THE COMPARISON OF MANGANESE (II) OXIDIZING MICROBES IN TWO CAVE SYSTEMS

A Thesis
by
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ABSTRACT

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Manganese (Mn) oxidizing microorganisms are abundant in nature and play an important role in biogeochemical cycles in the environment. Cave and karst systems are important natural resources because they provide significant sources of the world's drinking water and support unique biological communities. The microbial ecology of manganese oxide deposits is not well understood, and even less is known about the factors that stimulate Mn-oxidizing microbes *in situ*. A community analysis of LBB positive and negative control samples from two cave systems was conducted to provide insight into the types of microbes that were present in each site location. Extracted DNA from clay/rock samples of 20 sites in Daniel Boone Caverns, a pristine cave environment, and Worley's Cave, an anthropogenically impacted environment, was analyzed through Illumina amplicon sequencing to observe the differences in the bacterial/archaeal and fungal communities in different cave environments. The prokaryotic communities for the LBB positive samples in Daniel Boone Caverns were most dominated by Betaproteobacteria, Latescibacteria, and Gemmatimonadetes. The LBB positive samples in Worley's Cave varied based on location

within the cave. Samples located along the spur passage were most abundant with Gammaproteobacteria and Actinobacteria, while samples located along the river running through the cave were most abundant with Alphaproteobacteria, Actinobacteria, and Gemmatimonadetes. The medium for the assembly of bacterial communities can depend on cave niches and the nutrients available. Predicting how and where Mn oxides will grow is a vitally important tool for the production of reactive mineral species, which can be used in a variety of water filtration and bioremediation technologies. This data may provide insight into the community make-up and interactions of Mn oxide communities and how environmental factors can drive diversity.

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Finally, I would like to thank my parents, Dale and Kim Smith, for their continuous support. They are my ultimate role models whose love and guidance are with me in whatever I pursue. This accomplishment would not have been possible without them.

DEDICATION

I dedicate this thesis to my parents, Dale and Kim Smith, for always giving me the encouragement and support that I needed in order to be successful throughout my academic career.

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CHAPTER 1: INTRODUCTION

Cave systems and karst environments provide a relatively stable environment regardless of seasonal variation, but they can be sensitive to anthropogenic disturbance. These environments are nutrient limited and little is known about their microbial taxonomic diversity, and how microbial communities change over time (Engel et al., 2004). Cave environments hold some of the most diverse microbial communities on Earth (Barton and Jurado, 2007), yet they are relatively understudied. There have been more than 50 molecular studies on bacteria in caves (Engel, 2010) but only a few of those studies have been conducted in the southern Appalachian Mountains (Carmichael et al., 2013a; Carmichael et al., 2013b; Cloutier et al., 2017; Zorn, 2014), which is a national hot spot for caves. Predicting how and where Mn oxides will grow is a vitally important tool for the production of reactive mineral species, which can be used in a variety of water filtrations systems and bioremediation technologies.

Many caves contain dark brown to black manganese (Mn) oxide biofilms, deposited from Mn-oxidizing bacteria and fungi (see review by Carmichael and Bräuer, 2015). There are more than 30 Mn oxide minerals that occur in a wide array of geological settings (Post, 1999), and they serve as major components of rock crusts in some caves and streams (Carmichael and Bräuer, 2015; Tebo et al., 2007). They are some of the strongest oxidants found in the environment, impacting transport and speciation of metals, cycling of carbon, and flow of electrons within soils and sediments (Nealson et al., 1988; Tebo et al., 2005). Mn oxide minerals are ubiquitous in soils and clays and are involved in a variety of chemical reactions that affect groundwater and soil composition (Post, 1999); thus, they are important

in karst systems, which host a significant portion of the world's drinking water (Andreo et al., 2008). They are highly reactive due to their many vacancies, negatively charged surfaces, and adsorptive properties making them important biological tools for remediation of contaminated surfaces and groundwater (Hennebel et al., 2009; Luan et al., 2012; Santelli et al., 2010). In most surface and groundwater environments, Mn(II) is soluble in water unless it is complexed with specific ligands such as pyrophosphate or siderophores (Harrington et al., 2012; Madison et al., 2011; Trouwborst et al., 2006), or is part of a Mn(III/IV) oxide or oxyhydroxide mineral (Tebo et al., 2004). The most common valences of Mn found in nature include Mn(II) (the dissolved form of Mn), and Mn(III) and Mn(IV) (as Mn oxide minerals). The biologically mediated Mn oxide minerals are typically either birnessite or todokorite, although other minerals have been observed that consist of poorly crystalline MnO_6 complexes (Carmichael and Bräuer, 2015; Soldatova et al., 2012). These Mn oxide minerals also readily undergo cation-exchange reactions (Golden et al., 1986) which can greatly affect the chemistries of soils and aqueous solutions (Post, 1999).

Mn oxides associated with cave systems are often simply referred to as ferromanganese deposits (Carmichael et al., 2013b; Miyata et al., 2007; Northup et al., 2003; Zorn, 2014), and contain a higher concentration of Mn and Fe than the surrounding bedrock (Carmichael et al., 2013b; Frierdich et al., 2011; Northup et al., 2003; Spilde et al., 2005). Fe and Mn oxide minerals are similar in their geochemical properties, but they differ in their color, formation mechanism, mineralogical structure, and ability to separate heavy metals and trace elements (Frierdich et al., 2011). Fe and Mn deposits in caves are thought to be mediated primarily by microbial activity (Baskar et al., 2008; Kasama and Murakami, 2001). Overall, there is only limited knowledge regarding the microbial consortia associated with

cave ferromanganese deposits, located either in the southwest United States (Cunningham et al., 1995; Northup et al., 2003; Spilde et al., 2006; Spilde et al., 2005) or in the southeastern United States (Carmichael et al., 2013a; Carmichael et al., 2013b; Carmichael et al., 2015; Cloutier et al., 2017; Zorn, 2014).

Despite a widespread prevalence of Mn oxides within cave systems throughout the US (Northup et al., 2003; White et al., 2009), there is very little molecular data that can define the roles that Mn oxidizing microbes perform in caves (Carmichael and Bräuer, 2015; Engel, 2010). Bacteria, fungi, and algae have all been involved with Mn oxidation, but the archaeal domain has never been involved (Zhou and Fu, 2020). Until this year all of the Mn oxidizers that were isolated were heterotrophic (Carmichael and Bräuer, 2015; Yu and Leadbetter, 2020). Whether chemolithoautotrophic Mn-oxidizing microorganisms exist had been an open question for over a century (Beijerinck, 1913; Nealson et al., 1988; Tebo et al., 2005). Yu and Leadbetter (2020) have recently proven their existence, providing insight into the details and dynamics of the process at cellular, physiological, genomic, transcriptomic and isotopic levels.

Field evidence from epigenic caves in the southern Appalachian Mountains indicate that Mn oxidation is stimulated by exogenous carbon input (Carmichael et al., 2013a; Carmichael et al., 2015; Cloutier et al., 2017) into carbon limited environments. The majority of caves rely on allochthonous organic matter transported from the surface (Chen et al., 2009; Engel et al., 2004), because the absence of sunlight prevents photosynthetic primary production. In contrast, a near pristine cave is nutrient limited and often contain levels of organic carbon that are three orders of magnitude below that of the surface environments (Lavoie et al., 2017).

Previous work suggests that solid input of exogenous carbon into caves stimulates fungal Mn oxidation, while a liquid input of exogenous carbon stimulates bacterial Mn oxidation (Figure 1) (Carmichael et al., 2015; Cloutier et al., 2017). This discrepancy between community structures supports the idea that simple and complex sugars support fungally driven Mn oxidation, while organic acids support bacterially driven Mn oxidation (Carmichael et al., 2015). Culturing studies of Mn-oxidizing bacteria have resulted in the isolation of *Leptothrix*, *Janthinobacterium*, *Flavobacterium*, *Arthobacter*, *Acinetobacter*, and *Psudomonas* spp. from caves in the southern Appalachians (Carmichael and Bräuer, 2015). Fungal communities in cave environments have been even more understudied than bacteria, despite their importance as primary decomposers in soil ecosystems (Lindahl et al., 2007; O'Brien et al., 2005). Members from the Glomerales, Pleosporales, Ascomycota, Basidiomycota, and Zygomycota orders have been isolated from Carter Saltpeter Cave and Daniel Boone Caverns (Carmichael et al., 2015; Cloutier et al., 2017). Addition of glucose can cause changes to the fungal communities which suggests that microbial communities can be influenced by exogenous input and impacting ecosystems through bottom-up effects and altering the biogeochemical cycling of nutrients and metals (Zorn et al., 2013).

Hypogene caves of the southwestern USA are deeper and receive less organic input, resulting in reported autotrophic Mn oxidation (Northup et al., 2003; Tebo et al., 2005). It is also possible that Mn oxidizers simply rely on autotrophic organisms, such as Fe oxidizers, to provide organic substrates for oxidation (Spilde et al., 2005). This helps explain why Mn oxides can form black crusts associated with orange iron oxide layers that can be found in caves (Frierdich et al., 2011; Spilde et al., 2005) and deep-sea environments (Edwards et al., 2011; Templeton et al., 2005). These studies highlight the fact that the composition of Mn-

oxidizing bacteria may change dramatically depending on environmental conditions such as carbon source, or geologic substrate. Therefore, the focus of this work is to determine the relationships between mineral substrates and microbial assemblages in the field. For this study, we hypothesized that specific Mn-oxidizers and/or oxidizing communities may demonstrate a preference for specific mineral surfaces. This research will allow us to facilitate the research and management of cave ecosystems by identifying the Mn oxidizers that preferentially colonize quartz, calcite, and feldspar or clay minerals. Understanding how and where Mn oxides grow can be beneficial for the creating and reactive mineral species, which can be used in a variety of water filtration systems.

CHAPTER 2: METHODS

Sample Sites

To determine how Mn(II) oxidizing microbes preferentially colonize new surfaces, two caves were chosen for this study: Worley's Cave (WC) (Figure 2) and Daniel Boone Caverns (DBC) (Figure 3). Worley's Cave is an epigenic cave within the Upper Tennessee River Basin located in Sullivan County, Tennessee (Figure 4). It is frequently visited by humans on guided tours making it an anthropogenically impacted cave. It contains a subterranean creek system that exits the cave and flows to the south fork of the Holstein River. Worley's Cave is hydrologically connected to sinkholes in nearby farm fields that may contain agricultural runoff (Gao et al., 2006). In contrast to this anthropogenically impacted cave, Daniel Boone Caverns, located in Scott County, Virginia, is a private, gated, rarely visited, relatively pristine cave (Figure 4). Daniel Boone Caverns is located in an isolated forest on the top of a ridge and is not subject to agricultural runoff. The only source of water it contains is natural drip pools and drip networks. These caves are only 37 miles apart and therefore experience similar weather patterns and precipitation amounts and have identical bedrock geology as they are both located within the Ordovician upper Knox Dolomite.

To test for Mn positive deposits, areas that suggested the presence of Mn oxidation were tested with leucoberbelin blue (LBB), a redox indicator that oxidizes Mn(III) and Mn(IV) to produce a bright blue color change. In WC, quartz and calcite pebbles were ground into pieces approximately 5 mm in diameter and were placed in at least three areas in WC that were LBB positive. Additionally, a minimum of three areas in WC that were negative or only mildly positive were selected as control sites to incubate the quartz and calcite pieces. For samples that were incubated in water or drip pools, 3–5mm pieces of

quartz, calcite, and both K-feldspar and plagioclase feldspar were placed in tulle bags to prevent them from washing away. Worley's Cave contained 24 tulle bags placed for *in situ* incubation (6 containing quartz, 6 containing calcite, 12 containing potassium and plagioclase feldspar), and DBC still contains 16 tulle bags placed for incubation *in situ* (8 containing quartz and 8 containing calcite).

Baseline Community Sampling

Rock and clay samples that were Mn positive and Mn negative or mildly negative were collected in duplicate from each site before incubations. The samples were transferred on ice and stored at -80°C. DNA was extracted from the clay samples using the MP Biomedicals Fast DNA™ Spin Kit for Soil. DNA concentrations were measured using a Fisher Scientific NanoDrop 1000 spectrophotometer. The extracted DNA samples were then shipped to Molecular Research LB Mr. DNA (Shallowater, TX, USA) for PCR amplification and paired-end sequencing using a MiSeq sequencer. The extracted DNA was amplified using PCR and barcoded primers. Bacteria DNA samples were amplified using the 515F and 806R primers and ITS primers for fungal isolates.

Microbial Community Sampling

Upon receiving results, samples were demultiplexed into individual fastq files using Sabre (Lee, 2019). The individual fastq files were then processed with RStudio v.4.0.0 and Python to observe the quality of the reads. The DADA2 package executes a pipeline to turn paired-end fastq files from the sequencer into merged, denoised, chimera-free, sample sequences (Callahan et al., 2016b). The sample sequences were quality filtered and

assembled into error corrected amplicon sequence variants (ASVs) using DADA2 v1.12 (Callahan et al., 2016b), which represent unique prokaryotic taxa and fungal taxa. Taxonomic classifications were performed on the unique ASVs using DADA2 and the RDP classifier (Wang et al., 2007) for bacterial and archaeal classifications, and UNITE (Kõljalg et al., 2013) for fungal classifications.

The phyloseq R package is a tool used to import, store, analyze, and graphically display complex phylogenetic sequencing data that has already been clustered into operational taxonomic units (OTUs) (McMurdie and Holmes, 2013). Prior to beta-diversity analysis, the final data was transformed to proportions (Callahan et al., 2016a). The following packages were used to plot distance matrices in non-metric multidimensional scaling (NMDS) (Kruskal, 1964) in R: Vegan (Oksanen et al., 2010), ggplot2 (Wickham, 2016), phyloseq and RColorBrewer (Neuwirth and Brewer, 2014). Vegan and phyloseq were used to calculate alpha diversity Shannon and Simpson measurements and observed OTUs (Oksanen et al., 2010). Statistical significance ($\alpha = 0.05$) among taxon level abundance between sample sites before incubations was calculated with a Student's t-test in Microsoft Excel. Vegan, ggplot2 and phyloseq were also used to calculate PCoA unweighted unifrac (Lozupone and Knight, 2005) analysis of the bacterial and archaeal samples.

CHAPTER 3: RESULTS

Baseline Prokaryotic Community Analysis

For the prokaryotic community analysis, we recovered 29,292 OTUs, most of which were not identified to the genus level. The majority of the identified OTUs defined by the 97% sequence similarity, were affiliated with three archaeal phyla and 22 bacterial phyla. The most abundant phylum across all sample sites was Proteobacteria classified as Gammaproteobacteria (4.55 – 47.37%), with most communities dominant in DBC (9.41 – 22.26%). Additional phyla that were most abundant in DBC include Nitrospirae (0.29 - 10.15%), Chloroflexi (0.58 - 7.44%), and Euryarchaeota (0.01 - 6.53%). The second most abundant phylum across all sample sites was Acidiobacteria, which dominated community structure in WC sites (4.56% - 19.01%). Additional phyla that had high proportions in WC included Alphaproteobacteria (1.64 - 9.28%), and Plantomycetes (0.40 - 5.06%) (Figure 5).

Sites SF and STFF in WC were distinct in their prokaryotic community composition relative to the other sites in the cave. Site SF had a higher proportion of Bacteroidetes, with 33.98% in the clay samples and 28.78% in the Mn-oxidizing sample, while the remaining samples across all sites were less than 10%. Site STFF had a higher proportion of Gammaproteobacteria, with 31.12% in the clay sample and 47.37% in the LBB positive sample, while the remaining samples in WC were less than 14% (Figure 5). At the order level, site SF had a higher proportion of Sphingabacteriotes, which belongs to the phylum Bacteroidetes. Site STFF did not have significantly higher proportions of Chromatiles, which belongs to the phyla of Gammaproteobacteria. Site CAUS in both the positive and clay control sample in DBC had a higher proportion of Chromatiles when compared across all sites (Figure 6).

Comparing samples sites in DBC, the Mn positive samples were more dominant with Betaproteobacteria, Latescibacteria, and Gemmatimonadetes. Acidiobacteria was the only trend for the clay control samples. Comparing the sample sites in WC that were along the spur-passage in the cave which include sites, STFF, FSD, and DDE, the Mn positive samples had higher proportions of Gammaproteobacteria and Actinobacteria. The clay control samples had higher proportions of bacteria not assigned (NA), Latescibacteria, Deltaproteobacteria, Firmicutes, and Plantomycetes. The WC samples that were located along the river that flows through the cave included, CS, MA, SF, and CP. Site MA is further down the river, and is indicated to have a different variety of nutrients as it skews the trends for the clay control samples when they are all compared. Looking at sites CS, SF, and CP, which are located near each other, the Mn positive samples had higher proportions of Actinobacteria and Gemmatimondates. The clay control samples had higher proportions of Alphaproteobacteria, Bacteroidetes, and Nitrospirae (Figure 5).

Archaea ranged from 0.01 - 8.35% of the total prokaryotes across all sites (Figure 5). Woesearchaeota and Pacearchaeota relative abundances ranged from 0.01 - 1.83% and 0.01 - 1.14%. Euryarchaeota was the most abundant archaeal phylum with a relative abundance of 0.01- 8.35%. There was not a consistent trend for archaea across all sample sites.

Results from the alpha diversity measurements of bacterial and archaeal communities showed a significant difference between caves (Table 1). Richness measured through observed OTUs before treatment ranged from 425 at CAUS.CC in DBC to 6278 at MA.Mn.plus in WC. Analysis of measurements calculated with the Shannon diversity index suggests that most sites in WC were diverse. However, sites SF and STFF in WC were the least diverse samples across all sites. There was a significantly lesser amount of OTUs for

each of these sites, resulting in less diversity. For DBC site CAUS.CC had the least amount of diversity and also the least amount of OTUs when compared to the remaining sites. Results from the Simpson diversity index confirm that the baseline bacterial and archaeal communities across all sites were diverse (Table 1).

Results from the NMDS plot based on Bray Curtis beta diversity analysis revealed that microbial communities in both DBC and WC clustered together based on site location and not if Mn was present. These results indicated that as the medium for the assembly of bacterial communities, cave niches played a key role in determining cave bacterial diversity. This observation is reasonable, because different caves differ in nutrients. Different niches also represent unique nutrients as growing substrates for bacterial communities. Sites MCSB and FCP in DBC clustered significantly closer to each other than site CAUS (Figure 7). This is consistent with the alpha diversity measurements as the Shannon values for site CAUS were less. All sites in WC clustered together except for sites SF and STFF. The alpha diversity measurements support this as the Shannon values were significantly less for these two sites than the remaining WC sites.

Results for the principal coordinates analysis (PCoA) plot on the unweighted UniFrac distance matrix also represent microbial communities that are similar based on closeness of the points. The same trend followed for each site. They clustered together based on location and not if Mn was present (Figure 8). The WC samples clustered significantly closer together, excluding sites SF and STFF.

Baseline Fungal Community Analysis

Six fungal phyla were represented through sequencing. The most abundant phylum across all sample sites was Ascomycota (4.57 – 61.44%). The second most abundant phylum was Basidiomycota (9.00-71.92%), and then Mortierellomycota was also abundant (1.11 – 85.94%) in all sites except CAUS.CC. (Figure 9). At the order level, the DBC sites were more abundant with Agaricales (13.99 – 56.71), while WC was relatively more abundant with Unclassified Fungi (1.74 – 42.21%). Site SF in WC was most abundant with Trichosporonales, and site STFF, was most abundant with Mortierellales. Surprisingly, site FSD was significantly more abundant with Sordariales than the remaining sample sites (Figure 10).

Results from the alpha diversity measurements of the fungal communities indicated that there was not a significant difference between the cave types. Fewer OTUs were observed when compared to the prokaryotic assemblages. Comparing the Shannon diversity values across all sites, site CP.CC in WC had the largest value at 5.06 and site STFF.CC in WC with the lowest value at 1.47. Results from the Simpson diversity values indicates that the diversity is highly variable. Site STFF.CC in WC, had a Simpson value of 0.24 and site CAUS.CC in DBC, had a value of 0.85 (Table 2).

Results from the NMDS plot based on Bray Curtis beta diversity analysis revealed that microbial communities in both caves clustered based on cave type and not if Mn was present in the sample. WC samples clustered significantly closer together for the fungal communities than they did for the prokaryotic samples. All of the samples in DBC also clustered closer together except for site CAUS.Mn.plus (Figure 11). This sample had the least amount of diversity in DBC.

Results from the PCoA plot also supported the idea that all sites clustered together based on location and not if Mn was present (Figure 12). The same trend followed for site CAUS in DBC, the Mn positive sample did not group as close to the clay control sample. The FCP samples in DBC did not group as close together when compared to the NMDS plot. The FCP clay control sample was closer to the CAUS.Mn.positive sample rather than the Mn positive FCP sample (Figure 12).

CHAPTER 4: DISCUSSION

Comparison of Microbial Communities in Different Cave Environments

Cave nutrients can vary depending on the location of the cave and its surrounding environment. The vast majority of cave environments in the southern Appalachians and surrounding areas are formed in limestone or dolostone, but some caves elsewhere in the world are formed in a quartz-cemented sandstone, in areas such as Venezuela (Barton et al., 2014; Sauro et al., 2018). The soils in these types of caves are thin, leading to nitrogen-limited surface ecosystems (Klimchouk et al., 2000). Imawari Yeuta cave located in Venezuela, is formed in a quartz cemented sandstone and the microbial communities analyzed from this cave were similar to carbonate caves in the US (Sauro et al., 2018). The three phyla that dominated in the Imawari Yeuta cave were Proteobacteria (Alphaproteobacteria), Actinobacteria, and Acidiobacteria, and additional phyla present included Plantomycetes and Chloroflexi (Sauro et al., 2018). Similarly, to DBC and WC, the phyla Proteobacteria was most abundant, specifically Gammaproteobacteria in DBC, while Acidiobacteria was most abundant in WC. Members of the Alphaproteobacteria and Actinobacteria phyla were described to have Fe-oxidizing activity (Sauro et al., 2018), which has been observed in the southern Appalachian caves as well (Carmichael et al., 2013a). In the Venezuela study, a geochemical analysis suggested that an increase of metals other than iron (zinc, barium and calcium) were involved in the biomineralization process (Sauro et al., 2018). In epigene caves, such as DBC and WC, similar processes occur. In these caves the microbial communities can bio-weather the rock substrate for accessing reduced metals, such as Mn and Fe, that are oxidized by microbial activities and deposited on cave walls and floors (Sauro et al., 2018).

Bacterial Baseline Microbial Communities at the Phylum level

Bacteria and archaea constitute the majority of biodiversity in caves and are ubiquitous in various cave habitats such as soils, sediments, stream waters, and rock surfaces (Barton and Jurado, 2007; Engel et al., 2004). Bacterial phyla have been detected in cave environments by sequencing of 16s rRNA genes (Barton and Jurado, 2007; Engel et al., 2004; Ortiz et al., 2013), advancing our understanding of the microbial communities in cave systems (Roesch et al., 2007). For most phyla, the advantage of biogenic Mn(II) oxidation is not fully understood. However, Mn oxides can serve as a protective shield for bacteria by forming a redox barrier that acts as a barrier against other microorganisms and viruses (Tebo et al., 2004).

Many caves are largely dominated with Actinobacteria, Proteobacteria, Acidobacteria Firmicutes, and Bacteroidetes (Barton and Jurado, 2007; Cuezva et al., 2012; Pašić et al., 2009; Yasir, 2018). Previous studies have studied the types of microbial communities in caves, and of the phyla identified, Actinobacteria have been found to dominate most cave samples. Manao-Pee cave in Thailand identified 36 families of Actinobacteria with the most abundant being *Pseudoncardiaceae* (Wiseschart et al., 2019), while samples taken from Farkpuk Cave in India had an 81% abundance (De Mandal et al., 2015). Liu et al. (2019) also analyzed microbial communities in the air, water, rocks and sediment and determined that Actinobacteria dominated the sediment and rock samples, while Proteobacteria dominated water and air samples. Proteobacteria is another phylum that is notably abundant in cave microbial communities. Microbial communities analyzed from two caves located in the mountain range of Hindu Kush determined that Alphaproteobacteria and Gammaproteobacteria were most dominate in both caves (Yasir, 2018), and communities

analyzed in Kartchner Caverns, Arizona determined communities with higher diversity corresponded to Proteobacteria (Ortiz et al., 2013). Other limestone cave studies have shown that Proteobacteria was identified as the dominant phylum with Alpha-, Gamma-, and Betaproteobacteria classes being most common (Schabereiter-Gurtner et al., 2004; Zhou et al., 2007).

Sites analyzed for this study had relatively high concentrations of Gammaproteobacteria, Betaproteobacteria, Bacteroidetes, and Acidiobacteria, while Actinobacteria accounted for less than 15% of the communities (Figure 5). Gammaproteobacteria was the most abundant phylum in DBC across all sites (103.24%), and Acidiobacteria was most abundant across all sites in WC (186.19%) (Figure 5). Proteobacteria and its classifications play an important role in energy generation metabolisms, such as carbon fixation, nitrogen metabolism and sulfur metabolism (Kersters et al., 2006; Spain et al., 2009). Sites in WC had relatively low proportions of Gammaproteobacteria except for site STFF both in the clay control and Mn positive sample. Site STFF was located along a spur passage in the cave where less foot traffic may occur. Because DBC is considered a relatively pristine cave, the nutrients at site STFF may have been similar. Caves are known to contain levels of organic carbon that are three orders of magnitude below that of the surface environment (Lavoie et al., 2010) therefore, it was expected to see a large amount of Acidiobacteria. As a greater abundance was seen in WC and not DBC, it may indicate that WC has a lower pH, as this type of bacterium flourishes at a pH below 5.5 (Lavoie et al., 2017).

While Bacteroidetes are commonly identified in microbial communities in caves, this phylum accounted for 0.51- 33.98% relative abundance across all samples. Site SF.CC

(33.98%) and SF.Mn.plus (28.78%) in WC, had significantly higher proportions of Bacteroidetes when compared to all sample sites. A previous study identified an abundant amount of Bacteroidetes at different site locations in DBC (Cloutier et al., 2017), however, the relative abundance in our study for DBC ranged from 0.51 – 3.85%. A significantly larger abundance was expected in the Mn positive samples, because members of this phylum have been previously reported to oxidize Mn(II) (Ford and Mitchell, 1990; Nealson and Krumbein, 1978; Santelli et al., 2010) including in ferromanganese deposits in Carter Saltpeter Cave, a similar cave only 291 km away, located in Carter County, TN (Carmichael et al., 2013a).

Microbial Differences in Cave Types

Comparing the sample sites in DBC, the Mn positive samples were most dominant with Betaproteobacteria, Latescibacteria, and Gemmatimonadetes. Members of Betaproteobacteria are commonly associated with the species *Leptothrix,* which are known Mn-oxidizers in caves (Carmichael et al., 2013a). *Leptothrix* is also commonly associated with Fe-oxidizing microbes. Biogenic iron deposits and their biosignatures in caves can help decipher activities related to past aqueous activities, microbial metabolisms, and sources of energy supporting life-forms (Angert et al., 1998). *Leptothrix*-like morphologies are known to be characterized by their straight, hollow sheaths coated in Mn and Fe (Carmichael et al., 2013a).

Latescibacteria is a member of Fibrobacteres-Chlorbi-Bacteroidetes super phylum (Youssef et al., 2015). Species within this phylum are capable of utilizing nanoparticle inclusions for specific metabolic functions such as navigation based on magnetic field

(Blakemore, 1982; Chen et al., 2010). Magnetotactic bacteria are known to induce the formation of magnetite through solid-state transformation of precursor nanometric Fe(III)-oxides, or solid ferrous iron phases (Baumgartner et al., 2016; Baumgartner et al., 2013). From the best of our knowledge there is not much known about Mn oxidizers that are associated with Latescibacteria.

Gemmatimonadetes are typically found in soils that are highly contaminated (Fawaz, 2013). However, a recent study focused on Mn(II)-oxidizing microbial communities in a secondary subsurface Mn oxide deposit influenced by acidic (pH4.8) metal-rich groundwater in a former uranium mining area determined that Gemmatimonadetes were strongly associated with Mn oxides (Bohu et al., 2016), indicating that Mn oxides might sustain the indigenous bacterial community (Bohu et al., 2016).

While many studies have shown that Actinobacteria is a dominant phylum in cave systems, DBC had a low abundance ranging from 1.34 – 4.19%, and WC ranged from 0.78 – 14.49% (Figure 5). Consistently with our study, Cloutier et al. (2017) analyzed Mn oxides in the southern Appalachians and found similar results in that Actinobacteria accounted for less than 12% of the communities. Even though these cave environments contain low levels of organic carbon compared to surface environments, sites were chosen based on Mn(II) being actively oxidized, or sites that were negative or slightly negative for Mn oxidation. Actinobacteria may be involved in mediating mineralization processes and producing bioactive compounds, such as antimicrobials that allow the biotic control on other populations (Rangseekaew and Pathom-Aree, 2019). Several studies have suggested that microbial communities, such as Actinobacteria, can be found in the formation of Mn and Fe rich deposits on rock walls (Carmichael et al., 2013a; Spilde et al., 2005). Consistent with our

study, all Mn positive sites in WC were more abundant with Actinobacteria than the clay control sites. This phylum may serve an important ecological role in biogeochemical cycles of cave ecosystems.

Diversity Comparison and Microbial Communities

The amount of nutrients in cave systems can be a driving factor for the microbial communities that flourish in the area. The sample sites that had the least diversity in this study included: CAUS.CC and CAUS.Mn.plus in DBC, SF.CC and SF.Mn.plus in WC, STFF.CC and STFF.Mn.plus in WC (Table 1). Site CAUS in DBC, was part of the cave wall and the remaining samples were either in natural drip pools or on the cave floor. The samples SF and STFF in WC were not located near each other in the cave, indicating a lack of correlation between diversity and site location. Comparing the samples to each other, Betaproteobacteria and Gemmatimonadetes had higher proportions in the Mn positive samples, while Euryarchaeota, Woesearchaeota, and Verrucomicrobia had higher proportions in the clay control samples. Gemmatimonadetes were expected to have a higher proportion in the Mn positive samples, as they can be found in a variety of arid soil, such as pasture soil, and eutrophic lake sediments and alpine soils (Fawaz, 2013). Gemmatimonadetes also had a higher proportion in all DBC Mn positive samples, and sites CP, CS, and SF that are located near a water source in WC. This supports the idea that bacterially dominated Mn(II) oxidation can occur in cave systems with an input of exogenous carbon dissolved in groundwater (Carmichael et al., 2015). The presence of Verrucomicrobia located in these less diverse areas was not expected as this phylum is commonly found in terrestrial ecosystems and polluted soils (Navarrete et al., 2015). Across all of the least diverse samples,

Verrucomicrobias relative abundance ranged from 0.48 – 0.94%. It was not a significant amount in any of the sites, but still indicates that a source of anthropogenic impact may be affecting these caves.

Fungal Baseline Microbial Communities at the Phylum Level

Little is known about the fungal role in biogeochemical cycling in cave systems. Fungal communities in cave environments have been even more understudied than bacteria, despite their importance as primary decomposers in soil ecosystems (Lindahl et al., 2007; O'Brien et al., 2005). Slight progress has been made over the years identifying new fungal communities in cave systems. Since 2017, more than 1150 fungal species in 550 genera have been discovered in caves and mines worldwide (Vanderwolf et al., 2013; Zhang et al., 2017). Recently, 20 new species were described from caves, accounting for 49% new fungal species identified in cave systems (Zhang et al., 2017). Vanderwolf et al. (2013) collected data from 225 published papers and 10 books and reviewed the most abundant fungal phyla that occur in cave systems and mines. It was determined that Ascomycota is the most abundant phyla accounting for 69.1% (Vanderwolf et al., 2013). The second most abundant was Basidiomycota (20%), then Zygomycota (6.6%), Mycetozoas (2.6%), and Oomycota (1%) (Vanderwolf et al., 2013). For Ascomycota, several genera have been reported to oxidize Mn(II) through laccases or biologically produced superoxide (Hansel et al., 2012). Fungi that belong to the phylum Basidiomycota can oxidize Mn(II) into Mn(III) by manganese peroxidase (MnP) in the presence of H_2O_2 (Wariishi et al., 1992). The organic form of Mn(III) is then disproportionated to form Mn oxide phases (Zhou and Fu, 2020).

In this study the most abundant phyla identified were Ascomycota, Basidiomycota and Mortierellomycota, but Zygomycota was not identified in either cave system. Comparing this study to (Carmichael et al., 2015), Ascomycota, Basidiomycota and Zygomycota were identified as the most abundant phyla in DBC and Carter Saltpeter Cave. Similar to findings in this study, Basidiomycota was more abundant in DBC, while Ascomycota dominated in the anthropogenically impacted Carter Saltpeter Cave (Carmichael et al., 2015). Cloutier et al. (2017) also found that Basidiomycota was more abundant in DBC, and Ascomycota was more abundant in Carter Saltpeter Cave. Unlike this study, Cloutier et al. (2017) found that Ascomycetes had higher proportions at sites where Mn(II) oxidation was stimulated. There was not a trend between the clay control samples and Mn positive samples in any of the phyla in our study.

Unlike previous studies focused on the southern Appalachian caves, this study identified a significant percentage of Mortierellomycota present in both caves. There are few studies on how *Mortierella* adapt to subterranean or extreme environments (Burow et al., 2019). Their role is thought to be associated with the degradation of polymers and via production of organic acids or enzymes degrading the host rock, using the high proportion of organic carbon (Burow et al., 2019).

CHAPTER 5: ONGOING AND FUTURE WORK

The information that follows was intended to be studied however, due to the pandemic access to the lab was restricted. This is a summary of the background, methods and completed research that was originally supposed to be part of the thesis but had to be abandoned due to the lockdown.

Mechanisms of Mn Oxidation

A cave environment typically has a neutral pH ranging from 7.0-8.5 depending on the water quality (Sasowsky et al., 2005). Since abiotic oxidation cannot occur below pH 8 and Mn oxidizers readily oxidize Mn(II) at a pH ranging from 6.5-7.5 (Zhou and Fu, 2020), cave systems are highly favorable environments for Mn-oxidizing microbial reactions. This suggests that natural Mn oxides are primarily biogenic (Zhou and Fu, 2020). Mn oxidation can occur through a variety of metabolic pathways in both bacterial and fungal microorganisms. For bacteria, these include enzymatic reactions that use multicopper oxidase (MCOs) or haem peroxidase enzymes (Anderson et al., 2009; Tebo et al., 2005), superoxide-mediated reactions (Learman et al., 2011a) and siderophore reactions (Duckworth et al., 2009). Fungi can oxidize Mn(II) by producing MCO-like enzymes (Höfer and Schlosser, 1999), manganese peroxidase (MnP) (Kuan et al., 1993), versatile peroxidase (Ruiz-Duenas et al., 2007), or superoxide-mediated mechanisms (Hansel et al., 2012). The production of MnOx is thought to either benefit the utilization of organic nutrients by Mn oxidizing microbes or protect Mn oxidizing microbes against the toxicity of inorganic and organic species (Zhou and Fu, 2020).

In addition, a wide variety of organisms, including bacteria, fungi and algae have been used in the bioremediation process to remove heavy metals from water systems (Bahar et al., 2016; Jaiswal et al., 2018; Yin et al., 2017; Zeraatkar et al., 2016). Of all microbes that are used, manganese-oxidizing bacteria (MnOB) are gaining the most attention due to their unique characteristics (Shoiful et al., 2020). Heterotrophic MnOB are capable of oxidizing Mn(II) to Mn(III/IV), which can also be referred to as biogenic manganese oxides (Francis and Tebo, 2002). Biogenic manganese oxides can perform a variety of functions that include adsorbing cationic metals and oxidizing inorganic contaminants because of their poorly crystalline layered materials that can have many vacant sites (Bai et al., 2016; Droz et al., 2015; Meng et al., 2009; Tang et al., 2014). MnOB are widespread in nature and can be found in a variety of environmental conditions.

A previous study on caves in the Southern Appalachians determined that copper can be used to distinguish metabolic pathways of Mn oxidation, since copper has differential effects on some of these pathways (Cloutier et al., 2017). For example, microbes that increase Mn oxidation in the presence of Cu are typically using MCO (or MCO-related) enzymes (Brouwers et al., 2000), whereas those that are inhibited in the presence of Cu are likely using superoxide dismutase mechanisms (Hansel et al., 2012; Learman et al., 2011b). Some microbes can use multiple Mn oxidation pathways; for example *Psudeomonas putida* contains three different genes for Mn-oxidation that are differentially expressed, depending on the environmental conditions (Geszvain et al., 2012; Tebo et al., 2007). While many details of regulated functional pathways have emerged in the last two decades, the overall purpose of Mn(II) oxidation has yet to be determined, as there are a variety of mechanisms

and complex interactions between communities that can be utilized under different environmental conditions.

Mn Oxide Mineralogy

Despite the fact that Mn oxides have been studied for many years, the details of their structures and association with microorganisms remain poorly understood, in part because of the enormous diversity of microorganisms that produce them using a variety of mechanisms. Mn can occur in three different oxidation states (+4, +3, and +2) that are arranged in an octahedral building block that can share edges, corners or faces to construct a variety of tunnels and layer structures (Post et al., 2020). X-ray diffraction (XRD) is commonly used to analyze material properties like phase composition, structure, and texture in samples such as Mn oxides (Ermrich and Opper, 2013). However, XRD patterns exhibit broad peaks from disordered phases, making phase identification difficult (Post et al., 2020). Consequently, other methods are utilized to characterize Mn oxides. Other spectroscopic techniques include Raman spectroscopy, Fourier transform infrared spectroscopy (FT-IR), and X-ray absorption spectroscopy (XAS), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) (Post et al., 2020). However, these techniques also have experimental limitations and cannot be applied to *in situ* analysis (Post et al., 2020).

Raman spectroscopy has been used to identify and investigate changes in response to certain redox, cation-exchange, and other reactions (Bernard et al., 1993). Advantages of the micro-Raman system is that it can provide phase identification and structural information quickly from discrete areas on natural or synthetic Mn oxide samples (Post et al., 2020). Combining Raman microscopy and scanning electron microscopy (SEM) can provide

insights into the micro mineralogy and chemistry of complex samples (Post et al., 2020). While Raman spectroscopy is widely used for the recognition of MnOx, the results can be ambiguous due to the lack of literature supporting it (Bernardini et al., 2019).

Fourier-transform infrared spectroscopy (FT-IR) is a powerful technique that is used to characterize MnO_x complexes that are disordered and/or have poorly crystalline materials (Bernardini et al., 2019). However, the results that can come from this spectroscopy do not always allow simple and unique identification of the mineral, especially if there is a mixture (Bernardini et al., 2019).

X-ray absorption spectroscopy also includes x-ray absorption fine structure (EXAFS) spectroscopy and x-ray absorption near-edge structure (XANES) spectroscopy. EXAFS is widely used in the study of materials (Ormerod, 2001). It also provides reliable structural information regarding the chemical and electronic structure of specific sites within materials, including the number, chemical nature, and distance of neighboring atoms (Ormerod, 2001). XANES on the other hand, has near edge structures that arise from excitation of core electrons into discrete unoccupied valence orbitals (Ormerod, 2001). However, the interpretation is complex making it difficult to assign the observed transitions and to interpret the energy and intensities of the transitions of interest (Ormerod, 2001).

Electron microscopy

Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) have been used to determine where oxides are deposited in *in vitro* studies, while also imaging intracellular components (Hansel et al., 2012). TEM imaging of Mn-oxidizing microbes helps determine the spatial relationships between microbes and the minerals they

deposit, while SEM imaging can determine the relationship between microbial communities and their substrates (Carmichael et al., 2013a; Carmichael et al., 2013b; Cunningham et al., 1995; Frierdich et al., 2011; Spilde et al., 2005).

Mn oxides that are biologically deposited in cave environments typically form poorly crystalline, nanometer scale minerals that look like crumpled tissue paper (Carmichael et al., 2013a; Spilde et al., 2005), and micrometer scale boxwork shapes or sheets (Spilde et al., 2005). TEM imaging can be used to show that Mn oxides may be concealed within cell membranes at the tips and joints of fungal hyphae (Carmichael and Bräuer, 2015; Carmichael et al., 2013b), unlike the simple mineral coatings on microbial cells described in nearly all other studies. TEM imaging has also been used to link superoxide production and Mn oxidation, while also proving that Mn oxidation can be a result of both biotic and abiotic reactions (Learman et al., 2011b). SEM imaging is beneficial for visualizing microbial morphologies of environmental samples, especially in ferromanganese deposits (Carmichael et al., 2013a).

CHAPTER 6: CONCLUSION

The microbial communities for bacteria in DBC and WC clustered based on the sites within the cave, and fungi clustered based on site location and cave type, but none of the communities clustered based on if Mn oxides were present, indicating that the nutrient type and amount of nutrients for each LBB positive and negative site was similar. Shannon Index plots show that the diversity in cave systems can be a driving factor for the microbial communities that flourish in that area based off of the nutrients. Within the near-pristine cave environment of DBC, both LBB positive and negative bacterial samples produced lower observed OTUs, while the anthropogenically impacted cave WC produced greater numbers of observed OTUs and evenness. The fungal sites in DBC also produced lower observed OTUs compared to WC. There was a significant amount of diversity between the fungal samples in both cave types. The results also showed that the relative abundance of bacterial and fungal phyla in each cave was significantly different when compared to the other. Previous studies have shown that anthropogenic disturbances can change the microbial community diversity in cave systems, and this study was consistent with that finding. This research demonstrates that the different sources of nutrients can have a greater effect on the microbial communities in two different cave systems, than between Mn oxidizers in the same cave.

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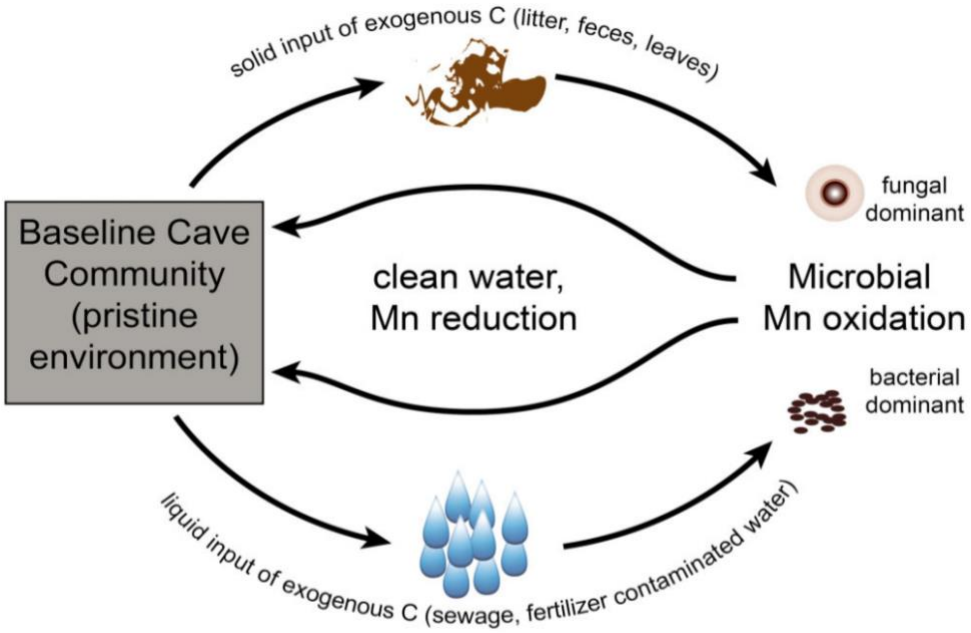

Figure 1. A working model demonstrating the stimulation of Mn oxidation in an anthropogenically impacted cave environment, versus a near pristine cave environment (Carmichael et al., 2015).

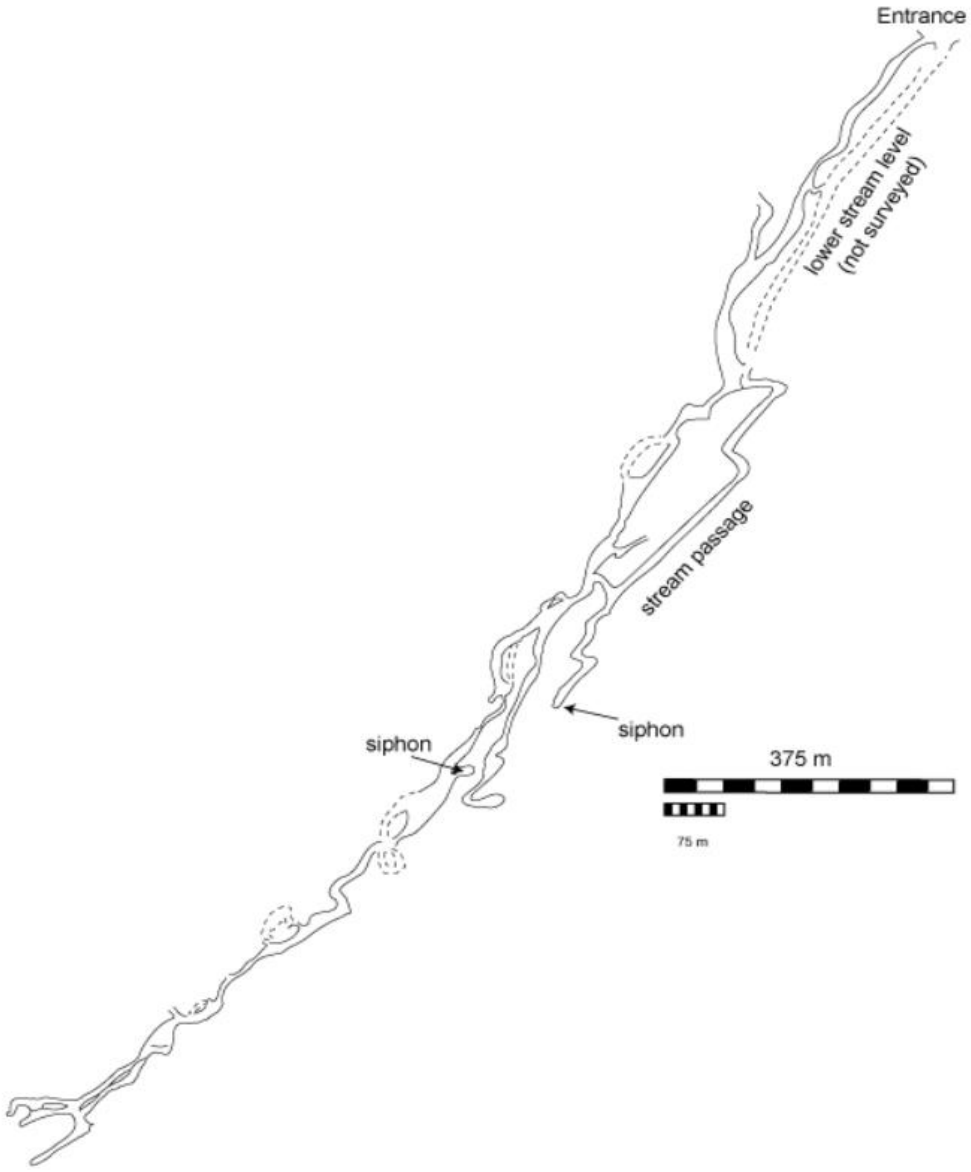

Figure 2. A map of Worley's Cave.

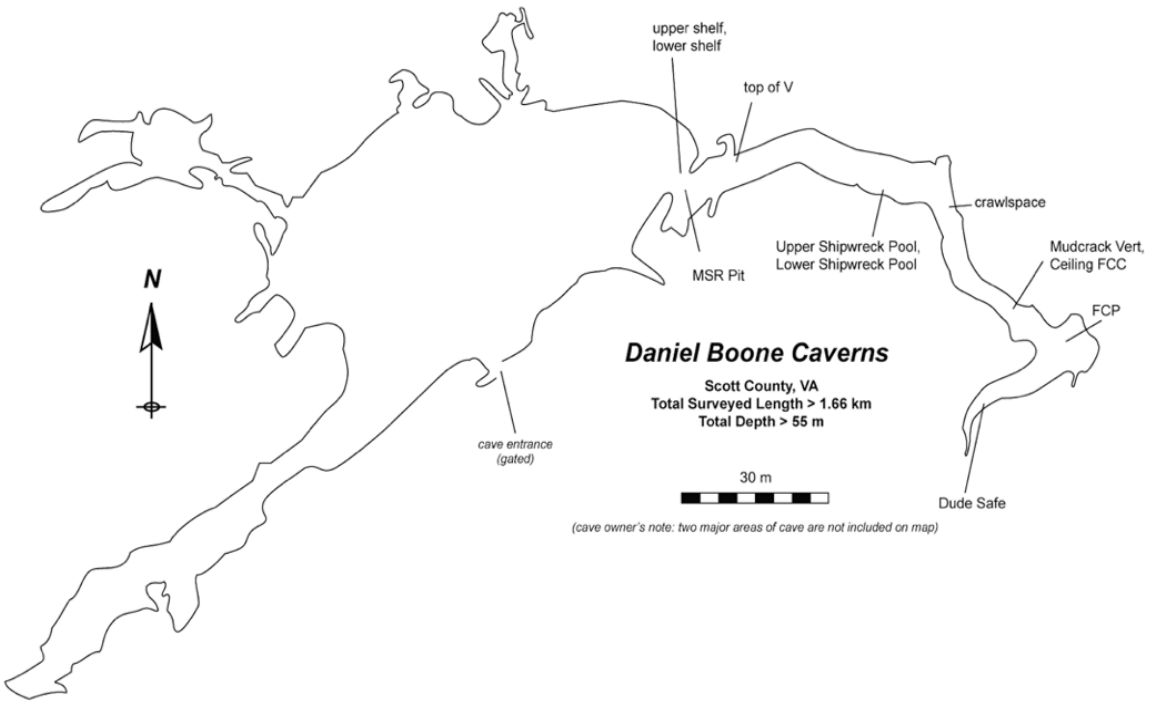

Figure 3. A map of Daniel Boone Caverns.

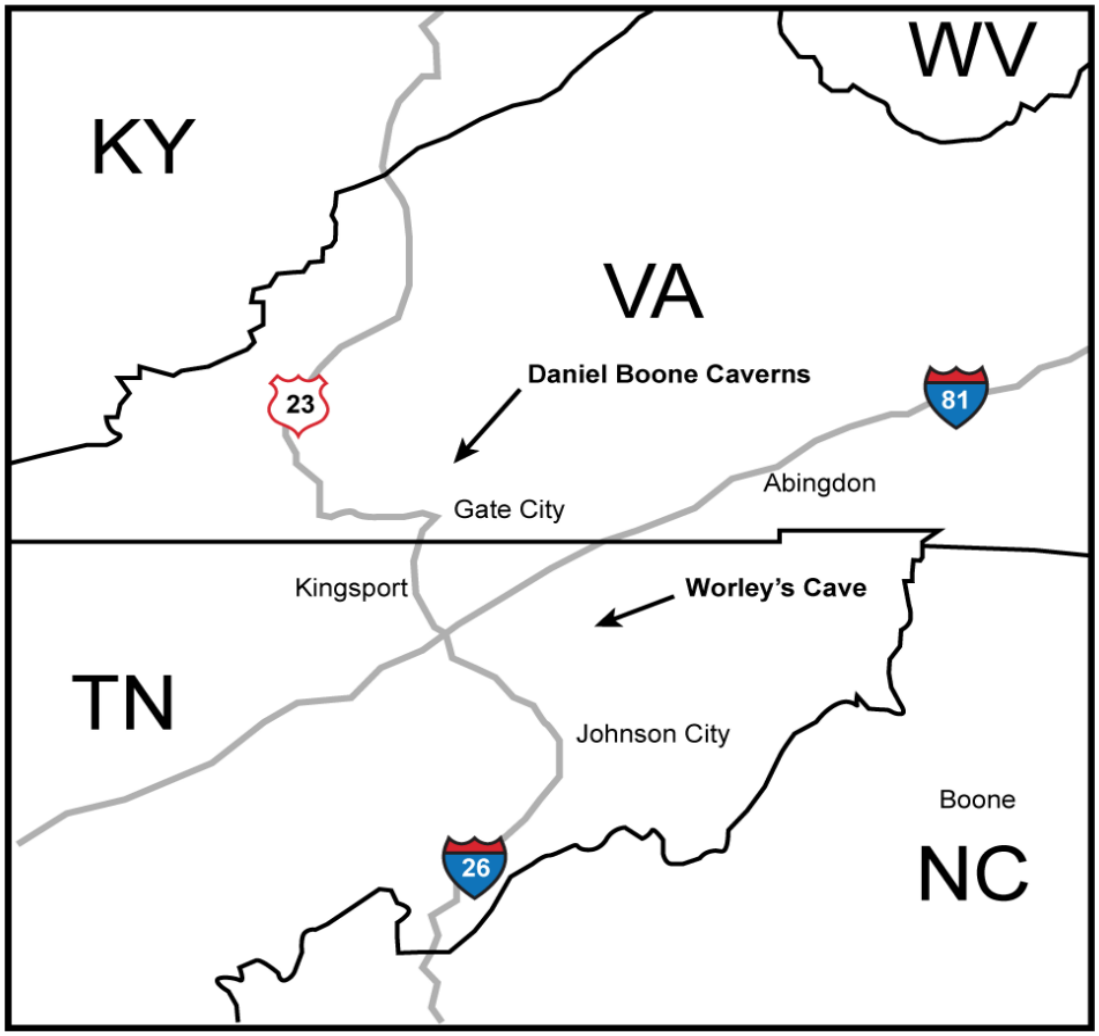

Figure 4. A map of the study site.

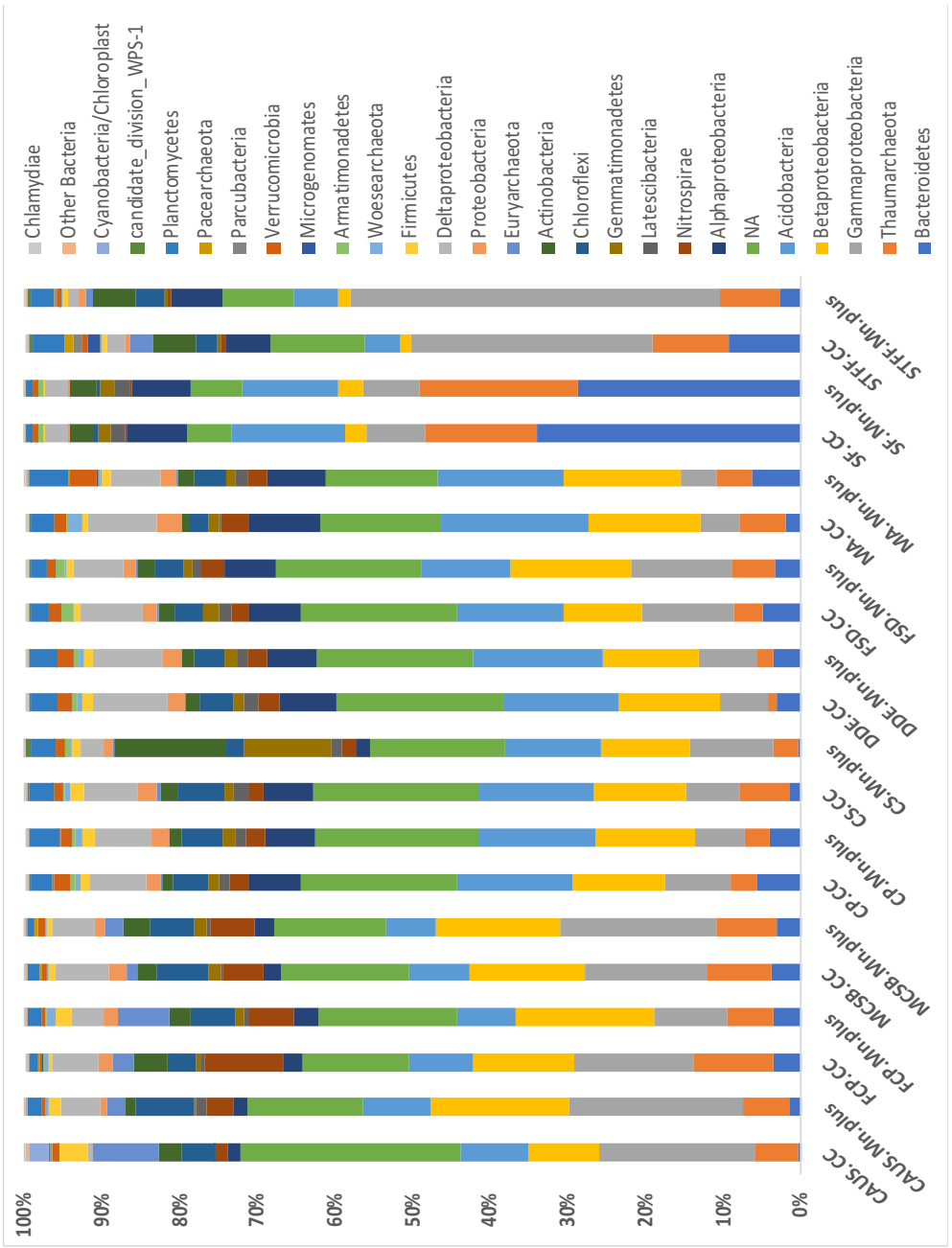

Figure 5. Phyla-level distribution of bacteria and archaea with a 1% abundance cut-off before mineral incubations.

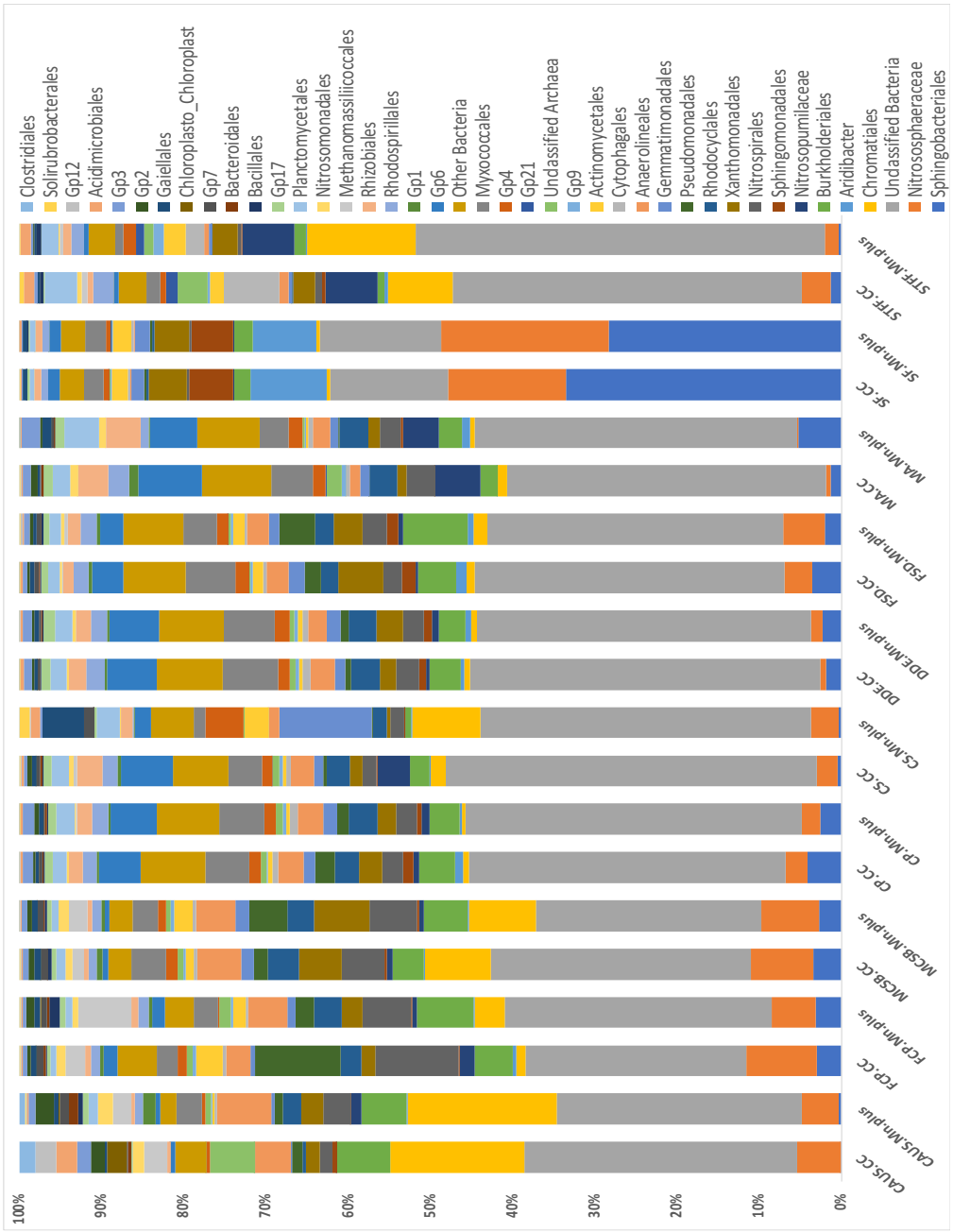

Figure 6. Order-level distribution of bacteria and archaea with a 1% abundance cut-off before mineral incubations.

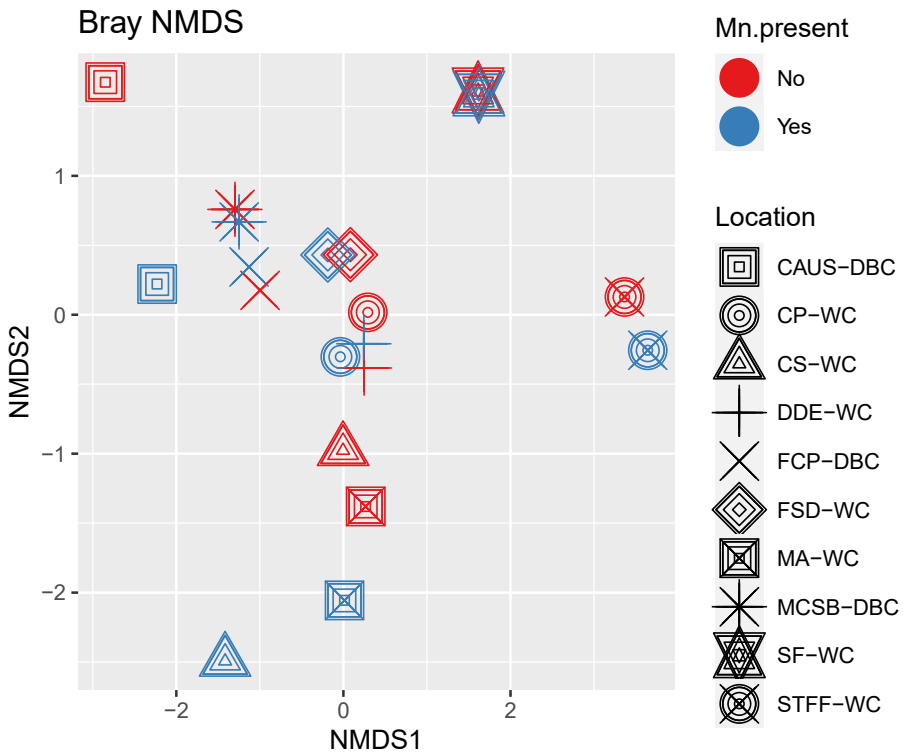

Figure 7. Beta–diversity analysis of baseline bacterial and archaeal communities at sites before mineral incubations using the Bray Curtis similarity matrix and non-metric multidimensional scaling.

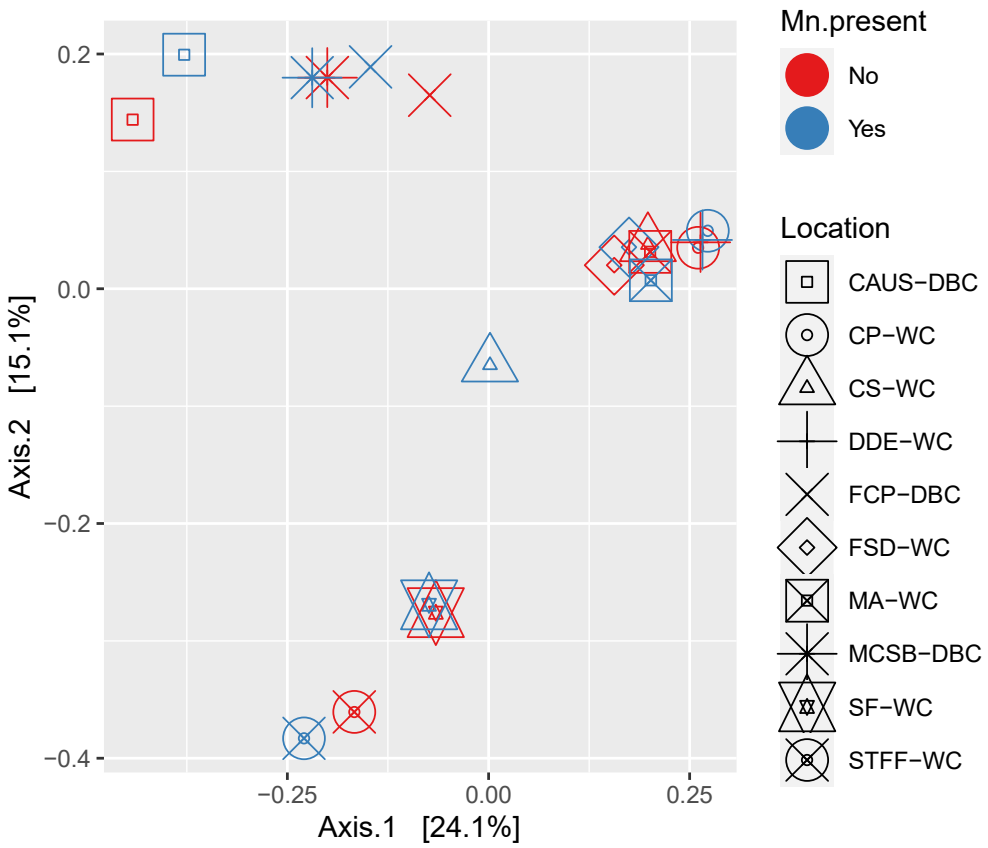

Figure 8. PCoA unweighted analysis of baseline bacterial and archaeal at sites before mineral incubations in DBC and WC.

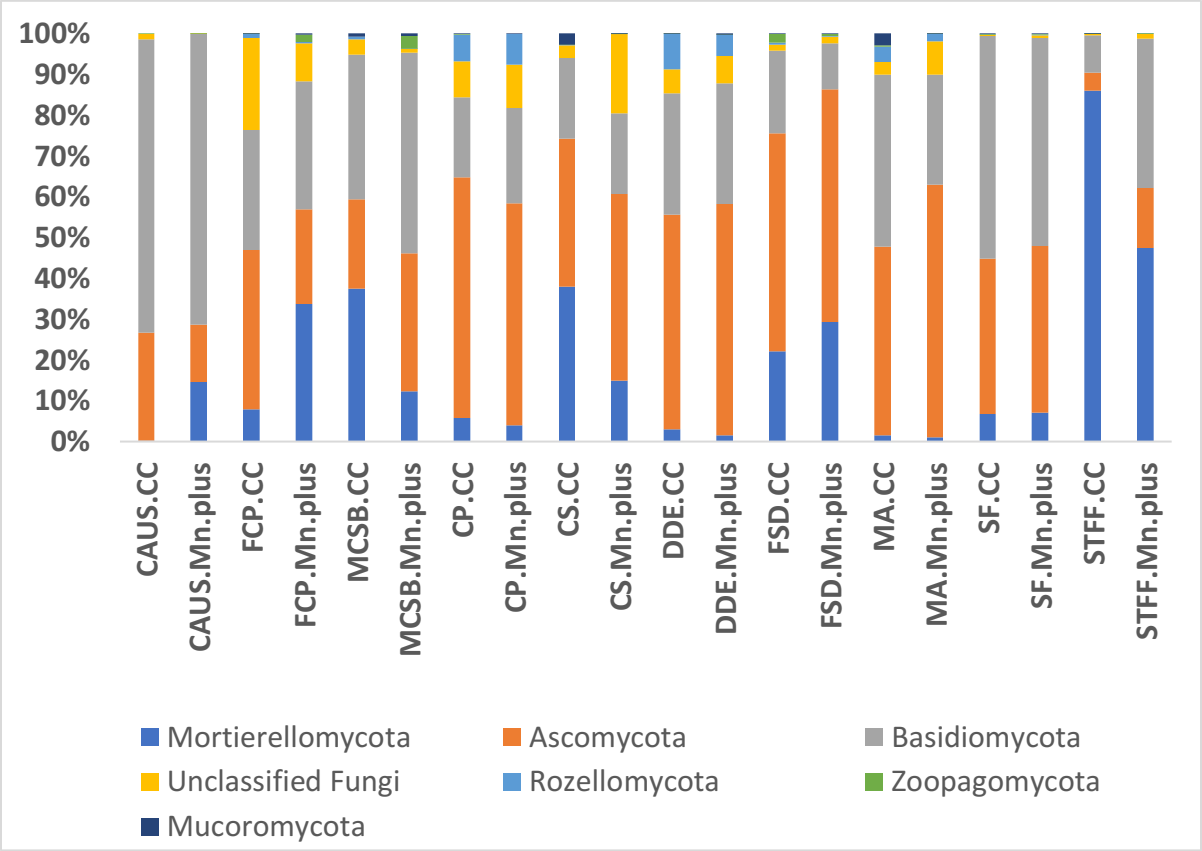

Figure 9. Phyla-level distribution of fungi with a 1% abundance cut-off before mineral incubations.

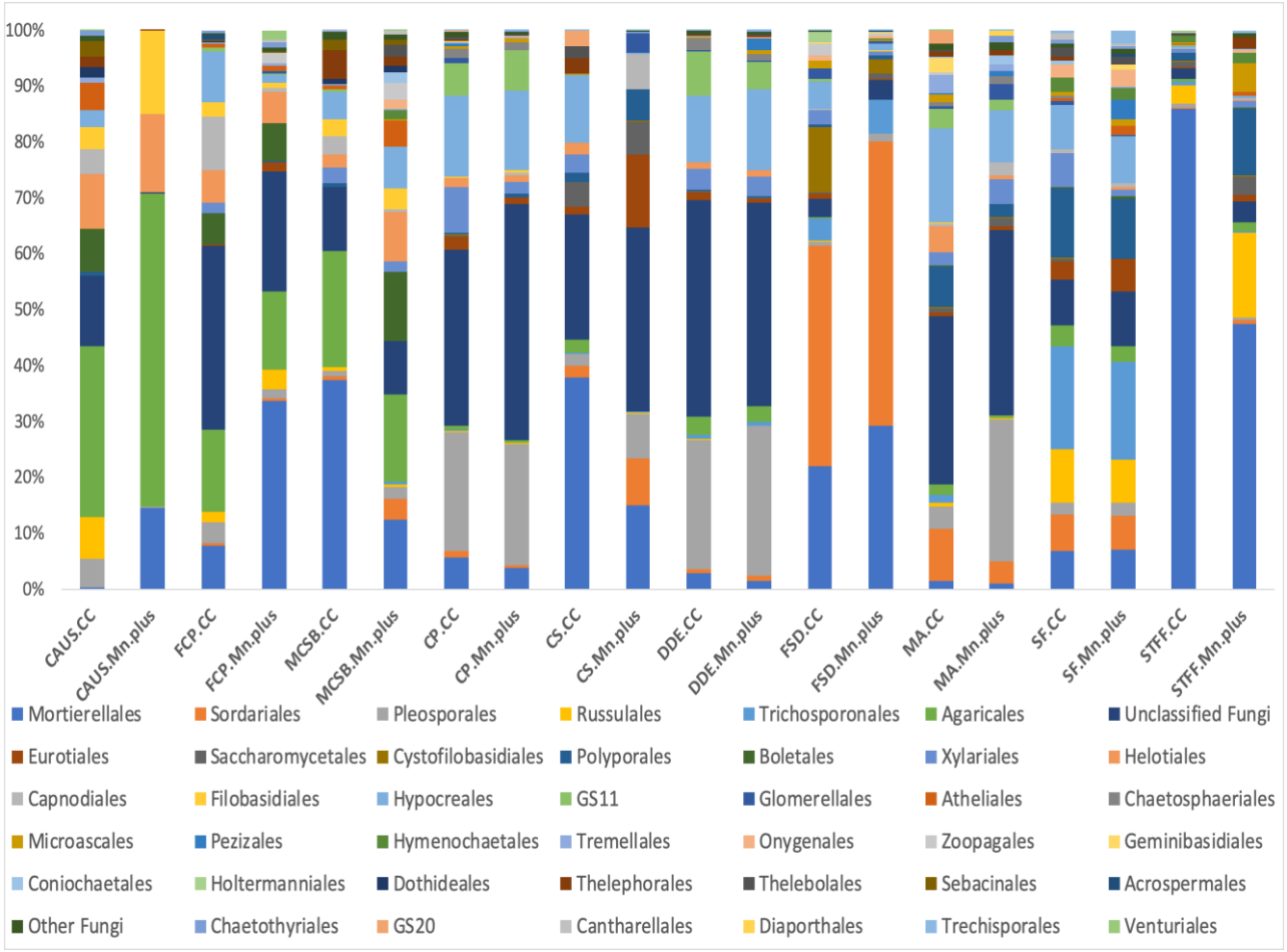

Figure 10. Order level of fungi with a 1% abundance cut-off before mineral incubations.

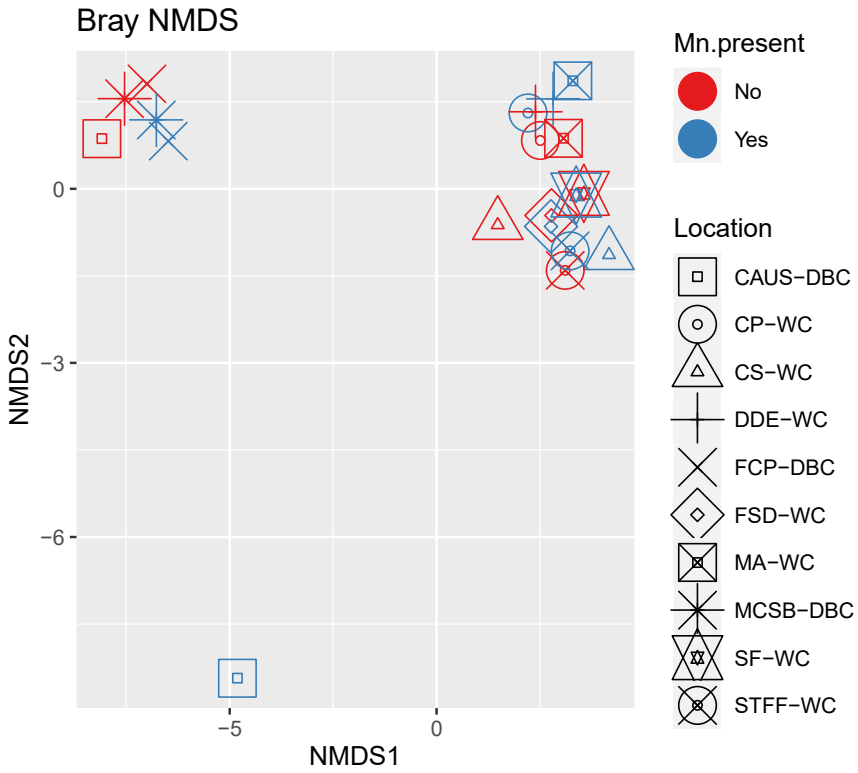

Figure 11. Beta–diversity analysis of baseline fungal communities at sites before mineral incubations using the Bray Curtis similarity matrix and non-metric multidimensional scaling.

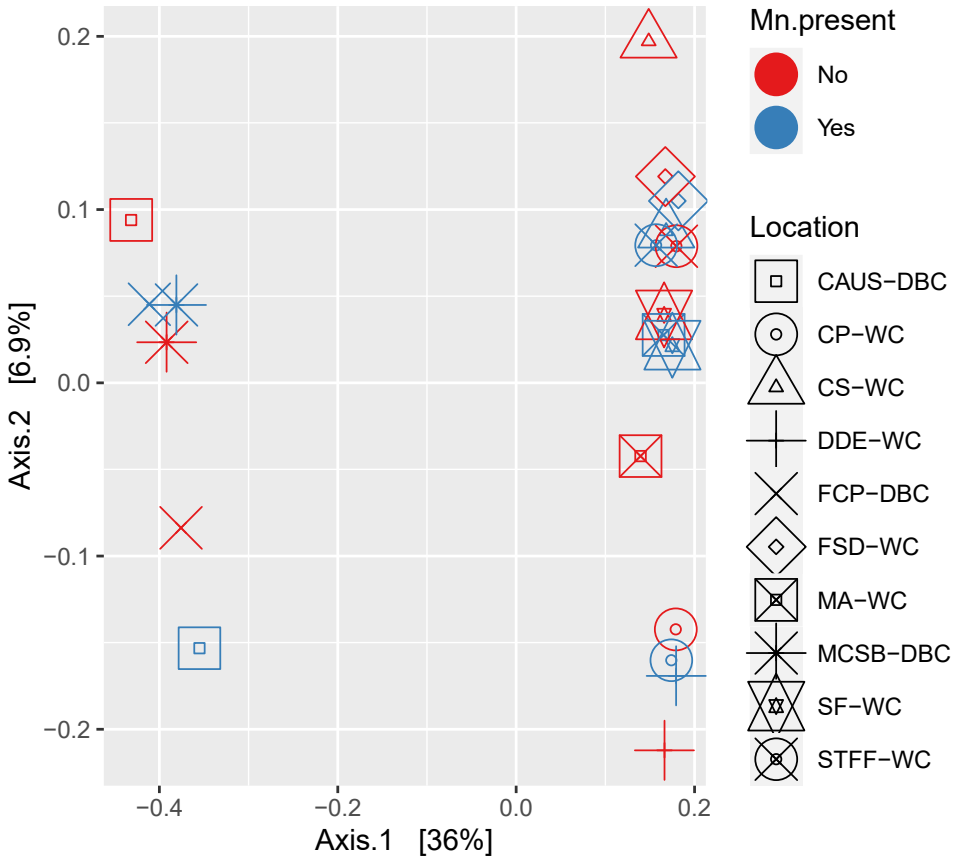

Figure 12. PCoA unweighted analysis of baseline fungi at sites before mineral incubations in DBC and WC.

Table 1. Alpha diversity estimates of bacterial and archaeal sites in DBC and WC. Statistical significance between caves, according to a student's t-test are indicated by either a single asterisk (*, <0.05) or a double asterisk (**, <0.01).

Cave	Site/Treatment	Observed OTUs **	Shannon *	Simpson
DBC	CAUS.CC	425	4.41	0.97
	CAUS.Mn.plus	914	5.46	0.98
	FCP.CC	2210	6.22	0.99
	FCP.Mn.plus	1915	6.24	0.99
	MCSB.CC	1823	5.87	0.99
	MCSB.Mn.plus	1696	5.85	0.99
WC	CP.CC	5731	7.54	1.0
	CP.Mn.plus	6150	7.60	1.0
	CS.CC	5185	7.46	1.0
	CS.Mn.plus	2765	6.18	0.99
	DDE.CC	5630	7.59	1.0
	DDE.Mn.plus	5953	7.65	1.0
	FSD.CC	4433	7.18	1.0
	FSD.Mn.plus	5164	7.31	1.0
	MA.CC	4883	7.31	1.0
	MA.Mn.plus	6278	7.57	1.0
	SF.CC	2220	4.43	0.92
	SF.Mn.plus	2152	4.40	0.92
	STFF.CC	1824	5.01	0.96
	STFF.Mn.plus	1302	4.46	0.93

Table 2. Alpha diversity estimates of fungi sites in DBC and WC.

Cave	Site/Treatment	Observed OTUs	Shannon	Simpson
DBC	CAUS.CC	140	4.20	0.85
	CAUS.Mn.plus	166	2.87	0.56
	FCP.CC	200	4.12	0.78
	FCP.Mn.plus	161	3.87	0.76
	MCSB.CC	161	3.60	0.71
	MCSB.Mn.plus	170	4.25	0.83
WC	CP.CC	608	5.06	0.79
	CP.Mn.plus	561	4.72	0.75
	CS.CC	278	3.30	0.59
	CS.Mn.plus	330	3.00	0.52
	DDE.CC	630	4.90	0.76
	DDE.Mn.plus	518	4.64	0.74
	FSD.CC	352	3.25	0.55
	FSD.Mn.plus	350	2.69	0.46
	MA.CC	502	4.82	0.77
	MA.Mn.plus	426	4.80	0.79
	SF.CC	455	4.60	0.75
	SF.Mn.plus	470	4.55	0.74
	STFF.CC	397	1.47	0.24
	STFF.Mn.plus	381	3.23	0.54

APPENDIX: COVID-19 EFFECTS ON RESEARCH

Culturing

Rock samples that were LBB positive were collected from each site for culturing experiments. Serial dilutions were prepared and streaked to the 10^{-8} on two different media types. The media that were prepared were: (1) FMO2 in an agar and nutrient broth, and (2) PYG at a pH of 5.5 and pH of 7.2 in an agar and nutrient broth. Cultures were continuously re-streaked on new plates until they were axenic. The plates were incubated at room temperature and continuously monitored for Mn(II) oxidation by individual bacterial or fungal colonies using LBB as a chemical indicator. Due to Covid-19 the cultures were no longer viable. Results would have demonstrated which culturable microbes selectively inhabit the LBB positive surface.

Sequencing

Isolates collected from culturing were to be PCR amplified with 27f and 1492r primers for bacterial isolates and ITS primers for fungal isolates. DNA would have been added to each reaction by touching an isolated colony with a sterile pipette tip and washing the tip in the reaction mixture. PCR products would be run through gel electrophoresis and imaged to ensure amplification was successful. Products that amplified would be purified with QIAGEN QIAquick PCR Purification Kit and the concentrations measured with a NanoDrop spectrophotometer to ensure purity. Purified samples would be sent for 24-hour processing by Eton Bioscience, Inc. Amplified sequences would be identified using a BLAST search (Altschul et al., 1990). Results would demonstrate which microorganisms were present in the cave before the quartz, calcite and feldspar were applied to the area.

Microscopy and Electron Microscopy

Clay and rock samples that were both LBB and/or LBB negative (or only mildly positive) were to be returned to the lab for fixation/preservation with glutaraldehyde for scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDS) microanalysis. Both *in situ* cultures and lab cultured isolates were to be analyzed using SEM. Samples would be critically point dried with liquid CO_2 in a Polaron critical point dryer. Samples would then be mounted on stubs and sputter coated with gold with a Denton Vacuum Evaporator and Desk III Sputter Coater. Samples would have been imaged and analyzed with a 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. Clay minerals would also be identified using X-ray diffraction on a Shimadzu 6000 X-ray diffraction system with Match Crystal identification software in the Department of Geological and Environmental Sciences at Appalachian State University. Samples would have been 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.

Community Analysis of Incubated Cultures

The crushed-up minerals that are incubating in the cave would have been collected and returned to the lab for DNA extraction using the MP Biomedicals Fast DNA™ Spin Kit for Soil. DNA concentrations would have been measured using a Fisher Scientific NanoDrop 1000 spectrophotometer. The extracted DNA samples would have been sent to Mr. DNA for PCR amplification and Illumina sequencing. The extracted DNA would have been amplified using PCR and barcoded primers. The bacterial DNA samples would have been amplified

using 515F and 806R primers and ITS primers for fungal isolates. The returned samples would have been formatted to analyze in R studio.

VITA

Morgan Olivia Smith was born in Asheville, North Carolina on the 20th of April 1996, and raised in Hickory, North Carolina. Morgan graduated from Appalachian State University with a B.S in Cell and Molecular Biology in 2018. During her undergraduate studies she realized her interest in microbiology and began research in Dr. Suzanna Bräuer's lab. She continued her education at Appalachian State University and completed her M.S in Cell and Molecular Biology in December 2020.