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Nutrient Input Influences Fungal Community Composition And Size And Can Stimulate Mn(II) Oxidation In Caves

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Abstract:
Little is known about the fungal role in biogeochemical cycling in oligotrophic ecosystems. This study compared fungal communities and assessed the role of exogenous carbon on microbial community structure and function in two southern Appalachian caves: an anthropogenically impacted cave and a near-pristine cave. Due to carbon input from shallow soils, the anthropogenically impacted cave had an order of magnitude greater fungal and bacterial quantitative-PCR gene copy numbers, had significantly greater community diversity and was dominated by Ascomycota fungal phylotypes common in early phase, labile organic matter decomposition. Fungal assemblages in the near-pristine cave samples were dominated by Basidiomycota typically found in deeper soils (and/or in late phase, recalcitrant organic matter decomposition), suggesting more oligotrophic conditions. In situ carbon and Mn(II) addition over 10 weeks resulted in growth of fungal mycelia followed by increased Mn(II) oxidation. A before/after comparison of the fungal communities indicated that this enrichment increased the quantity of fungal and bacterial cells yet decreased overall fungal diversity. Anthropogenic carbon sources can therefore dramatically influence the diversity and quantity of fungi, impact microbial community function, and stimulate Mn(II) oxidation, resulting in a cascade of changes that can strongly influence nutrient and trace element biogeochemical cycles in karst aquifers.

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Introduction

Pristine karst systems, including caves, are extremely nutrient limited and often contain levels of organic carbon that are three orders of magnitude below that of surface environments (Lavoie et al., 2010). Biogeochemical cycles in many of the deeper, actively forming, sulfur-rich hypogene cave ecosystems (characteristically deeper caves formed via sulfuric acid in ascending groundwater), are driven by chemolithoautotrophic communities dominated by Epsilonproteobacteria (see review(s) by Engel, 2007; Engel, 2010). In contrast, carbon cycles in shallow epigene cave systems (typically shallow caves formed via carbonic acid in descending meteoric water) primarily rely on nutrients that can enter the cave via hydrologic processes, as well as aerosols, plant roots, humans, bats, and other fauna (Culver and Pipan, 2009; Mulec et al., 2012). Karst systems also harbor some of the most fragile and diverse biological communities on Earth (Barton and Jurado, 2007), yet relatively little is known regarding the impact of terrestrial or anthropogenic nutrient input on microbial community structure and function in these unique ecosystems. More than 50 molecular studies have been conducted on bacteria in caves (Engel, 2010 and

references therein; De Leo et al., 2012; Herzog Velikonja et al., 2013; Tomova et al., 2013; Hathaway et al., 2014; Ortiz et al., 2014), compared to only five on fungi (Bastian et al., 2009; Bastian et al., 2010; Jurado et al., 2010; Adetutu et al., 2011; Vaughan et al., 2011). As fungi are the primary decomposers in soil ecosystems (O'Brien et al., 2005; Lindahl et al., 2007; Kellner and Vandenbol, 2010) they are a fundamental yet historically overlooked component of biogeochemical cycling in caves.

It can be difficult to visually assess microbial function. However, microbial Mn(II) oxidation, which produces dark brown or black cryptocrystalline material, is visible in the field and has been shown to develop in response to nutrient input in shallow epigene caves, forming a massive Mn-oxidizing biofilm (Carmichael et al., 2013a; Carmichael et al., 2013b). While input of organic acids common in sewage such as acetate, butyrate, and propionate (Cummings, 1981) encouraged growth of a bacterially-driven Mn-oxidizing community, the input of solid litter appeared to stimulate Mn(II) oxidation primarily by fungi (Carmichael et al., 2013b). This suggests that simple and complex sugars (particularly in solid form) support fungally-driven Mn(II) oxidation, whereas organic acids (particularly in aquatic seepage) support bacterially-driven Mn(II) oxidation (Fig. 1, Carmichael and Bräuer, in press). This study represents the first molecular and culture-based study that compares the fungal populations in two southern Appalachian epigene caves: a heavily impacted cave (Carter Saltpeter Cave in eastern Tennessee, USA, herein referred to as CSPC) versus a near-pristine cave (Daniel Boone Caverns in southwestern Virginia, USA, herein referred to as DBC). In addition, we explore the role of carbon enhancement on microbial populations and microbial Mn(II) oxidation in nutrient-limited environments via field manipulations where cave sites were amended with carbon-based media targeting fungal Mn(II) oxidizers. Moreover, this study (initiated in August 2012) represents one of the first applications of Illumina sequencing technology to examine fungal ITS gene assemblages in a field setting.

Results

Molecular analysis of fungal communities

Roughly 470,000 sequences remained after sequence assembly, clean-up, and clustering in MOTHUR and QIIME, representing approximately 7% of the original 7 million reads. Overall, 877 unique OTUs were identified across both caves. Sequences were generally evenly distributed across sites within each cave, with approximately 7,000 sequences per sample within DBC and approximately 19,000 sequences per sample within CSPC. Analysis of alpha diversity indicated that microbial diversity in DBC was significantly lower compared to CSPC across all metrics tested (Table S1). In three dimensional principal coordinates analysis (PCoA) based on abundance-weighted Jaccard analyses, samples within each cave clustered together and a clear separation between cave samples was observed (Fig. S1). Nucleotide amplicon data from both caves indicated that dominant phyla (using the UNITE reference database) included Zygomycota, Ascomycota, and Basidiomycota and that significant unidentified fungi were also present. OTUs at the phylum level within each cave were similar for both Zygomycota spp. (CSPC: 5.74 ± 1.54%, DBC: 3.58 ± 0.97%) and unidentified fungi (CSPC: 13.11 ± 2.40%, DBC: 11.34 ± 4.24%), but differed significantly for both Ascomycota spp. (CSPC: 57.94 ± 5.24%, DBC: 47.51 ± 5.94%) ($P = 0.002$) and Basidiomycota spp. (CSPC: 22.67 ±4.95%, DBC: 37.58 ± 3.54%) ($P = 0.003$) (Fig. 2). Interestingly, ectomycorrhizal

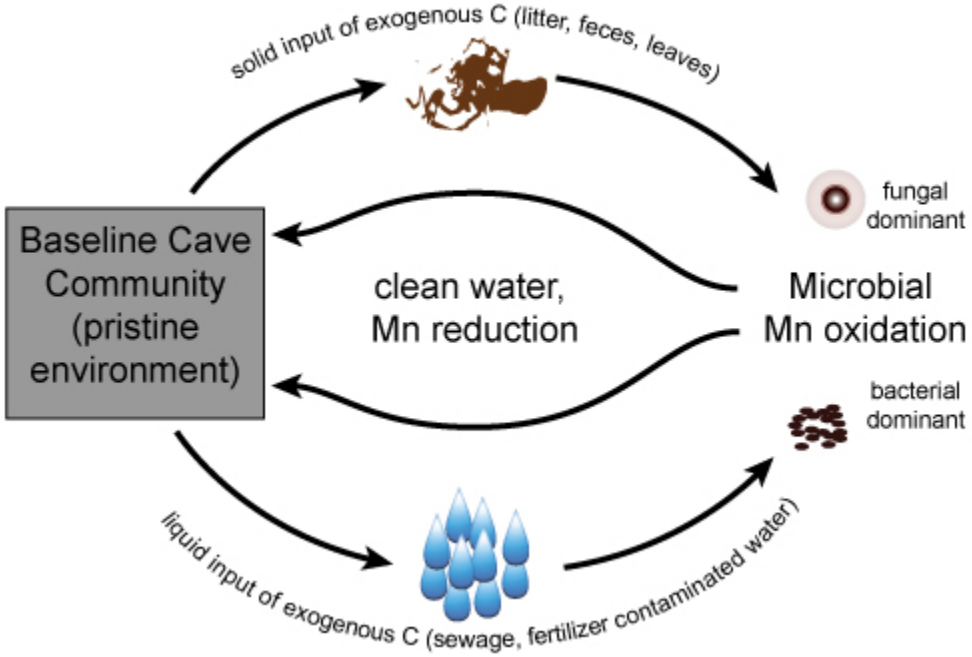

Fig.1 Hypothetical model predicting stimulation of Mn oxidation by exogenous carbon inputs in shallow, epigene cave environments and in cave environments susceptible to anthropogenic impact.

lineages within the Basidiomycota were particularly abundant in DBC, with 40% in the ectomycorrhizal-associated Agaricomycete class (96,915 out of 64,360 sequences), compared to only 12% in CSPC (20,550 out of 167,351 sequences).

The top seven OTUs for both DBC and CSPC were compared after sorting by total sequence count across all nine sites within each cave. Notably, the top seven OTUs within CSPC (Fig. S2) reflected approximately 30% of total sequences attained for all samples within CSPC, while the top seven OTUs within DBC reflected approximately 83%, suggesting greater species richness among CSPC than DBC, and providing support for the alpha diversity analysis (Table S1). Among the top OTUs, only Pseudogymnoascus verrucosus was shared between cave samples. Of the 13 most abundant families detected here, six were unidentified. For the remaining seven families in the top seven OTUs (Pseudeurotiaceae, Rhizopogonacea, Mortierellaceae, Psathyrellaceae, Dermateaceae, Trichocomaceae and Ganodermataceae), only Pseudeurotiaceae, Psathyrellaceae and Mortierellaceae have been detected in previous cave studies (Vanderwolf et al., 2013; Reynolds and Barton, 2014), highlighting the need for more molecular data. The data set was also evaluated for the presence of families detected across other cave studies that employed primarily culture-based techniques (Vanderwolf et al., 2013). All but three of the 16 total families reported by Vanderwolf et al. (2013) were present in DBC and CSPC, albeit at much lower relative abundances, generally by one to three orders of magnitude (Table S2). Interestingly, two families, Mortierellacea and Psathyrellaceae, were represented in higher relative abundances in our molecular data set compared to the primarily culture-based data set (Table S2).

Real-time qPCR of cave communities

Acceptable efficiencies were achieved for both bacteria (102-103%) and fungi (96-110%) with strong correlation coefficients between 0.96-0.99. The estimated number of fungi (calculated as ITS1 copy number per gram wet weight of soil) represented a low proportion of the overall community at each site, with fungal to bacterial gene copy ratios (per ng) of 0.004 +/- 0.027 in CSPC and 0.007 +/-0.069 in DBC. Bacterial gene quantification resulted in an estimated 3 x 108 - 2 x 1010 SSU rRNA gene copies per gram of soil, wet weight, within anthropogenically-impacted CSPC, whereas DBC was estimated to contain significantly (P = 0.007) fewer copies, ranging from 4 x 107 - 3 x 109 copies per gram wet weight of soil (Fig. 3). Quantification of fungal ITS amplicons within CSPC yielded an estimated 2 x 106 - 6 x 107 copies per gram wet weight of soil, while those within DBC were significantly lower (P = 0.012) at 2 x 104 - 9 x 106 copies per gram of soil, wet weight. Even with the lowest concentration sample NNDG removed, DBC still had significantly lower copy numbers of both fungal (P = 0.013) and bacterial (P = 0.008) genes.

Comparison of field-manipulated versus non-manipulated communities

Within two months, a glucose cast at the Terminus site (CSPC) was colonized by white fungal mycelia and then turned brown (Fig. 4). After nearly ten weeks of incubation, two additional locations in CSPC also demonstrated Mn(II) oxidation in response to field manipulation: SSSS showed a strong stimulation of Mn(II) oxidation while Mn Falls showed weak stimulation, according to empirical observations of LBB reactions of brown-black material on the casts (Fig. 4b and 4c). In all three sites, a net increase in bacterial abundance was observed (Fig. 5). Quantification of bacterial SSU rRNA gene copy number demonstrated an increase from an average of 6 x 109 copies to 2 x 1010 copies per gram of soil, wet weight.

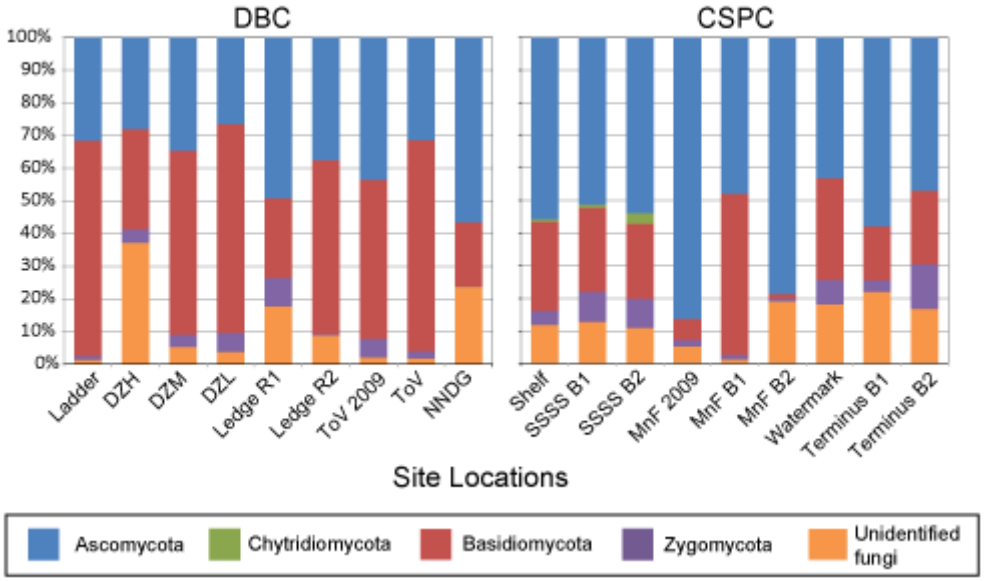

Fig.2. Phylum-level distribution of all sequences within the near-pristine cave DBC and the impacted cave CSPC (n = 9 for both Ascomycota and Basidiomycota). Sample names that are followed by a B1 or B2 indicate replicate samples taken before agar casts were added.

Fungal consortia changed more dramatically, at least among SSSS and Terminus, with an overall average increase from 2 x 107 copies in non-manipulated samples to 2 x 1010 copies in manipulated samples per gram of soil, wet weight. This enrichment in fungi over bacteria was also apparent in the fungal:bacterial ratios that increased from 0.002 to 0.513 at the SSSS site and from 0.003 to 0.007 at the Terminus site. In contrast, a net reduction in fungal gene copy number from 3 x 107 to 2 x 107 copies per gram of soil, wet weight was observed at the Mn Falls site between non-manipulated and manipulated samples, corresponding with a reduction in the ratio of fungi:bacteria from 0.009 to 0.004 for Mn Falls. This finding was not unexpected, as Mn Falls was previously found to contain blooms of bacteria that produced biogenic Mn oxides as a result of exogenous carbon input in the form of untreated sewage (Carmichael et al., 2013b).

Throughout CSPC, a strong shift in taxonomic distribution was observed among OTUs after field manipulations with significant changes in Ascomycota (56.07% ±4.77% decreasing to 27.50% ±10.68, P = 0.022) and Basidiomycota (23.17% ±6.35% decreasing to 2.82% ±1.18, P = 0.011), but a significant increase in Zygomycota (6.30% ±2.09% to 37.72% ±12.42, P = 0.031) (Fig. 6). In particular, sequences related to Mortierella spp., which only accounted for 1.9% of sequences before the field manipulation, represented the majority (35.0%) of sequences across all three sites after manipulation (Table S2). A significant (P < 0.05) decrease in Shannon and Simpson values was found for all sites (Table S3), although site SSSS also demonstrated a significant decrease in ACE and Chao1 values as well (P = 0.022 and 0.015, respectively). Further, PCoA using Bray-Curtis beta diversity analyses demonstrated a difference between microbial communities in the before and after samples at each of the three sites, although this difference was less pronounced for Mn Falls samples (Fig. S3).

Culturing of Mn-oxidizing fungi

All of the Mn(II)-oxidizing fungal isolates cultured from CSPC and DBC grouped within the phylum Ascomycota. Phylogenetic analysis of ITS1 sequences showed that three isolates clustered within Plectosphaerellaceae family of the Glomerellales order (Plectosphaerellaceae sp. DBCMVA, Acremonium sp. DCI1F and Acremonium sp. CSPCBetsoh) and three clustered within the Pleosporales order (Alternaria sp. DBC-AD, Pleosporales sp. MSW and Leptosphaerulina sp. DBCMVB; Fig. S4, S5 and Table S4). Additionally, three Mn(II)-oxidizing enrichment cultures were obtained for which a member of the Zygomycota phylum, Mortierella sp. (YECT1, YECT3, and Blackdot) was the dominant member (Fig. S4, S5 and Table S4).

Mn oxide characterization and distribution within cultures

Powder micro X-ray diffraction (μ-XRD) patterns of the Mn oxides coatings associated with selected fungal isolates (Mortierella sp. YECT1, Pleosporales sp. MSW, Alternaria sp. DBC-AD) indicated that the Mn oxides associated with these isolates are a combination of poorly crystalline Mn oxide phases with both layer and tunnel structures. μ-XRD data for Mn oxides associated with Pleosporales sp. MSW show broad peaks at 2.43 and 1.41 Å, indicating poorly crystalline buserite or vernadite (both

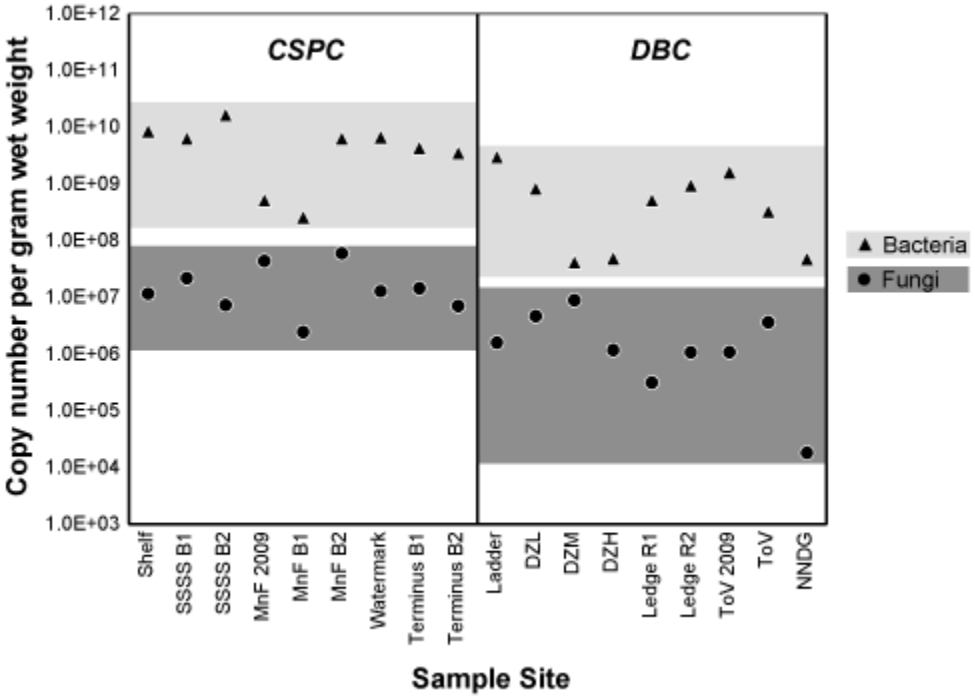

Fig. 3. qPCR-based gene copy number (n = 9) per gram wet weight of soil for the SSU rRNA gene for bacteria or the ITS1 region for fungi in Mn oxide rich sediment samples within Carter Saltpeter Cave and Daniel Boone Caverns.

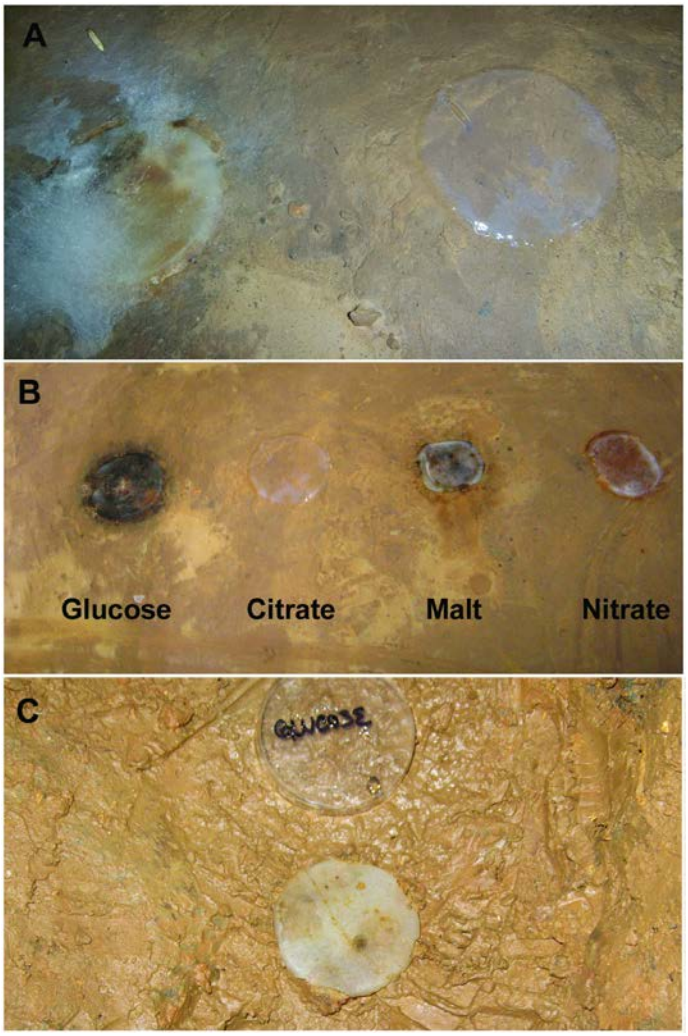

Fig. 4. Fungal colonization of media casts in situ. (A) Aerial hyphae colonizing glucose media in CSPC at the Terminus site, photo courtesy of Melanie Hoff. (B) In situ enrichment experiments at the Terminus site demonstrating significant stimulation of fungal Mn(II) oxidation by the agar cast made with glucose medium, mild stimulation by the cast made with malt medium, and no oxidation in response to agar casts made with citrate or nitrate media after the ca. ten week incubation. (C) A glucose medium agar cast demonstrating minimal stimulation of Mn(II) oxidation at the Mn Falls site.

layer structures) or todorokite (tunnel structure). Mn oxides associated with Alternaria sp. DBC-AD exhibited a sharp peak at 10.04Å and a smaller peak at 7.23Å, indicative of buserite (layer structure). Cultures of Mn oxides from Mortierella sp. YECT1 exhibited amorphous broad peaks at ~ 2.45, 2.03, and 1.41 Å, consistent with Mn oxide octahedral spacing in very poorly crystalline layer/tunnel structure combinations where individual mineral phases were not readily apparent from μ-XRD data alone. Most biological Mn oxidation is poorly crystalline (Tebo et al., 2004) and therefore the presence of poorly crystalline layer and tunnel structures such as buserite and todorokite in fungi are consistent with results from more detailed synchrotron-based X-ray methods such as X-ray absorption near edge structure (XANES) and extended X-ray absorption fine structure (EXAFS) (Miyata et al., 2006a; Petkov et al., 2009; Saratovsky et al., 2009; Grangeon et al., 2010; Santelli et al., 2011).

Electron microscopy data (Fig. S7) of Mortierella sp. YECT1, Leptosphaerulina sp. DBCMVB, and Plectosphaerellaceae sp. DBCMVA shows a variety of morphologies and Mn oxidation distribution. Leptosphaerulina sp. DBCMVB (Fig. S7a) exhibits fruiting bodies with Mn oxidation (not visible in image) at the base of the stalks. Plectosphaerellaceae sp. DBCMVA (Fig. S7b) shows enhanced Mn oxidation at the tips of spores compared to the spore interiors. SEM and TEM images of Mortierella sp. YECT1 (Fig. S7c, Fig. S7d) show enhanced Mn oxidation within the septa and junctions of hyphae.

Discussion

Fungal species composition

Molecular work using denaturing gradient gel electrophoresis has suggested that caves may harbor fewer fungal taxa per gram than top soils (Vaughan et al., 2011), but little is known regarding the abundance, function, or composition of fungal taxa in caves (Bastian et al., 2009). Current knowledge is primarily derived from culture-based studies, and as such is inherently biased toward Ascomycota (see review by Vanderwolf et al., 2013). Further, culture-based analyses are known to reveal as little as 0.6% (Hibbett et al., 2009) to less than 8% of the total fungal population (Hawksworth, 2001) in a particular environment. In the absence of sufficient molecular data from caves to which to draw direct comparisons, indirect comparisons can be made to studies of soil communities, which are relevant to cave systems in the southern Appalachians due to speleogenesis by descending percolation of groundwater and soil water. Previous work in forest soils has determined that microbial assemblages change (Baldrian et al., 2012) and become less diverse (Jumpponen et al., 2010) with soil depth and that Ascomycota dominate the upper-most (0.5-1 cm) litter layers whereas ectomycorrhizal Basidiomycota, including agarics, are more abundant in the deeper soil layers (Buée et al., 2009; Voříšková et al., 2014). Similarly, other researchers have determined that Ascomycota flourish in response to straw additions to soil (Ma et al., 2013), during the early phase of litter decomposition in soils (Kubartová et al., 2009; Voříšková and Baldrian, 2013), or on living leaves of Quercus macrocarpa (Jumpponen and Jones, 2009). Because the fungal community in the near-pristine DBC was dominated (84%) by Basidiomycota (particularly Agaricomycetes), it more closely mirrored fungal communities that are typical of deeper soil layers. This is not unexpected, as plant roots have been found to reach 20 m depths (McElrone et al., 2004) and root-associated fungi would certainly be expected to extend into the depths of the shallow (less than 32-55 m) caves assessed here. Supporting this interpretation, Coprinellus micaceus (representing 42% of the sequences) was the most

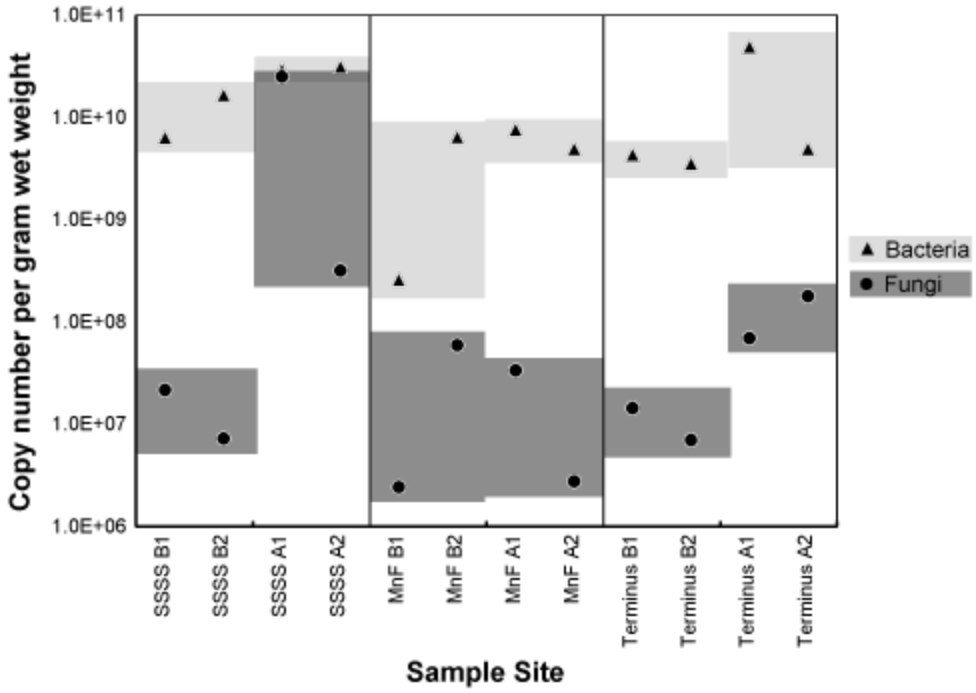

Fig. 5. qPCR-based gene copy number per gram wet weight of soil for the SSU rRNA gene for bacteria or the ITS1 region for fungi in Mn oxide rich sediment samples within Carter Saltpeter Cave taken before (B1, B2) and after (A1, A2) the ca. ten week carbon enrichment.

abundant OTU in DBC and is most commonly observed as a saprophytic fungus involved in the late phase decomposition of leaves, wood and dung (i.e., less labile organic matter sources), but has also been associated with tree roots (González, 2008 and references therein). The high abundance of Coprinellus micaceus in DBC is therefore likely a reflection of the highly limited nutrient input expected for a near-pristine cave.

In contrast, primarily saprophytic Ascomycota lineages (ca. 58%) dominated the fungal community in CSPC (the impacted cave). These lineages, including Geomyces, Dactylella, and Pseudogymnoascus, more closely approximate those in nutrient-rich litter layer of soils, suggesting that CSPC may either be receiving significant amounts of exogenous carbon, or that communities dominated by these lineages are particularly vulnerable to nutrient input. Similarly, a molecular mycological study of Lascaux Cave in France (a UNESCO World Heritage site and European tourist cave that is heavily impacted with hundreds of thousands of visitors per year), detected almost exclusively Ascomycota, suggesting that the proportion of this phylum may increase with degree of impact and nutrient input (Martin-Sanchez et al., 2012). Also of relevance, a recent study by Chaput et al. (2015) demonstrated that Ascoymcete fungi were more prevalent than Basidiomycota (largely Agaricomycetes) in passive remediation systems that were most effective in treating high concentrations of dissolved Mn emanating from abandoned coal mines. Although the Chaput et al. (2015) study did not specifically link the dominance of Ascomycota to systems with more labile organic sources, both studies suggest a possible link between the carbon and manganese biogeochemical cycles.

In addition to the Ascomycota:Basidiomycota relationship, a unique characteristic of CSPC when compared to DBC is the greater incidence of Chytridiomycota species Spizellomyces acuminatus and Powellomyces sp. Chytrids are common in aquatic ecosystems (Gleason et al., 2008), and the greater hydrologic input into CSPC compared to DBC may explain the presence of this small but significant population (0.7% of sequences from three samples in CSPC). Interestingly, one dominant OTU that was found in both caves, Pseudogymnoascus verrucosus, clusters with other Pseudogymnoascus species that include the causative agent of white nose syndrome in bats, Pseudogymnoascus destructans (Minnis and Lindner, 2013). Pseudogymnoascus verrucosus has not been linked to white nose syndrome, however. Members of this genus have been cultured from caves and show saprotrophic growth properties (Reynolds and Barton, 2014).

Fungal diversity within the impacted versus pristine cave

There is conflicting information on the effect of anthropogenic impact on microbial or fungal diversity in caves. Several studies have reported greater numbers of taxa in human-impacted caves (Mosca and Campanino, 1962; Somavilla et al., 1978; Vaughan-Martini et al., 2000), whereas others have detected lower diversity (Shapiro and Pringle, 2010). At least part of this discrepancy may be due to differences in culturing vs. molecular analyses, since one study found that human visitation in Australian caves

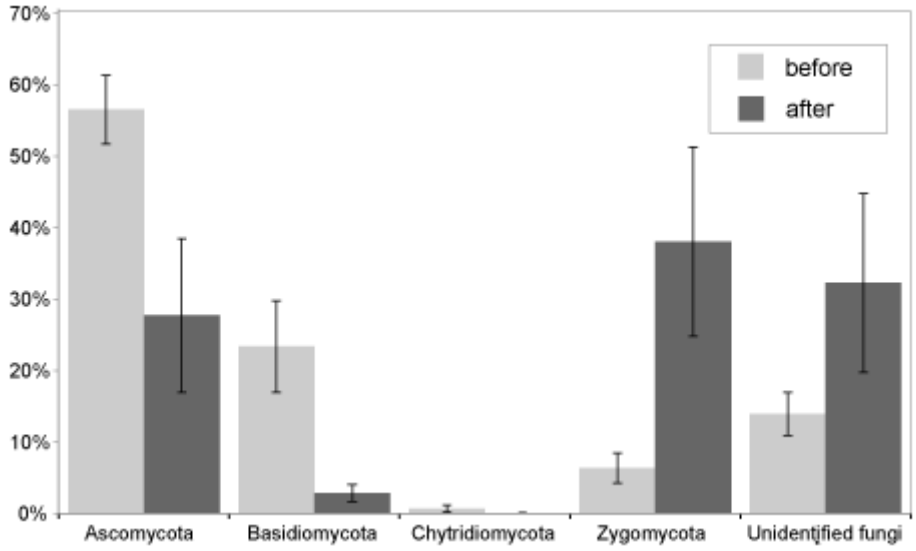

Fig. 6. Phylum-level distribution of sequences from CSPC (before) and enriched (after) communities. Error bars represent standard error and asterisks denote statistically significant ($P < 0.05$) differences according to a t-test.

resulted in a decrease in fungal diversity using culture-based methods, but an increase in fungal diversity using molecular-based methods (Adetutu et al., 2011). In this current study, molecular analyses also demonstrated significantly greater fungal diversity in the impacted cave samples compared to the near-pristine samples (Table S1), corroborating the majority of studies.

Quantification of microbial groups in the impacted and near-pristine cave

Although qPCR does not provide an estimate of biomass, it does allow researchers to compare relative gene copy numbers across multiple studies or across multiple sites or samples. Since there are no quantification data for fungi in caves to which to draw direct comparisons, indirect comparisons can be made to soil studies: qPCR has been used to estimate fungal:bacterial ratios of up to 3.5 for nutrient-rich forest soil (Fierer et al., 2005), ~0.3-1.0 for barren glacial soil (Brown and Jumpponen, 2014), or ~0.2-0.5 for desert and prairie soil (Fierer et al., 2005). Higher qPCR-based fungal:bacterial ratios of ~1 have been found for the top, litter-rich forest soil layers compared to the lower soil layers (below 1-4 cm) where fungal:bacterial ratios were ~0.30 (Baldrian et al., 2012). Extrapolating from results in the literature, the combined findings suggest that fungal:bacterial ratios may continue to decline with depth in soils, as the fungal:bacterial ratios for the 30 m deep cave sediments were 0.004 +/- 0.027 in CSPC and 0.007 +/- 0.069 in DBC (calculated based on raw copy number per nanogram of DNA). This is roughly an order of magnitude below even the lowest reported values of 0.03-0.08 for cultivated, pasture, hardwood, and pine-stand soil communities (Lauber et al., 2008). The lower values measured in this study are likely due to carbon limitation in caves (Barton and Jurado, 2007; Lavoie et al., 2010), since fungi are heterotrophic and rely on the presence of carbon substrates for growth. However, it is also important to note that abundance within an environment does not necessarily correlate with environmental relevance, as rare taxa, such as fungi, represent an important component of the functional diversity of an ecosystem (Curtis and Sloan, 2005; Sogin et al., 2006; Huber et al., 2007).

Our finding of higher cell abundance in samples within CSPC is consistent with field studies investigating nutrient loading in caves via tourism (Ikner et al., 2007), septic effluent (Simon and Buikema, 1997; Carmichael et al., 2013b), and input of carbon from guano, feces, and human traffic (Mulec et al., 2012). In the aforementioned studies, cultivable counts (determined by colony forming units) of total aerobic or fecal coliform bacteria were at least two orders of magnitude higher in high-impact compared to low-impact zones within the caves. Similarly, one study found increased microbial abundance from below detection to greater than 104 cells g-1 of sediment in response to urine (Johnston et al., 2012). Additionally, human presence has been strongly correlated with fungal quantity in caves in Spain (Docampo et al., 2010), China (Wang et al., 2010) and Russia (Kuzmina et al., 2012). Thus, our study lends support to a growing body of literature indicating that exogenous carbon can significantly impact total microbial biomass.

Population changes in response to field manipulations

In the current study, placement of glucose-containing agar-media casts resulted in an increase in total fungal and bacterial counts by roughly an order of magnitude over the 10 week timeframe. Corroborating our findings, experimental manipulations in Wind Cave, SD (USA), revealed an increase in bacterial biomass within plots fertilized by feces or a combination of clothing lint and feces, relative to plots fertilized with lint alone or control plots (Chelius et al., 2009). Similarly, laboratory manipulations of cave sediments from Lechuguilla Cave, NM (USA), resulted in increases in viable counts of fungal and bacterial cells by two to three orders of magnitude (Northup et al., 2000) and the addition of an allochthonous

carbon source in the form of human vomit in Castañar de Ibor Cave (Spain) resulted in the massive overgrowth of white fungal mycelia (Jurado et al., 2010).

The reduction in species richness/diversity in the enriched fungal communities (Table S3) may partially result from the simple substrate used (glucose) as well as the relatively short incubation time. In contrast, application of biocide treatments in Lascaux Cave resulted in an increase in overall fungal diversity—likely due to both the greater complexity of carbon substrates available from detrital biomass resulting from the biocide, as well as the longer timeframe of the study, 6 and 25 months after pesticide application (Martin-Sanchez et al., 2012).

Field and laboratory enrichment and culturing of Mn(II) oxidizing fungi

Although we expected changes in fungal assemblages in response to carbon input in the environment to provide clues regarding which organisms may be oxidizing Mn(II), groups known to contain Mn(II) oxidizers (such as Ascomycota and Basidiomycota) became less abundant relative to Zygomycota (members of which are not known to oxidize Mn). Given the timeframe and sugars used, it is not surprising that fast-growing saprophytic zygomycetous Mortierella spp. were enriched in the glucose-driven Mn(II)-oxidizing communities at all three sites (Table S5). Mortierella spp. favor simple sugars (Dyal and Narine, 2005) and are ubiquitious organisms that are readily isolated from soil (Streekstra, 1997 and references therein; Nagy et al., 2011) and caves (Mosca and Campanino, 1962; Vanderwolf et al., 2013). Although Mortierella spp. were also the dominant members in several Mn(II)-oxidizing enrichment cultures, and electron microscopy shows Mn enrichment within the septa and branching junctions of hyphae (Fig. S7c, S7d), the full contribution of Mortierella as an oxidizing member is obscured by its inconsistent and/or slow observed rates of Mn(II) oxidation compared to other cultures, and its presence in a mixed culture rather than an axenic one.

Aside from the presence of Mortierella spp., two of the six isolates obtained here are less than 90% identical to cultured strains across ~600 bp of the ITS sequences. These strains could only be identified down to either order (Pleosporales) or family (Plectosporallaceae), highlighting the unique diversity of cave microbial communities. The remaining four strains are approximately 98-99% identical to other cultured strains (across >487 bp regions of the ITS). Two of these share high identity to cultured Mn(II)-oxidizing strains from an acid mine drainage site (Santelli et al., 2010), and two are nearly identical to Acremonium napalense strains from Lascaux Cave (Martin-Sanchez et al., 2012), the Mn(II) oxidizing fungus later found in black coatings on cave sediment surfaces (Saiz-Jimenez et al., 2012). Notably, more than half (five out of the nine) cultured strains are at least 97% identical to sequences retrieved from molecular analyses indicating that these organisms are environmentally relevant; however all of these organisms were found in low abundance, highlighting the limitations of current culturing techniques.

The role of fungi in cave biogeochemical cycles

Biogeochemical cycling within microbial communities responsible for the formation of cave Mn oxide deposits is still poorly understood, especially for fungi. Though fungi regularly mediate key biogeochemical transformations within the environment (Sterflinger, 2000; Fomina et al., 2005; Dupont et al., 2007), the extent to which fungi may be involved in the formation of ferromanganese deposits (Taylor-George et al., 1983; Grote and Krumbein, 1992; Parchert et al., 2012) is the subject of long-standing debate (Schelble et al., 2005), at least in regard to Mn-oxide deposits (varnish) on desert rocks. The first detailed study of cave ferromanganese deposits suggested the occurrence of tightly coupled fungal-bacterial interactions whereby chemolithoautotrophic bacteria provide the organic base to support heterotrophic bacteria and fungi (Cunningham et al., 1995). Later studies of cave ferromanganese deposits either did not identify fungal participation in Mn(II) oxidation (Spilde et al., 2005), or only mentioned it briefly without a detailed molecular analysis of the fungal community structure (Saiz-Jimenez et al., 2012; Carmichael et al., 2013b). The importance of Mn(II)-oxidizing fungi as constituents of black coatings has recently been highlighted in studies of acid mine drainage remediation (Santelli et al., 2010; Chaput et al., 2015), freshwater pebbles (Miyata et al., 2006b; Grangeon et al., 2010), pond sediments (Santelli et al., 2014), desert varnish (Parchert et al., 2012) and in Lascaux cave (Saiz-Jimenez et al., 2012). While all of the Mn(II) oxidizers discovered to date are heterotrophic, the function of Mn(II) oxidation has remained enigmatic for decades. For example, researchers have speculated that Mn oxidation may be an energy yielding reaction (chemolithoautotrophic), or may serve to protect cells against UV radiation or oxidative stress (Tebo et al., 2005). Our results suggest that, at least in the caves studied here, Mn(II) oxidation is carbon-limited and the process appears to be dominated by heterotrophic organisms. This hypothesis is supported by recent work in the Madison Blue Spring cave system in Florida (USA) showing that Mn(II) oxidation in carbonate aquifers was directly due to an influx of reducing water containing dissolved organic carbon (DOC) and nitrate into an oxidized cave environment during flood events (Brown et al., 2014).

Conclusions

Whereas previous studies have demonstrated pH dependence of both fungal (Miyata et al., 2004) and bacterial (Zhang et al., 2002; Akob et al., 2014) Mn(II) oxidation, our current study highlights the importance of the availability of carbon and other nutrients as important drivers of Mn(II) oxidation in environments such as caves. In addition, we show that microbial community composition and abundance can be intricately linked with the quality, quantity and form of the carbon input. Our results further indicate that input of exogenous, anthropogenic carbon into oligotrophic cave ecosystems causes a cascade of changes that not only impact the function and physiology of microbial population dynamics but also strongly influence biogeochemical cycles. These changes are not restricted to the cave itself, but potentially can also influence both trace

element and nutrient cycling in the karst aquifers that host these cave systems.

Experimental Procedures

Site Descriptions and Sample Collection

Two epigenic caves in the upper Tennessee River Basin were selected for this study based on their similar geology and contrasting nutrient input. Both caves are located within the Ordovician Knox Dolomite Group, and experience nearly identical climate and rainfall patterns. The anthropogenically impacted study sites were located within CSPC (Carter Saltpeter Cave, Carter County, TN, USA; Fig. S6). CSPC is an epigenic cave system typical of the southern Appalachian region (approximately 30 m deep), and evidence of anthropogenic impact is widespread throughout the cave in the form of trash, sewage, and debris (Carmichael et al., 2013b). Additionally, there are many active seeps within CSPC and the cave is highly susceptible to flooding. The near-pristine study sites were located within DBC (Daniel Boone Caverns, Scott County, VA, USA; Fig. S6). DBC is a gated and rarely-visited cave where access is controlled by the landowner. Reaching 55 m depth, it is located in an isolated forest on the top of a ridge, and therefore is neither subject to flooding nor agricultural or municipal runoff. It contains several pools and drip networks, but does not appear to be part of an extensive subsurface hydrologic system. DBC has shown no geochemical evidence of anthropogenic impact nor of elevated nutrient levels (Carmichael et al., 2013b), thereby justifying its use as a pristine analogue of CSPC.

Sample substrates within CSPC and DBC testing positive for Mn(III/IV) oxides using Leucoberbelin blue indicator dye (Tebo et al., 2007) were collected, stored on ice and transported to the laboratory for processing within 2.5 hours. Samples were partitioned for culturing efforts or for DNA extraction following the manufacturer's protocols using the FastDNATM SPIN Kit for Soil (MP Biomedicals, LLC. Solon, OH. USA). DNA was stored at -20°C for subsequent usage.

Amplification and sequencing

The ITS1F primer (Gardes and Bruns, 1993) was modified with the addition of: 1) the reverse complement of the Illumina adapter 5'CAAGCAGAAGACGG CATACGAGAT), 2) a twelve base pair unique Golay barcode distinguishing sample site, 3) reverse primer pad of 5'AGTCAGTCAG, and 5'CC linker, all attached to the 5' end of ITS1F as described in the literature (Caporaso et al., 2011; Caporaso et al., 2012). Similarly, the 5.18R primer (Vilgalys and Hester, 1990) was modified with the addition of: 1) the Illumina adapter 5'AATGATACGGCCA CCACCAGATTACAC, 2) forward primer pad 5'TATGGT AATT, 3) primer linker 5'GT, all attached to the 5' end of the 5.8SR. Triplicate amplifications were performed in 25 µL reactions consisting of 12.5 µL of Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Inc. Ipswich, MA, USA), 2.5 µL of modified forward and reverse primers, 0.25 µL of BSA (New England Biolabs, Inc. Ipswich, MA, USA), 6.75 µL nuclease free water, and 1 µL of DNA. Thermocycling reactions included 95°C for 10 minutes, followed by 10 touchdown cycles at 94°C for 45 seconds, 68°C to 58°C (decrease of 1°C per cycle) for 45 seconds, followed by extension at 72°C for 75 seconds. An additional 27 cycles (or 29 cycles for sites with low yield) followed with 95°C for 45 seconds, 58°C for 45 seconds and a final additional extension of 72°C for 10 minutes. PCR products from all reactions were purified, pooled and shipped to West Virginia University's Genomics Core Facility (Morgantown, WV, USA) for paired-end sequencing on a MiSeq sequencer (Illumina, Inc., San Diego, CA, USA).

Data processing

MOTHUR was used to assemble and trim contigs after discarding sequences containing nucleotide ambiguities and regions with >11 homopolymers (Schloss et al., 2009). Chimeric sequences were identified de novo within QIIME using USEARCH6.1 and subsequently removed from the working data set (Caporaso et al., 2010; Edgar et al., 2011). Operational taxonomic units (OTUs) were clustered de novo within QIIME using UCLUST at a 97% similarity (Edgar, 2010). Classification of OTUs employed the BLAST algorithm through QIIME against the publicly available UNITE reference database (Version No. 6, Release date 2014-01-15, Kõljalg et al., 2005; Abarenkov et al., 2010). All other additional diversity analyses were performed within QIIME version 1.7.0 under nonphylogentic constraints (Caporaso et al., 2010). Results were evaluated for statistical significance (α=0.05) using a Student's t-test in Microsoft Excel. Sequences were deposited into the European Nucleotide Archive under project identification number PRJEB6581 with accession numbers ERS483455-ERS483478 for the 24 individual sample libraries. Additionally, eleven representative sequences with high identity to cultured strains in this study were deposited into GenBank with accession numbers KJ867237-KJ867247.

Real-time quantitative PCR

The primers 338F (Nadkarni et al., 2002) and 518R (Einen et al., 2008) were used to target bacteria (Johnson et al., 2012), and ITS1F and 5.8SR were used for fungi (Fierer et al., 2005). Standard curves were generated from linearized plasmid DNA containing either the Rhodobacter sp. CR07-74 (Bräuer et al., 2011) SSU rRNA gene sequence (range of 102–109 target copies/µL); or the Saccharomyces cerevisiae (range of 102-108 target copies/µL) ITS sequence. Triplicate samples were amplified as described previously for bacterial amplicons (Carmichael et al., 2013a). Thermocycling was performed according to Fierer et al. (2005) for fungi and according to Johnson et al. (2012) for bacteria. Results were evaluated for statistical significance (α=0.05) using a Student's t-test in Microsoft Excel.

All experimental procedures for culturing, X-ray diffraction of Mn oxides, and electron microscopy are located in the Supporting Information.

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Supporting Information: Experimental Procedures

Enrichment and culturing

Agar media was removed from 10 cm plates and the resulting casts were applied to approximately six locations within CSPC on September 6th, 2012, and to five locations within DBC on October 14th, 2012 (Fig. 4). Each location received a single set of agar casts consisting of one or more of the following media types, depending on the amount of space available: 1) glucose medium: 40 g L^{-1} dextrose, 10 g L^{-1} peptone, and 20 g L^{-1} agar; 2) AY medium (Santelli *et al.*, 2010); 3) malt medium: 20 g L^{-1} malt extract and 20 g L^{-1} agarose; 4) nitrate medium: 5 g L^{-1} peptone, 3 g L^{-1} meat extract, 1 g L^{-1} KNO_3, and 18 g L^{-1} agar; 5) citrate medium: 2.5 g L^{-1} NaCl, 2 g L^{-1} sodium citrate, 1 g L^{-1} ammonium sulfate, and 15 g L^{-1} agar. All media were supplemented with the following (filter sterilized and added after autoclaving): 100 μM $MnCl_2$ and 1 mL L^{-1} trace elements (10 mg L^{-1} $CuSO_4 \cdot 5H_2O$, 44 mg L^{-1} $ZnSO_4 \cdot 7H_2O$, 20 mg L^{-1} $CoCl_2 \cdot 6H_2O$, 13 mg L^{-1} $Na_2MoO_4 \cdot 2H_2O$) (Santelli *et al.*, 2011). On November 15th, 2012, after ca. ten weeks of incubation, duplicate samples were collected from the agar-sediment edge for DNA and sediment was partitioned for culturing from three field sites in CSPC that demonstrated Mn(III/IV) oxide production: Terminus, SSSS and Mn Falls. Mixed cultures that were positive for Mn(III/IV) oxide production using LBB were sub-cultured until deemed axenic via microscopy and sequencing. Six unique axenic cultures were obtained (Table S4, Fig. S4, S5); cultures containing *Mortierella* spp. cultures, however, would not grow and oxidize Mn axenically so were studied as a mixed culture (members of the phylum Zygomycota such as *Mortierella* spp. have not previously been reported to oxidize Mn(II) and were saved for further study). Bacterial SSU rRNA genes were amplified with the primers 8F/1492R (Lane, 1991). Fungal SSU rRNA genes, ITS regions and LSU rRNA genes were sequenced for each culture using the following primers: NS1/NS302 and NS3/NS5 (Takano *et al.*, 2006), ITS1F/ITS4 (O'Brien *et al.*, 2005), and LR0R/LR5-F (Tedersoo *et al.*, 2008), respectively. Sequences were assembled in Sequencher v. 5.2.4, Build 13548. Alignments and maximum-likelihood analyses were constructed in ARB (Ludwig *et al.*, 2004), and sequences were deposited in GenBank with accession numbers KJ867210-KJ867236.

Mn oxide characterization

Mn oxide crystal structures were determined using Fourier Transform Infrared Spectroscopy (FT-IR) and powder micro-XRD (μ-XRD) analysis. For μ-XRD analysis, both cultured samples and Mn oxide coatings scraped directly from substrates were analyzed using a Rigaku D/Max Rapid micro-X-ray diffractometer at the Smithsonian National Museum of Natural History.

Electron microscopy

Mn-oxidizing cultures were examined using a combination of scanning and transmission electron microscopy with energy dispersive X-ray spectrometry (SEM-EDS, TEM-EDS) in the Dewel Microscopy Facility at Appalachian State University. Selected samples for SEM-EDS were grown on 5 mm diameter slices of polycarbonate 0.45 μm filters. Filter slices were then dehydrated by soaking in a series of 50%, 75%, and 85% ethanol-water solutions, followed by soaking two times in 100% ethanol, for a minimum of two hours in each solution. Samples were then critically point dried using liquid CO_2 in a Polaron critical point dryer, were mounted on stubs with carbon tape, and gold coated. Other samples were mounted directly from the culture plate onto carbon tape and analyzed under low vacuum, environmental scanning electron

microscopy (ESEM) conditions. All samples were run between 15 and 20 keV with a 5 µm beam spot size. SEM-EDS and ESEM imaging and analysis were completed with a FEI Quanta 200 Environmental SEM with EDAX EDS detector. TEM-EDS samples were mounted on 100 mesh Carbon Type B formvar-coated copper 200 mesh grids (Ted Pella, Inc.), air dried, carbon coated, and analyzed using a JEOL JEM-1400 TEM with a G-135 Pentafet Sealed Window X-ray detector.

Supporting Information: Tables and Figures

Table S1. Alpha diversity estimates between CSPC and DBC across ACE, Chao1, Simpson, Shannon, and Observed Species metrics. Average values for each cave as well as and *P*-value results from a t-test comparison across caves is shown for each metric. Significant *P*-values are denoted by an asterisk (*).

Location	ACE	Chao1	Simpson	Shannon	Observed Species
CSPC					
Shelf	479.63	467.96	0.95	5.74	444
SSSS B1	524.04	521.28	0.97	6.54	482
SSSS B2	520.65	525.93	0.96	5.87	456
MnF 2009	104.56	102.33	0.55	2.72	61
MnF B1	54.74	49.00	0.70	2.57	44
MnF B2	145.03	130.25	0.84	3.13	107
Watermark	214.65	211.26	0.97	5.94	196
Terminus B1	205.46	203.25	0.94	5.02	186
Terminus B2	302.50	278.44	0.93	5.20	244
Average	*283.47*	*276.63*	*0.87*	*4.75*	*246*
DBC					
Ladder	81.24	71.00	0.55	1.93	61
DZL	101.07	95.00	0.61	2.48	72
DZM	203.44	120.50	0.90	4.22	43
DZT	172.04	122.60	0.81	3.43	56
Ledge R1	95.44	104.00	0.92	4.33	70
Ledge R2	153.99	133.50	0.79	3.42	102
ToV 2009	135.47	98.17	0.63	1.84	44
ToV	75.58	65.60	0.69	2.48	35
NNDG	81.47	78.27	0.79	2.74	51
Average	*122.19*	*98.74*	*0.74*	*2.99*	*59*
***P*-value**	**0.015***	**0.010***	**0.040***	**0.005***	**0.006***

Table S2. Relative percentages of top fungi reported in caves. The abundances of the most frequently isolated fungi, adapted from Vanderwolf et al (2013), were calculated as a percentage of the 1,813 isolates reported from the review (excluding the slime mold group Dictyosteliaceae). Also shown are the corresponding Illumina-based percentages averaged across the eighteen baseline samples taken within the CSPC and DBC cave systems studied here. Not detected is indicated by ND.

Family	Abundances as reviewed by Vanderwolf et al., 2013	Illumina-based estimates of abundances in CSPC and DBC
Trichocomaceae	34.197%	7.529%
Mucoraceae	8.163%	0.029%
Nectriaceae	5.847%	0.027%
Laboulbeniaceae	5.792%	ND
Pleosporaceae	4.744%	0.409%
Microascaceae	4.247%	0.004%
Hypocreaceae	4.247%	0.013%
Cordycipitaceae	4.192%	ND
Chaetomiaceae	3.971%	0.118%
Bionectriaceae	3.861%	0.009%
Davidiellaceae	3.530%	0.011%
Myxotrichaceae	2.758%	1.161%
Polyporaceae	2.758%	0.041%
Arthrodermataceae	2.648%	0.038%
Mortierellaceae	2.427%	5.213%
Clavicipitaceae	1.820%	0.006%
Fomitopsidaceae	1.655%	0.030%
Psathyrellaceae	1.600%	11.794%
Mycenaceae	1.544%	ND

Table S3. Alpha diversity estimates before (B1, B2) and after (A1, A2) carbon enrichment using ACE, Chao1, Simpson, Shannon, and Observed Species metrics. Average values across all three sites are shown for data collected both before and after enrichment. *P*-value results from a t-test comparing the before and after samples is shown for each metric. Significant *P*-values are denoted by an asterisk (*).

	ACE	Chao1	Simpson	Shannon	Observed Species
SSSS B1	524.04	521.28	0.97	6.54	482
SSSS B2	520.65	525.93	0.96	5.87	456
MnF B1	54.74	49.00	0.70	2.57	44
MnF B2	145.03	130.25	0.84	3.13	107
Terminus B1	205.46	203.25	0.94	5.02	186
Terminus B2	302.50	278.44	0.93	5.20	244
Average Before	**292.07**	**284.69**	**0.89**	**4.72**	**253**
SSSS A1	244.55	245.33	0.75	2.53	173
SSSS A2	202.82	214	0.64	2.22	136
MnF A1	126.70	109.5	0.83	2.91	90
MnF A2	210.00	214.55	0.71	3.21	140
Terminus A1	151.13	152.23	0.65	2.18	101
Terminus A2	142.57	147.33	0.77	2.79	106
Average After	**179.63**	**180.49**	**0.725**	**2.64**	**124**
P-value	*0.11183*	*0.13283*	*0.00561**	*0.01041**	*0.07126*

Table S4. Identification and description of Mn(II)-oxidizing fungal cultures in relation both to other cultured strains and to sequences retrieved from Illumina sequencing of PCR amplicons. N indicates a percent identity lower than 80%. Strains capable of Mn(II) oxidation are denoted with a plus (+), and those with inconsistent and/or excessive oxidation at times are denoted with a plus/minus (+/-).

Culture name	Cave	Status	Mn(II)-oxidation	Closest cultured strain	% ID to cultured fungus	Across # BP	Reference	Representative cultured strain	% ID to Illumina sequences	across # BP
Plectosphorellaceae sp. DBCMA	DBC	Pure	+	KF367475	89	604	(Oliveira et al 2013)	*Prosthecium* sp. BRO-2013	N	
Acremonium sp. DCI1F	CSPC	Pure	+	HE605218	99	569	(Martin-Sanchez et al 2012)	*Acremonium nepalense* LX Ma6-10	N	
Acremonium sp. CSPCBetsoh	CSPC	Pure	+	HE605212	99	545	(Martin-Sanchez et al 2012)	*Acremonium nepalense* LXM1-1	N	
Alternaria sp. DBC-AD	DBC	Pure	+	HM216210	99	487	(Santelli et al 2010)	*Alternaria alternata* SRC1lrK2f	99%	251
Pleosporales sp. MSW	DBC	Pure	+	HQ631028	86	594	(Shrestha et al 2011)	*Periconia* sp. TMS-2011	81%	240
Leptosphaerulina sp. DBCMVB	DBC	Pure	+	HM216194	98	618	(Santelli et al 2010)	*Leptosphaerula chartarum* DS1bioJ1b	96-98%	256-259
Mortierella sp. YECT1	CSPC	Enrichment	+/-	JF439484	99	690	(Han et al 2011)	*Mortierella alpina* T7	98-99%	254-260
Mortierella sp. YECT3	CSPC	Enrichment	+/-	JX975938	99	594	(Wagner et al 2013)	*Mortierella minutissima* CBS 277.71	N	
Mortierella sp. BlackDot	CSPC	Enrichment	+/-	HQ630316	99	606	(Nagy et al 2011)	*Mortierella dichotoma* CBS 221.35	79-80%	259-264

Table S5. Relative abundance of the top three OTUs from respective sites before and after enrichment.

Site name and top OTUs	Relative abundance	**Site name and top OTUs**	Relative abundance
SSSS Before		**SSSS After**	
Pholiota burkei	1.79%	*Mortierella biramosa*	7.12%
Trichosporon moniliiforme	1.74%	*Epicoccum nigrum*	0.21%
Pseudogymnoascus verrucosus	0.59%	*Cladophialophora chaetospira*	0.20%
MnF Before		**MnF After**	
Oidiodendron maius	4.23%	*Mortierella* sp.	6.40%
Amanita bisporigera	3.76%	*Penicillium polonicum*	5.04%
Umbelopsis dimorpha	3.42%	*Jahnula aquatica*	4.79%
Terminus Before		**Terminus After**	
Pseudogymnoascus verrucosus	5.56%	*Mortierella biramosa*	7.76%
Rhizopogon pseudoroseolus	5.00%	*Trichosporon wieringae*	1.36%
Jahnula aquatica	1.89%	*Pseudogymnoascus verrucosus*	0.22%

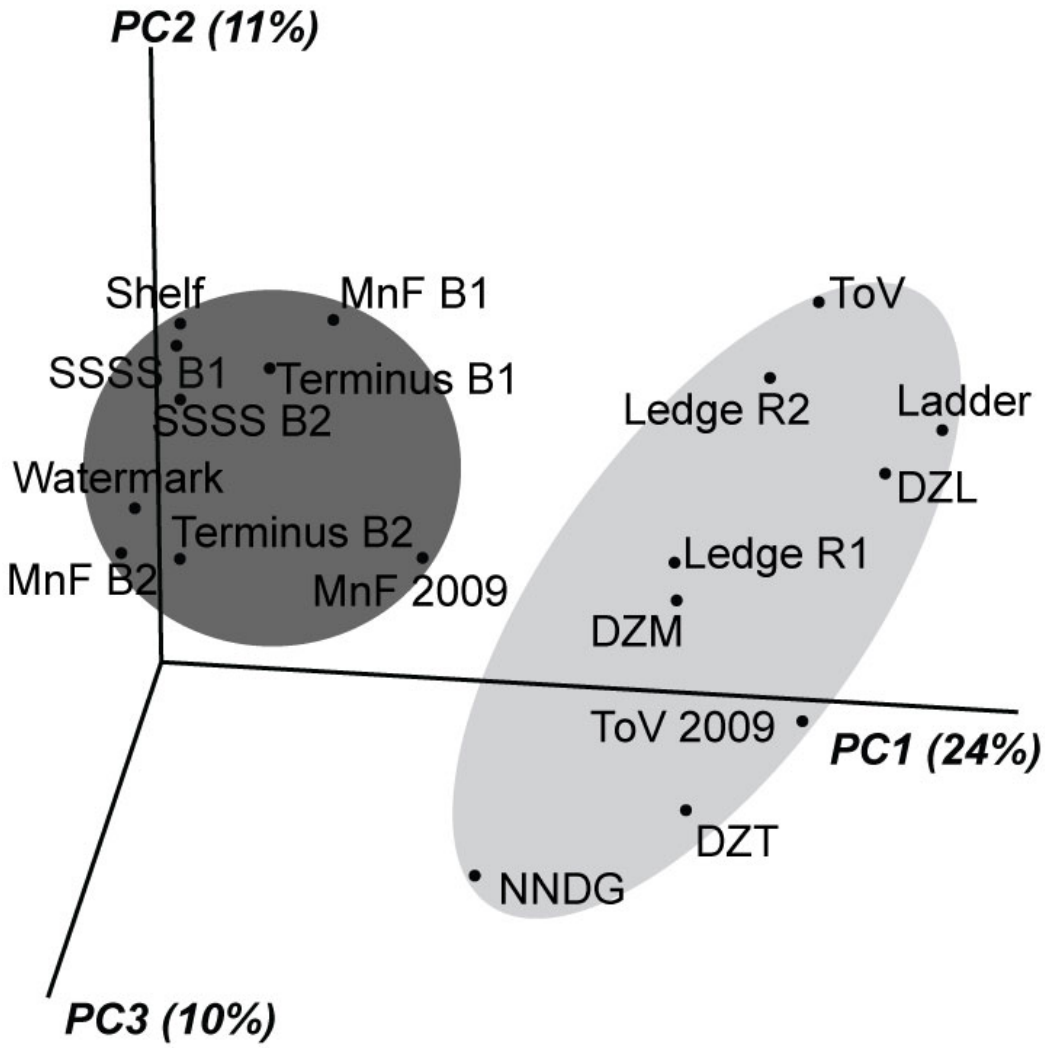

Fig. S1. Principal coordinate analysis based on abundance weighted Jaccard matrices of microbial communities sampled from either CSPC sediments (dark shade) or DBC sediments (light shade).

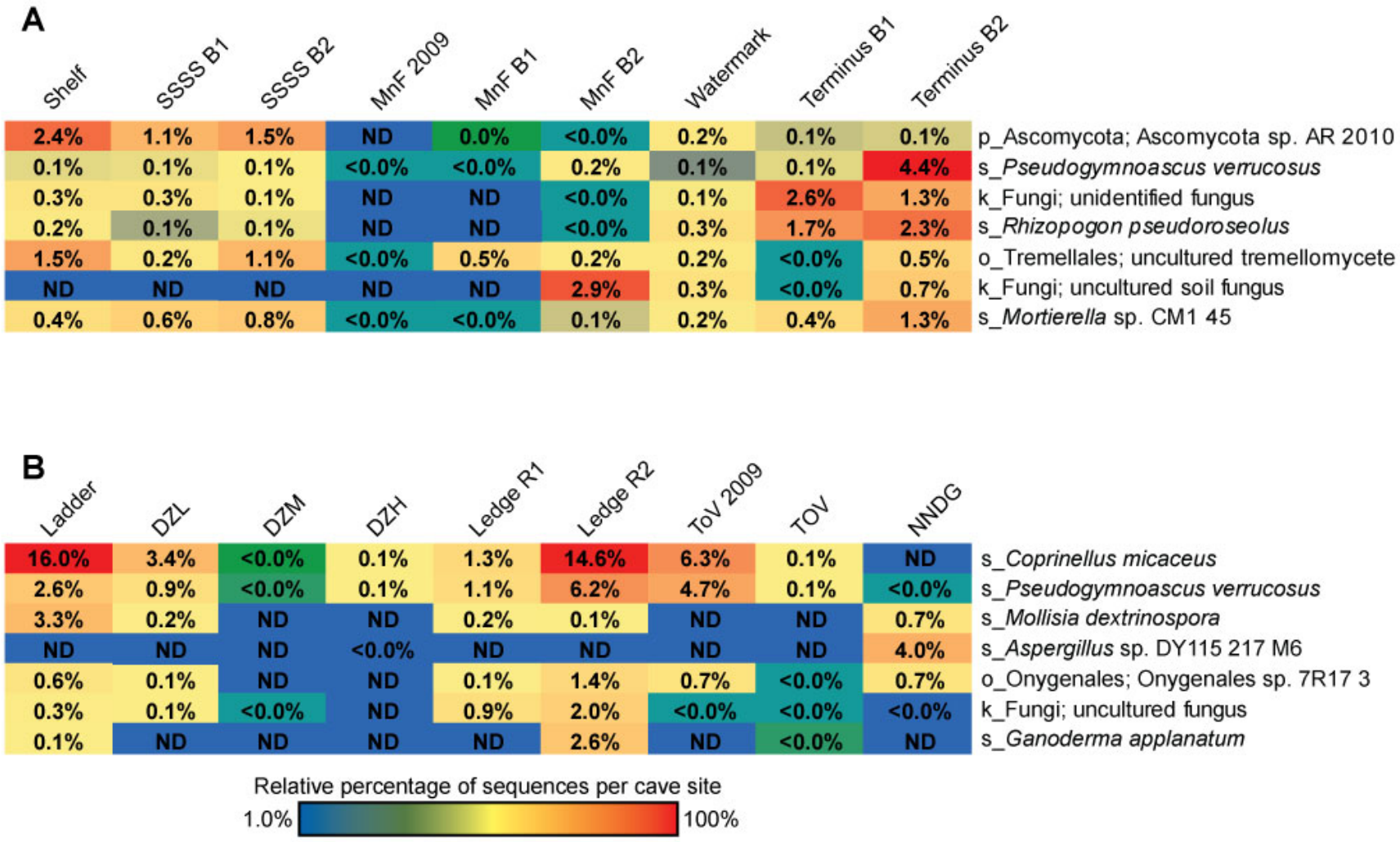

Fig. S2. Heatmaps of the dominant seven OTUs within (A) CSPC and (B) DBC. OTUs are ranked by the sum of sequences associated with them across all sites ranked in ascending order. The lowest taxonomic rank to which the sequence could be assigned is shown, followed by an underscore: k_kingdom; p_phylum; o_order; s_species.

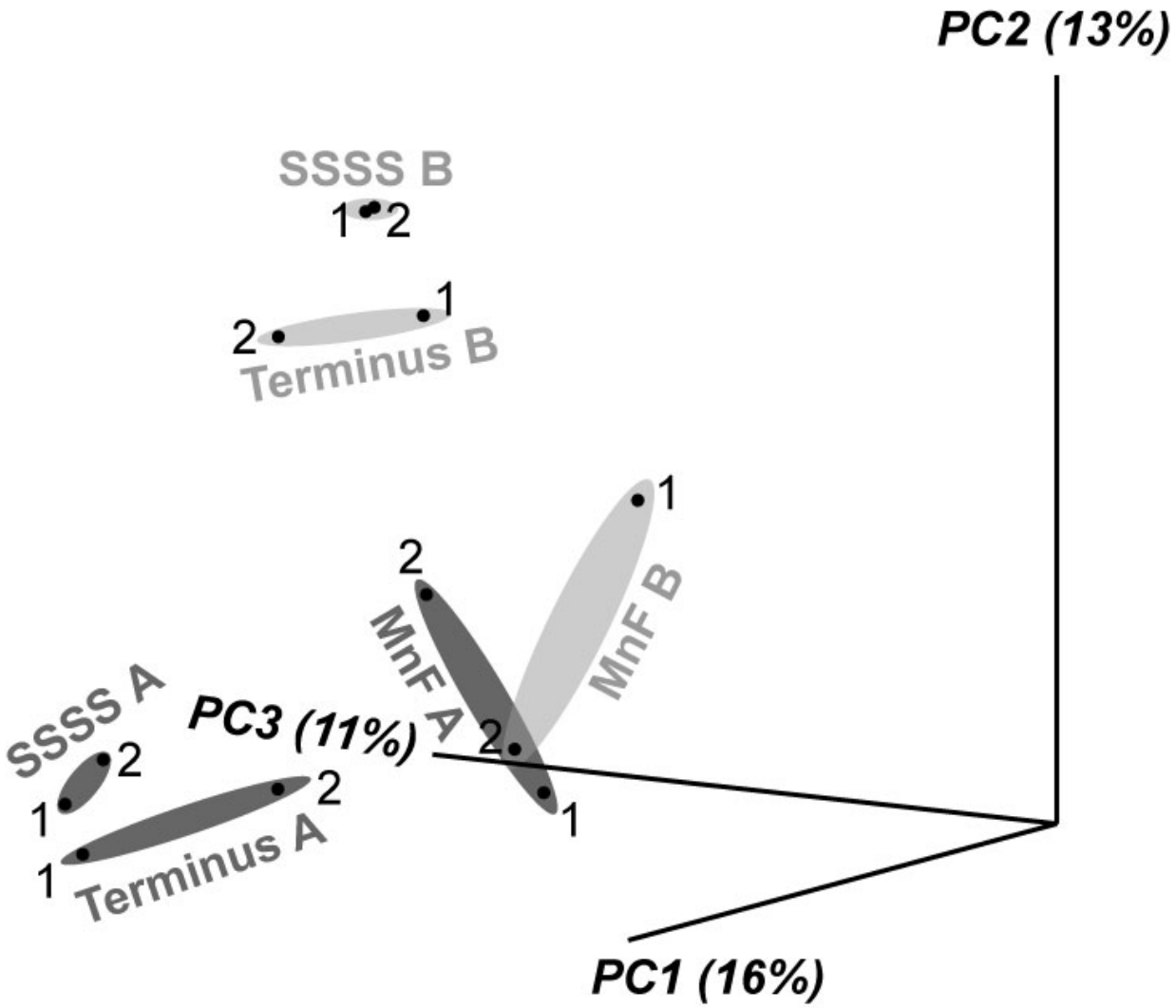

Fig. S3. Principal coordinate analysis based on Bray-Curtis dissimilarity matrices of microbial communities sampled from CSPC sediments either before (light shaded ellipses) incubation with exogenous carbon and stimulation of Mn(II)-oxidation or after (dark shaded ellipses).

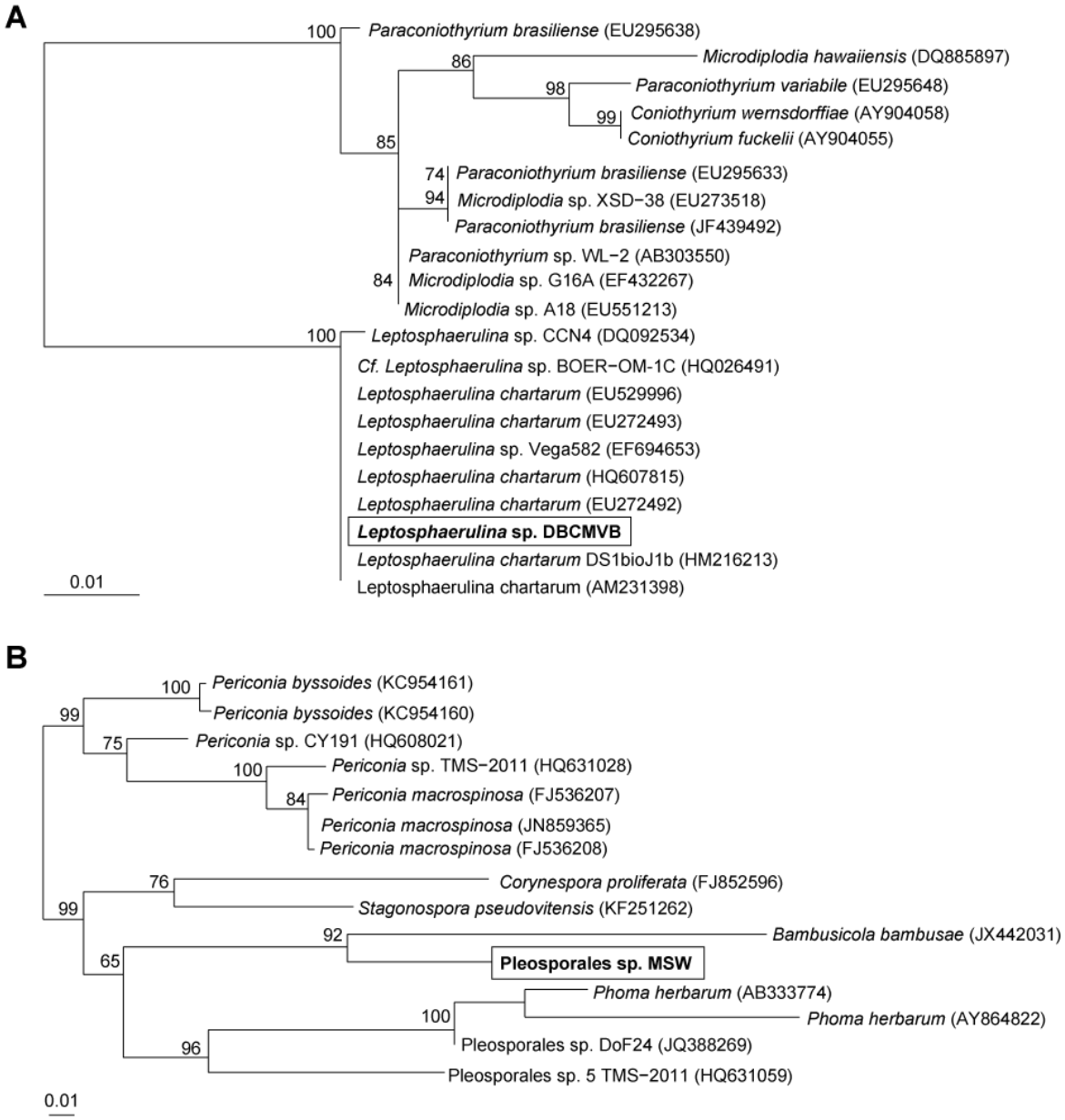

Fig. S4. Maximum likelihood phylogenetic trees based on ITS region analysis of Mn(II)-oxidizing fungal isolates (bolded and boxed). Panels show Ascomycota sequences within the A) Didymellaceae and B) *genus incertae sedis* families of the Pleosporales order. Bootstrap values for nodes with greater than 50% support, determined with 1,000 replicates, are displayed as percentages. Genbank accession numbers are in parentheses. Scale bars represent 0.01 substitutions per nucleotide site.

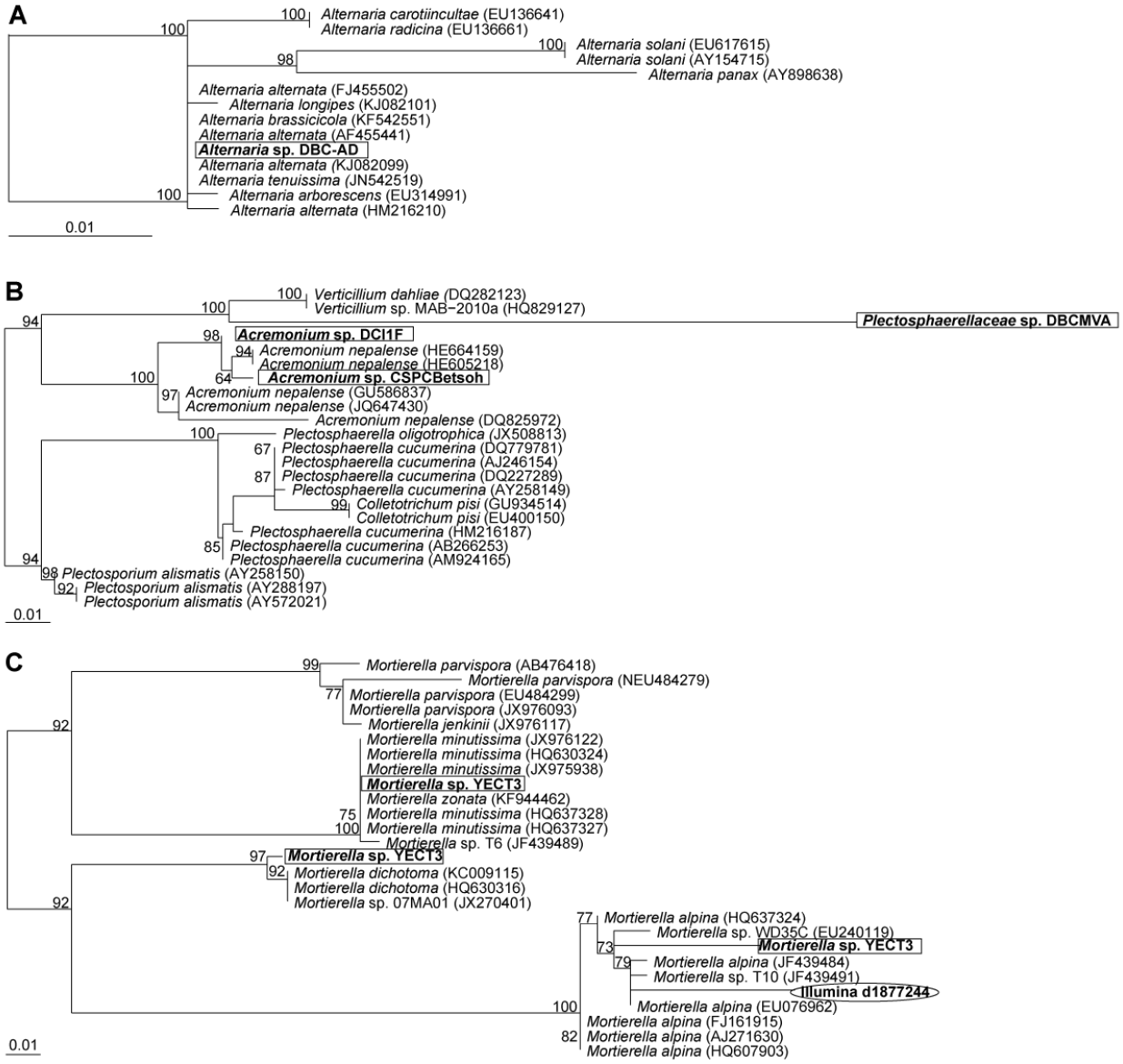

Fig. S5. Maximum Likelihood phylogenetic trees based on ITS region analysis of Mn(II)-oxidizing fungal cultures (bolded and boxed). Panels show sequences for A) isolates within the Pleosporaceae family of the Pleosporales order of the Ascomycota phylum; B) isolates within the Plectosphaerellaceae family of the Glomerellales order of the Ascomycota phylum; and C) *Mortierella* sp. enrichment cultures grouping within the Zygomycota phylum. Also shown in C) is a representative Illumina sequence (bolded and circled) for an abundant OTU (d1877244) with high identity (98-99%) to the cultured *Mortierella* species, YECT1. Bootstrap values for nodes with greater than 50% support, determined with 1,000 replicates, are displayed as percentages. Scale bars represent 0.01 substitutions per nucleotide site.

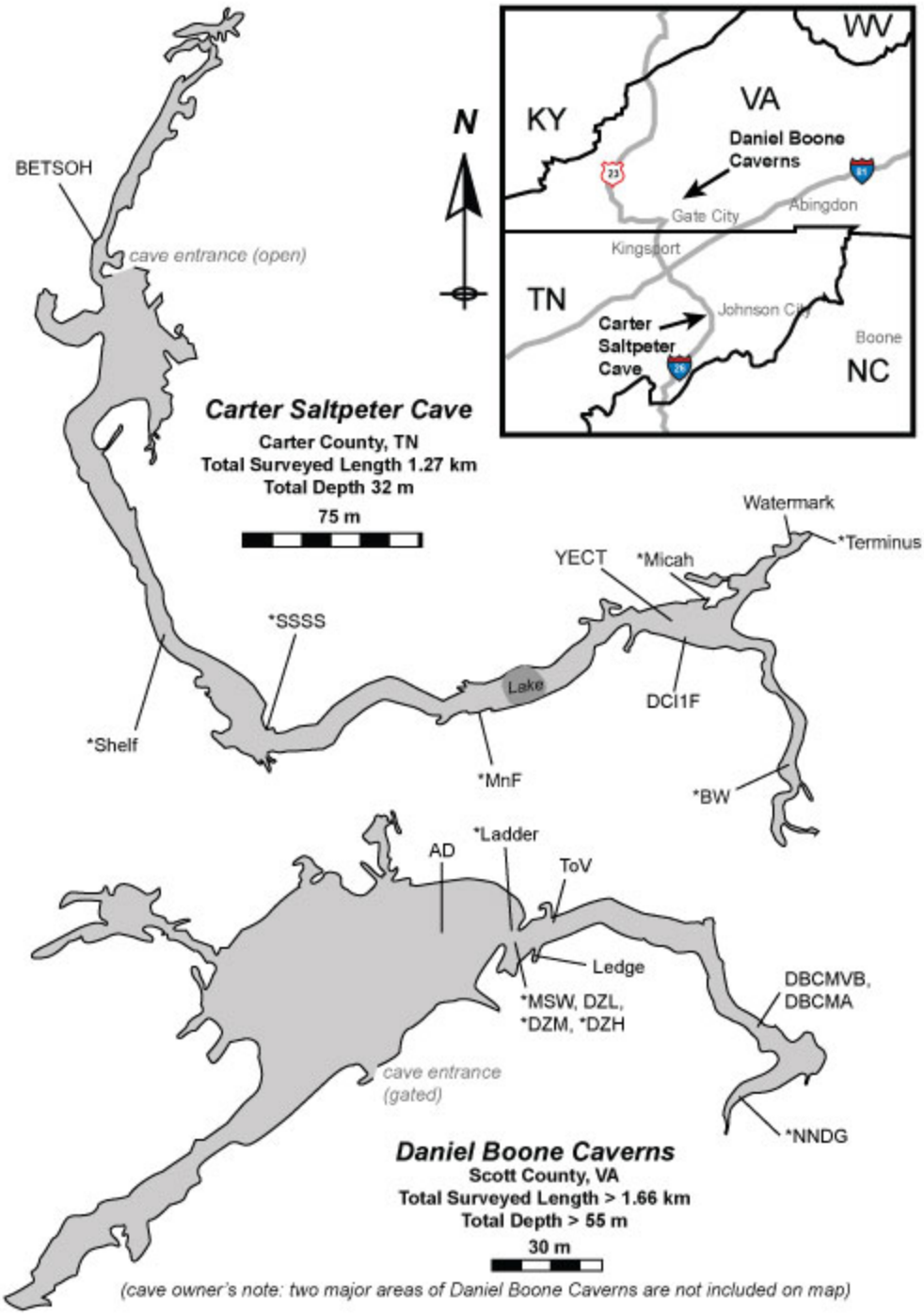

Fig. S6. Maps and sampling locations within Carter Saltpeter Cave (CSPC) and Daniel Boone Caverns (DBC). Asterisks denote nutrient supplementation sites. Cave survey of CSPC conducted on 02/08/81 by L. Adams, R. Knight, R. Page, and T. Wilson. Initial cave survey of Daniel Boone Caverns conducted in 1969 by M. Starnes, B. Lucas, D. Breeding, C. Stowers, and B. Balfour, and an additional survey was conducted from July-November 1996. Two substantial passages in the cave have not yet been surveyed. All cave maps were adapted by S.K. Carmichael.

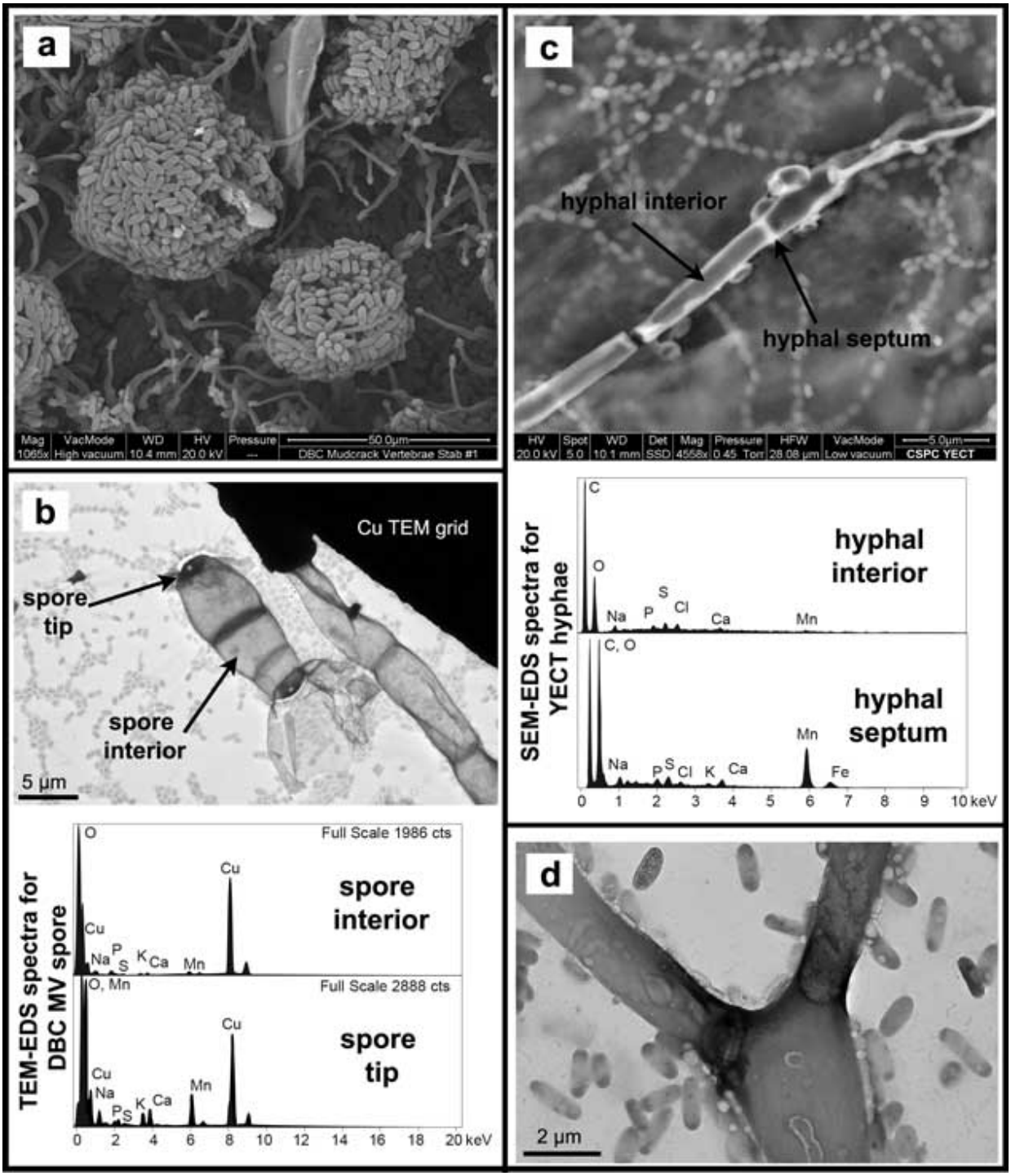

Fig. S7. Microscopic images of cultured organisms: (a) backscattered scanning electron microscopy (SEM) image for critically point dried (CPD) isolate *Plectosphaerellaceae* sp. DBCMVA, showing fruiting bodies; (b) transmission electron microscopy (TEM) image for isolate *Leptosphaerulina* sp. DBCMVB with energy dispersive X-ray spectroscopy (EDS) spectra showing enhanced Mn oxidation at the tips of spores; (c) backscatter ESEM image of enrichment culture *Mortierella* sp. YECT1 with EDS spectra, showing Mn oxidation at septa rather than hyphal interiors (modified from Carmichael et al., 2013b); (d) TEM image *Mortierella* sp. YECT1 showing Mn oxidation in hyphal junctions compared to hyphal interiors.

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