MICROBIAL COMMUNITY ANALYSIS COUPLED WITH GEOCHEMICAL STUDIES REVEAL FACTORS AFFECTING BIOTIC MN(II) OXIDATION IN SITU

A Thesis
by
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Abstract

MICROBIAL COMMUNITY ANALYSIS COUPLED WITH GEOCHEMICAL STUDIES REVEAL FACTORS AFFECTING BIOTIC MN(II) OXIDATION IN SITU

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Identifying the factors that affect biogeochemical cycling has important implications for maintaining ecosystems that are susceptible to anthropogenic input such as cave systems. The oligotrophic and carbon-limiting conditions of most cave environments can limit microbial growth and other processes, yet microbes can actively participate in the biogeochemical cycling of elements, including Mn. The extent to which the type, quality and quantity of carbon can modulate the pathways and degree/amount of Mn cycling in caves is largely unknown. Herein, microbial community analyses coupled with geochemical analyses have constrained the specific environmental parameters that may be affecting the oxidation of Mn(II) in caves. Bacterial, archaeal, and fungal communities associated with Mn(III/IV) oxide deposits were assessed in both relatively pristine as well as anthropogenically impacted caves in the southern Appalachians. Differences observed in the bacterial and archaeal community structure before stimulation appeared to be driven by long-term exogenous carbon loading from either anthropogenic sources or natural carbon sources. In contrast,

fungal community composition at sites experiencing pulses of exogenous carbon were similar to sites recycling endogenous carbon, suggesting that fungal assemblages are independent of carbon infiltration.

Cave sites were amended with various carbon sources that are commonly associated with anthropogenic input in order to determine whether exogenous input such as lint, food crumbs, wood and/or sewage would stimulate biotic Mn(II) oxidation *in situ.* Carbon treatments that stimulated Mn(II) oxidation resulted in significant changes to the microbial communities, indicating that anthropogenic input can both enhance biotic Mn(II) oxidation and potentially shape community structure and diversity. Geochemical analyses of sediment substrates suggest that some biotic Mn(II) oxidation pathways, like MnP mediated oxidation, can be limited by carbon and low C:N ratios.

Additional carbon sources amended with copper were incubated at cave sites to test the role that Cu(II) plays in *in sit*u biotic Mn(II) oxidation. Bacterially selective Mn(II)-oxidizer media supplemented with 100 µM Cu(II) inhibited Mn(II) oxidation, whereas the fungally selective media with Cu(II) stimulated Mn(II) oxidation. Thus, it was inferred that the bacteria in these cave systems were using superoxide to oxidize Mn(II) while fungi were using MCO enzymes for Mn(II) oxidation.

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Chapter 1: Background

Mn(III/IV) oxides are ubiquitous in nature and have the potential to be used as bioindicators of exogenous carbon input in ecosystems that are generally considered oligotrophic (Bastian et al. 2010; Carmichael, Carmichael, Strom, et al. 2013; Carmichael et al. 2015). Reduced Mn(II) can be transformed to Mn(III/IV) oxides through abiotic or microbially catalyzed oxidation reactions. Following their discovery nearly 80 years ago (Gerretson 1937), biologically mediated Mn(III/IV) oxides have been found in many different environments (Carmichael and Brauer 2015; Dick et al. 2009; Santelli et al. 2010). Abiotic oxidation of Mn(II) with O_2 is thermodynamically unfavorable at the pH levels most commonly found in the natural environment (Luther 2010), but biotic Mn(II) oxidation can be up to several orders of magnitude faster than abiotic oxidation (Tebo et al. 2004).There are more than 30 known Mn oxide minerals (Post 1999); the biologically-mediated Mn oxide minerals include birnessite, todokorite, buserite, hausmannite, and other poorly crystalline Mn_xO_x complexes. Mn(II) oxidation occurs either by membrane/cell surface-associated processes, which results in Mn(III/IV) oxides surrounding cells (Carmichael, Carmichael, Santelli, et al. 2013; Carmichael et al. 2015; Larsen et al. 1999; Santelli et al. 2011; Soldatova et al. 2012; Tang et al. 2013), or extracellular processes, which results in Mn(III/IV) oxides deposited among extracellular matrices (Carmichael, Carmichael, Santelli, et al. 2013; Santelli et al. 2011; Tang et al. 2013). Leading researchers to propose that the enzymatic pathway utilized by the microorganism to oxidize Mn(II) can be inferred by where the oxides are deposited within cultures, so if oxides surround the microbial cell then Mn(II) is being oxidized by a cell-surface associated enzyme (Santelli et al. 2011; Tang et al. 2013).

Among the soluble forms of manganese in surface and groundwater environments, Mn(II) was previously thought to predominate, although new detection methods have shown that Mn(III) may represent up to 90-100% of the total Mn in anoxic waters or sediments when complexed with specific ligands (Madison et al. 2013; Trouwborst et al. 2006). These ligands may include pyrophosphate or siderophores such as desferrioxamine B (Harrington et al. 2012; Madison et al. 2013; Soldatova et al. 2012; Trouwborst et al. 2006). Mn(IV) is completely insoluble and is found as part of Mn oxide or oxyhydroxide minerals (Tebo et al. 2004). Biotic Mn(II)→Mn(III) oxidation appears to be followed by either an abiotic or biotic Mn(III)→Mn(IV) oxidation reaction since Mn(III) is typically unstable and will quickly reduce or oxidize to form a more stable complex (Duckworth et al. 2008; Hansel and Francis 2006; Learman et al. 2013; Su et al. 2014). Biogenic Mn(III/IV) oxides can affect the cycling of other elements like Zn, Co, Pb and As by sequestration (Chang et al. 2014; Sasaki et al. 2008; Yin et al. 2011). The instability of Mn(III) coupled with the highly oxidative, reactive and sorptive properties of biogenic Mn(III/IV) oxides contributes to the environmental and economic importance of Mn cycling (see review by Carmichael and Brauer 2015; Post 1999).

Despite decades of research, the reasons for biotic Mn(II) oxidation have long been uncertain. Some Mn(II)-oxidizing microbes become encased in biologically mediatedMn(III/IV) oxides (Ghiorse and Hirsch 1979; Santelli et al. 2011; Soldatova et al. 2012; Tang et al. 2013), leading researchers to propose that these Mn(III/IV) oxide coatings may act as a protective mechanism against a variety of environmental threats (Akob et al. 2014; Banh et al. 2013). Mn(III/IV) oxide coatings may also provide a selective advantage in habitats with abundant heavy metal ions (Akob et al. 2014). Mn(II) can also act as an antioxidant when incorporated inside cells by protecting cellular functions via reducing

reactive oxygen species (ROS), a product formed during cellular respiration (Barnese et al. 2012; Sobota and Imlay 2011). Sunda and Kieber (1994) suggest that Mn(II) oxidation may provide an evolutionary and/or competitive advantage because Mn(III/IV) oxides can degrade humic substances into smaller organic substrates that can be used for microbial growth. Fungal Mn(II) oxidation has been correlated with fungal virulence of a plant pathogenic species, *Gaeumannoyces graminis* var. *tritici* isolate 1079-1 (Thompson et al. 2006). Still, some researchers have proposed that Mn(II) oxidation is a byproduct of other reactions and serves no evolutionary advantage (Hansel et al. 2012; Learman et al. 2011).

Bacterial Mn(II) oxidation has been linked to all of the following: production of superoxide (Learman et al. 2011), haeme-peroxidases (Wariishi et al. 1992), bacterially produced siderophores (Duckworth et al. 2009; Harrington et al. 2012; Parker et al. 2007) and/or multicopper oxidase (MCO) enzymes (Butterfield et al. 2013; Dick et al. 2009; Geszvain et al. 2013; Soldatova et al. 2012; Su et al. 2014). Even within the same organism, evidence suggests that multiple mechanisms for Mn(II) oxidation may be employed under different conditions. For example, strains of *Pseudomonas putida* that can oxidize Mn(II) contain two genes which encode for enzymes implicated in Mn(II) oxidation, McoA and MnxG (Geszvain et al. 2013). Single deletions of either gene in Mn(II)-oxidizing strains of *P. putida* results in different Mn(II)-oxidizing activity when grown either on liquid media or on solid agar, indicating that the MnxG gene may oxidize Mn(II) during planktonic cellular growth and McoA may oxidize Mn(II) while in biofilm states (Geszvain et al. 2013). The diversity of genes and pathways involved suggests a large degree of variability among processes and purposes for Mn(II) oxidation among bacteria.

Mn(II)-oxidizing fungi have been identified as Ascomycetes, Basidiomycetes, or Zygomycetes and the Mn(II)-oxidizing pathways utilized by fungi vary between these phyla. Ascomycetes can oxidize Mn(II) through the production of MCO enzymes (Hofer and Schlosser 1999; Miyata, Maruo, et al. 2006; Soldatova et al. 2012; Thompson et al. 2006) or superoxide (Hansel et al. 2012; Tang et al. 2013), while manganese peroxidase (MnP) is the main enzymatic mechanism used by Basidiomycetes and one Zygomycete (Bonugli-Santos et al. 2009; Kuan et al. 1993; Palma et al. 2000; Schlosser and Hofer 2002; Urzua et al. 1998; Wariishi et al. 1992). Typically, mycogenic Mn(III/IV) oxides are deposited either directly onto hyphae, onto other minerals, or into extracellular matrices (Santelli et al. 2011; Tang et al. 2013), resulting in diverse types of Mn(III/IV) oxide deposits among fungal species (Akob et al. 2014; Santelli et al. 2011; Tang et al. 2013).

Carbon in Caves

Carbon can be the limiting factor for biotic reactions occurring in subterranean environments that are not chemolithoautotrophic (Carmichael et al. 2015). Depending on cave morphology, carbon sources can potentially infiltrate the caves through drip water networks, underground water sources, or flooding. Studies addressing carbon infiltration through water sources in caves have focused on the effects of dissolved organic matter (DOM) or sewage (Birdwell and Engel 2010; Carmichael, Carmichael, Strom, et al. 2013; Simon and Buikema Jr 1997; van Beynen et al. 2000). Simon and Buikema Jr (1997) found that bacterial biomass was increased in pools that were polluted with sewage, while fungal biomass was trivial. Carmichael, Carmichael, Strom, et al. (2013) found that sewage input stimulated bacterial growth and Mn(II) oxidation. DOM can also infiltrate cave systems

through drip water networks and include terrigenous or autochthonous carbon sources. Terrigenous carbon sources include humic or humic-like compounds that are derived from humus, coal or peat and are generally considered to recalcitrant. Autochthonouse carbon sources found in cave systems includes proteins such as tyrosine and tryptophan and are considered to be a labile carbon source (Birdwell and Engel 2010; van Beynen et al. 2000). DOM entering limestone aquifers and cave systems may have a greater proportion of protein-like DOM than humic or humic-like DOM (Birdwell and Engel 2010; van Beynen et al. 2000). Microbes can catabolize DOM in stagnant pools in cave systems (Shabarova et al. 2014). The composition of DOM entering dolomite hosted bedrock caves (commonly found throughout the southern Appalachian Mountains) remains unknown.

Carbon can also be introduced to cave ecosystems through recreational caving and includes trash, human waste, shedding of skin cells/hair, and lint from clothing. Researchers have found significant differences among microbial communities associated with areas that have heavy foot traffic in comparison to areas of the caves that are closed or have limited access (Ikner et al. 2007; Shapiro and Pringle 2010). Several studies have found that species richness decreased at sites that were heavily impacted by humans (Ikner et al. 2007; Shapiro & Pringle 2010). Human traffic can have contrasting impacts on colony forming units (Griffin et al. 2014; Mulec et al. 2012; Wang et al. 2010).

Human Impacts on Biotic Mn(II) Oxidation

Mn(III/IV) oxides have been described from caves across multiple continents. While much of their origin remains uncertain, several studies have suggested that biotic Mn(II) oxidation in caves is stimulated when exogenous carbon enters the cave. Mn(II)-oxidizing

bacteria and fungi have been cultured from various carbon sources associated with anthropogenic input and include batteries, electrical tape, socks, fireworks and Mn(III/IV) oxide deposits that were associated with sewage infiltration into Cater Saltpeter Cave in the southern Appalachian Mountains (Carmichael, Carmichael, Strom, et al. 2013). Another example of anthropogenic impacts on microbial communities and processes includes Lascaux Cave, France, were a fungal outbreak that was synchronous with the appearance of black stains (Jurado et al. 2010). Further research of the black stains revealed that species within the genera, *Ochroconis*, were in part responsible for the stains. Researchers speculated that the stains were due to fungal growth which was stimulated with the addition of carbon sources, possibly from biocide treatments (Bastian et al. 2010). Culture studies from those Mn(III/IV) oxide deposits in Lascaux Cave found that an *Acremonium* species was in part, responsible for the oxides observed (Saiz-Jiminez et al. 2012). More recently, Carmichael et al. (2015) incubated cave sites with simple carbon compounds to stimulate Mn(II) oxidation *in situ* and found that fungal assemblages significantly increased in Zygomycota but decreased in both Ascomycota and Basidiomycota abundances.

This study further addresses how humans may affect the biogeochemical cycling of Mn in cave systems through a variety of *in situ* carbon incubations, comparison of microbial communities before and after treatments and identification of exogenous and endogenous nutrient sources that may be inhibiting or enhancing biotic Mn(II) oxidation. We hypothesized that Mn(II) oxidation *in situ* would be stimulated with 1) organic acids that are commonly associated with sewage contamination, and 2) simple sugars and complex carbon sources that are associated with common cave litter. Constraining factors that affect Mn cycling in cave systems is of importance, as Mn(III/IV) oxides can sequester heavy metals

and can potentially be used as a bioindicator of anthropogenic input in both soils and in groundwater sources.

Chapter 2: Methods

Sample Sites

Four caves within the upper Knox Dolomite were chosen for this study: Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC), Obey's Creek (OC) and Rye Cove-Cox Ridge (RCCR) (Figure 1). CSPC and OC are considered to be impacted caves, while DBC and RCCR are classified as near-pristine caves (sensu- Carmichael et al. 2015). Samples were also taken for a limited, undergraduate-based culturing study from Cranberry Iron Mine, Cranberry, N.C., which a part of the Cranberry Gneiss.

Within each cave, between five and six sites were chosen for carbon incubation experiments (Figure 1). Sites that were chosen for carbon treatments tested slightly positive for Mn(II) oxidation via leucoberbelin blue (LBB) tests, since the goal of the incubations was to stimulate/enhance oxidation occurring at low levels. For brevity, sample sites herein will be referred to first by the cave in which the sample is from and then by the site name (i.e. site MNF in CSPC will be referred to as CSPC-MNF).

Baseline Community Sampling

Sediment samples were taken in triplicate from each site before incubations, transferred on ice and stored at -80^{o}C. DNA was extracted from the sediment samples using the MP Biomedicals Fast DNA^{TM} Spin Kit for Soil. DNA concentrations were measured using a Fisher Scientific NanoDrop 1000 spectrophotometer. Extracted DNA was amplified using PCR and barcoded primers. Fungal DNA samples were amplified using the protocol outlined by Zorn (2014) and bacteria were amplified following the protocol used by Caporaso et al. (2012). Fungal primers ITS1F and ITS2R, along with bacterial primers 515F

and 806R were modified with Illumina adapters, a unique Golay barcode, a reverse primer pad and a linker. Following PCR amplification, samples were standardized, pooled, and gel purified. Purified samples were shipped to the West Virginia University's Genomic Core Facility (Morgantown, WV, USA). Paired-end sequencing was performed on the samples using a MiSeq sequencer (Illumina, Inc., San Diego, CA, USA).

Carbon Stimulation

Nutrient agar casts were deployed at all of the sites chosen for carbon incubations (Figure 1). FMO2 nutrient agar casts that were intended to stimulate bacterial Mn(II) oxidation were supplemented with organic acids (arabinose, casamino acids, and succinic acids) and followed the outline by Carmichael, Carmichael, Santelli, et al. (2013) to stimulate fungal Mn(II) oxidation, agar casts were supplemented with malt extract, glucose, and yeast extract (AY) (Santelli et al. 2010). All agar casts were augmented with 100 µM $MnCl_2$. Complex carbohydrates including sawdust, cotton, and cellulose were also deployed to stimulate fungal oxidation; however, Mn was not added to these complex carbon sources. Agar casts aimed at stimulating bacterial Mn(II) oxidation were incubated for 10 weeks and fungal carbon sources were incubated for 16 weeks. Additional sets of nutrient media were appended with 100 µM $MnCl_2$ and 100 µM $CuCl_2$ to test whether biotic Mn(II) oxidation *in situ* would be inhibited with Cu(II). To test whether Mn(II) oxidation at these sites was limited by Mn(II) or carbon availability, agar casts were supplemented with 100 µM $MnCl_2$ but were not augmented with carbon. LBB tests were then performed and triplicate DNA samples were taken from each nutrient agar that tested positive for Mn(II) oxidation.

Bacterial and Archaeal Community Analysis

PANDAseq commands (Masella et al. 2012) in QIIME (Caporaso et al. 2010) were used to build contigs from forward and reverse bacterial sequence reads and to discard sequences shorter than 245 bp or longer than 335 bp. Sequences were dereplicated with USEARCH (Edgar 2010), while UPARSE was used in conjunction with USEARCH to cluster operational taxonomic units (OTUs) at a 97% identity (Edgar 2010; Edgar 2013). UCHIME (Edgar et al. 2011) was used to filter chimera's using the RDP Gold database (Wang et al. 2007). Taxonomic classifications were performed on the clustered OTUs with QIIME and the Greengenes database (DeSantis et al. 2006). Singletons were then discarded using a QIIME supported command (Caporaso et al. 2010).

PyNAST was used to align sequences (Caporaso et al. 2010) and a maximum likelihood phylogenetic tree was built with FastTree (Price et al. 2010). QIIME was used to calculate alpha diversity metrics with Shannon, Simpson, and observed OTUs (Caporaso et al. 2010). Statistical significant (α=0.05) among taxon level abundance between sample sites and sites before and after stimulation was calculated with a Student's t-test in Microsoft Excel.

OTU tables were modified to BIOM formatted tables (McDonald et al. 2012) and imported into R (Team 2015) using the phyloseq package (McMurdie and Holmes 2013). Vegan was used to calculate Bray-Curtis, and Jaccard distance matrices in R (Oksanen et al. 2016). Vegan (Oksanen et al. 2016), ggplot2 (Wickham 2009),and RColorBrewer (Neuwirth 2014) were used to plot distance matrices in non-metric multidimensional scaling (NMDS) (Kruskal 1964). A permutational multivariate analysis of variance (PERMANOVA) was completed using the Adonis function within the Vegan package in R (Oksanen et al. 2016).

Fungal Community Analysis

PANDAseq (Masella et al. 2012) and QIIME (Caporaso et al. 2010) were used to align forward and reverse fungal sequences and to discard sequences shorter than 200 bp and longer than 300 bp. Sequences were dereplicated with USEARCH and singletons were discarded (Edgar 2010). The UPARSE-OTUref algorithm was employed in USEARCH to cluster OTUs at a 97% similarity cutoff (Edgar 2010; Edgar 2013). UCHIME was used in conjunction with the UNITE reference database to discard chimeric sequences (Abarenkov et al. 2010; Edgar et al. 2011). Taxonomy was assigned to OTU clusters using the BLAST method and database (Altschul et al. 1990).

QIIME was used to calculate alpha-diversity metrics with Shannon, Simpson, and observed OTUs (Caporaso et al. 2010). Statistical significance (α=0.05) among taxon level abundance between sites and sites before and after stimulation was calculated with a Student's t-test in Microsoft Excel. OTU tables were modified to BIOM formatted tables (McDonald et al. 2012) and imported into R using the phyloseq package (McMurdie and Holmes 2013). Vegan was used to calculate Bray-Curtis, and Jaccard distance matrices in R (Oksanen et al. 2016; Team 2015). RColorBrewer (Neuwirth 2014), and ggplot2 (Wickham 2009) were used to plot distance matrices in NMDS format. The Adonis function (Vegan package) (Oksanen et al. 2016) was used to determine differences in microbial communities by site and nutrient treatments.

Culturing

Samples were collected from each site before carbon incubations for culturing experiments. Samples were also taken from an iron sheet, iron gate, and rusty ceiling that tested positive for Mn(II) oxidation with LBB from Cranberry Iron Mine. Samples were streaked aseptically on nutrient agar casts including AY and citrate agar and were supplemented with 100 µM $MnCl_2$. Cultures were continually re-plated using the stab and swipe transfer method until they were axenic. Cultures were kept in the dark at room temperature and were continually monitored for Mn(II) oxidation using LBB as a chemical indicator. Isolated cultures that tested positive for oxidation were colony PCR amplified using 12.5 µL New England BioLabs One Taq Hot Start 2X Master Mix with Standard Buffer, 0.5 µL 10 mM forward primer (either ITS1F or Edel-Hermann nu-SSU-08), 0.5 µL 10 mM reverse primer (either ITS4 or Edel-Hermann nu-SSU-153), and 11.5µL PCR water. Thermocycler parameters were set to 94^0C for 10 min., 30 cycles of 94^0C for 30 s, 54^0C for 45 s, 68^0C for 60 s, followed by 68^0C for 5 min., and 4^0C hold. PCR products were purified, prepped for quick lane sequencing, and sent to Beckman Coulter Genomics (Danvers, MA, USA). Amplified sequences were identified using a BLAST search (Altschul et al. 1990). To test whether or not a Sr or N may be inhibiting Mn(II) oxidation, Mn(II)-oxidizing isolates were cultured on nutrient media that contained 50 or 100 µM SrCl2 or $NH_4^+$and were tested for Mn(II) oxidation via LBB.

Electron Microscopy

Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDS) was performed on isolated Mn(II)-oxidizing cultures. Cultures were preserved with

glutaraldehyde and dehydrated with ethanol. Samples were then subjected to critical point drying with liquid CO_2 in a Polaron critical point dryer. Samples were then mounted on stubs and gold coated with a Denton Vacuum Evaporator and Desk III Sputter Coater. Samples were imaged and analyzed with an FEI Quanta 200 Environmental SEM with an EDAX Genesis XM energy dispersive X-ray spectrometer in the William and Ruth Dewel Microscopy Facility at Appalachian State University.

Geochemical and Mineralogical Analysis

Whole rock geochemistry (WRG) was performed on sediment samples to determine which metals may be inhibiting or stimulating Mn(II) oxidation at sites in DBC, CSPC, and RCCR. WRG samples were taken from sites where agar casts supplemented with Cu(II) were positive for Mn(II) oxidation, sites where carbon sources that were not supplemented with Mn(II) eventually tested positive for biotic Mn(II) oxidation after stimulation, and sites where fungal growth was observed but did not exhibit oxidation (Table 1). Control samples were also collected from calcite flowstones and Mn(III/IV) oxide deposits that were not manipulated by this study. Samples for WRG were collected in sterile vials and shipped to ActLabs, Inc. (a commercial laboratory in Ancaster, Ontario) for analysis using the 4E-Research Total Ident package. Principal component analysis (PCA) was performed in R to determine relationships between sediment samples and caves. Powder X-ray diffraction (XRD) was also performed on the sediments at site BR in CSPC in order to constrain the mineralogy of the site. XRD analyses were performed with a Shimadzu XRD 6000 X-ray diffractometer with a PDF/4+ Minerals database at Appalachian State University. Samples

were ground in a diamonite mortar and pestle and scanned at 5-80° 2Θ, using a Cu tube X-ray source operated at 40kV and 30mA.

Sediment samples were taken from several sites chosen for carbon incubations in all three caves. Samples were dried, homogenized, and approximately 0.020-0.025 g of each sample were weighed in tin foil on a Sartorius CP2-P microbalance. Standards for total carbon (TC) and total nitrogen (TN) in soils included LECO soil and atropine. Sediment TC and TN were analyzed using a FlashEA 1112 NC Analyzer. A Student's t-test was used to identify differences among TC and TN at sites. TC was converted to soil organic matter (SOM) using the conversion TC%*1.724 =SOM proposed by van Bemmelen (1891).

Water Analysis

Three sites in CSPC, DBC, and RCCR were chosen for water collection (Figure 1). At each site dissolved oxygen (DO), temperature, pH, and total dissolved solids (TDS) were measured with a YSI multiparameter handheld water chemistry meter. Water samples for ion chromatography (IC) were filtered into HDPE sterile bottles with a 0.45 µm filter. Sulfate, phosphate, nitrate, fluoride, chloride, and bromide ion concentrations were measured for each sample using an Agilent 6850 Series II Gas Chromatograph in the Chemistry Department at Appalachian State University. Water samples for total organic carbon (TOC) measurements were collected in sterile amber glass bottles and were acidified to a pH < 2 with HCl upon arrival at the lab. Samples for TOC were shipped to the Arizona Laboratory for Emerging Contaminants at the University of Arizona (Tuscon, AZ, USA). Water chemistry parameters and water samples were sampled approximately every six weeks for one year. Water was unable to be collected from two DBC sites (CL and FCP) from June-November, 2015, as the

sites were dry. IC and TOC analyses were compared to Quality Controlled Local Climatological Data taken by NOAA (http://www.ncdc.noaa.gov/qclcd) and discharge data collected by USGS of the north fork of the Holston River near Gate City, VA (http://help.waterdata.usgs.gov).

Chapter 3: Results

Baseline Prokaryotic Community Analysis

After removing singletons, we recovered 20,063 OTUs, most of which were unable to be identified at the species level. Within RCCR a total of 4,767 OTUs were amplified, while 6,693 were amplified from DBC and 7,101 OTUs were found within CSPC. In total, three archaeal phyla and 59 bacterial phyla were represented through sequencing. At the phylum level, Proteobacteria accounted for 23.43-41.34% of the bacterial and archaeal communities across all samples before incubations (Figure 2). The second most abundant phylum was site dependent. Bacterial and archaeal communities at sites RCCR-AB, RCCR-WWWT, and DBC-LL were distinctly different than DBC-CL and all CSPC sites. Site RCCR-AB, RCCR-WWWT, and DBC-LL had higher relative abundances of Chloroflexi, Nitrospirae, and NC10 and lower relative abundances of Actinobacteria, Bacteroidetes and Verrucomicrobia when compared to sites DBC-CL and all CSPC sites (Figure 2). At the order-level, bacterial and archaeal communities at RCCR-AB, RCCR-WWWT, and DBC-LL had higher relative abundances of Alteromonadales and lower relative abundances of Burkholderiales, Saprospirales, and Actinomycetales, when compared to all of the sites at CSPC and at site DBC-CL (Figure 3).

Archaea ranged from 0.06-13.5% of the total prokaryotic sequences across all sites and treatments (Figure 2). Crenarchaeotal and Euryarchaeotal relative abundances ranged from 0.01-7.2% and 0.01-5.6%. Thaumarchaeota and Thermoplasmata were the most abundant archaeal classes across all sites and treatments. The most abundant archaeal genus was Nitrososphaera and accounted for up to 1.8% of the prokaryotic communities.

Results from alpha-diversity measurements of bacterial and archaeal communities showed no significant differences among caves (Table 2). Richness measured through observed OTUs before treatments ranged from 1,552 at RCCR-WWWT to 2,683 at DBC-CL. Analysis of measurements calculated with the Shannon diversity index suggests that all sites were diverse, as the sites ranged from 7.43 at RCCR-WWWT to 8.31 at DBC-CL. Results from the Simpson diversity index confirm that the baseline bacterial and archaeal communities across all sites were diverse (Table 2).

Results from the Bray-Curtis beta-diversity measurements from PERMANOVA calculations suggest that the bacterial and archaeal communities at sites within DBC, CL and LL were not different than the sites within the anthropogenically impacted cave, CSPC (Table 3). In contrast, Jaccard measurements from PERMANOVAs indicate that DBC-LL is different ($P<0.05$) than all sites except for CSPC-WM. Results from the pairwise comparisons between DBC-LL and RCCR-WWWT suggest that the two sites have significantly different OTUs present (Jaccard $P=0.032$) but do not have different OTU abundances (Bray-Curtis $P=0.072$) (Table 3). This comparison provides additional evidence that both sites, DBC-LL and RCCR-WWWT, most likely shared common abundant OTUs but had different rare taxa. Analysis of the beta-diversity plots computed with the Jaccard similarity matrix and NMDS corroborates the PERMANOVA results from the Jaccard distance matrix (Figure 4).

Baseline Fungal Community Analysis

Four fungal phyla were represented through sequencing. All sites were dominated by either Ascomycota (4.3-92.8%), Basidiomycota (1.4%-66.7%), and/or Zygomycota (3.1%-

49.8%), while Glomeromycota accounted for less than 1% across all sites (Figure 5). Pezizomycetes dominated site CSPC-MNF (74.4%), Zygomycota Incertae sedis dominated site RCCR-AB (49.8%) and CSPC-NOOK (55.7%), and Agaricomycetes were in high abundance at site CSPC-BR (66.5%) (Figure 6). Sordariomycetes were also in relatively high abundance at sites RCCR-AB, DBC-LL, RCCR-PUD, and CSPC-WM. At the species level, 934 fungal OTUs were assigned, but similar to prokaryotes, most OTUs could not be identified at the species level. *Mortierella, Pseudaleuria* and *Piloderma* were in relatively high abundances across at least one sample (Figure 7). *Mortierella* was in highest abundance at site CSPC-NOOK (55%), *Pseudaleuria* was highest at CSPC-MNF (68%), and *Piloderma* was highest at CSPC-BR (37%).

There appeared to be no significant differences between the alpha-diversity of fungal communities between sites within caves (Table 2). Observed OTUs were several orders of magnitude lower for fungal communities compared to prokaryotic assemblages. Analysis of the fungal diversity measured with the Shannon diversity index indicates that WM, a site at CSPC, had the highest diversity (4.36), and MNF, another site at CSPC, had the lowest diversity (1.76). Results from the Simpson diversity of fungal communities also suggests the same, CSPC-MNF had a Simpson value of 0.42, while WM had a Simpson value of 0.91, indicating that the fungal diversity within CSPC is highly variable. Observed OTUs did not always correlate with increased or decreased diversity.

Before stimulation, fungal communities showed some differences among treatments according to PERMANOVAs calculated with the Bray-Curtis and Jaccard indices ($P<0.05$, Table 3). There were several inconsistences between the Jaccard and Bray-Curtis PERMANOVA measurements, which suggests that rare taxa may be significantly

influencing the beta-diversity at some sites and OTU abundances are driving the differences between the communities at other sites ($P<0.05$).Overall, results from the PERMANOVAs suggest that beta-diversity was highly variable among sites and did not correlate with the caves in which the samples were taken. Fungal communities at two sites, CSPC-BR and CSPC-MNF, were different than each other and all other sites tested. The Jaccard NMDS biplot showed similar patterns to those found with the Jaccard PERMANOVAs (Figure 8).

Geochemical Analyses

PCA results of whole rock geochemistry at sites suggests that CSPC sites cluster together, RCCR sites cluster together and DBC sites cluster together, with the exception of DBC-CL, which clusters more closely with the site WWWT at RCCR. Cu(II) concentrations ranged from 2-50 ppm with the highest concentration found at site RCCR-AC, and the lowest at a flowstone sampled near DBC-FCP (Figure 10), reflecting differences in substrate mineralogy. Sulfur concentrations at CSPC-BR and the non-oxide site at CSPC sampled near WM were much higher than S concentrations at other sites (Figure 10). Strontium concentrations at site CSPC-BR were an order of magnitude higher than the other sites (661 ppm compared to < 65 ppm) (Figure 10). The Mn(II)-oxidizing capability of fungal isolates from CSPC and CBM was not inhibited with various concentrations of Sr or NH_4^+in *in vitro* culture experiments. XRD analysis of the sediment sample taken from BR shows that the sample is mostly quartz, and orthoclase silt with minor goethite.

Nitrogen levels ranged from 0.03-0.35% with most of the sites containing less than 0.10% nitrogen and the highest concentrations were found at CSPC-BR and CSPC-MudTrap. Average soil organic matter (SOM) concentrations from sites ranged from 0.29-6.29 g C kg^{-1}

and was less than 1 g C kg^{-1} at three CSPC sites (BR, NOOK and WM) (Figure 11). Carbon and nitrogen ratios were highest at DBC-LL (103.88) and lowest at CSPC-BR (1.57), CSPC-NOOK (3.60), and CSPC-WM (5.21) (Figure 11).

Water Chemistry

Water chemistry parameters can be found in Table 4. At all water sites, phosphate and bromide levels were below the detection limit for all samples. Across sites, fluoride concentrations ranged from 0.11-1.70 ppm, chloride concentrations ranged between 0.37-81.10 ppm, sulfate concentrations ranged from 1.73-14.75 ppm, and nitrate concentrations ranged from 0.048-33.50 ppm. (Figure 12). Streamflow data collected from north fork of the Holston River near Gate City, VA did not correlate with precipitation data taken from the Virginia Highlands Airport, Abington, VA (Figure 12).

TOC analysis from water samples taken from sites ranged from 280-35326 µg/L (Figure 13). TOC concentrations typically did not exceed 1500 µg/L, with the exception of isolated excursions in February, March, and May. TOC did not appear to correlate with ion concentrations or precipitation data.

Carbon Incubations

Incubated carbon sources chosen for this study stimulated biotic Mn(II) oxidation at multiple sites within CSPC, DBC, and RCCR, with the exception of the organic acid enrichments supplemented with Cu(II) and yeast extract (Table 1). Carbon treatments at all sites in Obeys Creek Cave were not able to be used for this study, as most of the nutrient media had been eaten or was missing from the sites. Bacterial growth was not visibly

observed on or around any plates *in situ*. Fungal growth was observed with simple sugars and complex carbon sources at ten sites (Table 1). Each of the organic acids stimulated Mn(II) oxidation at six sites and glucose and malt extract stimulated Mn(II) oxidation at 10 total sites within CSPC, DBC, and RCCR. Fungal Mn(II) was stimulated with cotton at three sites, with sawdust at four sites, and with cellulose filter paper at one site. Glucose supplemented with Cu(II) stimulated Mn(II) oxidation at eight sites and malt extract supplemented with Cu(II) stimulated Mn(II) oxidation at one site (Table 1). Additionally, media plates that lacked a carbon source but were supplemented with $MnCl_2$ did not stimulate Mn(II) oxidation.

Prokaryotic Communities after Stimulation

At the bacterial and archaeal phyla-level, sites RCCR-AB, DBC-LL, and RCCR-WWWT experienced more significant shifts in individual phyla abundances than site CL and sites at CSPC (Figure 2). Alphaproteobacterial, Gammaproteobacterial, Bacteroidetes and Verrucomicrobial relative read abundances increased ($P< 0.05$) at RCCR-AB, RCCR-WWWT, and DBC-LL across most treatments. Acidobacteria and Chloroflexi decreased significantly with most treatments at site RCCR-AB, RCCR-WWWT, and DBC-LL and decreased, but not significantly, at DBC-CL and the CSPC sites. Crenarchaeota and Planctomycete abundance decreased with all treatments and sites. Betaproteobacteria and Gammaproteobacteria increased significantly across some treatments and accounted for 4.3-13.6% and 4.7-26.6% before treatments and 6.8-32.7% and 11.5-45.8% after treatments, respectively. Saprospirae were also relatively high after treatments and accounted for 30% of the prokaryotic assemblages. At the order-level, Saprospirales, Burkholderiales, and

Pseudomonadales increased across all treatments with simple sugars (Figure 3). Flavobacteriales and Burkholderiales increased with most simple sugars. Chromatiales was more abundant at RCCR-AB, DBC-LL, and RCCR-WWWT before treatments and decreased with almost all treatments. In contrast, Actionmycetales was most abundant at DBC-CL, CSPC-MNF, CSPC-NOOK, and CSPC-WM before treatments and increased the most at RCCR-AB, DBC-LL, DBC-CL, and CSPC-MNF. In general, archaeal abundance decreased with carbon treatments, with the exception of casamino acids at RCCR-AB, CSPC-MNF, and CSPC-WM.

The four most abundant OTUs for each sample can be found in Figure 14. The most abundant OTU was OTU 1, which belongs to the *Polaromonas* genus and accounted for 0.37-35.25% of all sequences within samples. OTU 1's abundance seemed to increase with the addition of carbon sources with the exception of CSPC sites MNF and WM with simple sugars and site CSPC-NOOK. The second most abundance OTU, OTU 2, belongs to the Pseudomonadaceae family and made up 0.09-29.58% of samples. Both OTU 1 and 2 abundance increased with the carbon treatments except for simple sugar incubations at CSPC-MNF and CSPC-WM and organic acids at CSPC-NOOK. Most of the treatments were dominated by a few OTUs, while the before samples were more evenly distributed. For example, at site WM the top four OTUs with the before sample and treatment with succinic acid accounted for 19.47% and 63.58% of the total sequences, respectively. Three of the top OTUs across all sites were archaeal and included OTU 71, 39, and 150. OTU 71, which was identified to the order, Crenarchaeales, was most abundant at RCCR-AB and DBC-LL before treatments, OTU 39 which is part of the Methanomassiliicoccaceae family was most

abundant at DBC-CL before treatments, and OTU 150 could be identified to the Cenarchaecaceae Family and was most abundant at RCCR-AB

Observed OTUs diversity both decreased and increased with treatments, and there were no obvious pattern among sites, treatments, or species count (Table 2). Increases in observed OTUs did not always correlate with increased Shannon or Simpson alpha-diversity. CSPC-MNF had approximately 2,137 observed OTUs before treatments and a Shannon diversity index of 8.02. After treatment at CSPC-MNF with arabinose total OTU count increased to 4,050 observed OTUs and the Shannon index value for CSPC-MNF decreased to 7.28. After Mn(II) oxidation had been stimulated there were significant decreases in Shannon and Simpson diversity across several sites and treatments.

Stimulation of bacterial Mn(II) oxidation with various carbon sources impacted the beta-diversity of bacterial and archaeal communities across all sites with the exception of site CSPC-NOOK, with both the Jaccard and Bray-Curtis calculations (PERMANOVA, $P<0.05$) (Table 3). Also, every organic acid and simple sugar that had stimulated Mn(II) oxidation resulted in a shift in beta-diversity at at least one site. In general, calculations from the Jaccard similarity PERMANOVAs were similar to those calculated from the Bray-Curtis dissimilarity index, with the exception of four treatments. Results from the Jaccard NMDS biplot were mostly consistent with the results from both the Bray-Curtis and Jaccard PERMANOVAs (Figure 15-17).

Fungal Communities after Stimulation

After stimulation, phyla level abundance shifted the most with cotton and sawdust treatments (Figure 7). In general, Ascomycotal abundances increased with treatments with

the exception of incubations at CSPC-MNF and CSPC-WM and cotton treatment. Basidiomycetes decreased across all sites and treatments except casamino acids, malt extract, and sawdust at CSPC-MNF. Zygomycotal abundance decreased with most recalcitrant carbon sources treatments (sawdust and cotton) and increased with nearly all simple sugar treatments (malt extract, glucose and glucose+Cu). DBC-LL treated with cotton and sawdust and RCCR-PUD treated with sawdust were almost completely dominated by the Sodariomycetes fungal class (94.9-98.9%), most of which was from the Microascales order (41.0-98.8%) (Figure 6). Microascales abundance increased at site DBC-LL and RCCR-PUD with sawdust ($P<0.05$) and Hypocreales increased with cotton treatments at DBC-LL and CSPC-WM. Mortierellales abundance increased with organic acid and simple sugar treatments and decreased with most complex carbon incubations. Significant decreases ($P<0.05$) in Mortierellales were observed with cotton and sawdust at DBC-LL. Agaricales increased at CSPC-MNF and CSPC-WM with several treatments ($P<0.05$). *Pseudaleuria*, *Acremonium*, and *Mortierella* were the most abundant genera identified (Figure 7), while most genera and species could not be identified. *Pseudaleuria* was in highest abundance before and after treatments at CSP-MNF, and *Mortierella* was in relatively high abundances across multiple sites before and after treatments.

The four most abundant OTUs for each sample can be found in Figure 18. Across three samples, fungal communities before treatments were less even than the baseline prokaryotic communities. After most treatments, fungal communities were dominated by a few OTUs. Of those OTUs, OTU 2 was the most abundant OTU across all samples and was identified as *Pseudogymnoascus sp*. UFMGCB 5981 (originally isolated from macroalgae communities in the Antarctic (Goncalves et al. 2014). Before treatments this OTU accounted

for 0.09-58.51% of the fungal communities and after treatments its abundance ranged from 0.00-82.34%. The second most abundant fungal OTU was OTU 1 which was identified as a species within the *Pseudaleuria* genus, which was highly distributed at site CSPC-MNF and ranged from 0.20-83.98%. Approximately 61,119 of the 61,604 sequences associated with OTU 1 were amplified from site CSPC-MNF. Across other sites and treatments, the abundance of OTU 1 did not exceed 2%. Of the top OTUs across all sites, 11 OTUs belonged to the Zygomycota phyla and accounted for up to 70% of a fungal community.

Observed OTUs increased across most treatments, with the exception of arabinose, casamino acids, and malt extract at CSPC-MNF, and succinic acid at CSPC-NOOK. Shannon and Simpson diversity indices decreased with most treatments, while significant decreases ($P<0.05$) were only measured with cotton and sawdust at DBC-LL and sawdust at RCCR-PUD. The lowest diversity measurements were found at CSPC-MNF with arabinose treatment and the highest diversity measurements were at RCCR-PUD treated with glucose+Cu and malt extract.

Complex carbon incubations resulted in some shifts in fungal communities (PERMANOVA, $P<0.05$), while simple sugar and organic acid treatments did not significantly impact the communities (Table 3). Interpretations of the Jaccard NMDS biplot suggested that malt extract treatments impacted the fungal community and other simple sugars and organic acid treatments did not impact the community (Figure 19). Analysis of Jaccard PERMANOVA measurements from fungal communities indicate differences between communities before and after treatment with sawdust at all three sites ($P<0.05$) (Table 3), which was consistent with analyses of the Jaccard similarity matrix plotted with NMDS (Figure 20). Bray-Curtis PERMANOVAs of sawdust treatments showed differences

in only two of the three sites where sawdust stimulated Mn(II) oxidation (Table 3), indicating that recalcitrant carbon stimulation at DBC-LL had significant impacts on OTU presence/absence but not on OTU abundances.

Mn(II)-Oxidizing Isolates

Axenic cultures were isolated from caves CSPC, DBC, and RCCR, and from CBM. Most Mn(II)-oxidizing microbes isolated from these sites were assigned to the fungal genera *Acremonium*, *Plectosphaerella*, *Pleosporales*, or *Alternaria* and bacterial genera *Pseudomonas*, and *Flavobacterium*. All of which have been previously isolated from either CSPC or DBC (Carmichael, Carmichael, Santelli, et al. 2013; Carmichael et al. 2015). Fungi that have yet to be identified as Mn(II)-oxidizers include an isolate that is 99% identical to *Trichosporon spp.*, which belongs to the Tremellales order and Basidiomycota phyla, and an isolate that is 99% identical to *Dictyosporium spp*., which belongs to Pleosporales order and Ascomycota phyla (Table). SEM images were taken from Mn(II)-oxidizing isolates and EDS analyses confirmed the presence of Mn(III/IV) oxides associated with the cell wall of the fungal hyphae (Figure 21-23).

Chapter 4: Discussion

Microbial Community Analysis Prior to Carbon Treatments

Past research regarding microbial communities in cave systems has been focused on culturing techniques or clone libraries (Vanderwolf et al. 2013), while only a handful of studies have used high-throughput sequencing to identify microbial communities (Mandal, Panda, et al. 2015; Mandal, Sanga, et al. 2015; Ortiz et al. 2012; Wu et al. 2015). Of those, only one has identified fungal assemblages (Carmichael et al. 2015). Microbial community involvement in biogeochemical cycling in cave systems is important to identify, as several studies have found that microbes can affect host-rock geochemistry (Barton and Jurado 2007; Gradzinksi et al. 1995; Jones et al. 2008; Sáiz-Jimenez et al. 2012; Spilde et al. 2005).

Previous studies analyzing bacterial communities from cave samples identified 11-33 bacterial phyla (Mandal, Panda, et al. 2015; Mandal, Sanga, et al. 2015; Ortiz et al. 2012; Wu et al. 2015), which is much lower than the 59 bacterial phyla described from this study. Of the phyla found in other caves, Actinobacteria have been found to dominate most of the cave samples taken from Farkpuk Cave with 81% abundance (Mandal, Sanga, et al. 2015), Khuengcherapuk Cave with 64% abundance (Mandal, Panda, et al. 2015), and at Kartchner Caverns accounting for up to 60% of the sequences (Ortiz et al. 2012). Within Kartchner Caverns, sites not dominated by Actinobacteria had high abundances of Deltaproteobacteria (Ortiz et al. 2012), a class that accounted for less than 5% of the bacterial and archaeal communities identified in this study. Community analysis at Jinjia Cave showed Actinobacteria in highest abundance (33%) at sites closest to a sinkhole, while Gammaproteobacterial accounted for 40% of the sequences in the deepest, least accessible sites of the cave (Wu et al. 2015). Sites analyzed for this study had relatively high

concentrations of Gammaproteobacteria, Betaproteobacteria, Bacteroidetes, and Acidobacteria, while Actinobacteria accounted for less than 12% of the communities before treatments (Figure 2). Discrepancies observed between phyla-level abundances may be due to the locations of sample sites within caves. For example, almost all samples sites chosen in Kartcher Caverns were speleothems (Ortiz et al. 2012), whereas samples sites chosen for this study had a variety of substrates where Mn(II) was being actively oxidized and Mn(III/IV) oxides were present.

Prokaryotic communities based on OTU presence/absence at RCCR and one of the two sites at DBC were significantly distinct from the assemblages at CSPC prior to carbon incubations (Jaccard, $P<0.05$). Bacteroidetes, commonly associated with the mammalian gut (Thomas et al. 2011), were the most abundant at DBC-CL and the CSPC sites. The increased abundance of Verrucomicrobia at DBC-CL and CSPC sites is not surprising, as Verrucomicrobia are commonly found in soils or waters that are polluted or eutrophic (see review by Schlesner et al. 2006). These results support the notion that CSPC is experiencing 'sustained anthropogenic impact', which was proposed by Carmichael, Carmichael, Strom, et al. (2013). Bacterial and archaeal assemblages, as well as fungal communities in cave systems can be affected by anthropogenic impact, which can either be direct, such as tracking in exogenous microbes from outside of the cave (Griffin et al. 2014) or littering in caves (Carmichael, Carmichael, Strom, et al. 2013; Carmichael et al. 2015; Ikner et al. 2007), or indirect, such as when groundwater becomes contaminated with human waste or municipal water sources (Carmichael, Carmichael, Strom, et al. 2013; Simon and Buikema Jr 1997). IC analysis from water samples suggests that CSPC sites experienced increased nitrate and

chloride loading, which is similar to the findings of Carmichael, Carmichael, Strom, et al. (2013).

Caves are generally considered oligotrophic unless they experience increased exogenous input or are sustained by chemolithoautotrophic microbes. Carmichael, Carmichael, Strom, et al. (2013) hypothesized that SOM from sediment samples at CSPC would be elevated in comparison to DBC or RCCR, as CSPC has experienced exogenous carbon infiltration through anthropogenic impact. Localized carbon loading has had significant impacts on microbial communities and culturable microbes from caves (Adetutu et al. 2012; Carmichael et al. 2015; Ikner et al. 2007; Jurado et al. 2010), thus it was expected that increased SOM would be correlated with distinct microbial communities. Results from this study do not support this hypothesis, as SOM was significantly higher at DBC-LL than any other site tested (Figure 11). Based on results from the Jaccard similarity index, OTUs present at DBC-LL were different than every site except for CSPC-WM ($P<0.05$) and OTU abundances at DBC-LL were similar to several other sites (Bray-Curtis $P>0.05$). Additionally, increased carbon sources were also expected to decrease microbial diversity (Ikner et al. 2007; Shapiro and Pringle 2010; Silva Taylor et al. 2013), which is not what was observed during this study. For example, DBC-LL had the highest SOM in sediments and also had higher microbial alpha-diversity measurements than several other sites tested. DBC-CL also indicated relatively high amounts of TOC from water infiltration and had the most diverse prokaryotic community based on alpha-diversity calculations. Nitrogen has also been suggested to be a limiting nutrient for microbial processes (Chelius et al. 2009), however, there are no patterns between total nitrogen concentrations and alpha-diversity or relative abundances associated with microbial communities. Previous studies have concluded that

microbial community composition is significantly correlated with C:N ratios (Wan et al. 2015; Zhang et al. 2014), which was likewise not supported in this study. If C:N ratios were correlated to microbial assemblages then beta-diversity at sites would be grouped into four categories, as follows: 1) CSPC-MNF, DBC-CL, and RCCR-AB, 2) RCCR-PUD and RCCR-WWWT, 3) CSPC-BR, CSPC-NOOK, and CSPC-WM, 4) DBC-LL. However, bacterial communities at DBC-CL, and CSPC sites MNF, NOOK and WM grouped together, RCCR-AB was significantly different from all other sites and DBC-LL and RCCR-WWWT clustered together (Figure 4). In fact, no obvious patterns were observed between C:N ratios and alpha-diversity measurements. This lack of correlation between SOM or C:N ratios to microbial alpha and beta-diversity therefore indicates that other factors are affecting assemblage structure within these cave systems.

Previous studies have found that bacterial communities within cave systems are dependent on geochemistry of the bedrock (Barton et al. 2007; Wu et al. 2015). However, geochemistry at site DBC-CL more closely resembled that at site RCCR-WWWT (in an entirely different cave) (Figure 9). If host rock geochemistry was driving the prokaryotic community diversity within CSPC, DBC and RCCR, then DBC-CL should have a similar microbial community` to RCCR-WWWT, which was not observed. Actually, DBC-CL had similar bacterial and archaeal beta-diversity to sites at CSPC, the impacted cave. Another explanation for the similar beta-diversities found at site DBC-CL and CSPC sites is reflected in the result that site DBC-CL experienced significantly higher TOC infiltration through drip water sources than any other site during two sampling events (Figure 13). We speculate that increased exogenous TOC input, either through natural (i.e. autochthonous/allochthonous) carbon sources or anthropogenic sources (i.e. lint, skin cells, sewage, food crumbs, hair

follicles) has long term effects on the prokaryotic communities within cave systems. This hypothesis would explain why CSPC, which is an historically anthropogenically impacted cave, has low levels of SOM and similar prokaryotic communities to DBC-CL, which is a relatively 'pristine' cave that experienced high amounts of TOC during several sampling events, and DBC-LL, which had elevated levels of SOM. Thus, it is hypothesized that microbial communities in caves may represent carbon input, either as exogenous carbon input or recycled endogenous carbon. Further studies addressing community changes over a longer period (4 months vs 12 months), will need to be performed in order to test long term effects of carbon on biotic Mn(II) oxidation and microbial communities.

Prokaryotic Community Responses to the Stimulation of Mn(II) Oxidation

Bacterial and archaeal communities at sites experiencing endogenous carbon (sites – RCCR-AB, RCCR-WWWT and DBC-LL), were most sensitive to the direct addition of carbon compared to sites experiencing long term exogenous carbon input (DBC-CL and CSPC sites). Proteobacteria increased across most treatments with organic acids and simple sugars (Figure 2). We predicted that stimulation with organic acids would enrich for Proteobacteria, Bacteroidetes, Actinobacteria, and Firmicutes, as all known Mn(II)-oxidizing bacteria are members of these phyla (Table 6). Mesocosm experiments identifying bacterial communities before and after direct addition of labile carbon sources by Goldfarb et al. (2011) and Eilers et al. (2010) likewise found an increase in Proteobacteria after carbon addition. Eilers et al. (2010) observed relatively higher increases in Betaproteobacterial abundances for citric acid treatments and glucose treated soils in comparison to the results

reported by Goldfarb et al. (2011). Gammaproteobacteria also increased with citric acid treatments with coniferous forest soils (Eilers et al. 2010). Results from this study were similar, as Proteobacterial abundance increased the most with the most labile carbon sources (organic acids) versus the less labile carbon sources (simple sugars). Betaproteobacteria and Gammaproteobacteria accounted for the majority of the increases in phyla/class level distribution observed with treatments (Figure 2). In contrast, Bacteroidetes increased the most with simple sugars vs. organic acids with the exception of arabinose treatment at CSPC-WM (Figure 7). Bacterial orders that increased with the addition of labile carbon sources from forested soils (Goldfarb et al. 2011) were very similar to those that increased with simple sugars and organic acids from this study and included Actinomycetales, Alteromonadales, Burkholderiales, Pseudomonadales, Saprospirales, Xanthomonadales (Figure 3), which all have Mn(II)-oxidizing species representatives with the exception of Saprospirales (see Table 6 for taxonomic classification of known Mn(II)-oxidizing bacteria).

Most studies addressing microbial communities have found a decrease in diversity with the addition of carbon sources (Ikner et al. 2007; Shapiro and Pringle 2010). Therefore, diversity was expected to decrease with the addition of carbon sources. Most treatments did decrease the prokaryotic diversity, with a few exceptions. Overall, alpha-diversity based on Shannon's diversity index was still relatively high even after treatments, ranging from 5.52-8.47 (Table 2). Changes to alpha-diversity are represented visually with the prokaryotic heatmap created from the top OTUs at each site (Figure 14). Before treatments, the four most abundant OTUs ranged from 13.57-29.99% of the community at a given site. *Polaromonas spp.* increased the most after treatments, followed closely by OTU 2, which was identified as a species within the Pseudomonadaceae family. Previous studies identifying bacterial

communities in caves have also found *Polaromonas spp.*, including Jinjia Cave, China (Wu et al. 2015), Tjuv-Ante's Cave, Sweden (Mendoza et al. 2016), and Kartchner Caverns, AZ, USA (Ikner et al. 2007).

Fungal Community Responses to the Stimulation of Mn(II) Oxidation

Previous metagenomics research from Mn(III/IV) oxide deposits found in Ashumet Pond and Mn removal beds from passive remediation systems in acid mine drainage sites found that fungal communities were dominated by Ascomycota, followed by Basidiomycetes, while Zygomycetes accounted for < 5% of the assemblages (Chaput et al. 2015; Santelli et al. 2014). Carmichael et al. (2015) found that Ascomycota was the dominant phyla at CSPC and fungal communities at DBC had higher concentrations of Basidiomycetes. Zygomycetes were in relatively low abundance at both CSPC and DBC did not account for more than 10% of the communities (Carmichael et al. 2015). Unlike results in Carmichael et al. (2015) fungal communities identified in this study did not show significant patterns between the fungal distributions and caves (Figure 8). In contrast, community structure appeared to explain whether or not Mn(II) oxidation could be stimulated. This study found that Ascomycetes were in higher abundance at sites where Mn(II) oxidation was stimulated (Figure 5), which was likewise a finding for Carmichael et al. (2015) where sites at CSPC had significantly higher Ascomycete abundances than DBC and Mn(II) oxidation was only stimulated at CSPC sites during a 10 week incubation period. The increased relative abundance of Ascomycetes may have led to a more readily stimulated fungal Mn(II)-oxidizing community at sites where oxidation was stimulated. Alpha-diversity for fungal communities before treatments were lower than that observed for bacterial communities,

which is analogous to fungal and bacterial diversity found in other caves (see review by Vanderwolf et al. 2013). Fungal diversity decreased with the addition of carbon sources at most sites, which is not surprising as diversity has been shown to decrease with nutrient input (Vanderwolf et al. 2013). We hypothesized that more complex carbon sources like sawdust and cotton would have the most negative effect on diversity, because the ability of microbes to breakdown lignin and cotton is not universal (Erden et al. 2009; Hofrichter et al. 2010), therefore, only a subset of the fungal community would be able to utilize these carbon sources. This was mostly true, with the exception of sawdust at CSPC-MNF where fungal evenness decreased by only 0.0001. Malt extract treatments at CSPC-MNF and RCCR-PUD resulted in a higher fungal diversity, which could be due to an increased number of fungal species that were able to utilize malt extract as their carbon source.

Carmichael et al. (2015) found that Ascomycota and Basidiomycota decrease in abundance, while Zygomycota and unidentified fungi increase in cave environments when Mn(II) oxidation is stimulated with glucose plates. Thus, we also hypothesized that Ascomycota and Basidiomycota would decrease in abundance with Mn(II) oxidation stimulated with simple sugars. Results from this study support the results from Carmichael et al. (2015), because Zygomycota increased with most glucose, glucose+Cu, and malt extract treatments. Zygomycetes also increased at CSPC-WM with organic acids and cotton. In this study, however, Ascomycota did not always decrease. For most of the treatments, Basidiomycetes decreased and Ascomycota were relatively unchanged in abundance with the exception of stimulation with sawdust and cotton (Figure 5). Differences observed between fungal community responses to nutrient input could be due to the fact that different sites were used for each of the studies, with the exception of CSPC-MNF (Carmichael et al. 2015).

It was hypothesized that stimulation with sawdust and cotton would enrich for Mn(II)-oxidizing Basidiomycetes due to their ability to oxidize cellulose and Mn(II) via the production of cellulases (Baldrian and Valášková 2008) and the lignin-oxidizing enzyme, MnP (Hofrichter 2002). There were three sites where sawdust stimulated Mn(II) oxidation. Of those three sites, only CSPC-MNF had an increase in Basidiomycetes after treatment with sawdust. Site DBC-LL and RCCR-PUD increased in Ascomycota, specifically in Microascales, which are mostly saprobic fungi found in soils (Zhang et al. 2006). Species within the Hypocreales order use MCO enzymes and superoxide to oxidize Mn(II) (Hansel et al. 2012; Miyata, Tani, et al. 2006) and enzymes cellobiohydrolase I, cellobiohydrolase II, and endoglucanase II to break down cellulose (Kubicek et al. 1996). Therefore, the increased abundance of Hypocreales from < 2% to 54% abundance after treatments with cotton is not surprising.

Pseudogymnoascus species have adapted to cold, nutrient depleted habitats, including caves, and have the ability to utilize various carbon sources (see review by Hayes 2012), so their relatively high abundances before and after treatments are not unexpected. An unidentified species within the *Pseudaleuria* genus was also found to be in relatively high abundance before and after treatments, which is interesting because the genus has yet to be identified from any other cave. The absence of *Pseudaleuria* from cave microbial ecology studies could be the result of the lack of high-throughput sequencing methods identifying fungal assemblages in caves, as most studies focus on the bacterial communities. In addition, cave microbiologists tend to employ limited culturing efforts, as most studies focus on culturing specific types of fungi (Carmichael et al. 2015; Sáiz-Jimenez et al. 2012; Vanderwolf et al. 2013). Nevertheless, *Pseudaleuria* was found to be in relatively high

abundance, accounting for up to 83.98% of the fungal species at CSPC-MNF. Of the known Mn(II)-oxidizing isolates (see Table 7 for comprehensive list) three Ascomycetes and eight Basidiomycetes could be identified to the genera level and accounted for at least 1% of the fungal abundance at one or more sites (Figure 7).

Environmental Parameters Affecting Biotic Mn(II) Oxidation *in situ*

The role that copper plays in the enzymatic oxidation of Mn(II) *in situ* has yet to be determined. However, in *in vitro* studies Cu(II) has played a conflicting role in biotic Mn(II) oxidation due to its both stimulatory and inhibitory effect on specific pathways in which microbes use to oxidize Mn(II). Mn(II) oxidation has been shown to be enhanced with the addition of 2-180 µM Cu(II) for bacteria that oxidize Mn(II) through MCO enzymes (Brouwers et al. 2000; Brouwers et al. 1999; El Gheriany et al. 2011; Larsen et al. 1999; van Waasbergen et al. 1996). In contrast, Mn(II) oxidation by microbes that utilize superoxide production is inhibited with 100 µM Cu(II) because superoxide scavenges Cu(II) (Andeer et al. 2015; Hansel et al. 2012; Learman et al. 2011). For this study, carbon sources supplemented with 100 µM Cu(II) aimed at stimulating bacterial Mn(II) oxidation (arabinose, succinic acid or casamino acids) inhibited Mn(II) oxidation *in situ*, whereas the same carbon sources without Cu(II) promoted oxidation suggesting that bacteria are oxidizing Mn(II) with superoxide production and not MCOs. Fungal Mn(II) oxidation was stimulated with 100 µM Cu(II) supplemented malt extract or glucose suggesting that the fungal species oxidizing Mn(II) are most likely using MCOs and not superoxide. This hypothesis is supported by the presence of Mn(III/IV) oxides associated with the cell wall of fungal hyphae observed on the Mn(II)-oxidizing isolates from this study (Figure 21-23).

MCO catalyzed Mn(II) oxidation is the only oxidizing mechanism associated with the cell wall that has lead researchers to infer that MCOs are responsible for oxidizing Mn(II) if the oxide deposits are cell wall associated (Santelli et al. 2011; Tang et al. 2013).

There were two sites where fungal Mn(II) oxidation was expected to be stimulated, yet Mn(II) oxidation tests with LBB were negative. Both sites were at CSPC and include site NOOK and site BR. At NOOK, simple sugars agar casts were incubated for 19 months and fungal Mn(II) oxidation was not stimulated. Interestingly, when organic acids were incubated at NOOK, Mn(II) oxidation was stimulated after just 10 weeks of incubation. BR was originally chosen as a study site due to a bottle rocket (BR) being present and Mn(II) oxidation occurring on that bottle rocket. Simple sugar treatments at BR lead to the visible growth of fungal hyphae but did not stimulate Mn(II) oxidation, which was unique as every other site where fungal growth was detected Mn(II) oxidation was also stimulated. Geochemical analyses performed at BR showed increased concentrations of Sr, S, and N, leading to the hypothesis that one of these elements may be inhibiting biotic Mn(II) oxidation. Bottle rockets normally contain S, Sr, and N, so their increased abundance at BR was not surprising. The Mn(II)-oxidizing capability of fungi previously isolated from other sites/caves was not inhibited with the addition of Sr or N. This lack of correlation between Sr/N and Mn(II)-oxidizing isolates could mean that Sr/N does not inhibit biotic Mn(II) oxidation or that Sr/N inhibits specific Mn(II)-oxidizing biotic reactions but not others (i.e. MnP vs superoxide).

However, additional geochemical analyses have led to the formation of another, more plausible speculation. Fungal communities at NOOK and BR were dominated by either Basidiomycetes or Zygomycetes, which can oxidize Mn(II) through the production of MnP

(Bonugli-Santos et al. 2009; Hofrichter 2002). MnP is a high molecular weight extracellular enzyme that is produced to sequester recalcitrant carbon from the environment (Hofrichter 2002). Modelling performed by Allison (2005) found that microbes will use approximately 1% of the carbon from the product consumed to produce enzymes, and approximately 10% of the mass of the enzyme being produced will be respired during the production of that enzyme. Therefore, microbes will use ~1.1% of the carbon consumed to produce enzymes. Carbon concentrations at BR (0.77 g C kg^{-1}) and NOOK (0.29 g C kg^{-1}) (Figure 11) are below the SOM concentrations commonly found in soils (1.2-70.5 g C kg^{-1}) (Batjes 1996; Bowles et al. 2014; Chen et al. 2009; Matsuura et al. 2012). Additionally, enzymes have a C:N ratio of ~ 3.5:1 and microbes need to consume products that have a C:N > 3.5:1 due to the relatively low carbon use efficiency (0.3-0.6) suggested for soil microbes (Allison 2014; Moorhead et al. 2013; Sinsabaugh et al. 2013). Due to the low SOM content and low C:N ratios found at BR and NOOK (<3.7:1), we speculate that the fungi at BR and NOOK are not actively producing MnP, which is precluding them from actively oxidizing Mn(II). Previous *in vitro* studies of microbes isolated from oligotrophic environments have lead researchers to conclude that the microbes have reduced genomes lost genes in order to adapt and survive in such conditions (Carini et al. 2013; García-Fernández et al. 2004; Williams et al. 2009). It is possible, that due to the extreme oligotrophic environments at BR and NOOK, microbes have lost the ability to produce extracellular enzymes, including MnP and haeme peroxidases. It is also important to note that Basidiomycetes and Zygomycetes may be producing MnP at sites like RCCR-AB and RCCR-LL where SOM concentrations and C:N ratios are higher than those found at CSPC-BR and CSPC-NOOK. Alternatively, BR and NOOK, and possibly other sites within CSPC, DBC and RCCR, may have lacked the recalcitrant carbon needed to

induce MnP production by Basidiomycetes and Zygomycetes. For example, MnP production by *Pleurotus sajor-caju* was increased with recalcitrant carbon sources and MnP was not detected with several carbon sources (Massadeh et al. 2010). Further research would need to be performed in order to verify which enzymes are actively participating in Mn(II) oxidation *in situ*.

Chapter 5: Conclusion

Previous studies have identified changes to microbial community diversity in reponse to anthropogenic impact in cave systems, while this study has focused on how anthropogenic sources of input can *specifically* alter biogeochemical cycling in cave systems, either directly or indirectly. This study has demonstrated that microbial processes, like Mn(II) oxidation, are limited by carbon sources, while direct carbon addition had significant impacts on the bacterial, archaeal, and fungal assemblages. By combining results from previous *in vitro* studies, this research was able to elucidate the mechanistic pathway in which microbes are actively oxidizing Mn(II) *in situ*. Identifying the enzymatic pathways utilized by microbes to oxidize Mn(II) allows for the determination of environmental parameters that may be inhibiting or enhancing the Mn(II)-oxidizing capability of those microbes. This research has important implications for groundwater health and the health of cave ecosystems, specifically those that are in urban areas or are used as show caves, as Mn(II) oxidation may be used as an indicator of contamination. Additionally, this research has potential to be used for sites that will benefit from increased Mn(II) oxidation rates, such as remediation sites for acid mine drainage systems or wastewater treatment plants.

Table 1. Sites where carbon sources were incubated.

		Carbon sources commonly associated with sewage used to stimulate bacterial			Carbon sources commonly associated with sewage used to stimulate bacterial Mn oxidation											
		S + Cu	C + Cu	A + Cu	S	C	A	AY	M	G	M+ Cu	G+ CU	Filter Paper	Sawdust	Cotton	Fungal Hyphae
CSPC Sites	BH						+		+	+		+				Y
	BR															Y
	K															N
	MNF				+	+	+		+	+		+		+		Y
	WM				+	+	+		+	+	+				+	Y
	NOOK				+	+	+									N
DBC sites	FCP															N
	LL				+	+	+		+	+		+		+	+	Y
	CL				+	+			+	+		+	+	+		Y
	NNDG															N
	UPS															N
	SD								+	+		+		+		N
RCCR Sites	AC								+	+		+				Y
	PUD								+	+		+		+	+	Y
	SW															N
	AB				+	+	+		+	+						Y
	WL															N
	WWWT								+	+		+				Y

+ denote that biotic Mn(II) oxidation was stimulated with that crabon source.

Organic acid carbon sources included Succinic acid (S), casamino acids (C), and Arabinose (A)

Simple sugars carbon sources included yeast extract (AY), glucose (G), and malt extract (M).

+Cu denotes the addition of Cu(II) to that carbon source

Table 2. Alpha-diversity estimates at sites at CSPC, DBC, and RCCR.

	Cave	Site/Treatment	Observed OTUs	Shannon	Simpson
Bacteria	RCCR	**AB.B**	**2566**	**8.23**	**0.99**
		AB.A	2078	7.83	0.98
		AB.C	2721	8.37	0.99
		AB.S	3335	8.44	0.99
		WWWT.B	**1552**	**7.43**	**0.97**
		WWWT.G	1798	6.52	0.95
		WWWT.GCU	1846	7.44	0.98
		WWWT.M	1842	7.81	0.98
	DBC	**LL.B**	**2053**	**7.53**	**0.98**
		LL.A	2406	6.98*	0.95
		LL.C	1917	6.31*	0.93
		LL.S	2024	6.76*	0.96
		LL.GCU	1931	6.89*	0.97
		LL.M	1505	6.71*	0.97
		CL.B	**2683**	**8.31**	**0.99**
		CL.C	1839	5.52*	0.91*
		CL.S	1342	5.78	0.94*
	CSPC	**MNF.B**	**2137**	**8.02**	**0.98**
		MNF.A	4050	7.28	0.95
		MNF.S	3534	7.08*	0.95*
		MNF.G	3270	7.74	0.98
		MNF.GCU	3483	8.43	0.98
		MNF.M	3517	8.45	0.99
		NOOK.B	**2219**	**7.92**	**0.98**
		NOOK.A	2899	7.08*	0.97
		NOOK.C	2833	7.90	0.98
		NOOK.S	3944	8.47	0.98
		WM.B	**1839**	**7.89**	**0.99**
		WM.A	2246	6.01*	0.92
		WM.C	3213	6.46*	0.91*
		WM.S	2455	5.60*	0.89*
		WM.G	1112	5.92	0.95*
		WM.M	1812	7.19	0.97
		WM.MCU	2342	7.14	0.95
Fungi	RCCR	**AB.B**	**50**	**3.46**	**0.84**
		PUD.B	**70**	**3.75**	**0.86**
		PUD.GCU	58	3.68	0.87
		PUD.M	51	3.75	0.87
		PUD.SAW	33	1.65*	0.52
	DBC	**LL.B**	**62**	**3.63**	**0.86**
		LL.COT	54	1.12*	0.29*
		LL.GCU	59	3.22	0.78

Table 2 (continued)

	Cave	Site/Treatment	Observed OTUs	Shannon	Simpson
Fungi	**DBC**	LL.SAW	38	1.72*	0.57*
	CSPC	**MNF.B**	**63**	**1.76**	**0.42**
		MNF.A	83	0.89	0.19
		MNF.C	100	2.64	0.61
		MNF.G	43	1.16	0.32
		MNF.M	112	3.38	0.73
		MNF.SAW	48	1.92	0.53
		WM.B	**97**	**4.32**	**0.91**
		WM.COT	42	2.56	0.53
		WM.M	25	1.51	0.43
		NOOK.B	**32**	**2.57**	**0.67**
		NOOK.S	84	3.02	0.70
		BR.B	**202**	**4.36**	**0.84**

Significant differences in Shannon and Simpson diversity before and after treatments is denoted by *
Stimulation of Mn(II) oxidation by arabinose (.A), casamino acids (.C), succinic acid (.S), malt extract (.M), glucose (.G), glucose+Cu (.GCU), malt extract+Cu (.MCU), cotton (.COT), and sawdust (.SAW)
Numbers represent average values for each calculation

Table 3. PERMANOVA pairwise comparisons of microbial communities.

	Pairwise Comparisons		Jaccard		Bray-Curtis	
			R^2	Pr(>F)	R^2	Pr(>F)
Bacterial and archaeal communities	AB.B	AB.A	0.250	0.003*	0.383	0.001*
	AB.B	AB.C	0.137	0.084	0.152	0.126
	AB.B	AB.S	0.204	0.001*	0.247	0.013*
	AB.B	CL.B	0.105	0.004*	0.364	0.003*
	AB.B	LL.B	0.088	0.024*	0.226	0.097
	AB.B	MNF.B	0.114	0.002*	0.142	0.005*
	AB.B	NOOK.B	0.116	0.001*	0.146	0.006*
	AB.B	WM.B	0.102	0.004*	0.126	0.01*
	AB.B	WWWT.B	0.131	0.001*	0.149	0.004*
	CL.B	CL.C	0.310	0.003*	0.434	0.007*
	CL.B	CL.S	0.247	0.036*	0.379	0.022*
	CL.B	LL.B	0.079	0.05*	0.099	0.044*
	CL.B	MNF.B	0.032	0.903	0.026	0.894
	CL.B	NOOK.B	0.041	0.701	0.030	0.826
	CL.B	WM.B	0.046	0.551	0.041	0.615
	CL.B	WWWT.B	0.108	0.003*	0.137	0.006*
	LL.B	LL.A	0.134	0.006*	0.164	0.005*
	LL.B	LL.C	0.156	0.001*	0.191	0.001*
	LL.B	LL.S	0.157	0.001*	0.187	0.003*
	LL.B	LL.GCU	0.176	0.001*	0.232	0.001*
	LL.B	LL.M	0.153	0.003*	0.226	0.001*
	LL.B	MNF.B	0.083	0.049*	0.101	0.055
	LL.B	NOOK.B	0.093	0.024*	0.116	0.022*
	LL.B	WM.B	0.075	0.067	0.092	0.068
	LL.B	WWWT.B	0.086	0.032*	0.087	0.072
	MNF.B	MNF.A	0.095	0.017*	0.124	0.011*
	MNF.B	MNF.S	0.076	0.121	0.094	0.092
	MNF.B	MNF.G	0.077	0.097	0.106	0.047*
	MNF.B	MNF.GCU	0.084	0.044*	0.101	0.053
	MNF.B	MNF.M	0.088	0.039*	0.108	0.044*
	MNF.B	NOOK.B	0.032	0.910	0.022	0.932
	MNF.B	WM.B	0.039	0.775	0.033	0.773
	MNF.B	WWWT.B	0.111	0.002*	0.139	0.006*
	NOOK.B	NOOK.A	0.108	0.189	0.098	0.353
	NOOK.B	NOOK.C	0.082	0.679	0.087	0.47
	NOOK.B	NOOK.S	0.102	0.239	0.119	0.189
	NOOK.B	WM.B	0.040	0.701	0.038	0.645
	NOOK.B	WWWT.B	0.126	0.001*	0.165	0.002*
	WM.B	WM.A	0.111	0.213	0.116	0.213
	WM.B	WM.C	0.166	0.023*	0.175	0.04*
	WM.B	WM.S	0.156	0.04*	0.193	0.026*
	WM.B	WM.G	0.124	0.046*	0.195	0.003*
	WM.B	WM.M	0.096	0.352	0.108	0.251
	WM.B	WM.MCU	0.120	0.064	0.140	0.08
	WM.B	WWWT.B	0.105	0.004*	0.133	0.008*
	WWWT.B	WWWT.G	0.246	0.013*	0.368	0.002*
	WWWT.B	WWWT.GCU	0.262	0.004*	0.375	0.002*
	WWWT.B	WWWT.M	0.145	0.125	0.153	0.173
Fungal communities	AB.B	BR.B	0.090	0.001*	0.184	0.002*
	AB.B	LL.B	0.070	0.170	0.065	0.428
	AB.B	MNF.B	0.079	0.035*	0.111	0.024*
	AB.B	NOOK.B	0.068	0.252	0.080	0.166

Table 3 (continued)

	Pairwise Comparisons		Jaccard		Bray-Curtis	
			R^2	Pr(>F)	R^2	Pr(>F)
Fungal communities	AB.B	PUD.B	0.070	0.682	0.050	0.692
	AB.B	PUD.B	0.070	0.158	0.060	0.54
	AB.B	WM.B	0.059	0.682	0.050	0.692
	BR.B	LL.B	0.099	0.001*	0.189	0.001*
	BR.B	MNF.B	0.093	0.004*	0.223	0.001*
	BR.B	NOOK.B	0.084	0.014*	0.162	0.002*
	BR.B	PUD.B	0.082	0.022*	0.164	0.002*
	BR.B	WM.B	0.099	0.001*	0.185	0.001*
	LL.B	LL.COT	0.151	0.016*	0.153	0.09
	LL.B	LL.S	0.095	0.575	0.082	0.617
	LL.B	LL.GCU	0.099	0.485	0.110	0.318
	LL.B	LL.SAW	0.153	0.013*	0.148	0.11
	LL.B	MNF.B	0.095	0.001*	0.170	0.003*
	LL.B	NOOK.B	0.082	0.024*	0.095	0.104
	LL.B	PUD.B	0.076	0.083	0.099	0.073
	LL.B	WM.B	0.055	0.802	0.060	0.492
	MNF.B	MNF.A	0.026	0.981	0.008	0.994
	MNF.B	MNF.C	0.036	0.878	0.040	0.644
	MNF.B	MNF.G	0.022	0.993	0.016	0.968
	MNF.B	MNF.M	0.062	0.382	0.106	0.104
	MNF.B	MNF.SAW	0.116	0.018*	0.255	0.002*
	MNF.B	NOOK.B	0.086	0.009*	0.159	0.001*
	MNF.B	PUD.B	0.086	0.007*	0.162	0.006*
	MNF.B	WM.B	0.089	0.004*	0.147	0.006*
	NOOK.B	PUD.B	0.067	0.332	0.066	0.416
	NOOK.B	WM.B	0.083	0.012*	0.101	0.079
	PUD.B	PUD.GCU	0.092	0.976	0.062	0.986
	PUD.B	PUD.M	0.127	0.412	0.114	0.615
	PUD.B	PUD.SAW	0.163	0.029*	0.202	0.02*
	PUD.B	WM.B	0.061	0.595	0.058	0.559
	WM.B	WM.COT	0.235	0.333	0.220	0.400
	WM.B	WM.M	0.293	0.067	0.374	0.444

Before (.B) and after arabinose (.A), casamino acids (.C), succinic acid (.S), glucose (.G), glucose+Cu (.GCU), malt extract (.M), cotton (.COT), or sawdust (.SAW)

*denote significant P-value

Table 4. Water chemistry field measurements at CSPC, DBC, and RCCR.

Cave	Site	Collection Date	Temperature	pH	ORP	µs/cm	TDS	DO
CSPC	Lake	2/7/2015	12.52	7.71	262.80	0.38	0.25	8.82
		3/21/2015	13.36	7.12	319.20	0.44	0.29	8.49
		5/2/2105	13.51	6.72	119.80	0.44	0.29	9.94
		6/27/2015	13.00	6.58	231.20	0.30	0.20	8.46
		8/8/2015	13.35	7.24	395.20	0.32	0.21	7.82
		10/14/2015	13.85	7.32	495.20	0.42	0.32	6.81
		11/21/2015	13.64	7.37	367.50	0.43	0.33	8.65
		1/10/2016	13.58	7.54	330.80	0.48	0.24	10.41
	Unweathered Closet	2/7/2015	13.32	7.70	282.40	1.48	0.92	8.48
		3/21/2015	13.64	7.42	282.50	0.46	0.30	7.86
		5/2/2105	13.53	6.46	172.20	0.44	0.28	8.68
		6/27/2015	13.33	6.64	322.10	0.27	0.17	8.96
		8/8/2015	13.42	7.20	310.60	0.32	0.21	9.51
		10/14/2015	13.73	7.31	473.50	0.41	0.30	6.95
		11/21/2015	13.73	7.12	486.70	0.46	0.29	7.65
		1/10/2016	13.58	6.81	402.80	0.48	0.31	10.23
	WM	2/7/2015	13.84	7.40	249.80	0.49	0.32	9.04
		3/21/2015	13.70	7.09	327.00	0.47	0.30	8.85
		5/2/2105	13.55	6.49	160.80	0.44	0.29	9.08
		6/27/2015	13.78	6.72	363.70	0.53	0.35	8.95
		8/8/2015	13.82	7.31	356.70	0.54	0.35	9.22
		10/14/2015	13.58	7.58	512.60	0.33	0.30	8.23
		11/21/2015	13.69	7.34	485.50	0.40	0.30	9.76
		1/10/2016	13.64	6.78	432.70	0.48	0.31	11.65
DBC	CL	2/7/2015	11.77	8.08	190.10	0.69	0.46	9.53
		3/21/2015	12.03	7.67	163.80	0.27	0.17	9.70
		5/2/2105	12.16	6.68	453.70	0.30	0.19	9.01
		6/27/2015	N/A	N/A	N/A	N/A	N/A	N/A
		8/8/2015	N/A	N/A	N/A	N/A	N/A	N/A
		10/14/2015	N/A	N/A	N/A	N/A	N/A	N/A
		11/21/2015	N/A	N/A	N/A	N/A	N/A	N/A
		1/10/2016	12.24	7.65	348.60	0.31	0.20	8.28
	USP	2/7/2015	11.51	8.19	222.60	0.42	0.27	9.65
		3/21/2015	11.68	7.56	180.20	0.31	0.20	8.72
		5/2/2105	11.81	6.39	428.50	0.31	0.20	9.25
		6/27/2015	11.84	7.28	402.50	0.29	0.19	10.66
		8/8/2015	11.92	7.76	570.00	0.31	0.20	10.65
		10/14/2015	12.18	7.79	534.40	0.35	0.24	8.76
		11/21/2015	11.97	7.81	489.60	0.33	0.26	9.23
		1/10/2016	11.71	7.72	432.60	0.31	0.20	9.20
	FCP	2/7/2015	12.76	8.28	235.20	1.65	11.06	10.94
		3/21/2015	11.14	7.54	201.70	0.19	0.12	10.54
		5/2/2105	11.24	6.69	446.60	0.17	0.11	9.90
		6/27/2015	N/A	N/A	N/A	N/A	N/A	N/A
		8/8/2015	N/A	N/A	N/A	N/A	N/A	N/A
		10/14/2015	N/A	N/A	N/A	N/A	N/A	N/A
		11/21/2015	N/A	N/A	N/A	N/A	N/A	N/A
		1/10/2016	11.61	7.43	327.80	0.24	0.16	9.92

Table 4 (continued)

Cave	Site	Collection Date	Temperature	pH	ORP	µs/cm	TDS	DO
RCCR	Stalagmite Puddle	2/7/2015	10.42	8.39	219.80	1.03	0.65	7.11
		3/21/2015	10.05	7.58	199.10	0.34	0.22	9.14
		5/2/2105	10.56	6.52	281.60	0.34	0.22	7.94
		6/27/2015	13.84	6.98	308.80	0.34	0.22	9.89
		8/8/2015	15.11	6.84	490.10	0.39	0.25	5.80
		10/14/2015	14.98	7.90	521.80	0.37	0.25	4.20
		11/21/2015	13.45	7.09	456.20	0.39	0.26	5.46
		1/10/2016	12.02	5.39	429.50	0.41	0.25	5.99
	Upper Pool	2/7/2015	11.92	8.29	229.40	0.59	0.38	8.92
		3/21/2015	11.76	7.86	184.60	0.36	0.23	9.19
		5/2/2105	11.73	7.40	309.40	0.35	0.23	9.05
		6/27/2015	11.81	7.25	426.00	0.37	0.24	9.13
		8/8/2015	12.02	7.43	500.00	0.36	0.23	9.70
		10/14/2015	12.44	7.41	567.80	0.28	0.18	7.42
		11/21/2015	12.14	7.83	412.40	0.30	0.22	8.12
		1/10/2016	11.95	7.88	326.80	0.32	0.21	7.88
	Double Puddle	2/7/2015	11.68	8.49	222.50	0.39	0.25	9.28
		3/21/2015	11.62	7.51	246.30	0.30	0.19	8.91
		5/2/2105	11.66	6.56	307.50	0.28	0.18	9.04
		6/27/2015	11.84	6.63	307.60	0.28	0.18	9.33
		8/8/2015	12.08	8.13	488.30	0.28	0.18	9.80
		10/14/2015	12.09	7.27	556.90	0.36	0.23	6.24
		11/21/2015	11.94	7.63	478.30	0.36	0.25	7.25
		1/10/2016	11.80	7.79	402.10	0.35	0.23	8.90

Table 5. Top three BLAST hits for isolated cultures.

	Sequenced Region	Bp	Top three selected BLAST hits	Max Score	Query Cover	Identity	Accession
CBM-S #4	18S	730	*Acremonium antacrticum stram LF389*	1315	100%	99%	KM096192
			Plectosphaerella sp. MF464	1315	100%	99%	KJ939312
			Verticilium dahliae VdLs.17	1315	100%	99%	CP010980
	ITS	590	Uncultured fungus isolate OUT 52	939	94%	97%	KT328868
			Uncultured soil fungus clone A23	898	99%	94%	HM037659
			Uncultured verticillium	893	99%	94%	HG935549
CBM-S #2	18S	733	*Alternaria sp. PMK2*	1349	100%	99%	KT192438
			Alternaria sp. Y17-1	1349	100%	99%	KP872520
			Alternaria sp. Y16-1	1349	100%	99%	KP872519
	ITS	493	Uncultured Pleosporales clone 8WF1cc01	905	100%	99%	GU910697
			Fungal sp. Strain OTU2	902	99%	99%	KT923239
			Uncultured Alternaria	902	99%	99%	HG936477
CBM-RC #33	18S	710	*Dictyosporium heptasporum CBS 396.59*	1306	100%	99%	DQ018082
			Dictyosporium toruloides CBS209.65	1306	100%	99%	DQ018081
			Dictyosporium alatum ATCC 34953	1306	100%	99%	DQ018080
	ITS	601	*Dictyosporium toruloides CBS209.65*	1007	91%	99%	DQ018093
			Dictyosporium sp. 19VA07	979	96%	97%	JX270548
			Dictyosporium toruloides CBS209.65	894	88%	97%	HF677181
MNF Malt Citrate #14	18S	747	Uncultured fungus clone SZ030406	1358	99%	99%	FN557508
			Trichosporon mycotoxinovorans strain CBS9756	1354	100%	99%	KF036718
			Trichosporon guehoae strain CBS8521	1354	100%	99%	KF036717
	ITS	524	*Trichosporon mycotoxinovorans isolate 504481/2011*	952	100%	99%	JQ266092
			Trichosporon mycotoxinivorans strain R-4272	952	100%	99%	FJ416595
			Trichosporon mycotoxinivorans ACBR HB 1175	952	100%	99%	NR07335

Table 5 (continued)

	Sequenced Region	Bp	Top three selected BLAST hits	Max Score	Query Cover	Identity	Accession
MudTrap #18	16S	1377	*Flavobacterium oncorhynchi strain 631-08*	2534	100%	99%	NR117031
			Flavobacterium oncorhynchi strain 646B-08	2532	99%	99%	FR870076
			Flavobacterium sp. YO64	2529	99%	99%	DQ778317
Citrate Saw MNF #5	16S	1388	*Pseudomonas sp. FBF100*	2355	100%	97%	HG805771
			Pseudomonas sp. PN1059	2353	100%	97%	KC822799
			Pseudomonas sp. SAP1059.2	2351	100%	97%	JX067736

Table 6. Taxonomic assignments of previously isolated Mn(II)-oxidizing bacteria.

Phyla	Class	Order	Family	Genus	Source
Actinobactera	Actinobactera	Actinomycetales	Cellulomonadaceae	*Cellulomonas*	(Carmichael and Brauer 2015)
			Corynebacteriaceae	*Corynebacterium*	
			Microbacteriaceae	*Agromyces*	
				Leifsonia	
				Microbacterium	
			Micrococcaceae	*Arthrobacter*	
			Mycobacteriaceae	*Mycobacterium*	
			Nocardiaceae	*Nocardia*	
				Rhodococcus	
			Pseudonocardiaceae	*Pseudonocardia*	
			Streptomycetaceae	*Streptomyces*	
		Geodermatophilales	Geodermatophilaceae	*Geodermatophilus*	
		Micrococcales	Intrasporangiaceae	*Lapillicoccus*	
				Terrabacter	
			Microbacteriaceae	*Frigoribacterium*	(Akob et al. 2014)
				Frondihabitans	
		Propionibacteriales	Propionibacteriaceae	*Propionibacterium*	(Carmichael and Brauer 2015)
Bacteroidetes	Bacteroidetes	Bacteroidales	Bacteroidaceae	*Cytophaga*	
	Flavobacteria	Flavobacteriales	Flavobacteriaceae	*Flavobacterium*	
				Riemerella	
	Sphingobacteria	Sphingobacteriales	Chitinophagaceae	*Terrimonas*	
			Erythrobacteraceae	*Erythrobacter*	
			Sphingomonadaceae	*Sphingobacterium*	
				Sphingomonas	(Xuezheng et al. 2008)
				Sphingopyxis	
Firmicutes	Bacilli	Bacillales	Bacillaceae	*Bacillus*	(Carmichael and Brauer 2015)
				Exiguobacterium	
				Lysinibacillus	
			Paenibacillaceae	*Brevibacillus*	(Mayanna et al. 2015)
			Planococcaceae	*Planococcus*	(Carmichael and Brauer 2015)
			Staphylococcaceae	*Staphylococcus*	
Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	*Brevundimonas bullata*	(Santelli et al. 2014)
				Caulobacter	(Carmichael and Brauer 2015)
		Rhizobiales	Aurantimonadaceae	*Aurantimonas*	
				Fulvimarina	
			Bradyrhizobiaceae	*Afipia*	

Table 6 (continued)

Phyla	Class	Order	Family	Genus	Source
Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	*Bosea thiooxydans*	(Mariner et al. 2008)
			Hyphomicrobiaceae	*Hyphomicrobium* *Pedomicrobium* *Methylobacterium*	(Carmichael and Brauer 2015)
			Phyllobacteriaceae	Mesorhizobium	(Bohu et al. 2015)
			Rhizobiaceae	*Agrobacterium* *Rhizobium* *Shinella* *Sinorhizobium*	(Carmichael and Brauer 2015)
		Rhodobacterales	Rhodobacteraceae	*Methylarcula* *Paracoccus* *Rhodobacter* *Roseobacter* *Sulfitobacter*	
	Betaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	
		Burkholderiales	Alcaligenaceae	*Achromobacter*	
			Burkholderiaceae	*Burkholderia* *Cupriavidus/-Ralstonia*	
			Comamonadaceae	*Albidiferax* *Caldimonas* *Leptothrix* *Variovorax*	
			Oxalobacteraceae	*Duganella* *Janthinobacterium* *Oxalicibacterium*	
		Neisseriales	Neisseriaceae	*Chromobacterium*	
	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	*Alteromonas* *Marinobacter* *Microbulbifer*	
			Colwelliaceae	*Colwellia*	(Xuezheng et al. 2008)
			Pseudoalteromonadaceae	*Pseudoalteromonas*	(Carmichael and Brauer 2015)
			Shewanellaceae	*Shewanella*	
		Enterobacteriales	Enterobacteriaceae	*Citrobacter* *Enterobacter* *Escherichia* *Klebsiella* *Pantoea* *Proteus*	

Table 6 (continued)

Phyla	Class	Order	Family	Genus	Source
Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Raoultella*	(Santelli et al. 2014)
		Oceanospirillales	Alcanivoracaceae	*Alcanivorax*	(Xuezheng et al. 2008)
		Oceanospirillales	Halomonadaceae	*Halomonas*	(Carmichael and Brauer 2015)
			Oceanospirillaceae	*Marinomonas*	(Xuezheng et al. 2008)
				Oceanospirillum	(Carmichael and Brauer 2015)
		Pseudomonadales	Moraxellaceae	*Acinetobacter*	
				Pyschrobacter	(Xuezheng et al. 2008)
			Pseudomonadaceae	*Pseudomonas*	(Carmichael and Brauer 2015)
		Vibrionales	Vibrionaceae	*Vibrio*	
		Xanthomonadales	Rhodanobacteraceae	*Luteibacter*	
			Xanthomonadaceae	*Pseudoxanthomonas*	(Ashassi-Sorkhabi et al. 2012)

Table 7. Taxonomic assignments of previously isolated Mn(II)-oxidizing fungi.

Phyla	Class	Order	Family	Genera	Source
Ascomycota	Ascomy-cetes	Erysiphales	Erysiphaeceae	*Oidium*	(Carmichael and Brauer 2015)
	Dothideomycetes	Capnodiales	Davidiellaceae	*Cladosporium*	(Carmichael and Brauer 2015)
				Davidiella tassiana	(Cahyani et al. 2008)
		Incertae sedis	Incertae sedis	*Leptosphaerulina*	(Carmichael et al. 2015)
		Pleosporales	Didymosphaeriaceae	*Paraconiothyrium*	(Takano et al. 2006)
				Paraphaerosphaeria	(Cahyani et al. 2008)
			Incertae sedis	*Phoma*	(Carmichael and Brauer 2015)
				Pyrenochaeta	
			Leptosphaeriacea	*Coniothyrium*	
				Leptosphaeria	(Cahyani et al. 2008)
				Ophiobolus	(Carmichael and Brauer 2015)
			Phaeosphaeriaceae	*Phaeosphaeriopsis*	(Parchert et al. 2012)
			Pleosporaceae	*Stagonospora*	(Carmichael and Brauer 2015)
				Alternaria	
				Bipolaris	
				Pithomyces	
				Pleosporales	(Carmichael et al. 2015)
				Curvularia	(Timonin et al. 1972)
	Eurotiomycetes	Chaetothyrio-mycetidae	Chaetothyriales	*Cyphellophora*	(Carmichael and Brauer 2015)
		Eurotiales	Trichocomaceae	*Aspergillus*	(Wei et al. 2012)
				Paecilomyces	(Timonin et al. 1972)
				Penicillium	(del la Torre and Gomez-Alarcon 1994)
		Onygenales	Onygenaceae	*Chrysosporium*	(Timonin et al. 1972)
	Sordariomycetes	Glomerellales	Plectosphaerellaceae	*Plectosphaerellaceae*	(Carmichael and Brauer 2015)
		Hypocreales	Hypocreaceae	*Acremonium*	(Timonin et al. 1972)
				Gliocladium	
			Incertae sedis	*Cephalosporium*	

Table 7 (continued)

Phyla	Class	Order	Family	Genera	Source
Ascomycota	Sordariomycetes	Hypocreales	Incertae sedis	*Stilbella*	(Carmichael and Brauer 2015)
			Nectriaceae	*Cylindrocarpon*	(Timonin et al. 1972)
				Fusarium	
				Nectria	(Carmichael and Brauer 2015)
		Magnaporthales	Magnaporthaceae	*Gaeumannomyces*	(Thompson et al. 2006)
		Microascales	Halosphaeriaceae	*Periconia*	(Timonin et al. 1972)
		Ophiostomatales	Ophiostomataceae	*Sporothrix*	
		Sordariales	Chaetosphaeriaceae	*Gonytrichum*	
		Xylariales	Xylariaceae	*Xylariales*	(Miyata, Maruo, et al. 2006)
		Xylariomycetidae	Xylariales	*Microdochium*	(Carmichael and Brauer 2015)
Basidiomycota	Agaricomycetes	Agaricales	Agaricaceae	*Agaricus sp.*	(Hofrichter 2002)
				Coprinus atramentarius	(Erden et al. 2009)
				Lepiota spp.	
			Cortinariaceae	*Cortinarius spp.*	
			Entolomataceae	*Leptonia lazunila*	
			Inocybaceae	*Inocybe spp.*	
			Lyophyllaceae	*Lyophyllum subglobisporium*	
			Marasmiaceae	*Clitocybula*	(Hofrichter 2002)
				Marasmius	
			Nidulariaceae	*Cyathus*	
			Omphalotaceae	*Lentinula*	
			Physalacriaceae	*Armillaria*	
			Pleurotaceae	*Pleurotus*	
			Pluteaceae	*Volvariella sp.*	(Erden et al. 2009)
			Psathyrellaceae	*Panaeolus*	(Hofrichter 2002)
			Strophariaceae	*Agrocybe spp.*	
				Hypholoma	
				Kuehneromyces	
				Nematoloma	
				Stropharia	

Table 7 (continued)

Phyla	Class	Order	Family	Genera	Source
Basidiomycota	Agaricomycetes	Agaricales	Tricholomataceae	*Clitocybe sp.*	Erden et al. 2009)
				Lepista nuda	
		Atheliales	Atheliaceae	*Piloderma*	(Chen et al. 2001)
				Tylospora	
		Auriculariales	Auriculariaceae	*Auricularia*	(Hofrichter 2002)
		Boletales	Rhizopogonaceae	*Rhizopogon luteus*	(Erden et al. 2009)
			Serpulaceae	*Serpula himantioides*	(Wei et al. 2012)
				Trametes spp.	
		Gomphales	Gomphaceae	*Ramaria stricta*	(Erden et al. 2009)
	Agaricomycetes	Hymenochaetales	Hymenochaetaceae	*Phellinus*	(Hofrichter 2002)
		Phallales	Phallaceae	*Phallus*	
		Polyporales	Fomitopsidaceae	*Phaeolus*	
			Ganodermataceae	*Ganoderma carosum*	
			Meripilaceae	*Physisporinus*	
				Rigidoporus	
			Meruliaceae	*Abortiporus biennis*	
				Bjerkandera	
				Irpex lacteus	
				Merulius	
				Phlebia	
			Phanerochaetaceae	*Ceriporiopsis*	
				Phanerochaete chrysosporium/-Sporotrichium pulvervulentium	
			Polyporaceae	*Coriolopsis*	
				Dichomitus	
				Panus	
				Perenniporia	
				Trametes	
		Russulales	Bondarzewiaceae	*Heterobasidion*	
			Russulaceae	*Lactarius delicious*	(Erden et al. 2009)
				Russula spp.	
	Uredinio-mycetes	Sporidiales	Incertae Sedis	*Rhodotorula graminis*	(Staudigel et al. 2006)
Zygomycota	Mucormy-cotina	Mucorales	Mucoraceae	*Mucor*	(Bonugli-Santos et al. 2009)

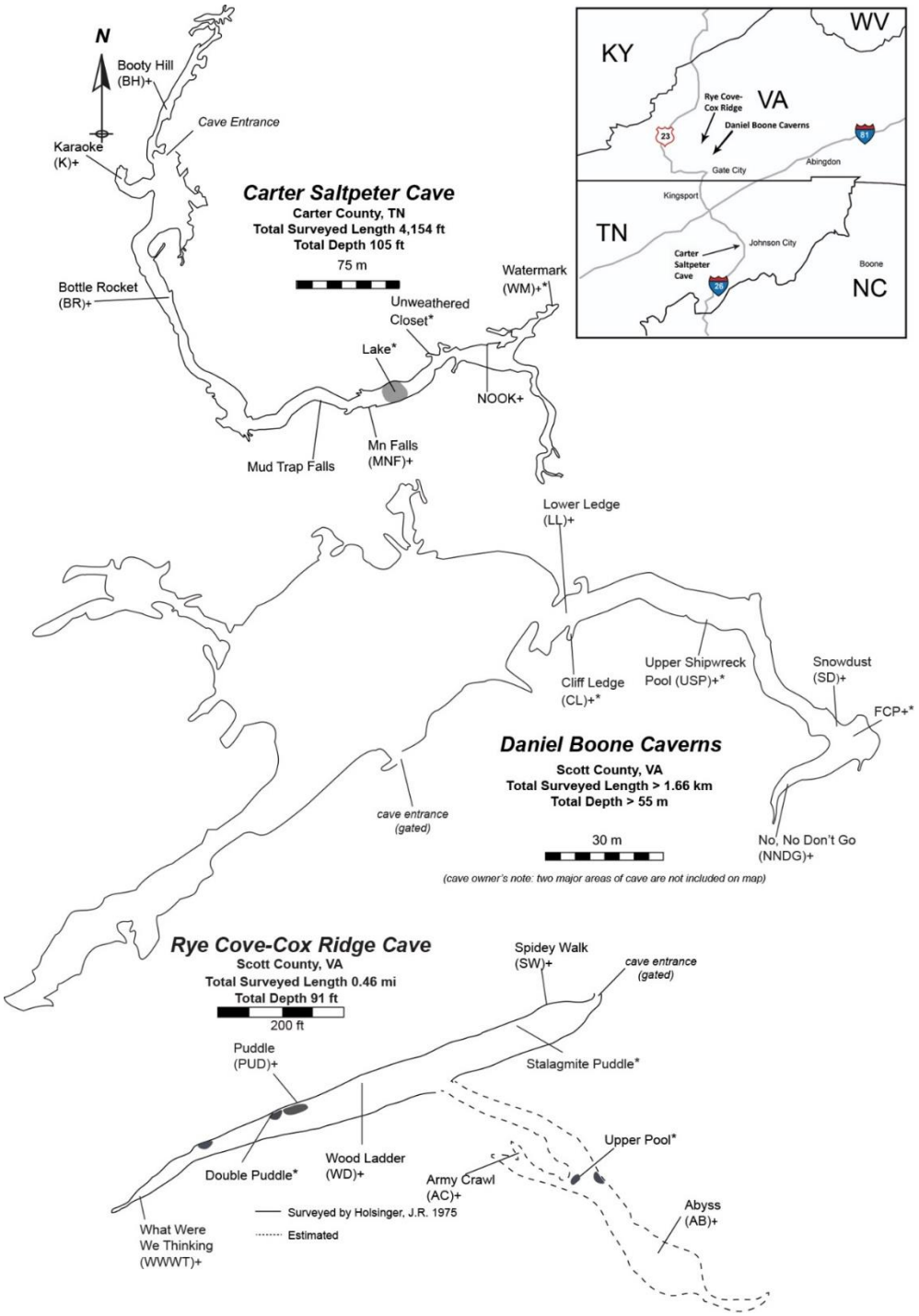

Figure 1. Maps of Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC), and Rye Cove-Cox Ridge (RCCR). Maps modified with arrows to indicate sampling locations, + to indicate carbon incubations sites and * to denote sites for water sampling. Regional map shows the relative location of the three caves. Cave survey of CSPC performed on 02/08/81 by L. Adams, R. Knight, R. Page, and T. Wilson. Initial cave survey of DBC 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 subsequent passages in DBC have not been surveyed. Cave survey of RCCR performed by Holsinger (1975).

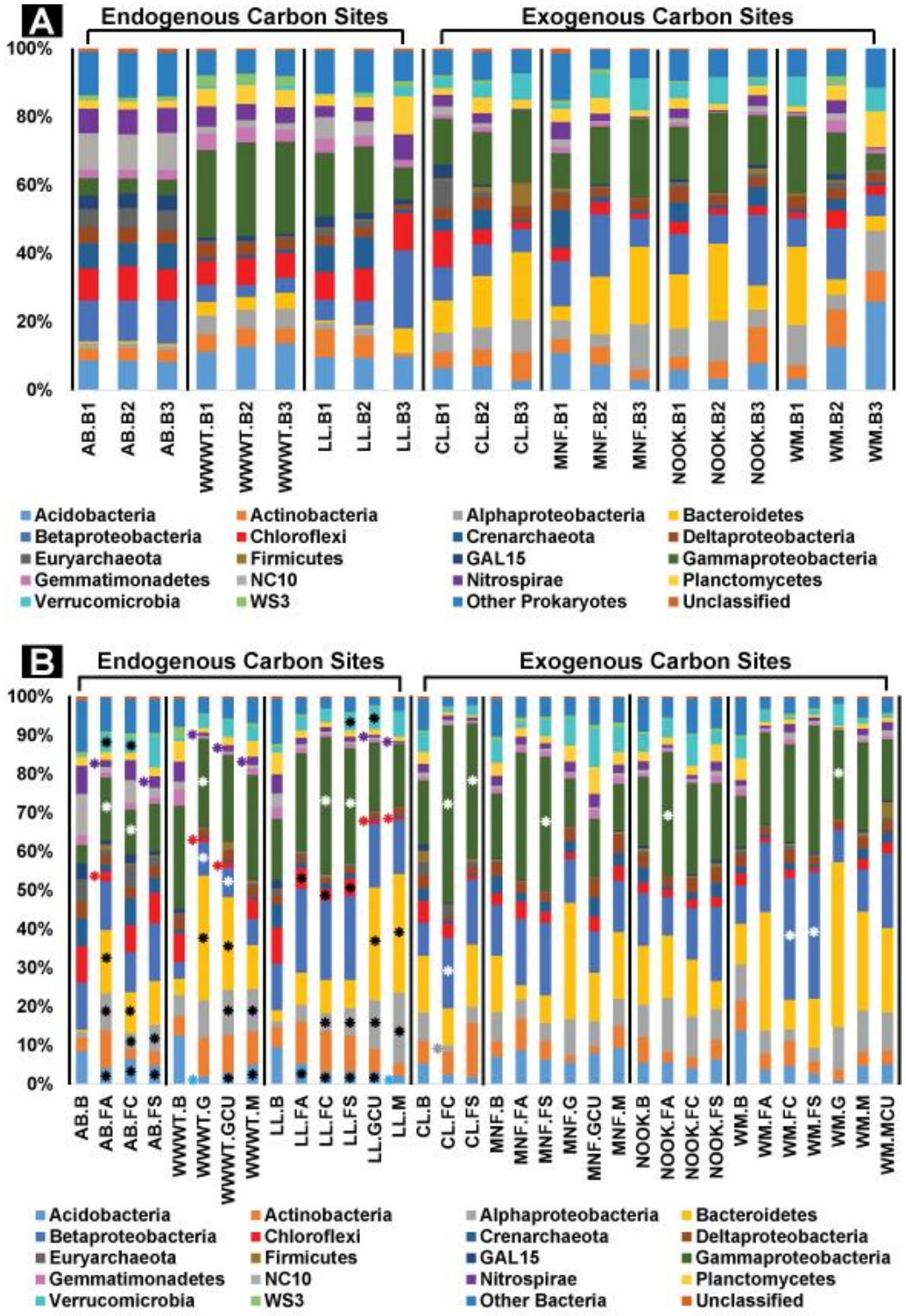

Figure 2. Phyla-level distribution of bacteria and archaea with a 1% abundance cut-off (A) before stimulation (.B) and (B) shows averages of samples for each site and * denote significant increases or decreases ($P < 0.05$) in phyla before (.B) and after stimulation with with arabinose (FA), casamino acid (FC), succinic acid (FS), glucose (G), glucose+Cu (GCU), malt extract (M), malt extract+Cu (MCU).

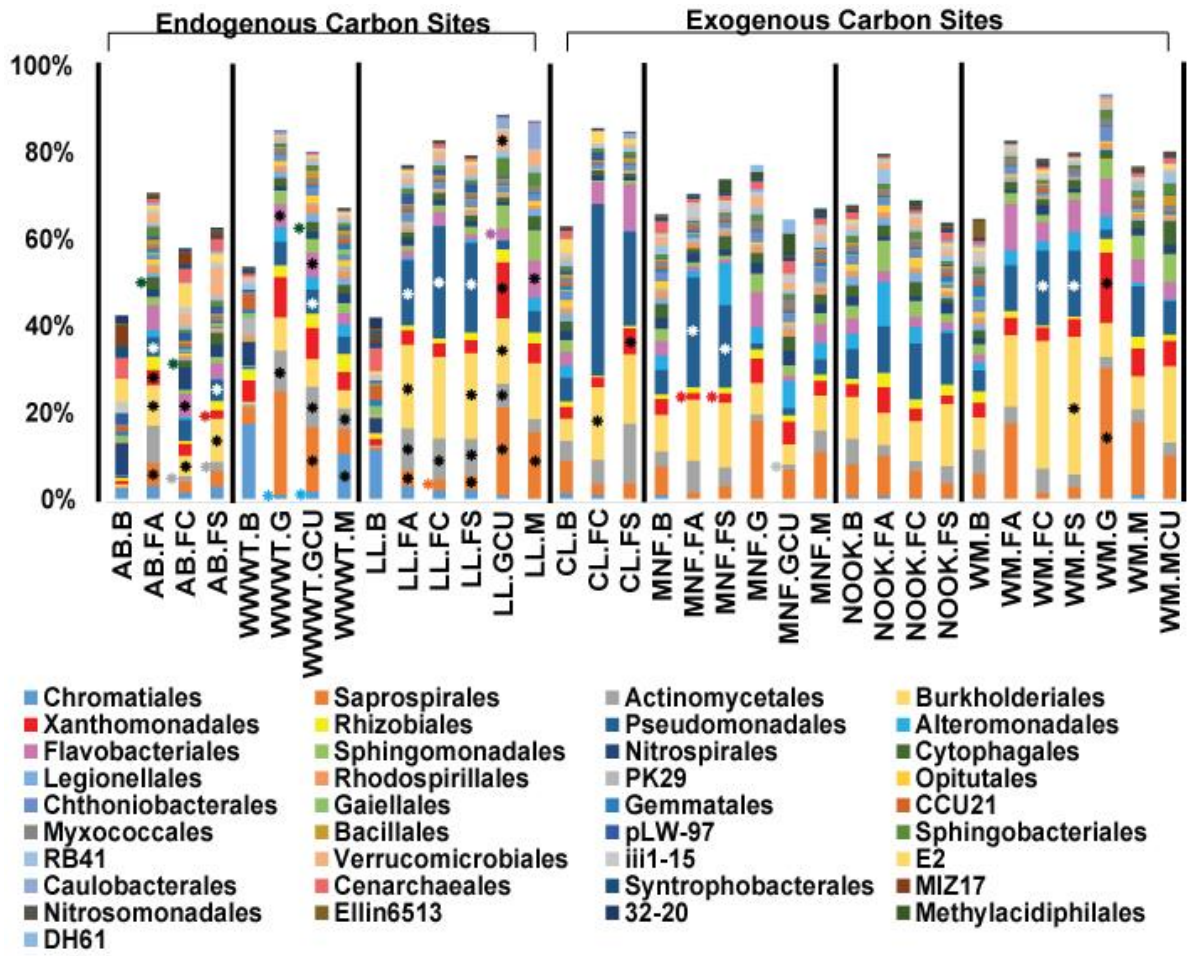

Figure 3. Order-level abundance of bacteria and archaea with a 2% abundance cut-off before (.B) and after stimulation with arabinose (FA), casamino acid (FC), succinic acid (FS), glucose (G), glucose+Cu (GCU), malt extract (M), malt extract+Cu (MCU).

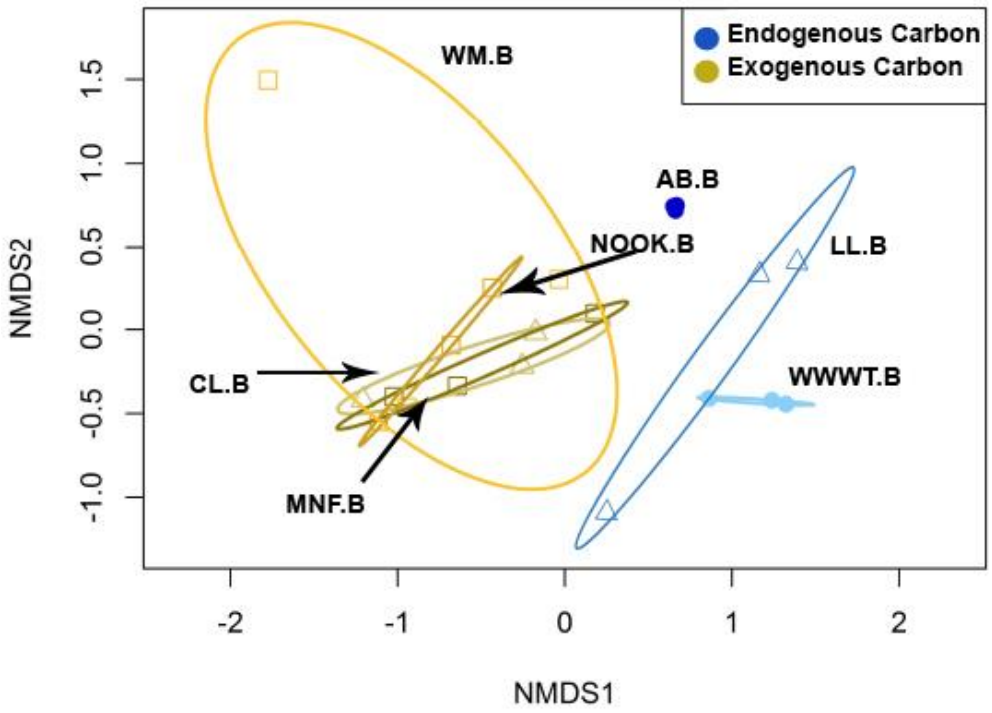

Figure 4. Beta-diversity analysis of baseline bacterial and archaeal communities at sites before incubations using the Jaccard similarity matrix and non-metric multidimensional scaling. Ellipses represent 95% confidence intervals.

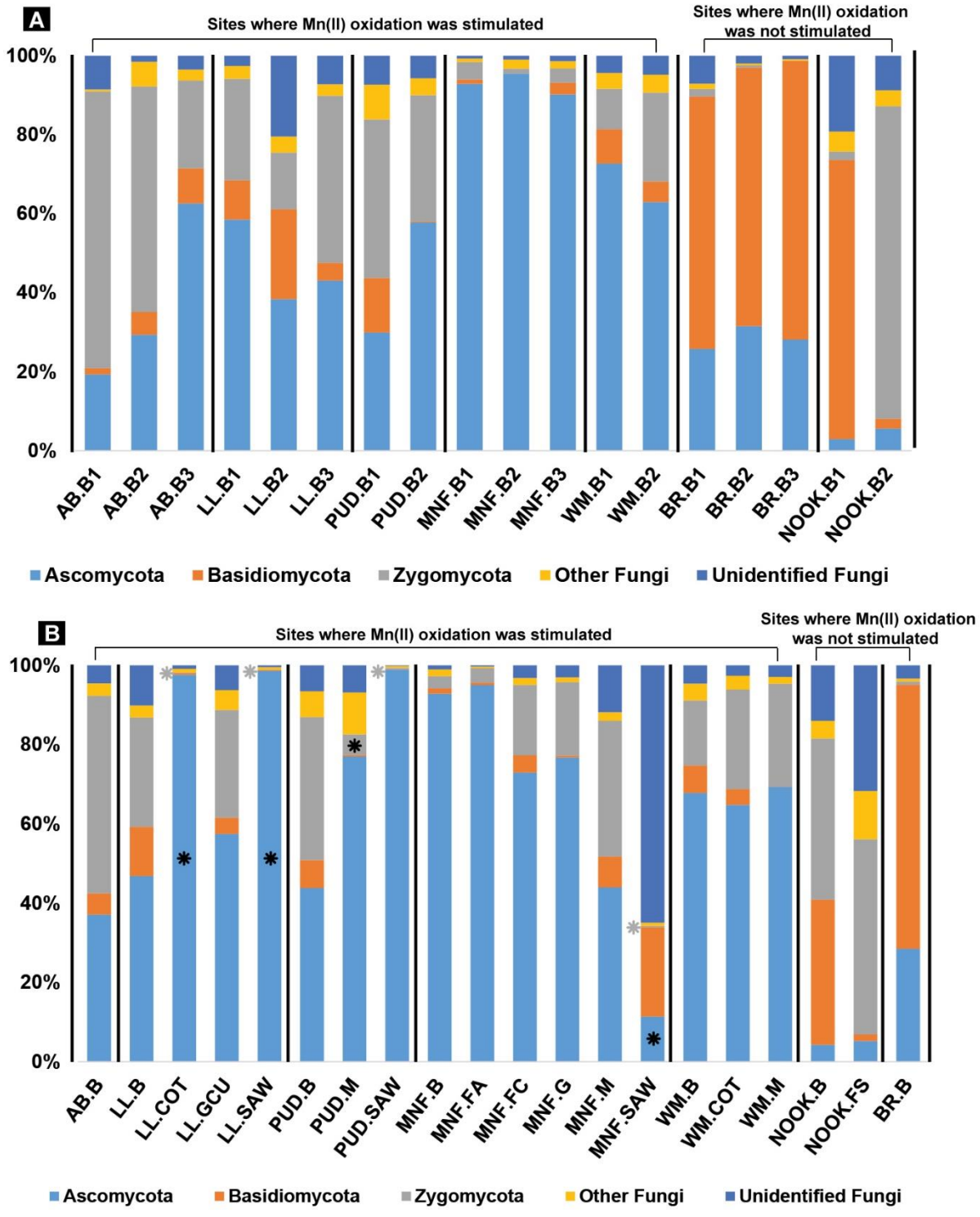

Figure 5. Phyla-level distribution of fungal communities before (A) stimulation with carbon sources. (B) before (.B) and after stimulation with cotton (COT), sawdust (SAW), arabinose (FA), casamino acid (FC), succinic acid(FS), glucose (G), glucose+Cu (GCU), malt extract (M) with a 1% abundance cut-off. * denote significant increases or decreases in phylum abundance after treatment with carbon source.

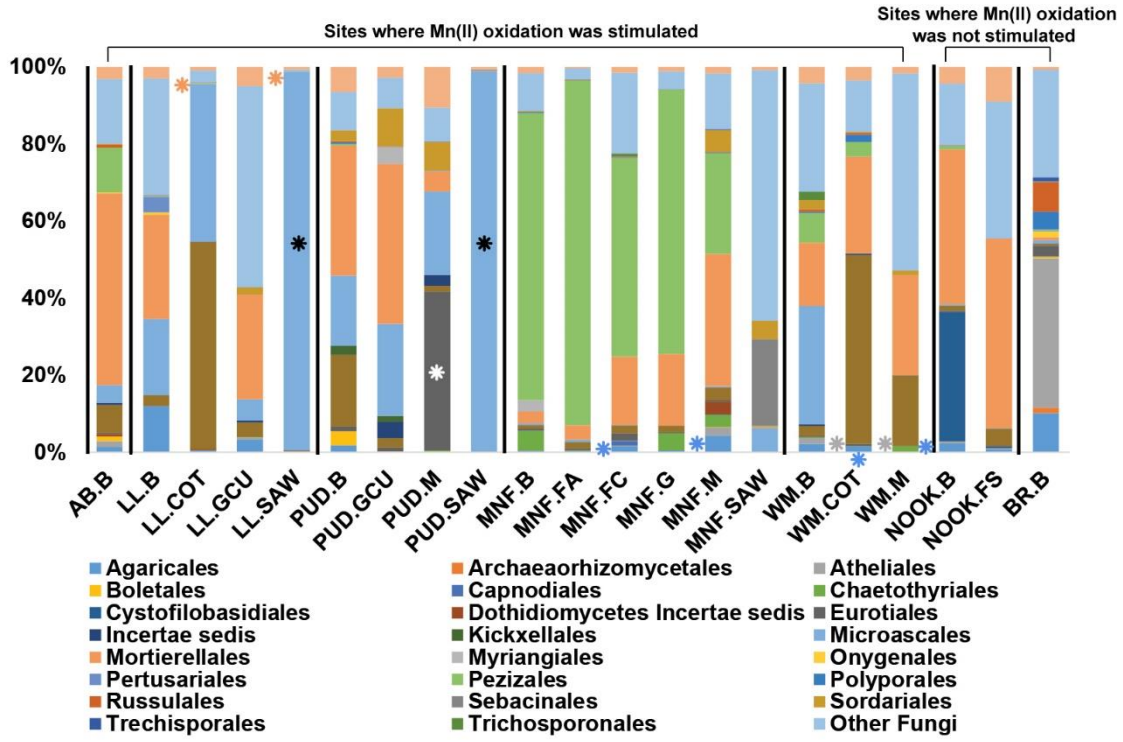

Figure 6. Order-level distribution of fungal communities before stimulation (.B) and after stimulation with cotton (COT), sawdust (SAW), arabinose (FA), casamino acid (FC), succinic acid(FS), glucose (G), glucose+Cu (GCU), malt extract (M) with a 1% abundance cut-off. * denote significant increases or decreases in phylum abundance after treatment with carbon source.

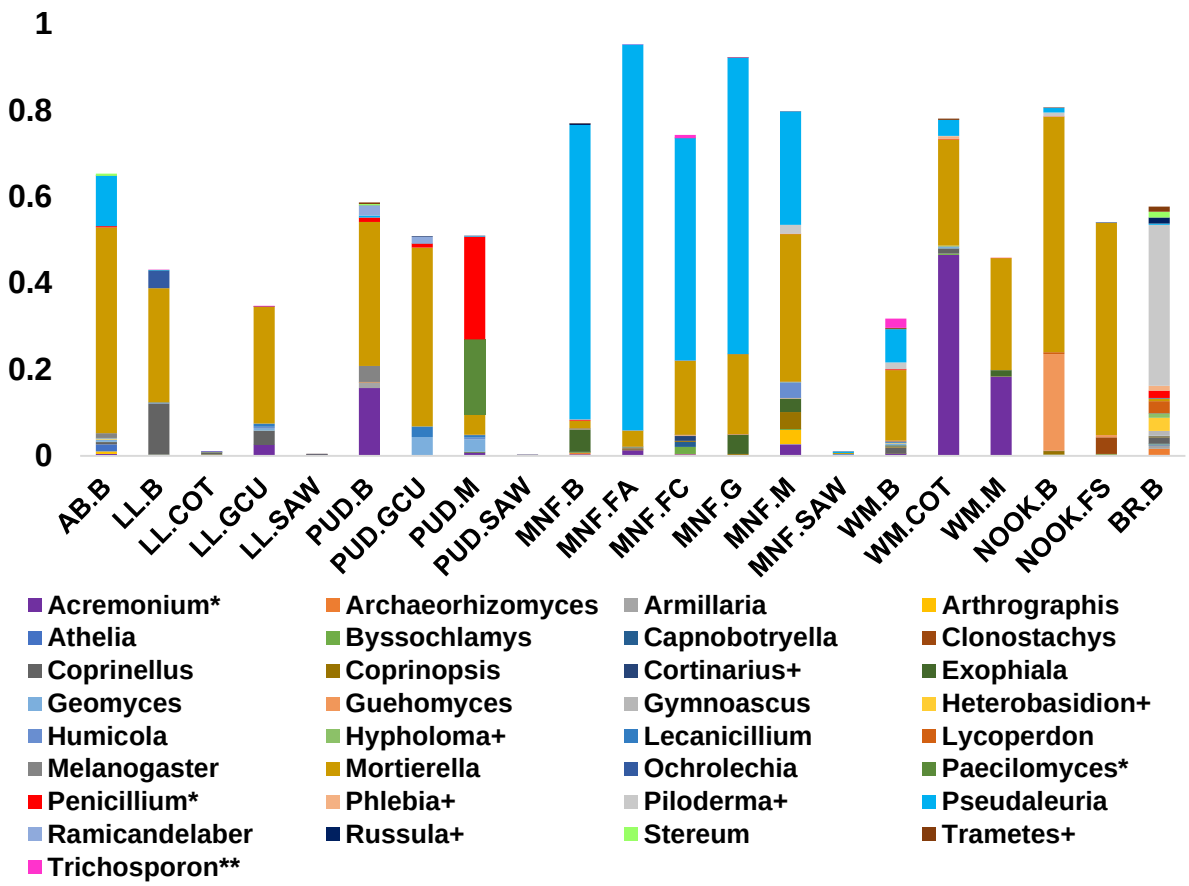

Figure 7. Genera-level distribution of fungal communities before stimulation (.B) with carbon sources and after stimulation with cotton (COT), sawdust (SAW), arabinose (FA), casamino acid (FC), succinic acid (FS), glucose (G), glucose+Cu (GCU), malt extract (M) with a 1% abundance cut-off. * denotes known Mn(II)-oxidizing fungi in the Ascomycota phylum and + denotes known Mn(II)-oxidizing fungi in the Basidiomycota phylum. ** denotes a new Mn(II)-oxidizing fungi from the Basidiomycota phylum isolated in this study.

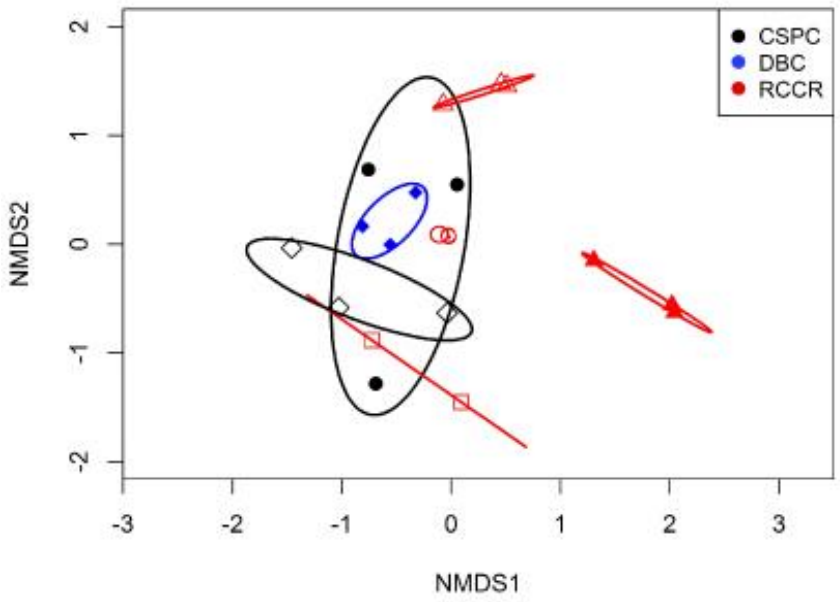

Figure 8. Beta-diversity analysis of baseline fungal communities compared with the Jaccard similarity matrix and non-metric multidimensional scaling. Ellipses represent 95% confidence intervals. Shapes denote separate sites within each cave.

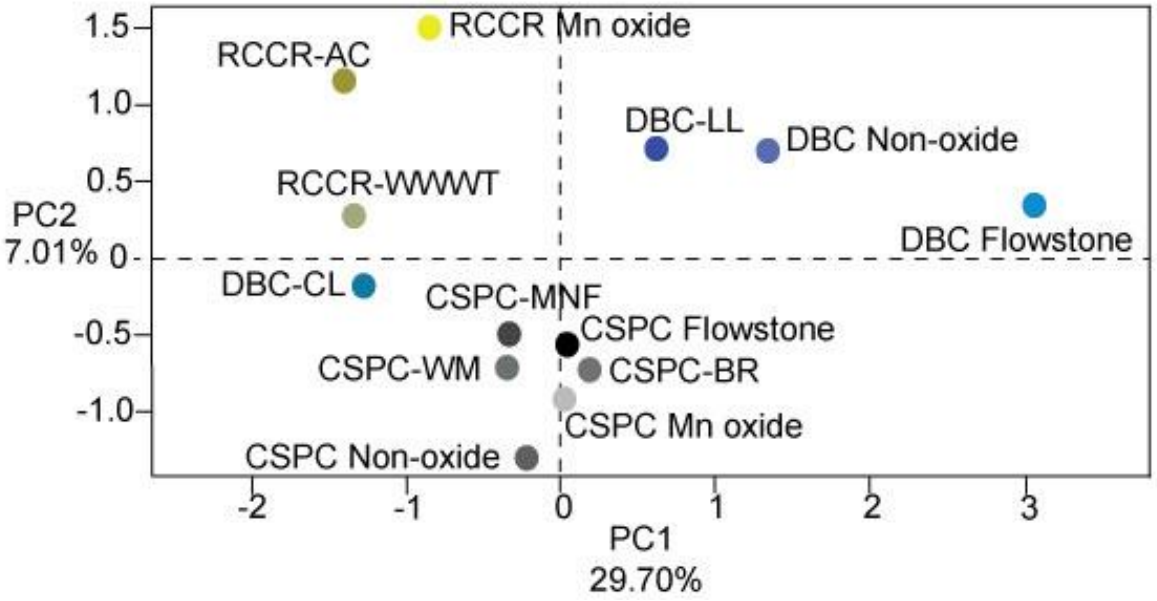

Figure 9. PCA of geochemical analysis performed on sites in CSPC, DBC, and RCCR. Blue shaded data points are CSPC sites, pink shaded data points are RCCR sites and red shaded data points are DBC sites.

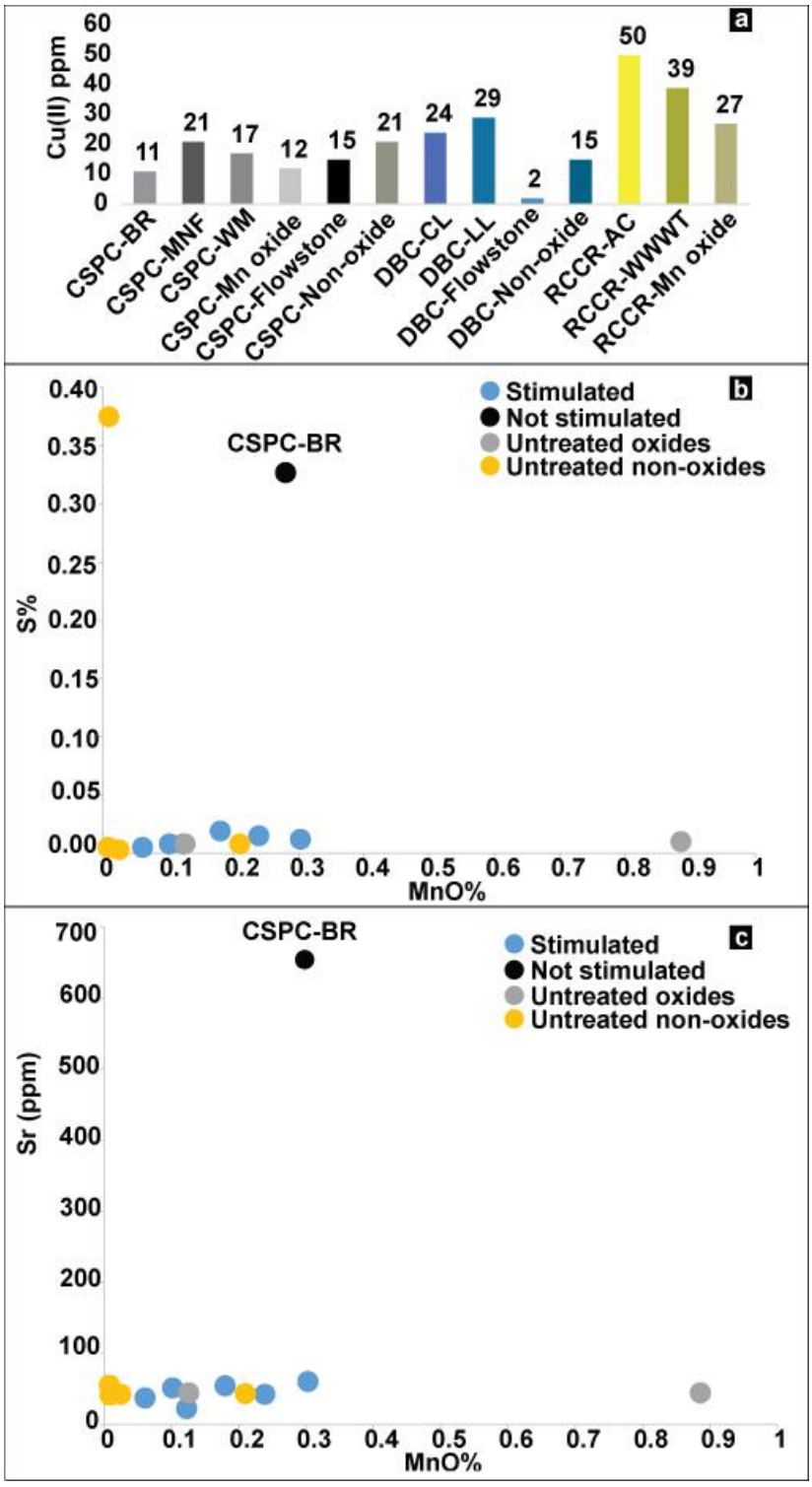

Figure 10. Geochemical analysis of sites at CSPC, DBC, and RCCR where Mn(II) oxidation was stimulated, sites were oxidation was not stimulated (BR), sites where Mn(III/IV) oxides were naturally occurring, and sites where Mn(III/IV) oxides were not present. (A) shows Cu(II) concentrations at CSPC sites in blue shades, DBC sites in red shades, and RCCR sites in pink shades. (B) shows S and MnO concentrations and (C) displays Sr and MnO concentrations.

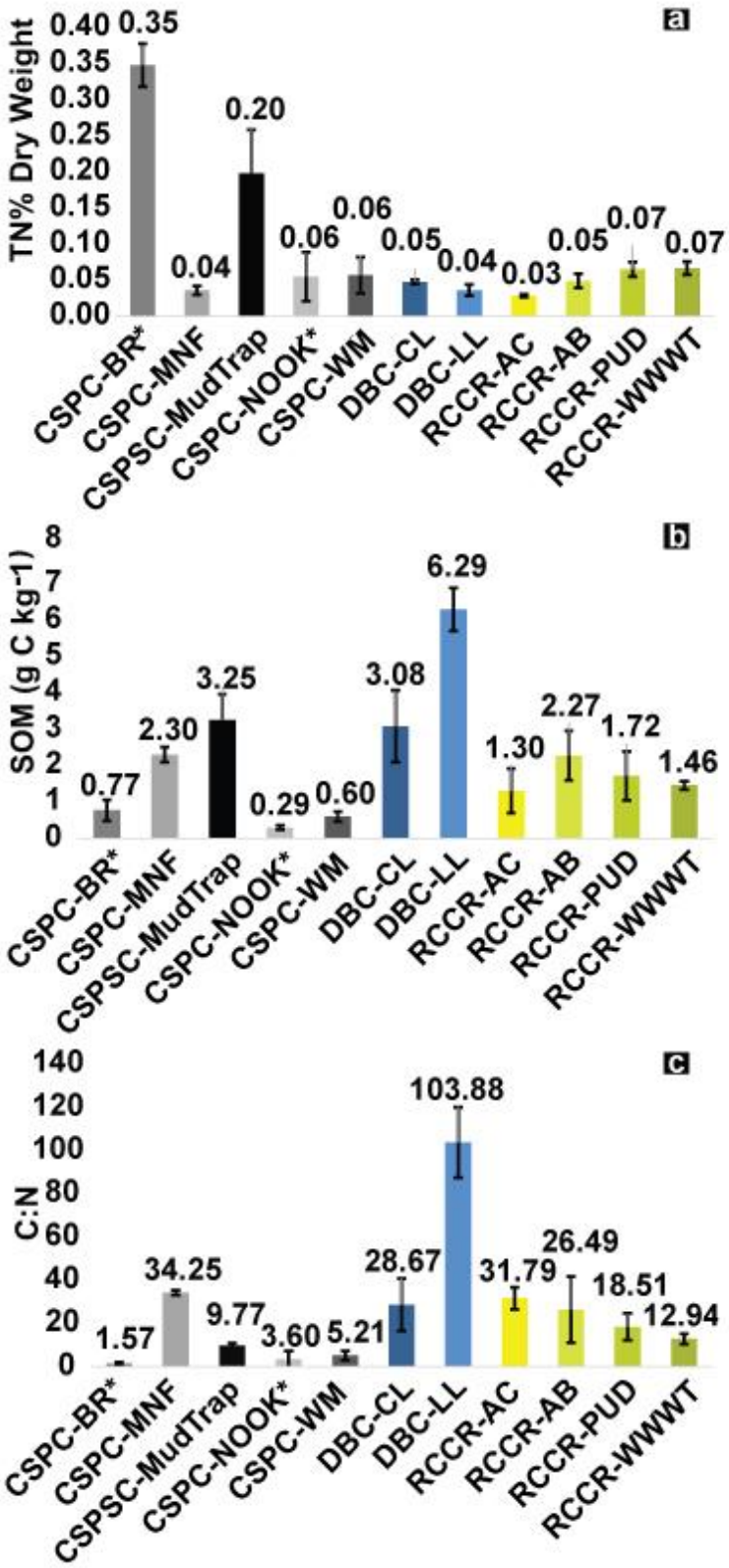

Figure 11. Nutrient concentrations at sites where Mn(II) oxidation was stimulated and where Mn(II) oxidation was not stimulated represented with *. (a) shows TN% from sites (b) is of SOM concentrations at sites and (c) shows C:N ratio's calculated from TC% and TN%.

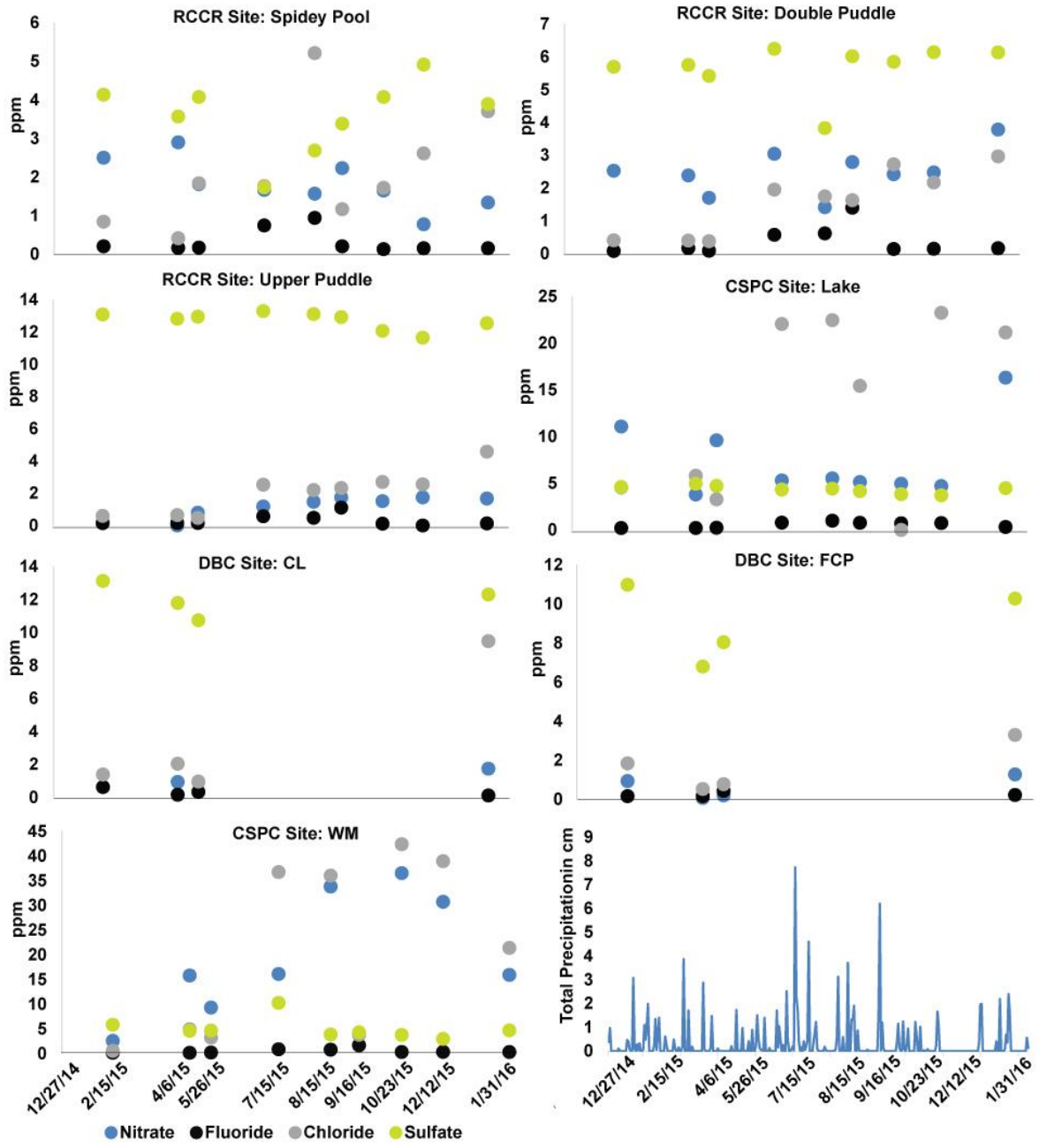

Figure 12. Ion concentrations from water samples collected from CSPC, DBC, and RCCR every six weeks from February 2015 to January 2016 and precipitation data from the Quality Controlled Local Climatological Data taken by NOAA at the Virginia Highlands Airport, VA.

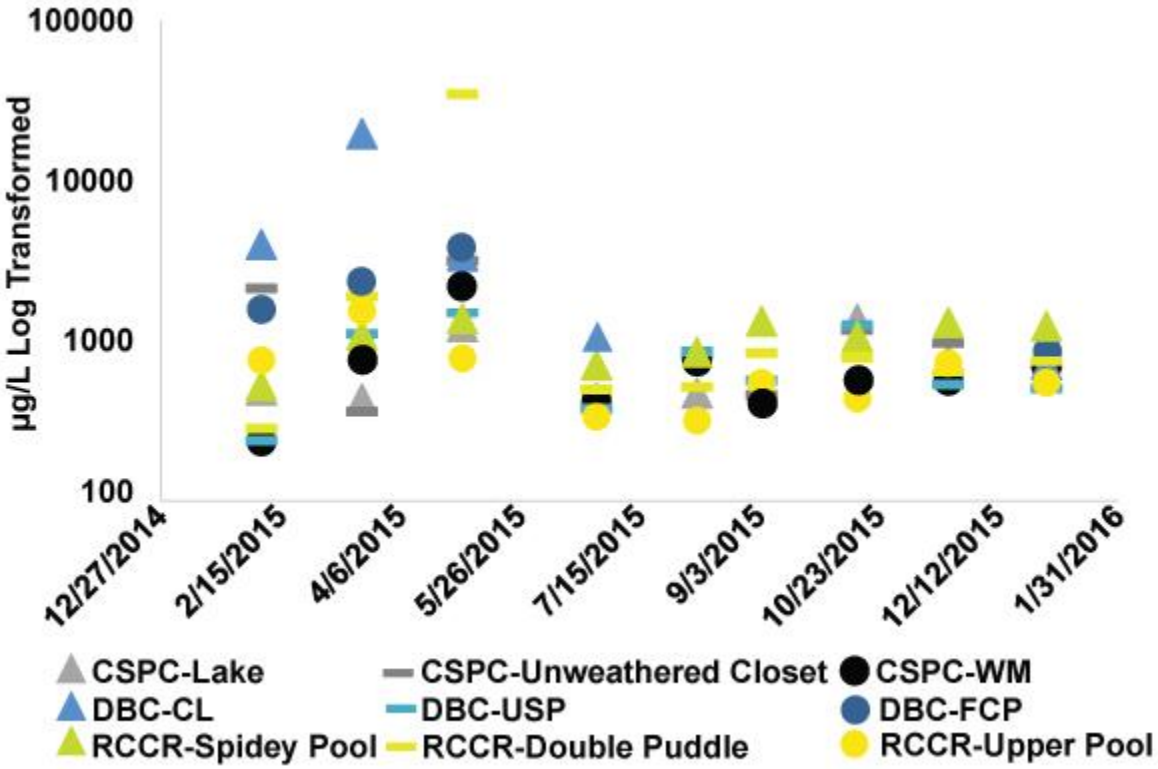

Figure 13. Log transformed TOC concentrations (µg/L) from water samples collected approximately every six weeks from February 2015 to January 2016 at CSPC, DBC, and RCCR.

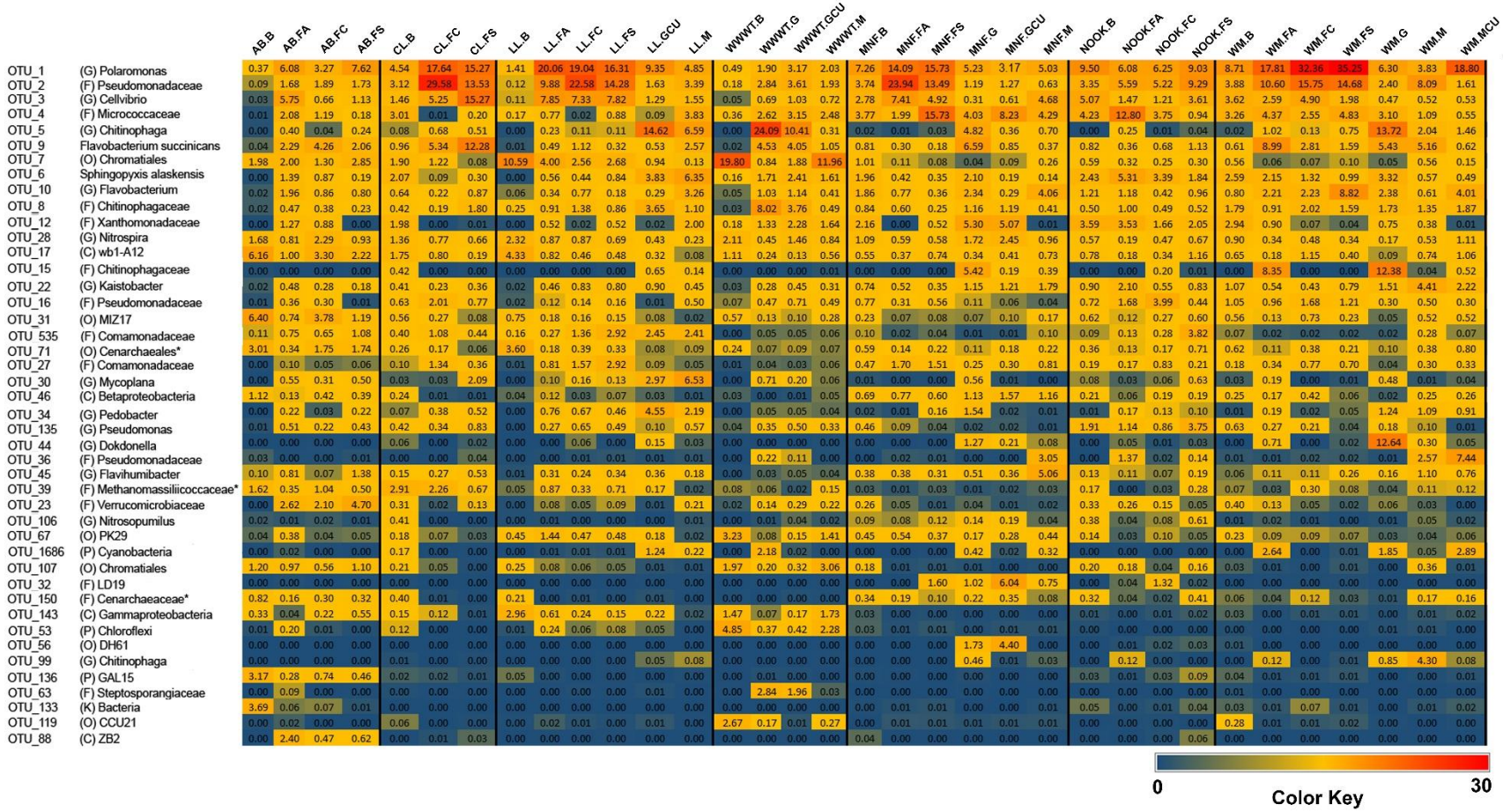

Figure 14. Heatmap of the four most abundant bacterial and archaeal OTUs for each site before (.B) and after stimulation with with arabinose (FA), casamino acid (FC), succinic acid (FS), glucose (G), glucose+Cu (GCU), malt extract (M), malt extract+Cu (MCU). () represents the level that the OTU could be identified to. (G) is genus, (F) is family, (O) is order, (C) is class, (P) is phylum. * denote archaeal OTUs. Numbers represent relative abundance as a %.

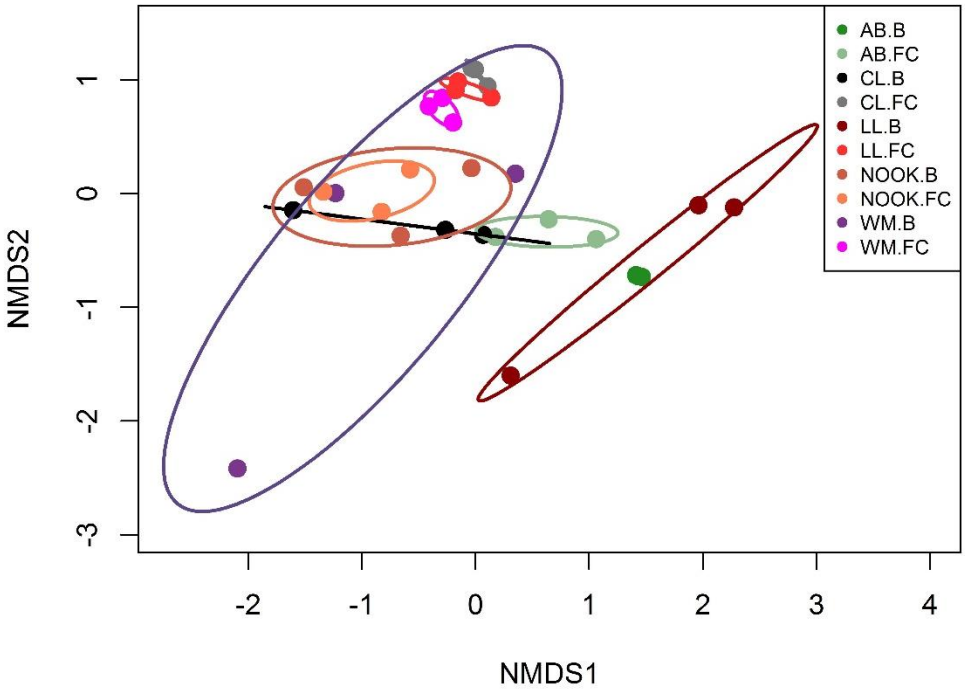

Figure 15. Beta-diversity analysis of sites before and after casamino acid treatments. Samples before (.B) and after treatment with casamino acids (.FC) were compared with the Jaccard similarity matrix and non-metric multidimensional scaling. Ellipses represent 95% confidence intervals.

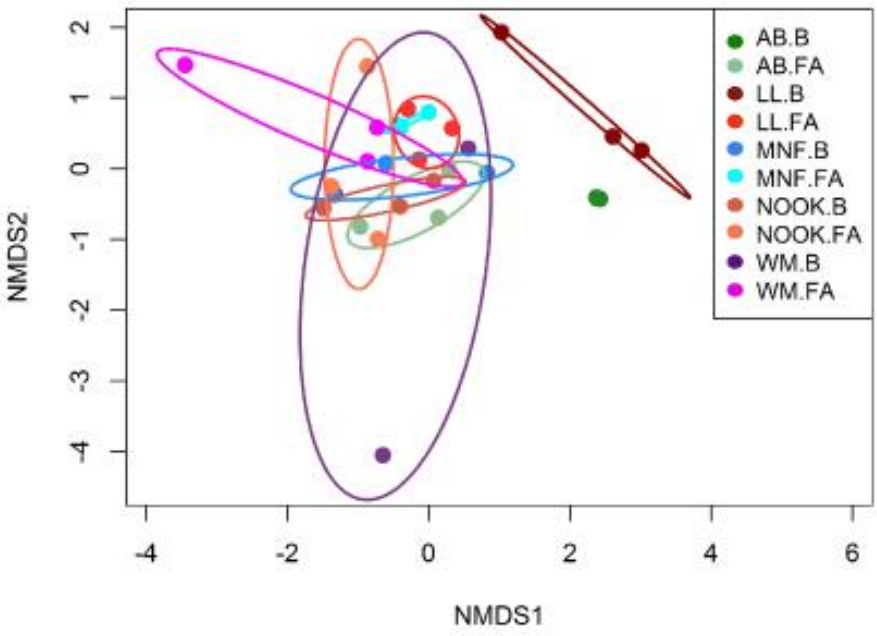

Figure 16. Beta-diversity analysis of sites before and after arabinose treatments. Sites Before (.B) and after stimulation with arabinose (.FA) were plotted using the Jaccard similarity matrix and non-metric multidimensional scaling. Ellipses represent 95% confidence intervals.

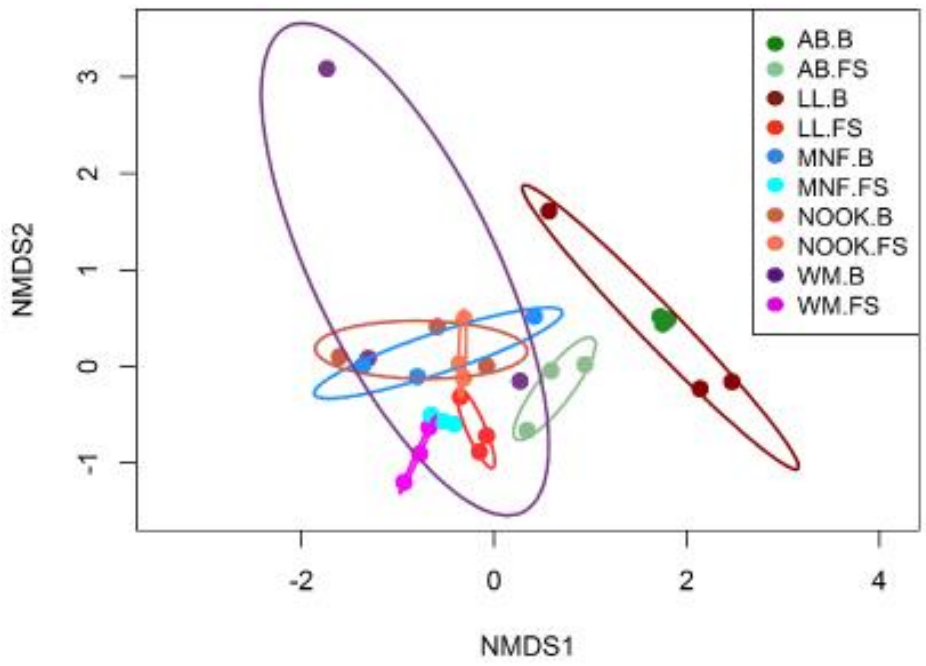

Figure 17. Beta-diversity analysis of sites before and after succinic acid treatments. Before (.B) and after samples treated with succinic acid (.FS) were plotted using non-metric multidimensional scaling and the Jaccard similarity matrix. Ellipses represent 95% confidence intervals.

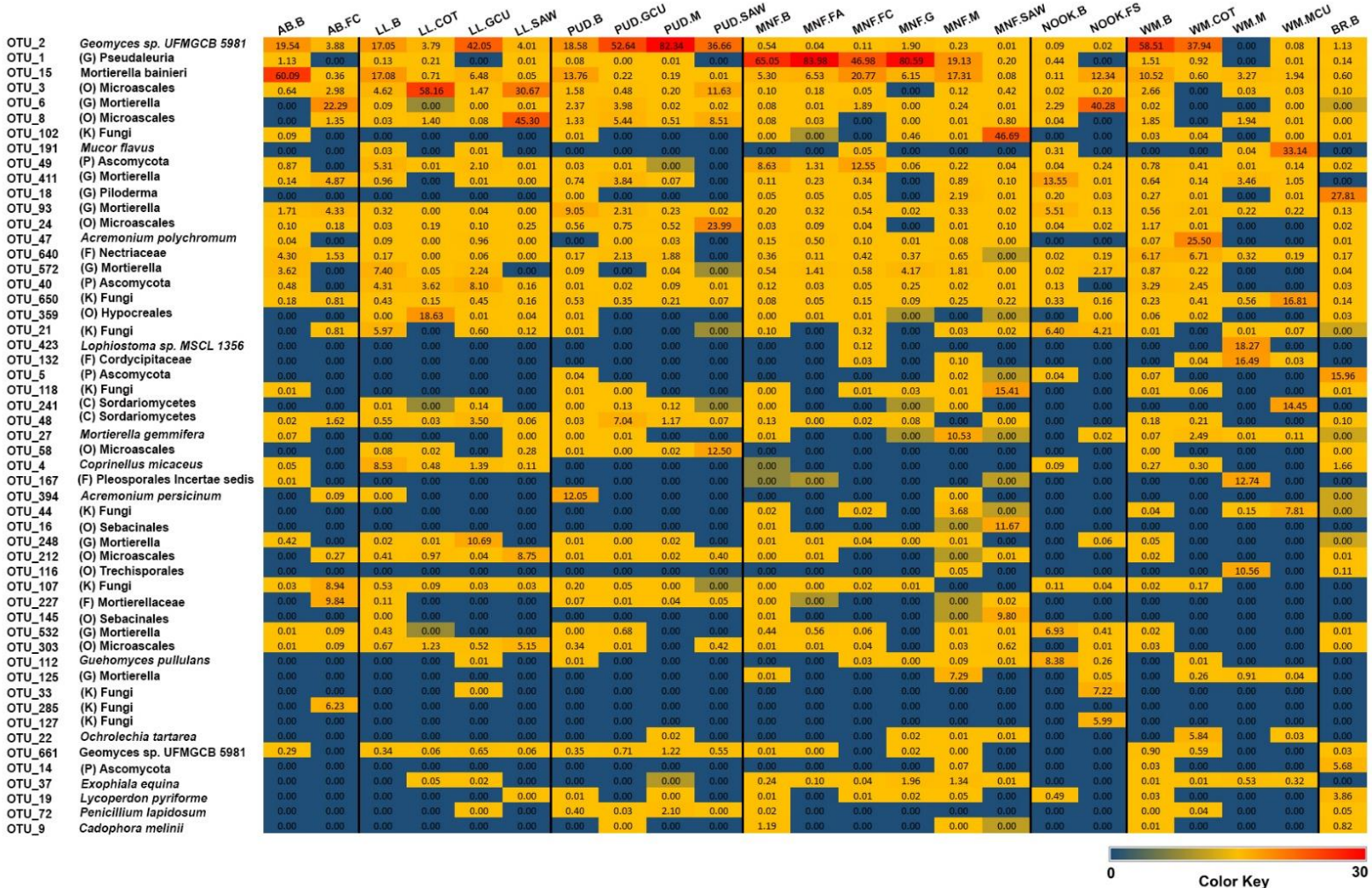

Figure 18. Heatmap of the top most abundant fungal OTUs for each site before (.B) and after stimulation with cotton (COT), sawdust (.SAW), arabinose (FA), casamino acid (FC), succinic acid (FS) glucose (G), glucose+Cu (GCU), malt extract (M). () represents the level that the OTU could be identified to. (G) is genus, (F) is family, (O) is order, (C) is class, (P) is phylum. Numbers represent relative abundance as a %

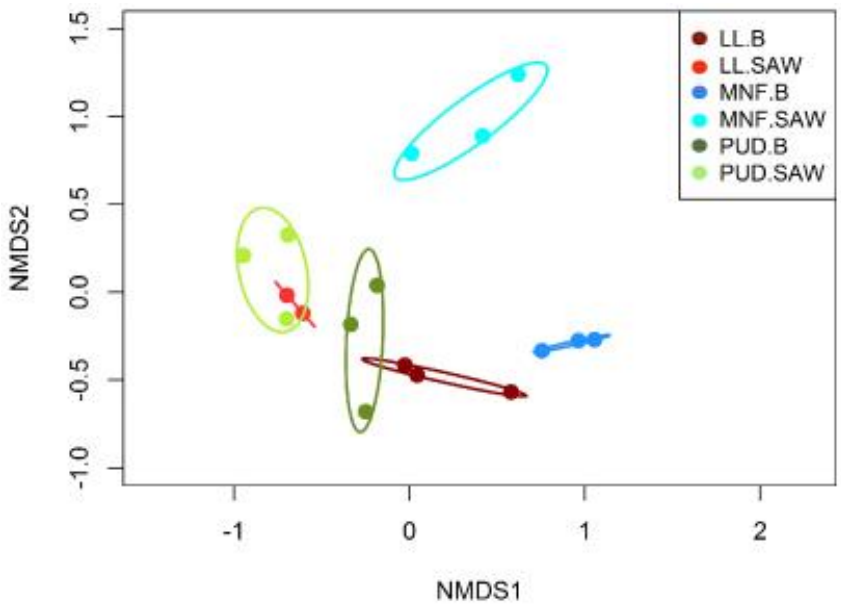

Figure 19. Beta-diversity of fungal communities before (.B) and after sawdust (.SAW) treatments computed with the Jaccard similarity matrix and plotted with non-metric multidimensional scaling. Ellipses represent 95% confidence intervals.

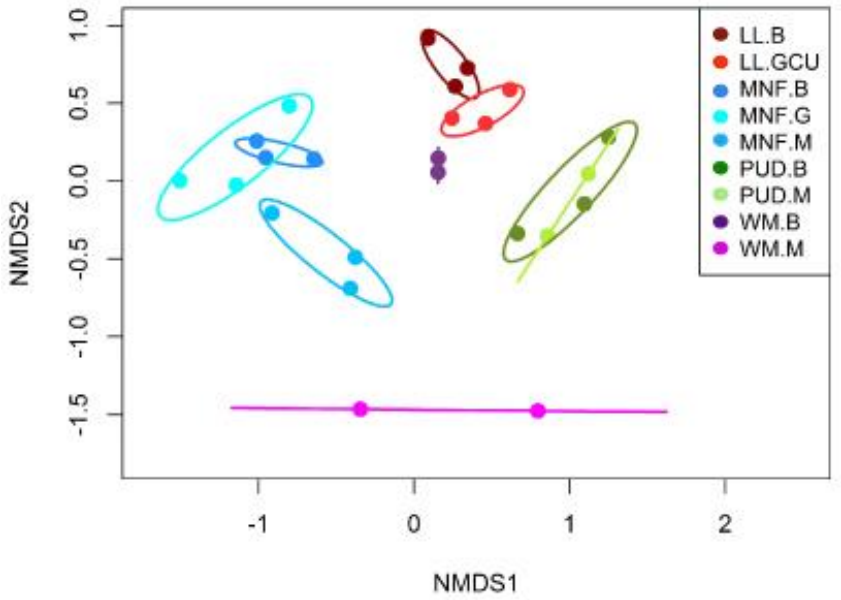

Figure 20. Beta-diversity analysis of fungal communities before and after treatments with simple sugars. Calculated with the Jaccard similarity matrix of fungal communities before (.B) and after stimulation with glucose (.G), glucose+Cu (.GCU), or malt extract (.M) and plotted with non-metric multidimensional scaling. Ellipses represent 95% confidence intervals.

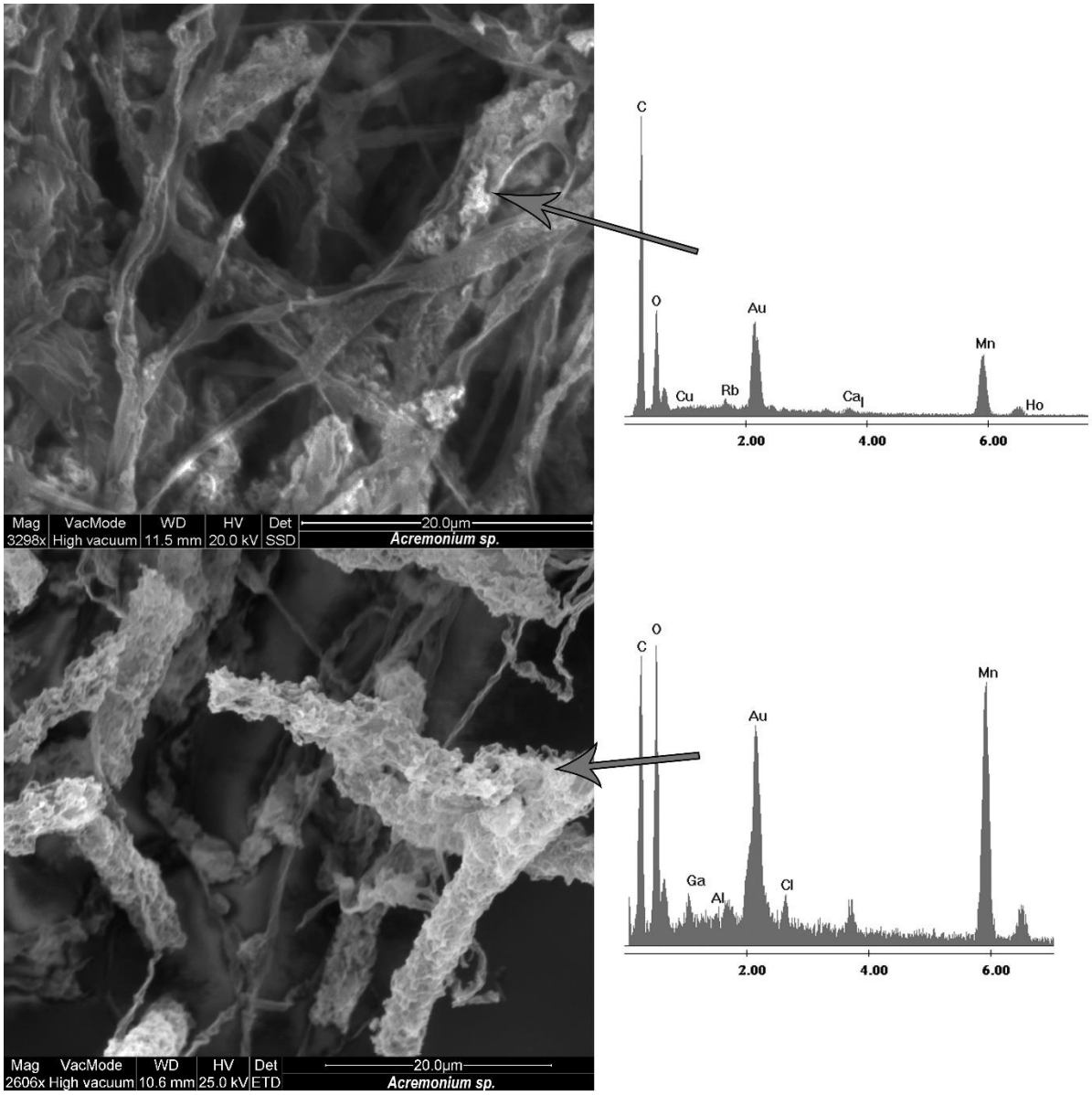

Figure 21. SEM images of two Mn(II)-oxidizing *Acremonium* species isolated from two different sites, one from RCCR-PUD and the other from CSPC-NOOK. EDS elemental analysis confirms Mn(III/IV) oxides surrounding fungal hyphae.

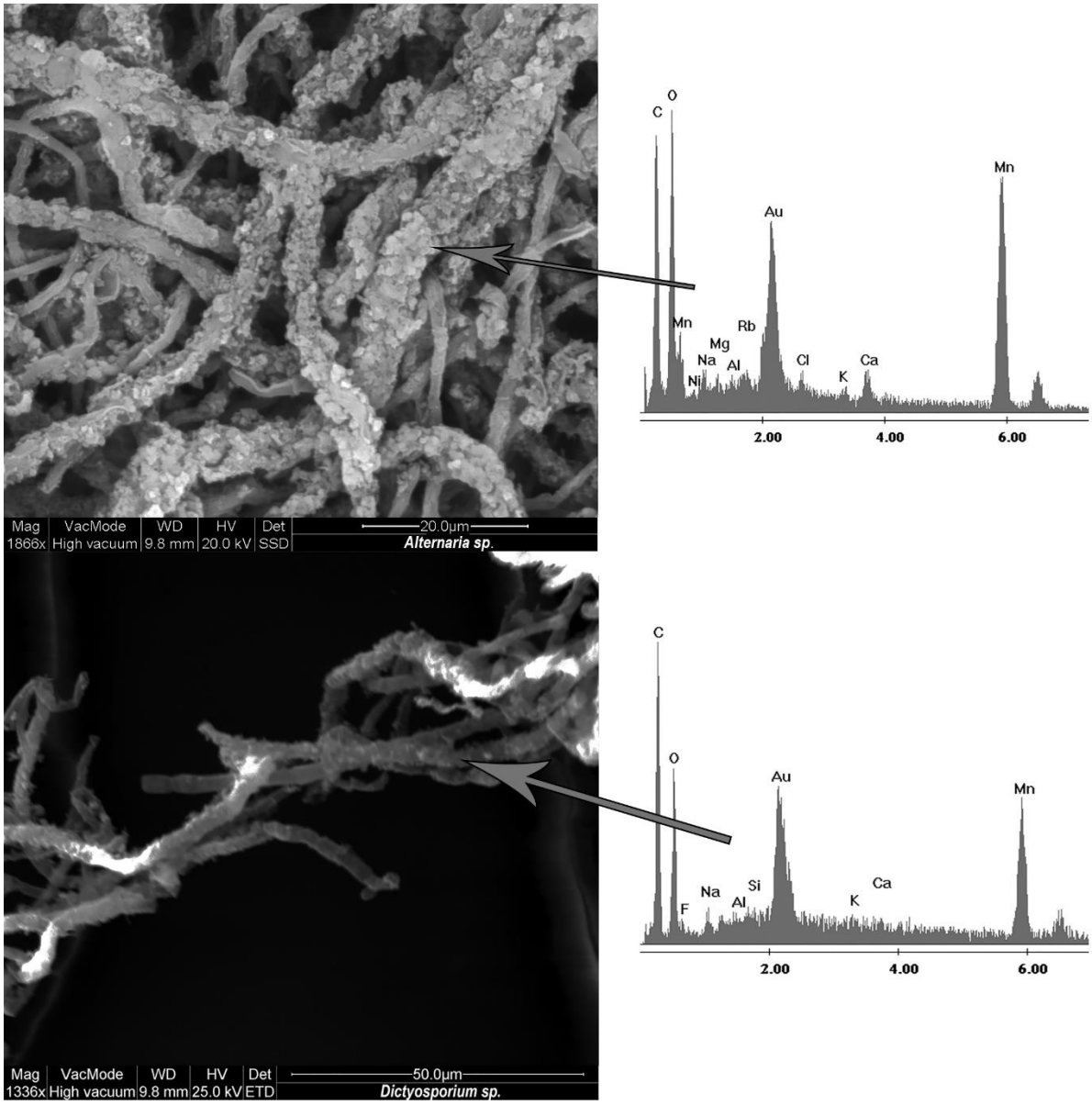

Figure 22. SEM images of Mn(II)-oxidizing *Alternaria* sp. (top) and *Dictyosporium* sp. (bottom) isolated from CBM. EDS elemental analysis shows Mn(III/IV) oxides surrounding fungal hyphae.

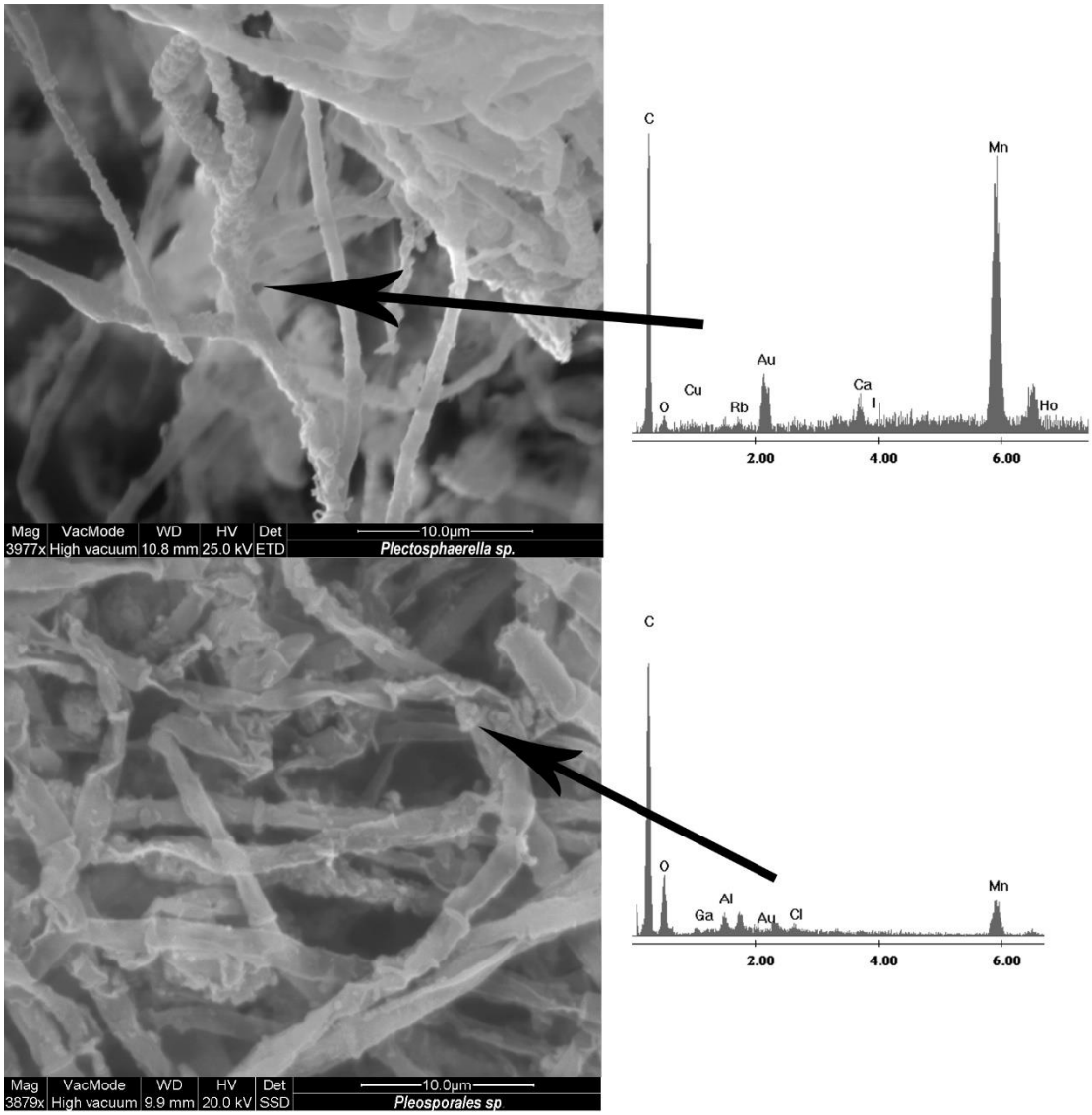

Figure 23. SEM images of Mn(II)-oxidizing *Plectosphaerella* sp. (top) and *Pleosporales* sp. (bottom) isolates from CSPC-MNF. EDS elemental analysis shows Mn(III/IV) oxides associated with the fungal hyphae.

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Vita

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