). At the time of sampling, PBS and SRC1 were removing nearly 100% of influent Mn(II), DS2 was removing 50% and DS1 was not removing any influent Mn(II). We aimed to determine i) whether similar microbial communities are found in different MRBs or whether each MRB develops a distinct community, ii) whether differences in community structure are apparent within MRBs, from the influent (where dissolved Mn(II) concentrations are highest) to the effluent (where they are lowest), and iii) whether communities in MRBs are distinct from those in the surrounding uncontaminated soil. These results will ultimately contribute to improving Mn(II) remediation technologies by increasing our understanding of MRB community diversity and highlighting key taxa present in MRBs with near-complete Mn(II) removal.

Materials and Methods

Sample collection

Four limestone-filled, geotextile-lined Mn(II) removal beds (MRBs) in western Pennsylvania were sampled in November 2010: PBS, Saxman Run bed C1 (SRC1), and De Sale phases I and II (DS1 and DS2). These systems, described in earlier studies (2, 5, 20), treat coal mine drainage with considerable dissolved manganese concentrations by promoting microbiologically-catalyzed oxidation of aqueous Mn(II)

compounds to sparingly soluble Mn(III/IV) oxide minerals that are retained within the beds. DS1, PBS and SRC1 receive influent Mn(II) concentrations averaging 18-20 mg L^{-1}, whereas DS2 has a higher mean influent Mn(II) concentration, 31.2 mg $L^{-}1$ (Table S1)(2). PBS and SRC1 are highly effective in attenuating Mn, with over 90% removal, resulting in mean effluent concentrations of 0.29 and 1.3 mg $L^{-}1$, respectively. DS2 is attenuating approximately 50% of dissolved Mn (mean effluent concentration of 17.1 mg $L^{-}1$), and DS1 showed minimal Mn attenuation in the year preceding sampling (Table S1)(2). DS1 and DS2 are in close proximity to each other (roughly 250 m) but are treating different influents. SRC1 and PBS are roughly 100 km and 150 km from the DS sites, respectively, and are separated by 50 km.

MnO_X-rich samples (limestone pebbles, sediment and organic debris) were collected near the influent, in the middle of the bed, and near the effluent, though not every location was accessible in each MRB. There is no influent sample for DS2 and no effluent sample for PBS, but DS1 and DS2 each have two middle samples. In DS1 and DS2, the Mn oxides occurred as loose soil-like sediments that were collected with sterile spatulas. In PBS and SRC1, Mn oxides formed varnish-like coatings on the limestone cobbles so entire cobbles were collected. In addition, a control soil sample was taken outside each MRB. Samples were immediately flash-frozen in a dry ice/ethanol bath in the field and stored on dry ice during transportation to the laboratory, where they were kept at -80°C until processing.

DNA extraction, amplification and pyrosequencing

129 Mn oxide sediments and fragments of Mn-coated limestone were crushed with a
130 sterile pestle and mortar. For DS1 and DS2 samples, a single 0.5 g aliquot of crushed
131 sediment yielded sufficient DNA for amplification and sequencing. However, samples
132 from PBS and SRC1 were more resistant to extraction. Four 0.25 g aliquots of each
133 sample were extracted separately and subsequently pooled and concentrated with
134 ethanol precipitation, using glycogen as a carrier. With these samples, smaller amounts
135 of starting material (half that recommended by the kit manufacturer) gave the best
136 yields, suggesting the difficulties were due to inhibition by metal cations rather than to
137 low biomass. DNA was extracted with the FastDNA SPIN Kit for Soil (MP Biomedicals),
138 with the following modifications. Polyadenylic acid (200 µg per sample) was added to
139 the lysis buffer to reduce inhibition by metal cations. Two homogenization steps on the
140 FastPrep instrument (MP Biomedicals) were carried out with a five-minute incubation on
141 ice in between. The initial centrifugation step to remove sediments and cell debris was
142 extended to 15 minutes, and the binding matrix incubation was extended to 10 minutes.
143 Elution was carried out by resuspending the binding matrix in 100 µL nuclease-free
144 sterile water and incubating at 55°C. Extracts were quantified using the Qubit dsDNA
145 HS Assay Kit (Life Technologies) with the Qubit 1.0 fluorometer, and those exceeding
146 15 ng μL^{-1} were diluted to that concentration. Each extract was then divided into four
147 aliquots, one for each of the four target amplifications.
148 Tag-encoded FLX amplicon pyrosequencing (TEFAP) was carried out directly
149 from aliquots of total community DNA at the Research and Testing Laboratory
150 (Lubbock, TX) as previously described (29). Four target groups were amplified and
151 sequenced from each of the sixteen samples: i) bacterial 16S rRNA with primers 28F

(modified from 30) and 519R (modified from 31), ii) archaeal 16S rRNA with primers 349F and 806R (32), iii) fungal ITS1-5.8S-ITS2 with primers ITS1F and ITS4 (33, 34), and iv) algal plastid 23S rRNA with primers AlgaeF and AlgaeR (35)(Table S2). Although the algae are polyphyletic and therefore not a true phylogenetic group, these plastid 23S rRNA primers amplify cyanobacteria and six eukaryotic algal lineages: Chlorophyta (green algae), Rhodophyta (red algae), Bacillariophyta (diatoms), Phaeophyceae (brown algae), Xanthophyceae (yellow-green algae) and Euglenida (euglenids) (35).

Processing of raw pyrosequencing reads

Raw pyrosequencing reads were processed as described in (36), using mothur 1.31.1 (37) and following the recommendations of (38). Algal plastid flow files were trimmed to a minimum and maximum length of 350, whereas other target flow files were trimmed to 400.

Bacterial and archaeal sequences were aligned to the Silva bacterial and archaeal SSU rRNA reference alignments (39) available in mothur. Algal sequences were aligned to the Silva LSURef 111 database, with metazoa sequences removed (which greatly improved the alignment performance). Only the sequences spanning the targeted regions were kept, and all sequences were trimmed to the same length. For bacteria and archaea, this was determined by optimizing alignment end and minimum length so as to keep 90% of sequences. For algae, the start and end positions were manually set based on the alignment statistics. Data were further denoised by clustering together sequences with 1 bp mismatch per 100 bp, and chimeras were removed using

the mother implementation of uchime (40), with the more abundant sequences as reference. Bacterial sequences were further cleaned up by classifying against the Silva reference database using the Wang method (41) with kmer size 8, 100 bootstrap iterations and a threshold of 60% for taxonomic assignment. Following this step, sequences identified as mitochondria, chloroplasts, Archaea or Eukarya were removed. For fungal sequences, following the primer, barcode and homopolymer removal outlined in the previous paragraph, the ITS1 region was extracted using ITSx (42) on the PlutoF Workbench (43), and sequences shorter than 100 bp following this step were discarded. Chimeras were removed as described above.

Diversity analyses and taxonomic assignment

Within each target, the number of sequences per sample was normalized to the size of the sample with the lowest number of sequences. For bacteria, archaea and algae, OTU clustering was carried out in mothur using the multiple sequence alignments generated during sequence processing. However, since multiple sequence alignments of fungal ITS are problematic for all but the most closely-related species, OTU clustering of fungal sequences was carried out based on pairwise distance values calculated in mothur, with consecutive gaps treated as one and ignoring gaps at the ends of pairs.

mothur was used to generate OTU-by-sample tables at all OTU clustering levels between 0.00 and 0.25, and to calculate the Simpson evenness index (44). R (45) was used for plotting and subsequent analyses. The vegan package (46) was used to calculate Bray-Curtis distances from relative abundance matrices and Jaccard distances from presence/absence matrices (47), followed by non-metric

multidimensional scaling. Community variation was partitioned between the two factors in this study (MRB and location within bed) and their interaction using permutational analysis of variance based on distance matrices (48), which is implemented by the adonis function in the vegan package, with 999 permutations. Bray-Curtis and Jaccard distances were subsequently used in pairwise comparisons of the four target groups (bacteria, archaea, fungi, algae) to look for evidence of biotic coupling, which would be suggested by positive correlations in distance values. Significance of pairwise correlations was assessed using the Mantel test with 999 permutations.

Bacterial and archaeal sequences were classified against the Silva reference databases available in mothur, as described above, with a threshold of 60%. Algal sequences were classified against the Silva LSURef 111 database, with metazoan and fungal sequences removed and a threshold of 60%. For fungal ITS1 sequences, the UNITE+INSDC fungal ITS database (49) version 27.01.13 (downloaded February 2013 from http://unite.ut.ee/repository.php) was used as a reference for classification, with the modifications described in (36). Differences in the relative abundances of taxonomic groups were assessed in R using t-tests (soils versus MRBs) and ANOVA (four MRBs), with *p*-values corrected for multiple comparisons.

All sequence data were deposited, with MIMARKS-compliant metadata, in the NCBI Sequence Read Archive under BioProject number PRJNA229802, BioSample numbers SAMN02404598 to SAMN02404613. The metadata are also available separately as a MIMARKS-formatted supplementary table.

Results

In total, 228,444 sequences passed a highly stringent quality control (Table 1 and Supplemental Results). After clustering into operational taxonomic units (OTUs), richness and evenness of bacteria, fungi and algae were plotted (Figs. S1, S2) but no consistent patterns were observed (see Supplemental Results), suggesting that at a coarse level, microbial diversity is similar within and between MRBs, and is comparable to that in the surrounding soil. Statistical comparisons of observed and estimated (Chao1) richness among the different MRBs (ANOVA) and between the MRBs and the soils (t-test) are shown in Table S3.

Archaea

Of the four targets, the archaeal sequence data proved most problematic and resulted both in the smallest percentage passing QC (18.4%; Table 1) and in the smallest sample size (n=254). When classified against the Silva archaeal reference database, 70.8% of sequences remained unclassified at the phylum level. Of the sequences that were placed in an archaeal phylum, fewer than 1% (46 sequences in total) were classified further. However, when classified against a combined bacterial/archaeal Silva database with a higher bootstrap threshold (80 instead of 60), all sequences were found to be bacterial (Fig. S3). Most were placed into the phyla *Chlamydiae*, *Planctomycetes* and *Verrucomicrobi*a, with only 4.6% remaining unclassified at the phylum level. Given that the archaeal data set appears to consist entirely of non-target bacterial amplicons, it was excluded from further analyses. Further work with different primer sets will be needed to determine whether Archaea are truly so

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scarce as to be undetectable or whether their absence from our data set was due to their signal being overwhelmed by non-target amplification.

Influence of Mn(II) removal bed and location within beds

Non-metric multidimensional scaling (NMDS) was performed to cluster samples based on the similarity of their microbial communities. NMDS based on Bray-Curtis distance of OTU relative abundances showed that, with the three target groups (bacteria, fungi, algae), samples clustered largely by MRB (Fig. 1) and not by their location within each bed (at the influent, where Mn concentrations would be highest, in the middle, or at the effluent, which would have the lowest Mn concentration). The soil samples from each site, however, formed a separate cluster rather than grouping with their corresponding treatment bed, except for fungi, whose soil samples did not form a separate group. Figure 1 shows clustering based on OTUs defined at the 0.03 level, but similar patterns were observed at all OTU clustering levels between 0.00 and 0.20 (data not shown). Furthermore, the same patterns were observed with NMDS based on Jaccard distance calculated from presence/absence matrices as opposed to relative abundance (data not shown).

To test whether the clustering observed in NMDS plots indicated significant differences, we carried out permutational analysis of variance based on distances, which partitioned the sample variance among the two factors, MRB (DS1, DS2, PBS, SRC1) and location within bed (influent, middle, effluent), and their interaction (Table S4). The effect of the MRB was significant for bacteria, fungi and algae based on Bray-Curtis distances calculated from relative abundance matrices, and was significant for

bacteria and algae based on Jaccard distances calculated from presence/absence matrices (p<0.05; Table S4). The location within beds (influent, middle or effluent) had a significant effect only on the bacterial communities with presence/absence data, and there was no significant interaction between these two factors in any of the three target groups. Similar results (data not shown) were obtained at the other OTU clustering levels (0.00 to 0.20).

Taxonomic profile

Proteobacteria accounted for 46.9% of all bacterial sequences (36.9-57.9% per sample), more than all other phyla combined, not including the 11.0% of bacterial sequences that remained unclassified at the phylum level (Fig. 2). They were significantly more abundant in the MRBs than in the soil samples (mean proportion of 49.1% in beds versus 40.6% in soils, p=0.031, Table S5). The predominant proteobacterial classes, orders and families are shown in Figures S4, S5 and S6, respectively. Most *Proteobacteria* belonged to the *Alpha-* and *Beta-* classes (Fig. S4), and there were no significant differences in the relative abundance of the classes between the soil and beds, or between the four beds. The *Alphaproteobacteria* were dominated by the order *Rhizobiales* in all samples except SRC1-influent (Fig. S5), and within this one order, fourteen families were detected (Fig. S6), pointing to considerable diversity at increasingly fine resolution. *Betaproteobacteria* sequences were mostly split between the orders *Burkholderiales* and *Nitrosomonadales* (Fig. S5), though the ratio of these two orders varied widely between and within MRBs (e.g. *Betaproteobacteria* in PBS were mostly *Nitrosomonadales*, whereas those in SRC1 were mostly

Burkholderiales). In all soil samples and in the PBS MRB samples, all *Nitrosomonadales* sequences belonged to the family *Nitrosomonadaceae* (Fig. S6); however, samples from inside the other MRBs also included varying proportions (2-100%) of the family *Gallionellaceae* (iron-oxidizing chemolithotrophs).

Of the other bacterial phyla detected, five accounted for 5-10% each of total bacterial sequences (*Actinobacteria*, *Chloroflexi, Acidobacteria*, *Cyanobacteria* and *Bacteroidetes*), and three (*Planctomycetes*, *Gemmatimonadetes* and *Firmicutes*) accounted for 1-5% each. The relative abundance of these eight phyla was similar in the four MRBs and in the soil samples, except for the *Acidobacteria*, which were significantly more abundant in the soil samples (mean relative abundance of 3.4% in beds versus 13.6% in soils, $p<0.01$). An additional 15 phylum-level groups were detected with overall relative abundances below 1% (*Nitrospirae*, *Verrucomicrobia, Fusobacteria*, *Chlorobi*, *Fibrobacteres*, *Deinococcus-Thermus*, *Spirochaetes*, WCHB1-60, BD1-5, GOUTA4, and candidate divisions OD1, OP10, TM6, TM7 and WS3).

The fungal sequences belonged mostly to the phyla *Basidiomycota* (47.4% overall) and *Ascomycota* (38.8% overall), with 9.7% of all fungal sequences remaining unclassified at the phylum level (Fig. 2). Three other fungal phyla occurred only sparsely (3.6% *Zygomycota*, 0.4% *Chytridiomycota* and 0.1% *Glomeromycota*). Unlike in the bacterial sequence data, stark differences were observed between the MRBs at the phylum level in the fungal data (Fig. 2), with *Ascomycota* and *Basidiomycota* relative abundances being significantly different among the four beds ($p<0.001$). DS1 and DS2 bed samples were heavily dominated by *Basidiomycota* (which accounted for 80.3% and 73.1% of fungal sequences, respectively), whereas for PBS and SRC1 samples,

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312 these proportions were 31.3% and 17.6%, respectively. Conversely, DS1 and DS2 had
313 lower relative abundances of *Ascomycota* sequences (10.9% and 7.0%, respectively)
314 compared with PBS (35.1%) and SRC1 (62.2%). Also in contrast to the bacterial
315 taxonomic profiles, which showed some clear differences between MRBs and soil
316 samples (consistently more *Acidobacteria* and fewer *Proteobacteria* in soils, for
317 example), none of the fungal classes differed significantly in relative abundance
318 between MRB samples and soil samples. Overall, most *Ascomycota* sequences
319 belonged to the class *Dothideomycetes* (Fig. 2) and most of these were further grouped
320 into the subclass *Pleosporomycetidae* (Fig. S7). *Basidiomycota* sequences belonged
321 almost entirely to the class *Agaricomycetes*, subclasses *Agaricomycetidae* and incertae
322 sedis (encompassing the orders *Cantharellales*, *Polyporales*, *Russulales*, *Sebacinales*
323 and *Thelephorales*). A much higher proportion of *Basidiomycetes* than *Ascomycetes*
324 remained unclassified at the subclass level and beyond (Fig. S7).

325 Almost half of algal sequences (40.4%) belonged to the phylum *Chlorophyta*
326 (green algae; Fig. 2). Most green algae (79.3% of *Chlorophyta* sequences) could not be
327 further classified. Those that could were placed in the classes *Chlorophyceae* (orders
328 *Sphaeropleales*, *Chlamydomonadales* and *Oedogoniales*) and *Trebouxiophyceae*
329 (orders *Chlorellales*, *Microthamniales* and *Coccomyxaceae*). After the *Chlorophyta*, the
330 next most abundant phyla were the *Bacillariophyta* (diatoms), accounting for 18.9% of
331 total sequences, and the PX clade (encompassing the yellow-green algae phylum
332 *Xanthophyceae*), accounting for 11.3% overall. 14.9% of algal sequences were
333 unclassified at the phylum level. All the *Bacillariophyta* sequences that could be
334 classified to genus level (39.2%) were placed in the genus *Fistulifera*, and all the PX

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clade *Xanthophyceae* sequences belonged to the genus *Vaucheria*, filamentous yellow-green algae. Of four main phyla detected in the algal sequence data, only the *Bacillariophyta* appeared to differ among the four MRBs, with higher abundances in beds DS1 and DS2 (26.4% and 37.6%, respectively) compared with PBS (6.7%) and SRC1 (6.9%); however, following correction for multiple comparisons, the difference was not significant (p=0.197). Similarly, *Bacillariophyta* appeared to differ between bed samples (21.0%) and soil samples (10.3%), but the difference was not significant (p=0.249).

Occurrence of known Mn(II)-oxidizing organisms

Four Mn(II)-oxidizing bacterial strains were previously isolated from these MRBs (20), but no sequences with 100% similarity to these isolates were detected in the MRB sequence data, except for one *Pseudomonas* sp. DS3sK1h sequence in SRC1. At a similarity level of 97.0 to 99.9%, these isolates accounted for 0.11% of DS1 bacterial sequences, 0.06% of DS2 sequences, 0.20% of SRC sequences and 0.13% of soil sequences (all soil sites combined). Relatives of the Mn(II)-oxidizing bacterial isolates were not detected in the PBS sequence data, even at a similarity level of only 95%.

Overall, nine Mn(II)-oxidizing fungi isolated from these MRBs (20) accounted for a greater proportion of the sequence data than did the Mn-oxidizing bacterial isolates, with several fungal isolates individually exceeding 1% of sequences at a similarity level of 100% within some treatment beds (Fig. S8). SRC1 had the greatest abundance of known Mn(II)-oxidizing fungi - eight of the nine isolates were detected, with *Phoma* sp. DS1wsM30b accounting for 10.52% of sequences and *Alternaria alternata* SRC1lrK2f

accounting for 2.81%. In total, known Mn(II)-oxidizing fungal isolates accounted for 14.78% of SRC1 fungal sequences. In contrast, these fungal isolates together accounted for 1.92% of DS1 fungal sequences, 1.63% of DS2 sequences and 1.43% of PBS sequences (Fig. S8). Eight of the nine isolates were detected in the control soils, and together they accounted for 4.11% of soil fungal sequences.

Biotic coupling

Previous analyses were carried out separately with the three target groups (bacteria, fungi and algae). With this final analysis, we attempted to determine whether the community structures of the three target groups were correlated or whether they were independent. In other words, are samples that are more similar with respect to one target group also more similar with respect to the other target groups?

Significant positive correlations were found between all pairs of target groups (Mantel r=0.446-0.665, p=0.001; Fig. 3). Samples with more similar communities of one target therefore tend to have more similar communities of the other targets - for example, samples with more similar bacterial communities typically have more similar fungal and algal communities as well, whereas samples with very different bacterial communities also generally have very different fungal and algal communities. Figure 3 shows Bray-Curtis distances calculated from relative abundance of OTUs clustered at the 0.03 level, but the significant positive correlations between target groups hold at all OTU clustering levels between 0.00 and 0.20, as well as when Jaccard distances based on presence/absence data are used instead. Overall, the three targets examined here

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showed strong biotic coupling, both in their membership and in the relative abundance of their community members.

Discussion

Microbial activity contributes greatly to the removal of Mn(II) from coal mine drainage (2), and understanding the communities involved could improve remediation efforts. We profiled the bacteria, archaea, fungi and algae in four Mn(II) removal beds (MRBs) to determine i) whether each bed develops a unique community or whether similar communities arise in different beds, ii) whether differences are observed within each bed (from the influent to the effluent), and iii) whether MRB communities are distinct from the surrounding CMD-free soil. Our four MRBs remove varying proportions of their influent's dissolved Mn (Table S1): nearly 100% in PBS and SRC1, 50% in DS2 (which, granted, has a higher influent concentration than the other MRBs) and 0% in DS1 (2). Of particular interest is whether microbial communities differ substantially between MRBs that remove the majority of influent Mn (PBS and SRC1) and those that do not (DS1 and DS2).

Of the four microbial groups profiled, only the fungi showed stark differences at the phylum level between the MRBs that remove the majority of influent Mn (PBS and SRC1) and those that do not (DS1 and DS2) (Fig. 2). The similarity of DS1 and DS2 MRB samples could be due to the geographic proximity of these two beds (roughly 250 m separates them), though if this were the only factor, their corresponding soil samples would also be expected to show similarities to each other, which is not the case (Fig. 2). *Ascomycota* sequences, belonging mainly to the classes *Dothideomycetes* and

403 *Sordariomycetes*, were significantly more abundant in PBS and SRC1, whereas
404 *Basidiomycota* sequences, almost exclusively in the class *Agaricomycetes*, heavily
405 dominated the fungal profiles of DS1 and DS2. Both *Ascomycota* and *Basidiomycota*,
406 and specifically the main classes identified in our four MRBs, contain known Mn(II)-
407 oxidizing members. However, previous efforts to isolate Mn(II)-oxidizing fungi from
408 these beds yielded only *Ascomycota* (5, 20), as did culturing efforts in other Mn(II)-rich
409 environments such as Ashumet Pond, Massachusetts, USA (15), Mn nodules in
410 Japanese rice fields (12), Mn oxide-coated stream bed pebbles in Japan (17), and
411 another Mn attenuation system in the UK (19). Most of the isolates obtained in these
412 culturing studies belonged to the *Ascomycota* classes *Dothideomycetes* and
413 *Sordariomycetes*, the same two fungal classes that dominated the MRBs with near-
414 complete Mn(II) removal in our study (PBS and SRC1). Furthermore, the Mn(II)-
415 oxidizing *Ascomycota* isolates obtained by (20) from these MRBs were more abundant
416 in the sequence data from SRC1 than from PBS, DS1 and DS2. Interestingly, nearby
417 soils had a higher proportion of these isolates than three of the four MRBs, suggesting
418 that the Mn(II)-oxidizing fungi that colonize MRBs are not specific to aquatic sediment
419 environments and that the fungal communities in MRBs are seeded in part from nearby
420 soil.

421 The *Basidiomycota* class *Agaricomycetes*, which dominated fungal profiles in
422 DS1 and DS2 (Fig. 2), is usually found in terrestrial environments, though it was also
423 abundant in a culture-independent survey of fungi in Mn oxide-rich sediments in
424 Ashumet Pond, USA (15). Several species of wood-rot or litter-decaying
425 *Agaricomycetes* can oxidize Mn(II), notably *Phanerochaete chrysosporium* (50),

426 *Stropharia rugosoannulata* (51) and *Pleurotus eryngii* (52). However, no close relatives
427 of these known Mn(II)-oxidizing *Agaricomycetes* were present in the four MRBs, with
428 the exception of a single sequence in DS2 with 100% similarity to *S. rugosoannulata*.
429 The bulk of the *Agaricomycetes* sequences that dominate DS1 and DS2 therefore do
430 not belong to known Mn(II) oxidizers, though it is possible that the taxa present can
431 oxidize Mn(II) but have remained resistant to cultivation. Previous culturing attempts
432 failed to obtain Mn(II)-oxidizing isolates from this class (20).

433 Interestingly, the stark differences in fungal taxonomic profiles between MRBs
434 that remove the majority of Mn(II) and those that do not were not mirrored in the other
435 groups (Fig. 2). Bacteria are thought to be the main drivers of biological Mn(II) oxidation
436 in many environments, but if that were the case in our MRBs, we would expect some
437 differences in bacterial taxonomic profiles between MRBs that remove the majority of
438 Mn(II) and those that do not, as well as within each MRB, correlating with dissolved
439 Mn(II) concentrations. The similarity in bacterial communities across all four MRBs, in
440 contrast to the drastic differences in fungal communities, suggests that the latter are
441 important drivers of Mn removal in these MRBs. This supports the findings of Burgos et
442 al. (5), who, in a series of MRB sediment incubation experiments with and without
443 fungicides, showed that fungal activity accounted for over 80% of Mn(II) oxidation in the
444 Fairview MRB in Pennsylvania. However, in later experiments, the same research group
445 found that fungi were not dominant contributors to Mn(II) oxidation in sediments from
446 that same MRB as well as from seven others (2), suggesting that Mn(II) oxidation is a
447 dynamic process affected by hydrological and other factors (though as these authors
448 noted, their laboratory growth conditions may have inhibited fungal activity).

Taxonomic profiles alone cannot rule out a prominent role for bacterial Mn(II) oxidation in our four MRBs, since differences between the beds in the proportion of influent Mn(II) removed could be due to environmental conditions that favour or inhibit the activation of necessary metabolic pathways without changing taxonomic distributions. *Proteobacteria* were the most abundant bacterial phylum in the MRBs (Fig. 2). The same has been reported in most culture-independent surveys of Mn-rich environments, including Mn oxide-rich freshwater sediments in the USA (15, 53), Mn nodules in Japanese rice fields (54), ferromanganese deposits in caves (55), and in biofilters treating Fe, Mn and ammonia-containing water (56, 57). The abundance of *Proteobacteria* is not unusual. This widespread, highly diverse phylum is dominant in many environments, notably soils (including the four control soil samples in this study, though at a significantly lower abundance than in the MRBs). It also encompasses numerous confirmed examples of Mn(II) oxidation in the *Alpha*-, *Beta*-, *Gamma*- and *Deltaproteobacteria* (10, 11, 14, 15, 24, 55). Indeed, many of the model Mn(II)-oxidizing bacteria used to elucidate mechanisms of oxidation belong to this phylum (24, 25, 58, 59). However, since the relative abundance of proteobacterial taxa did not differ significantly between MRBs, even at increasingly fine taxonomic resolution, and nor did the relative abundance of any of other bacterial phyla, the taxonomic profile of the bacterial communities cannot be correlated with differences in the proportion of Mn(II) removed by the four MRBs. Of course, since these are DNA-based profiles that include dead and dormant cells, it is possible that the active components of the bacterial communities show more pronounced differences between or within the MRBs that could only be detected with RNA-based profiling.

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At first glance, algal taxonomic profiles appeared to differ between MRBs that remove the majority of dissolved Mn(II) (PBS and SRC1) and those that do not (DS1 and DS2) (Fig. 2), with more *Stramenopiles* sequences (diatoms and yellow-green algae) in DS1 and DS2 than in PBS and SRC1, which contained mostly *Viridiplantae* sequences (green algae and plants). Due to the high variability within each bed and the small number of samples per bed, these differences were not statistically significant, so we cannot draw any conclusions regarding the influence of algae on the proportion of Mn(II) removed by these systems. However, it is likely that they contribute to Mn removal in some capacity, either indirectly (by providing fixed carbon for Mn(II)-oxidizing fungi and bacteria) or by oxidizing Mn(II) directly. Algae, including green algae such as *Klebsormidium*, *Chara*, *Ulothrix* and *Chlamydomonas*, as well as diatoms, are often observed to thrive in metal-contaminated environments (60, 61), and a limestone treatment pond with a mixed green algae and microbial mat removed far more dissolved Mn than limestone alone (62). Not only can algae accumulate Mn up to 90,000 mg/kg dry weight (63), but they can also catalyse its oxidation directly (64–66). This is thought to occur through the creation of pH microenvironments, whereby dense populations of algal cells, through photosynthesis, increase local pH above 9.0, resulting in abiotic oxidation of Mn(II) by molecular oxygen (64). Furthermore, photosynthesis-linked pH modulation might not be the only mechanism of algal Mn(II) oxidation. Robbins et al. (65) hinted at this when, on glass slides submerged in a Mn-contaminated creek, they observed that some but not all diatoms were coated with Mn oxides, whereas all would be expected to produce Mn oxides if the mechanism were purely photosynthesis-linked pH modulation. Many algae are known to produce reactive oxygen species, notably

495 superoxide, as a by-product of photosynthesis, as antibacterial agents (67), and in
496 response to metal contamination (60). Some bacteria (25) and fungi (22) oxidize Mn(II)
497 via superoxide production, and it is possible that some of the algae in our MRBs can do
498 the same.

499 While the taxonomic profiles showed that only fungal communities differed
500 significantly between MRBs, OTU-based analyses painted a different picture. When
501 samples were clustered based on distance metrics (Bray-Curtis and Jaccard), clear
502 patterns emerged (Fig. 1). Firstly, distinctions were seen between soil and MRB
503 samples, with MRBs from different parts of Pennsylvania (as far apart as 150 km)
504 clustering more closely together than with their corresponding soil samples, suggesting
505 that MRBs in disparate areas select for similar communities, and MRB communities are
506 not simply a subset of those in their immediate surroundings. This pattern held for
507 bacteria and algae; however, fungi showed no separation of soil and MRB samples;
508 instead, soil samples were loosely clustered with those from their corresponding MRB,
509 suggesting a different community development history and perhaps different dispersal
510 mechanisms. Furthermore, while the patterns in bacterial and algal community structure
511 are clearly influenced by one or more of the factors that distinguish MRBs from nearby
512 soils (water-saturated versus drained, high metal load in MRBs, differences in carbon
513 and nutrient availability, etc.), clearly these factors are less influential drivers of fungal
514 community structure in these systems. Of importance might also be the use of ITS1 for
515 fungal profiling, compared with ribosomal subunits for the other groups. ITS1 is
516 degraded after transcription and has high variability, including intraspecific variability
517 that is more pronounced in some fungal lineages than in others (68). The greater scatter

in the fungal NMDS plot could be a result of the fundamentally different function of and selection pressures operating on ITS1.

The second pattern observed in the NMDS plots was some loose clustering by treatment bed, which occurred with all groups (Fig. 1). In several instances, DS1 and DS2 samples showed some overlap, which is not surprising given their geographic proximity (roughly 250 m separates them). Perhaps more interesting, PBS and SRC1 often showed some overlap as well (for example, with bacteria and algae in Fig. 1). These MRBs are over 50 km apart. Their main similarity, in the context of this study, is that they remove nearly 100% of influent Mn(II), compared with the other two MRBs, which remove far smaller proportions (50% and 0%). This suggests that, despite the bacterial and algal taxonomic profiles being similar in MRBs that remove different proportions of influent Mn(II), subtle differences at the OTU level that are consistent across highly-efficient beds could point to sub-groupings of microorganisms with metabolic functions that are more conducive to Mn(II) removal. A wider survey of MRBs would be required to confirm this finding, and a more targeted investigation focusing on the specific OTUs in highly-efficient MRBs could give insight into mechanisms of Mn(II) removal in mixed communities.

The sample clustering observed at the OTU level was not reflected in the taxonomic profiles, highlighting differences in the two approaches. Taxonomic profiling is coarse (especially when limited to phyla and classes), and it relies on accurate reference databases, whereas OTU-based analyses rely solely on sequence data. It is interesting that in this study, while different OTUs appear to occur in each MRB (with more similar OTUs being detected in the MRBs with near-complete removal of Mn(II)),

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their proportion remains fairly constant across the main bacterial and algal taxonomic groups (but not fungal ones).

We showed that microbial communities in MRBs are far more diverse than suggested by culturing or by culture-independent profiling of a single group. Similar to (15), known Mn(II) oxidizers, especially bacterial ones, accounted for a small proportion of the total community, suggesting that their activity is not coupled to their relative abundance or that many more organisms are capable of Mn(II) oxidation than have been cultured, including organisms such as algae that have not been directly associated with Mn(II) oxidation in these systems. Clear differences were observed between MRBs with near-complete removal of Mn(II) and those that remove smaller proportions of influent Mn(II). Future work should expand to include groups not covered in this study, notably heterotrophic eukaryotes, which could also be influencing Mn(II) oxidation, and examine more closely how microbial communities become established in new MRBs, with particular emphasis on differences between fungi and other community members. Furthermore, it is important to elucidate the mechanisms by which these organisms oxidize Mn(II), since this could inform remediation strategies. For example, since oxidation is linked to superoxide production, encouraging processes that produce superoxide, such as fungal cell differentiation, should result in greater Mn(II) removal. Finally, given that the groups examined here showed significant biotic coupling (suggesting a degree of interdependence), it is worth exploring whether seeding new systems with mixed communities is more effective than seeding with pure cultures or allowing communities to develop without seeding.

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References

1. **Cravotta III CA**. 2008. Dissolved metals and associated constituents in abandoned coal-mine discharges, Pennsylvania, USA. Part 1: Constituent quantities and correlations. Appl. Geochemistry **23**:166–202.

2. **Luan F, Santelli CM, Hansel CM, Burgos WD**. 2012. Defining manganese(II) removal processes in passive coal mine drainage treatment systems through laboratory incubation experiments. Appl. Geochemistry **27**:1567–1578.

3. **Berghorn GH, Hunzeker GR**. 2001. Passive treatment for remediating abandoned-mine drainage. Remediat. J. **11**:111–127.

4. **Johnson KL, Baker A, Manning DAC**. 2005. Passive treatment of Mn-rich mine water: Using fluorescence to observe microbiological activity. Geomicrobiol. J. **22**:141–149.

5. **Burgos WD, Tan H, Santelli CM, Hansel CM**. 2010. Importance of fungi in biological Mn(II) oxidation in limestone treatment beds, p. 71–88. *In* Barnhisel, R (ed.), National Meeting of the American Society of Mining and Reclamation. ASMR, Pittsburgh, PA.

6. **Luther GW**. 2010. The role of one- and two-electron transfer reactions in forming thermodynamically unstable intermediates as barriers in multi-electron redox reactions. Aquat. Geochemistry **16**:395–420.

7. **Madden AS, Hochella MF**. 2005. A test of geochemical reactivity as a function of mineral size: Manganese oxidation promoted by hematite nanoparticles. Geochim. Cosmochim. Acta **69**:389–398.

8. **Hansard SP, Easter HD, Voelker BM**. 2011. Rapid reaction of nanomolar Mn(II) with superoxide radical in seawater and simulated freshwater. Environ. Sci. Technol. **45**:2811–2817.

9. **Learman DR, Wankel SD, Webb SM, Martinez N, Madden AS, Hansel CM**. 2011. Coupled biotic–abiotic Mn(II) oxidation pathway mediates the formation and structural evolution of biogenic Mn oxides. Geochim. Cosmochim. Acta **75**:6048–6063.

10. **Templeton AS, Staudigel H, Tebo BM**. 2005. Diverse Mn(II)-oxidizing Bacteria isolated from submarine basalts at Loihi Seamount. Geomicrobiol. J. **22**:127–139.

11. **Anderson CR, Dick GJ, Chu M-L, Cho J-C, Davis RE, Bräuer SL, Tebo BM**. 2009. *Aurantimonas manganoxydans*, sp. nov. and *Aurantimonas litoralis*, sp. nov.: Mn(II) oxidizing representatives of a globally distributed clade of alpha-Proteobacteria from the order Rhizobiales. Geomicrobiol. J. **26**:189–198.

12. **Cahyani VR, Murase J, Ishibashi E, Asakawa S, Kimura M**. 2009. Phylogenetic positions of Mn^{2+}-oxidizing bacteria and fungi isolated from Mn nodules in rice field subsoils. Biol. Fertil. Soils **45**:337–346.

13. **Ozaki T, Wang X, Ohnuki T**. 2013. Manganese and arsenic oxidation performance of Bacterium-Yunotaki 86 (BY86) from Hokkaido, Japan, and the bacterium's phylogeny. Geomicrobiol. J. **30**:559–565.

14. **Yang W, Zhang Z, Zhang Z, Chen H, Liu J, Ali M, Liu F, Li L**. 2013. Population structure of manganese-oxidizing bacteria in stratified soils and properties of manganese oxide aggregates under manganese-complex medium enrichment. PLoS One **8**:e73778.

15. **Santelli CM, Chaput DL, Hansel CM**. 2014. Microbial communities promoting Mn(II) oxidation in Ashumet Pond, a historically polluted freshwater pond undergoing remediation. Geomicrobiol. J. **31**:605–616.

16. **Thompson IA, Huber DM, Guest CA, Schulze DG**. 2005. Fungal manganese oxidation in a reduced soil. Environ. Microbiol. **7**:1480–1487.

17. **Miyata N, Maruo K, Tani Y, Tsuno H, Seyama H, Soma M, Iwahori K**. 2006. Production of biogenic manganese oxides by anamorphic ascomycete fungi isolated from streambed pebbles. Geomicrobiol. J. **23**:63–73.

18. **Takano K, Itoh Y, Ogino T, Kurosawa K, Sasaki K**. 2006. Phylogenetic analysis of manganese-oxidizing fungi isolated from manganese-rich aquatic environments in Hokkaido, Japan. Limnology **7**:219–223.

19. **Mariner R, Johnson DB, Hallberg KB**. 2008. Characterisation of an attenuation system for the remediation of Mn(II) contaminated waters. Hydrometallurgy **94**:100–104.

20. **Santelli CM, Pfister DH, Lazarus D, Sun L, Burgos WD, Hansel CM**. 2010. Promotion of Mn(II) oxidation and remediation of coal mine drainage in passive treatment systems by diverse fungal and bacterial communities. Appl. Environ. Microbiol. **76**:4871–4875.

21. **Tebo BM, Johnson HA, McCarthy JK, Templeton AS**. 2005. Geomicrobiology of manganese(II) oxidation. Trends Microbiol. **13**:421–428.

22. **Hansel CM, Zeiner CA, Santelli CM, Webb SM**. 2012. Mn(II) oxidation by an ascomycete fungus is linked to superoxide production during asexual reproduction. Proc. Natl. Acad. Sci. U. S. A. **109**:12621–12625.

642 23. **Dick GJ, Podell S, Johnson HA, Rivera-Espinoza Y, Bernier-Latmani R,**
643 **McCarthy JK, Torpey JW, Clement BG, Gaasterland T, Tebo BM**. 2008.
644 Genomic insights into Mn(II) oxidation by the marine alphaproteobacterium
645 *Aurantimonas* sp strain SI85-9A1. Appl. Environ. Microbiol. **74**:2646–2658.

646 24. **Hansel CM, Francis CA**. 2006. Coupled photochemical and enzymatic Mn(II)
647 oxidation pathways of a planktonic *Roseobacter*-like bacterium. Appl. Environ.
648 Microbiol. **72**:3543–3549.

649 25. **Learman DR, Voelker BM, Vazquez-Rodriguez AI, Hansel CM**. 2011.
650 Formation of manganese oxides by bacterially generated superoxide. Nat.
651 Geosci. **4**:95–98.

652 26. **Miyata N, Tani Y, Sakata M, Iwahori K**. 2007. Microbial manganese oxide
653 formation and interaction with toxic metal ions. J. Biosci. Bioeng. **104**:1–8.

654 27. **Soldatova AV, Butterfield C, Oyerinde OF, Tebo BM, Spiro TG**. 2012.
655 Multicopper oxidase involvement in both Mn(II) and Mn(III) oxidation during
656 bacterial formation of MnO_2. J. Biol. Inorg. Chem. **17**:1151–1158.

657 28. **Tebo BM, Bargar JR, Clement BG, Dick GJ, Murray KJ, Parker D, Verity R,**
658 **Webb SM**. 2004. Biogenic manganese oxides: Properties and mechanisms of
659 formation. Annu. Rev. Earth Planet. Sci. **32**:287–328.

29. **Sun Y, Wolcott RD, Dowd SE**. 2011. Tag-encoded FLX amplicon pyrosequencing for the elucidation of microbial and functional gene diversity in any environment. Methods Mol. Biol. **733**:129–141.

30. **Frias-Lopez J, Zerkle AL, Bonheyo GT, Fouke BW**. 2002. Partitioning of bacterial communities between seawater and healthy, black band diseased, and dead coral surfaces. Appl. Environ. Microbiol. **68**:2214–2228.

31. **Lane DJ**. 1991. 16S/23S rRNA sequencing, p. 115–175. *In* Stackebrandt, E, Goodfellow, M (eds.), Nucleic acid techniques in bacterial systematics. John Wiley & Sons, New York.

32. **Takai K, Horikoshi K**. 2000. Rapid detection and quantification of members of the archaeal community by quantitative PCR using fluorogenic probes. Appl. Environ. Microbiol. **66**:5066–5072.

33. **Gardes M, Bruns TD**. 1993. ITS primers with enhanced specificity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol. Ecol. **2**:113–118.

34. **White TJ, Bruns T, Lee S, Taylor JW**. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, p. 315–322. *In* Innis, MA, Gelfand, DH, Sninsky, JJ, White, TJ (eds.), PCR Protocols: A Guide to Methods and Applications. Academic Press, Inc., New York.

679 35. **Sherwood AR, Presting GG**. 2007. Universal primers amplify a 23S rDNA plastid
680 marker in eukaryotic algae and cyanobacteria. J. Phycol. **43**:605–608.

681 36. **Cutler NA, Chaput DL, van der Gast CJ**. 2014. Long-term changes in soil
682 microbial communities during primary succession. Soil Biol. Biochem. **69**:359–
683 370.

684 37. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB,**
685 **Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B,**
686 **Thallinger GG, Van Horn DJ, Weber CF**. 2009. Introducing mothur: Open-
687 source, platform-independent, community-supported software for describing and
688 comparing microbial communities. Appl. Environ. Microbiol. **75**:7537–7541.

689 38. **Schloss PD, Gevers D, Westcott SL**. 2011. Reducing the effects of PCR
690 amplification and sequencing artifacts on 16S rRNA-based studies. PLoS One
691 **6**:e27310.

692 39. **Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J,**
693 **Glöckner FO**. 2013. The SILVA ribosomal RNA gene database project: improved
694 data processing and web-based tools. Nucleic Acids Res. **41**:D590–D596.

695 40. **Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R**. 2011. UCHIME
696 improves sensitivity and speed of chimera detection. Bioinformatics **27**:2194–
697 2200.

41. **Wang Q, Garrity GM, Tiedje JM, Cole JR**. 2007. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. **73**:5261–5267.

42. **Bengtsson-Palme J, Ryberg M, Hartmann M, Branco S, Wang Z, Godhe A, De Wit P, Sánchez-García M, Ebersberger I, de Sousa F, Amend AS, Jumpponen A, Unterseher M, Kristiansson E, Abarenkov K, Bertrand YJK, Sanli K, Eriksson KM, Vik U, Veldre V, Nilsson RH**. 2013. Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods Ecol. Evol. **4**:914–919.

43. **Abarenkov K, Tedersoo L, Nilsson RH, Vellak K, Saar I, Veldre V, Parmasto E, Prous M, Aan A, Ots M, Kurina O, Ostonen I, Jogeva J, Halapuu S, Poldmaa K, Toots M, Truu J, Larsson K-H, Koljalg U, Henrik H**. 2010. PlutoF—a web based workbench for ecological and taxonomic research, with an online implementation for fungal ITS sequences. Evol. Bioinforma. **6**:189–196.

44. **Maurer BA, McGill BJ**. 2011. Measurements of species diversity, p. 55–65. *In* Magurran, AE, McGill, BJ (eds.), Biological diversity: frontiers in measurement and assessment. Oxford University Press, Oxford.

45. **R Core Team**. 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

718 46. **Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB,**
719 **Simpson GL, Solymos P, Stevens HH, Wagner H**. 2013. vegan: Community
720 Ecology Package. R package version 2.0-8.

721 47. **Jost L, Chao A, Chazdon RL**. 2011. Compositional similarity and beta diversity,
722 p. 66–84. *In* Magurran, AE, McGill, BJ (eds.), Biological diversity: frontiers in
723 measurement and assessment. Oxford University Press, Oxford.

724 48. **Anderson MJ**. 2001. A new method for non-parametric multivariate analysis of
725 variance. Austral Ecol. **26**:32–46.

726 49. **Abarenkov K, Nilsson RH, Larsson K-H, Alexander IJ, Eberhardt U, Erland S,**
727 **Høiland K, Kjøller R, Larsson E, Pennanen T, Sen R, Taylor AFS, Tedersoo**
728 **L, Ursing BM, Vrålstad T, Liimatainen K, Peintner U, Kõljalg U**. 2010. The
729 UNITE database for molecular identification of fungi – recent updates and future
730 perspectives. New Phytol. **186**:281–285.

731 50. **Glenn JK, Akileswaran L, Gold MH**. 1986. Mn(II) oxidation is the principal
732 function of the extracellular Mn-peroxidase from *Phanerochaete chrysosporium*.
733 Arch. Biochem. Biophys. **251**:688–696.

734 51. **Schlosser D, Höfer C**. 2002. Laccase-catalyzed oxidation of Mn^{2+} in the
735 presence of natural Mn^{3+} chelators as a novel source of extracellular H_2O_2
736 production and its impact on manganese peroxidase. Appl. Environ. Microbiol.
737 **68**:3514–3521.

52. **Ruiz-Dueñas FJ, Morales M, Pérez-Boada M, Choinowski T, Martínez MJ, Piontek K, Martínez AT**. 2007. Manganese oxidation site in *Pleurotus eryngii* versatile peroxidase: a site-directed mutagenesis, kinetic, and crystallographic study. Biochemistry **46**:66–77.

53. **Stein LY, La Duc MT, Grundl TJ, Nealson KH**. 2001. Bacterial and archaeal populations associated with freshwater ferromanganous micronodules and sediments. Environ. Microbiol. **3**:10–18.

54. **Cahyani VR, Murase J, Ishibashi E, Asakawa S, Kimura M**. 2007. Bacterial communities in manganese nodules in rice field subsoils: Estimation using PCR-DGGE and sequencing analyses. Soil Sci. Plant Nutr. **53**:575–584.

55. **Carmichael MJ, Carmichael SK, Santelli CM, Strom A, Bräuer SL**. 2013. Mn(II)-oxidizing bacteria are abundant and environmentally relevant members of ferromanganese deposits in caves of the upper Tennessee River Basin. Geomicrobiol. J. **30**:779–800.

56. **Li X, Chu Z, Liu Y, Zhu M, Yang L, Zhang J**. 2013. Molecular characterization of microbial populations in full-scale biofilters treating iron, manganese and ammonia containing groundwater in Harbin, China. Bioresour. Technol. **147**:234–239.

57. **Yang L, Li X, Chu Z, Ren Y, Zhang J**. 2014. Distribution and genetic diversity of the microorganisms in the biofilter for the simultaneous removal of arsenic, iron and manganese from simulated groundwater. Bioresour. Technol. **156**:384–388.

759 58. **Dick GJ, Podell S, Johnson HA, Rivera-Espinoza Y, Bernier-Latmani R,**
760 **McCarthy JK, Torpey JW, Clement BG, Gaasterland T, Tebo BM**. 2008.
761 Genomic insights into Mn(II) oxidation by the marine alphaproteobacterium
762 *Aurantimonas* sp. strain SI85-9A1. Appl. Environ. Microbiol. **74**:2646–2658.

763 59. **Geszvain K, Butterfield C, Davis RE, Madison AS, Lee S-W, Parker DL,**
764 **Soldatova A, Spiro TG, Luther GW, Tebo BM**. 2012. The molecular
765 biogeochemistry of manganese(II) oxidation. Biochem. Soc. Trans. **40**:1244–
766 1248.

767 60. **Das BK, Roy A, Koschorreck M, Mandal SM, Wendt-Potthoff K, Bhattacharya**
768 **J**. 2009. Occurrence and role of algae and fungi in acid mine drainage
769 environment with special reference to metals and sulfate immobilization. Water
770 Res. **43**:883–894.

771 61. **Prasanna R, Ratha SK, Rojas C, Bruns MA**. 2011. Algal diversity in flowing
772 waters at an acidic mine drainage “barrens” in central Pennsylvania, USA. Folia
773 Microbiol. **56**:491–496.

774 62. **Phillips P, Bender J, Simms R, Rodriguez-Eaton S, Britt C**. 1995. Manganese
775 removal from acid coal-mine drainage by a pond containing green algae and
776 microbial mat. Water Sci. Technol. **31**:161–170.

777 63. **Kleinmann RLP**. 1990. Acid mine water treatment using engineered wetlands.
778 Mine Water Environ. **9**:269–276.

64. **Richardson LL, Aguilar C, Nealson KH**. 1988. Manganese oxidation in pH and 0_2 microenvironments produced by phytoplankton. Limnol. Oceanogr. **33**:352–363.

65. **Robbins EI, Corley TL, Conklin MH**. 1999. Manganese removal by the epilithic microbial consortium at Pinal Creek near Globe, Arizona, p. 1–14. *In* Proceedings of the Technical Meeting. U.S. Geological Survey Toxic Substances Hydrology Program, Charleston, SC.

66. **Schöler A, Zaharieva I, Zimmermann S, Wiechen M, Manke A-M, Kurz P, Plieth C, Dau H**. 2014. Biogenic manganese-calcium oxides on the cell walls of the algae *Chara corallina* : Elemental composition, atomic structure, and water-oxidation catalysis. Eur. J. Inorg. Chem. **2014**:780–790.

67. **Marshall J, Hovenden M, Oda T, Hallegraeff GM**. 2002. Photosynthesis does influence superoxide production in the ichthyotoxic alga *Chattonella marina* (Raphidophyceae). J. Plankton Res. **24**:1231–1236.

68. **Nilsson RH, Kristiansson E, Ryberg M, Hallenberg N, Larsson K-H**. 2008. Intraspecific ITS variability in the kingdom Fungi as expressed in the international sequence databases and its implications for molecular species identification. Evol. Bioinform. Online **4**:193–201.

799 **Figure Legends**

800 Fig. 1. Non-metric multidimensional scaling of Bray-Curtis distances from bacterial,
801 fungal and algal OTU relative abundance matrices. Threshold for OTU clustering was a
802 sequence divergence of 0.03 (i.e. 97% similarity). n = 1,166, 1,885 and 518 sequences
803 per sample for bacteria, fungi and algae, respectively.

804

805 Fig. 2. Taxonomic affiliation of bacterial, fungal and algal sequences. Bacterial profiles
806 (n=1,166 sequences per sample) are shown at the phylum level. For clarity, only phyla
807 accounting for at least 2% of sequences are shown, with the less abundant phyla
808 grouped under `other bacteria'. For fungi (n=1,885 sequences per sample), only classes
809 accounting for at least 1% of sequences are shown, with remaining grouped under
810 'other fungi'. For algae (n=518 sequences per sample), phyla belonging to the
811 *Viridiplantae* are shown in green, and phyla belonging to the *Stramenopiles* are shown
812 in purple. Labels on the horizontal axis indicate the sample location within each Mn(II)
813 removal bed (inf=influent, mid=middle, eff=effluent, soil=uncontaminated soil collected
814 outside the bed), and for display, black lines separate the soil samples from the Mn(II)
815 removal bed samples.

816

817 Fig. 3. Correlation of bacterial, fungal and algal communities in Mn(II) removal beds.
818 Scatter plots show pairwise Bray-Curtis distances for all samples, excluding SRC1-soil
819 (15 samples in total, yielding 105 pairwise data points), with increasing distance
820 indicating less similar communities. Distances were calculated from OTU relative
821 abundance matrices based on a clustering threshold of 0.03. Correlation statistics for

each pair of targets (bacteria, fungi, algae) were calculated using Mantel tests with 999 permutations. Histograms on the diagonal show the distribution of pairwise Bray-Curtis distance values of each target group.

825 ## Tables

826 Table 1. Amplicon pyrosequencing output for the four target groups, before and after sequence
827 processing.

828

Target	No. raw seqs	No. per sample	No. passing QC	Percent passing QC	Final subsampled no. per sample	Mean final seq length (bp)
Algae	221,986	1,986 – 44,231	79,491	35.8	518*	209.5
Archaea	110,177	1,850 – 14,016	20,256	18.4	254	226.7
Bacteria	167,808	2,817 – 20,535	50,095	29.9	1,166	210.7
Fungi	372,851	6,833 – 123,772	78,602	21.1	1,885	163.9
Total	872,822		228,444	26.2		

829 * One algal sample, SRC1-soil, was removed from the data set due to the low number of sequences
830 passing QC

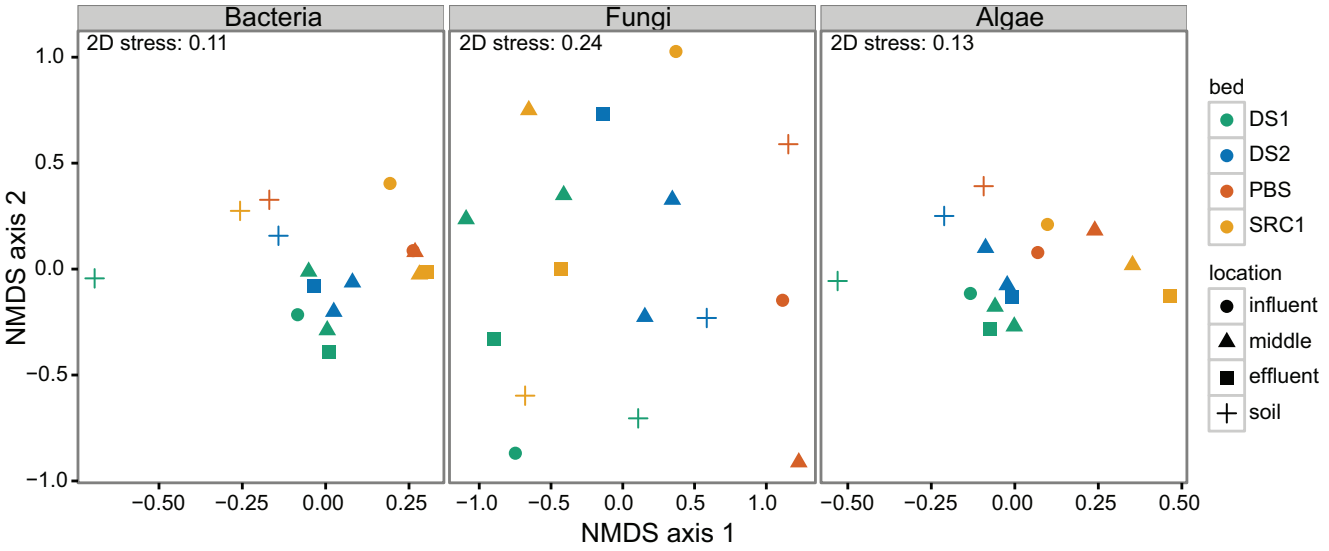

FIG 1

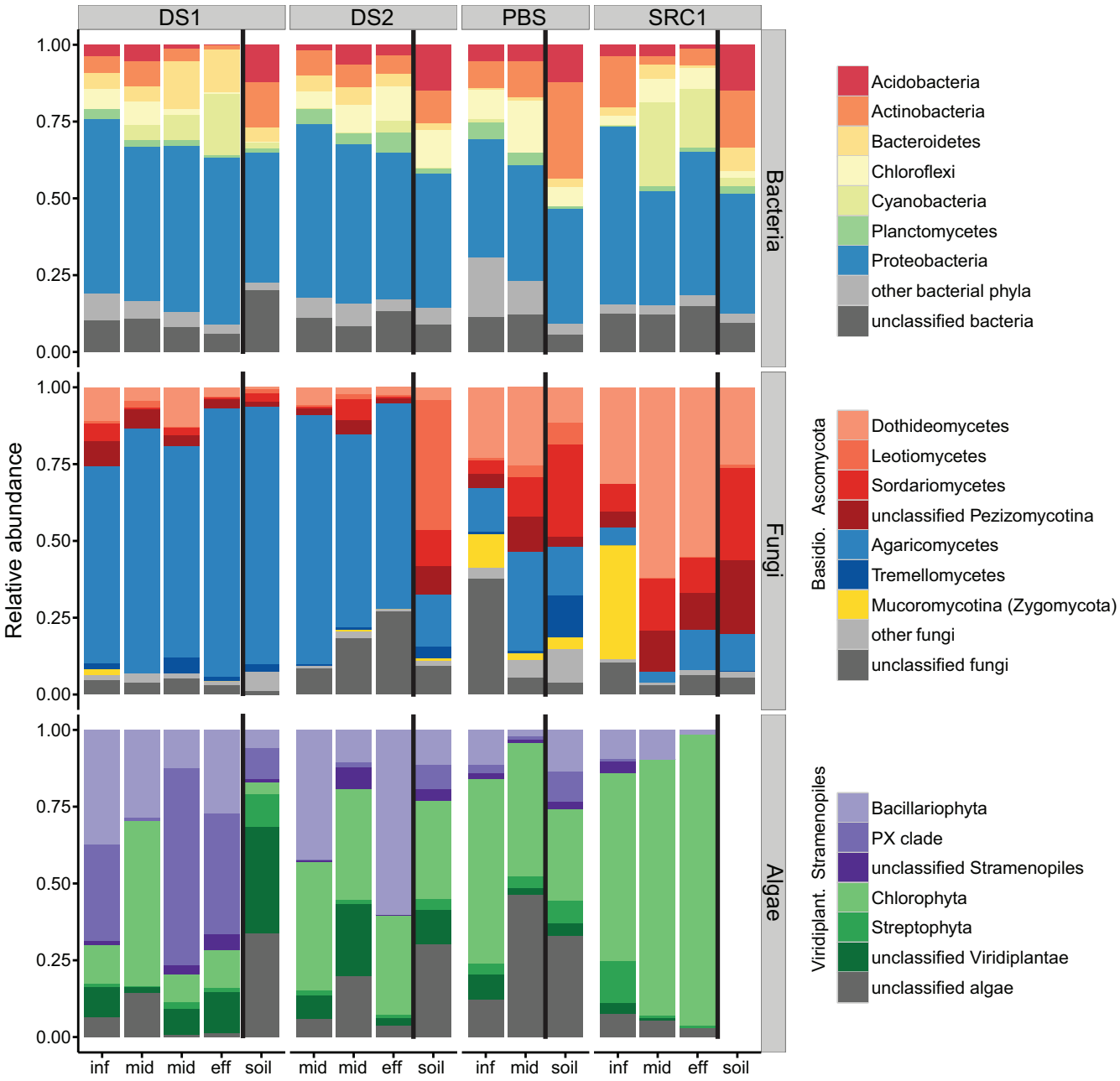

FIG 2

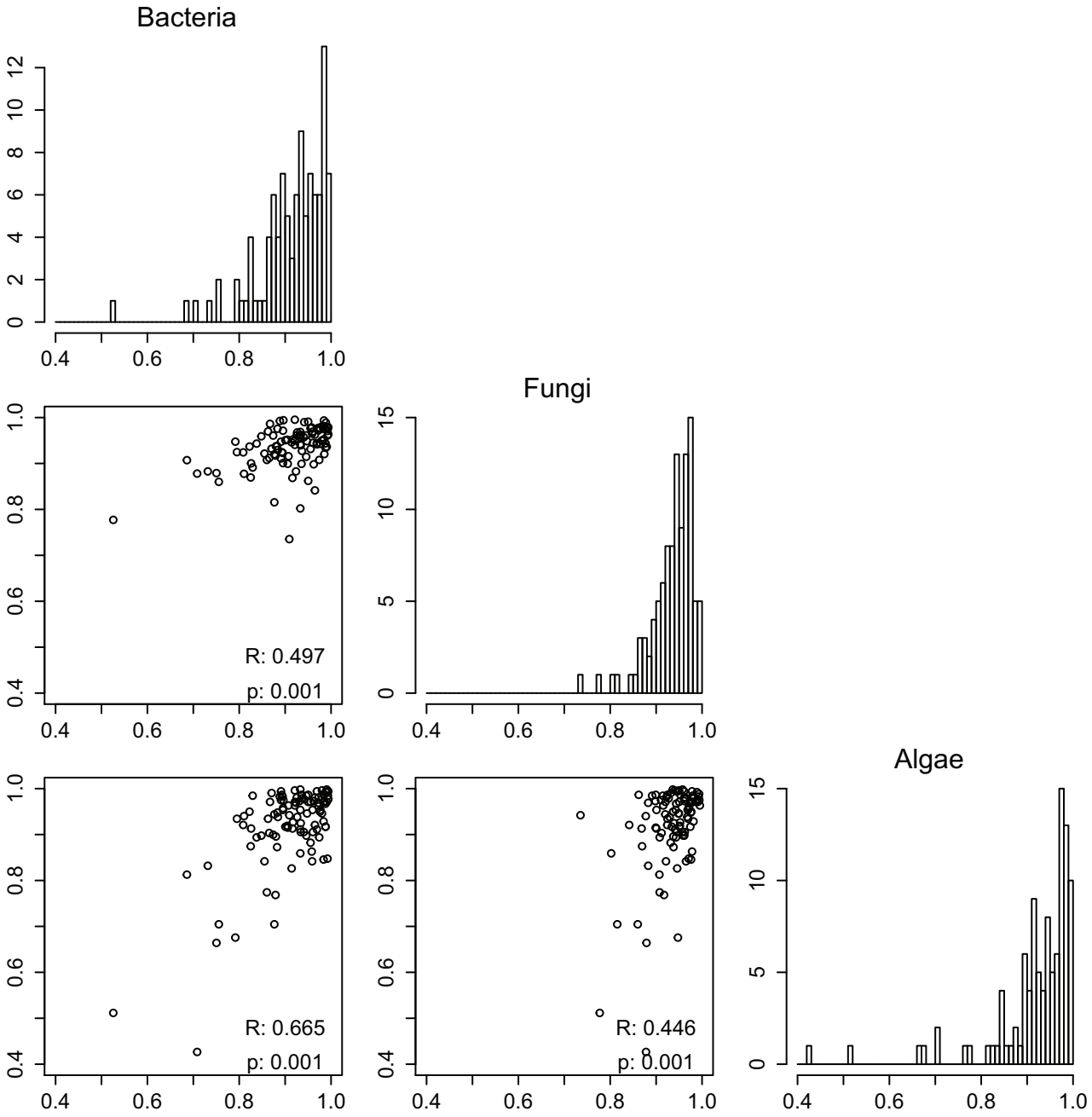

FIG 3