GEOMICROBIOLOGY OF FERROMANGANESE DEPOSITS IN CAVES OF THE UPPER TENNESSEE RIVER BASIN

A Thesis
by
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FOREWORD

The references, tables, and figures within this manuscript were prepared in accordance to the author submission requirements of *Environmental Microbiology*, the peer-reviewed journal co-published by the Society for Applied Microbiology and Blackwell Publishing, Limited. Chapters Two and Three are manuscripts that are intended for separate submission to the journal. As such, these two Chapters have been formatted independently.

ABSTRACT

GEOMICROBIOLOGY OF FERROMANGANESE DEPOSITS IN CAVES OF THE UPPER TENNESSEE RIVER BASIN

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Ferromanganese deposits in four epigenic cave systems (Carter Salt Peter Cave (CSPC), Daniel Boone Caverns, Rockhouse Cave, and Worley's Cave) located within the Knox Dolomite bedrock formation of the upper Tennessee River Basin were analyzed to determine the role of microbes in the cycling of manganese and the transformation of cave mineralogy. It was hypothesized that the Mn-oxidizing microorganisms present in these caves would most likely be phylogenetically distinct from those studied in cave systems in other parts of the world, and that novel Mn-oxidizing microorganisms may be present. ICP-OES measurements of Mn and Fe in cave ferromanganese deposits revealed Mn:Fe ratios of ca. 0.1-1.0. In cases where the Mn:Fe ratio approached 1.0, this represented an order of magnitude increase above typical Mn:Fe ratios for the bedrock, suggesting that Mn-biomineralization plays an important role in these sites. A SSU rRNA based molecular survey of one such site, Mn Falls (CSPC, Carter County, Tennessee), revealed that 21% of the 34 dominant OTUs were closely related to known metal-oxidizing bacteria or clones isolated from oxidized metal deposits. qPCR estimates of total bacterial SSU rRNA genes in biofilm samples from CSPC represented $9x10^9$ cells/g wet weight. Several cultures capable of Mn-biomineralization *in vitro* were isolated, some of which were obtained from high dilutions. SSU rRNA gene sequences of these isolates closely matched those of *Leptothrix, Pseudomonas, Flavobacterium, Arthrobacter,* and one species within a genus not previously known to oxidize Mn, *Janthinobacterium*. Thus, these results

expand our knowledge of the phylogenetic diversity of organisms capable of Mn(II) oxidation. Together, results from geochemical analyses, molecular surveys, and culture-dependent community surveys indicate that Mn(II)-oxidizing bacteria are abundant and environmentally relevant members of ferromanganese deposits in the study area, and that these cave systems harbor microbial communities unique from those found in caves in the southwestern United States.

Over the duration of the study period (July 2009-2011), a marked decline in the intensity of ferromanganese deposits in the Mn Falls biofilm (CSPC) was noted. Because a sewage discharge may have occurred at this site prior to July 2009, it was hypothesized that the decline may have been associated with the gradual abatement of a sewage discharge or another acute, point source of pollution. Additionally, clone library data from the Mn Falls site revealed many sequences closely related to those found in contaminated and/or nutrient impacted sites. Therefore, a second line of study was initiated to assess the potential of sustained contamination at the Mn Falls site. A molecular-based survey using primer sequences selective for the SSU rRNA gene sequences within the human group of *Bacteroides-Prevotella* demonstrated evidence of fecal contamination at Mn Falls throughout the duration of the study period. A greater percentage of sequences clustering within the human group was detected in DNA extracted from the biofilm in July 2009 as compared to July 2011. Culture-dependent most probable number assays supported molecular-based evidence: an increase in the total number of culturable heterotrophic microbes at the Mn Falls sites was observed when compared to an adjacent site (Mud Trap Falls) within the same cave system, suggesting that the microbes in Mn Falls were adapted to higher nutrient concentrations. The change in biofilm appearance combined with water chemistry data (S.K. Carmichael, unpublished data) as well as molecular-based and culture-dependent results suggests that the bloom of Mn(II)-oxidizing bacteria may have been associated with a nutrient loading event. More work is needed to determine if blooms of Mn(II)-oxidizing bacteria may be widespread where contamination is prevalent. However, if this hypothesis is validated, monitoring of Mn-oxidizing microbial communities in shallow cave systems would provide a cost-effective method of assessing anthropogenic impact.

DEDICATION

This thesis is dedicated to my parents, Jane and Richard Carmichael. I am deeply grateful for your love, support, and encouragement throughout my life.

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CHAPTER 1: INTRODUCTION

Karst terrain is a heterogeneous landscape that covers 20% of the Earth's dry land surface (White *et al.*, 1995). Carbonate rocks are the primary bedrock in karst regions (White *et al.*, 1995), and form the majority of the bedrock in eastern Tennessee (Oder, 1934). Other bedrock types, such as volcanic rocks, unconsolidated sediments, or clastic sedimentary rocks, may be just as easily weathered and exhibit external features and internal drainage complexities that are similar to those found in carbonate rocks (Veni, 2002; Goeppert and Goldscheider, 2011), and are therefore termed pseudokarst. Geomorphology in carbonate-hosted karst landscapes is determined by the influence of weathering along existing geologic features such as joints and bedding planes (White *et al.*, 1995; Northup *et al.*, 2000; Christman and Culver, 2001; Engel *et al.*, 2004; Ford, 2006). The characteristic features of karst regions include surface topography such as sinkholes, subsidence zones, and swallow holes. Subsurface features are characterized by the development of complex, dendritic internal drainage patterns (conduits) that can widen to form accessible regions known as caves and caverns (Palmer, 1991), which extend beyond the penetration of visible light (Northup and Lavoie, 2001).

Caves can form in a variety of bedrock substrates including carbonate rocks, evaporite rocks, and pseudokarst (Veni, 2002). Historically, cave ecology studies have been constrained to research in carbonate-hosted caves systems formed via one of two processes, sulfuric acid dissolution or carbonic acid dissolution. [However, it is important to note that research in pseudokarstic terrain is increasing, as lava tubes are recognized as astrobiological analogs (de los Ríos *et al.*, 2011; Northup *et al.*, 2011).] Sulfuric acid speleogenesis occurs via hypogenic processes as reduced sulfide rich groundwater upwells and comes in contact with the cave atmosphere. H_2S is off-gassed and becomes oxidized to form sulfuric acid (H_2SO_4), which dissolves the carbonate rock and may eventually precipitate gypsum, $CaSO_4$ (Engel *et al.*, 2004). On a global scale, carbonic acid speleogenesis is the most common method of cave

formation and is characterized by epigenic processes: the dissolution of limestone by carbon dioxide in solution (H_2CO_3) (White *et al.*, 1995). Microbes also play an active role in mineral weathering and precipitation in both sulfidic and epigenic caves, thus contributing to a variety of constructive and destructive processes within the cave environment (Jones, 2001; Warren and Kauffman, 2003; Engel *et al.*, 2004; Barton and Luiszer, 2005; Taboroši, 2006).

Caves represent a transition zone between the surface and subsurface environment (Pedersen, 2000). Much like the deep sea, cave zonation is defined by the extent to which light and surface conditions penetrate: (1) an entrance zone, close to the mouth of the cave and subject to surface conditions, (2) the twilight zone, a zone of transition, and (3) the deep zone, characterized by environmental stability with an absence of light, constant year-round temperature, and high humidity (Northup and Lavoie, 2001). The cave environment has long been recognized as unique milieu in which natural selection can exert its powerful forces (Poulson and White, 1969). Strong selective pressures have led to the evolution of endemic cave macrofauna (Porter, 2007), including a variety of troglophilic and troglobitic species with unique phenotypes such as lack of eyes and pigmentation, unique extrasensory organs, lengthened appendages, and slender body forms (Poulson and White, 1969; Engel, 2007; Fong, 2011) that are useful in the subterranean realm. Macrofaunal diversity in caves has been linked to cave systems with a high degree of either autochthonous energy production (e.g., chemolithoautotrophy) or allocthonous energy input (e.g., intersection with the phreatic zone, surface input) and larger cave systems with an increase in habitat availability (Culver and Sket, 2000). Caves also contain a diverse cave microflora (Barton and Jurado, 2007; Engel, 2007), which is known to vary in composition as a result of abiotic conditions (e.g., pH, nutrient availability, niche formation) within a cave system (Engel *et al.*, 2003; Barton and Luiszer, 2005; Macalady *et al.*, 2006; Barton *et al.*, 2007; Macalady *et al.*, 2007; Macalady *et al.*, 2008; Engel *et al.*, 2010).

Within the continental United States, there are five major cave regions (Christman and Culver, 2001) all located in karstified regions: the Florida Lime Sinks, the central and southern Appalachians (parts of West Virginia, Virginia, Tennessee, Georgia, and Alabama), the Interior Low Plateau (parts of

Illinois, Indiana, Kentucky, Tennessee, and Alabama), the Ozarks (parts of Missouri, Oklahoma, and Arkansas), and the Balcones Escarpment and Edwards Plateau (parts of Texas). Of these five regions, the Appalachians (Christman and Culver, 2001), and specifically the state of Tennessee (Barton and Jurado, 2007), represent one of the most cave-dense terrains in the continental United States. Despite the abundance of cave systems in the Appalachians, relatively little is known regarding the organisms that inhabit the subterranean realm therein.

A handful of studies have addressed the macrofaunal composition of Appalachian cave systems. Two recent surveys have elucidated macrofaunal community composition in caves within the Tennessee-Georgia-Alabama region (Campbell *et al.*, 2011b; Dixon and Zigler, 2011). In general, cave macrofauna exhibit a suite of characteristics that increase their vulnerability to disturbance (Fong, 2011; Humphreys, 2011). Due to the stability of the cave environment, anthropogenic impact and disturbance regimes have a quick and detrimental impact on cave communities (Fong, 2011; Gillieson, 2011; Humphreys, 2011; Northup, 2011). Anthropogenic related disturbance in cave environments is documented within the Appalachian region: (1) changes in cave macroinvertebrate populations in response to organic pollution events in Banners Corner Cave (Virginia) resulted in the elimination of sensitive species from highly degraded environments (Simon and Buikema Jr., 1997), and (2) the devastating impact of *Geomyces destructants* (Gargas *et al.*, 2009), the fungal species that is the reported cause of bat white nose syndrome and whose dispersion is thought to be partially linked to human traffic in cave systems, has resulted in the conversion of some cave systems into bat gravesites (Blehert *et al.*, 2011).

In addition, relatively little is known about the microbial communities of Appalachian caves. Relevant studies include a single assessment of fungal ecology in caves of Kentucky and Tennessee (Shapiro and Pringle, 2010), an examination of the role of stream biofilms in cave ecosystem energetics (Simon *et al.*, 2003), molecular characterizations of microbial mats in cave systems formed by sulfuric acid speleogenesis (Angert *et al.*, 1998; Engel *et al.*, 2001), and documentation of fecal coliforms in cave systems within Northern Georgia and Alabama (Campbell *et al.*, 2011a). However, none of these studies has addressed the geomicrobiology of a southern Appalachian cave system generated by carbonic acid

speleogenesis, the primary method of speleogenesis in karst systems worldwide (White *et al.*, 1995). The contents of this thesis represent the first analyses of the geomicrobiology of epigenic cave systems in the southern Appalachians, a cave-rich but vastly understudied region within the United States.

CHAPTER 2: MN(II)-OXIDIZING BACTERIA ARE ABUNDANT AND ENVIRONMENTALLY RELEVANT MEMBERS OF FERROMANGANESE DEPOSITS IN CAVES OF THE UPPER TENNESSEE RIVER BASIN

Summary

Ferromanganese deposits in the karst network of the upper Tennessee River Basin were examined using culture-dependent and independent techniques to determine the role of microbes in biogeochemical cycling of manganese and in the formation/transformation of cave mineralogy. Mn:Fe ratios, measured using ICP-OES, ranged from ca. 0.1 to 1.0 in Mn and Fe oxide-rich biofilms and mineral crusts from several different caves. At sites where the Mn:Fe ratio approached 1.0 this represents an order of magnitude increase above the bulk bedrock ratio, suggesting that biomineralization processes play an important role in these subsurface karst systems. Estimates of total bacterial SSU rRNA genes in ferromanganese biofilms measured approximately $9x10^9$ cells/g wet weight sample, a number that was confirmed via direct cell counts. A SSU rRNA based molecular survey of Mn Falls biofilm material revealed that 21% of the 34 dominant OTUs were closely related to known metal-oxidizing bacteria or clones isolated from oxidized metal deposits. Several isolates that promote the oxidation of Mn(II) compounds were obtained in this study, some from high dilutions (10^{-8}-10^{-10}) of cave biofilm material. SSU rRNA sequences of several of these Mn-oxidizing bacterial isolates most closely matched those of *Pseudomonas*, *Leptothrix*, *Flavobacterium,* and one species not previously known to oxidize Mn, *Janthinobacterium*. Combined data from geochemical analyses, molecular surveys, and culture-based experiments suggest that Mn(II)-oxidizing bacteria are abundant and environmentally

relevant members of ferromanganese deposits in the karst network of the upper Tennessee River Basin.

Introduction

Carbonate bedrock underlies 20% of the land area east of the Mississippi River (White *et al.*, 1995; Christman and Culver, 2001), forming an intricate karst hydrologic network that is riddled with caves. The southern Appalachians (e.g., West Virginia, Virginia, Tennessee, North Carolina, South Carolina, Georgia, and Alabama) contain numerous caves. Of the 50,000 cave systems known to exist in the United States, ca. 14% occur within the state of Tennessee (Barton and Jurado, 2007). Although cave geomicrobiology is an emerging field with recent studies involving caves in the southwestern United States and Europe (Northup *et al.*, 2003; Barton *et al.*, 2004; Porter *et al.*, 2009; Jones *et al.*, 2011), a paucity of information exists concerning the numerous cave systems of the Appalachian region.

To date, only a few studies describe the microbial communities of Appalachian cave systems. These include an assessment of fungal microbial ecology (Shapiro and Pringle, 2010), an examination of the role of stream biofilms in controlling cave ecosystem energetics (Simon *et al.*, 2003), molecular characterizations of microbial mats located in hypogene cave systems formed by sulfuric acid speleogenesis (Angert *et al.*, 1998; Engel *et al.*, 2001), and documentation of fecal coliforms in cave systems within Northern Alabama and Georgia (Campbell *et al.*, 2011). To our knowledge, our study represents the first characterization of the geomicrobiology of southern Appalachian cave systems created by the process of carbonic acid speleogenesis, the primary method of speleogenesis in karst systems worldwide (White *et al.*, 1995).

In past decades, the formation of cave mineral deposits and speleothems was thought to be primarily controlled by abiotic processes as a result of microsite environmental conditions (e.g., temperature, pH, solution chemistry), and changes in redox conditions (Northup and Lavoie, 2001; Engel

et al., 2004a; Barton and Northup, 2007). However, more recent geomicrobiology research lends support to the hypothesis that microbes play a role in the formation and dissolution of cave mineral deposits via direct and indirect metabolic activities and biomineralization processes (Northup *et al.*, 1997; Jones, 2001; Melim *et al.*, 2001; Barton and Luiszer, 2005; Spilde *et al.*, 2005; Cañaveras *et al.*, 2006; Taboroši, 2006; de los Ríos *et al.*, 2011). In particular, microbial reactions have been shown to promote the formation of cave manganese oxide and ferromanganese (mixed Fe and Mn oxides) deposits, such as corrosion residues (Northup *et al.*, 2000; Northup *et al.*, 2003; Spilde *et al.*, 2005), manganese flowstones (Gradziński *et al.*, 1995), rock coatings (Peck, 1986; Allouc and Harmelin, 2001), and manganese stromatolites (Rossi *et al.*, 2010).

These cave ferromanganese deposits may contain Mn oxide, Mn hydroxide, and Mn oxyhydroxide minerals (collectively referred to hereafter as Mn oxides), and the mineralogy of these deposits can be quite complex (Post, 1999; White *et al.*, 2009; Onac and Forti, 2011). The oxidation of Mn(II) to Mn(III) or Mn(IV), which is largely insoluble and precipitates out of solution as Mn(III/IV) oxide, is kinetically inhibited in the absence of a catalyst at near-neutral pH of most environments. Microorganisms are known to catalyze the oxidation of Mn(II) compounds, increasing reaction rates up to five orders of magnitude relative to abiotic oxidation rates (Nealson *et al.*, 1988; Dixon and Skinner, 1992; Francis and Tebo, 2002). Therefore, rapid Mn(III/IV) oxide depositional rates, especially those which exceed predicted abiotic reaction rates in a given environment, are a strong indication of microbial involvement in deposit formation (Nealson *et al.*, 1988).

The ability to oxidize Mn is a widespread trait among bacterial phylogenies, as members of the *Alphaproteobacteria* (Gebers and Hirsch, 1978; Anderson *et al.*, 2009), *Betaproteobacteria* (Emerson and Ghiorse, 1992), *Gammaproteobacteria* (Geszvain and Tebo, 2010), *CFB* (Nealson, 1978; Santelli *et al.*, 2010), and *gram-positive* (Dick *et al.*, 2006) lineages have demonstrated the ability to oxidize Mn. Fungal species are also capable of Mn biomineralization (Miyata *et al.*, 2006; Cahyani *et al.*, 2009; Santelli *et al.*, 2010; Santelli *et al.*, 2011). In most cases, the exact mechanism through which microbes induce the formation of Mn oxides remains enigmatic (Tebo *et al.*, 2005). However a few studies provide insight

into the complex biochemical pathways involved in Mn biomineralization, and it is generally recognized that microbial precipitation of Mn oxide minerals can be the result of passive (biologically-induced) or active (biologically-controlled) processes (Ghiorse and Ehrlich, 1992; Skinner and Fitzpatrick, 1992). In many cases, Mn-oxidation is either directly (Adams and Ghiorse, 1987; Boogerd and de Vrind, 1987) or indirectly (Learman *et al.*, 2011a) catalyzed by enzymes, and can occur via mechanisms that are associated with the structure of the microbial cell (e.g., membrane, sheath, or spore coat) (Boogerd and de Vrind, 1987; Larsen *et al.*, 1999; Francis and Tebo, 2002; Dick *et al.*, 2006) or via extracellular secretion of Mn-oxidases (Geszvain and Tebo, 2010; Santelli *et al.*, 2011). Therefore, biogenic oxides can either encrust microbial cells or accumulate in the immediate extracellular environment.

Regardless of their origin, Mn oxide minerals have highly charged surfaces and are biogeochemically active, demonstrating the ability to degrade humic substances (Sunda and Kieber, 1994), scavenge reactive oxygen species (Archibald and Fridovich, 1981; Daly *et al.*, 2004; Ghosal *et al.*, 2005; Learman *et al.*, 2011a), concentrate rare earth elements (Onac *et al.*, 1997), and influence trace metal (e.g., Co, Ni, Pb, Zn) bioavailability (Nelson *et al.*, 1999a,b; Post, 1999; Kay *et al.*, 2001; Manceau *et al.*, 2002; Villalobos *et al.*, 2005; Toner *et al.*, 2006) and speciation (Fendorf and Zasoski, 1992; White *et al.*, 2009). Biogenic oxides, which tend to have higher percentages of vacancies and smaller particle sizes (Webb *et al.*, 2005; Learman *et al.*, 2011b), demonstrate an increased sorptive capacity relative to abiotically produced oxides (Nelson *et al.*, 1999a). Therefore, biogenic Mn oxides may exert a greater impact on local geochemistry than abiotically generated deposits.

In this study, we examine the geomicrobiology of ferromanganese deposits in a cave system generated through carbonic acid speleogenesis, located in the cave-rich but poorly studied southern Appalachian karst region. The primary goal of this study is to better elucidate the role of microorganisms in the formation of cave ferromanganese deposits. Using a combination of molecular-based SSU rRNA analysis and culture-based methodologies, we demonstrate that Mn(II)-oxidizing bacteria are abundant and environmentally relevant constituents of ferromanganese deposits within cave systems of the upper Tennessee River Basin.

Results

Geochemistry and biogeochemical analyses

A variety of carbonate speleothem formations (flowstone, dripstone, soda straws, corrosion residue) occur throughout the cave systems in the upper Tennessee River Basin addressed in this study. All four cave systems (Carter Salt Peter Cave (herein CSPC), Worley's, Rockhouse, and Daniel Boone Caverns (herein DBC)) are particularly enriched in ferromanganese oxide deposits (Fig. S1), which are visible in the form of black or chocolate brown biofilms and mineral-rich crusts that coat cave walls and speleothems. The source of Fe(II) and Mn(II) necessary for the formation of these deposits is likely the Knox Dolomite bedrock, which contains ca. 88-445 ppm Mn and ca. 1340-7050 ppm Fe (Montañez, 1994). Studies in similar cave systems also indicate a bedrock source for these reduced compounds (Rossi *et al.*, 2010), or an aqueous source via groundwater percolation (Moore, 1981).

Weathering and corrosion of the Knox Dolomite bedrock by infiltration of meteoric water containing carbonic acid produces a nontronite (smectite-type) clay residuum in addition to calcite speleothems in all four study sites, including flowstones, rimstones, and popcorn-like micronodules (cave coral). We observe ferromanganese crusts and/or biofilms occurring on these nontronite substrates, on weathered dolostone bedrock, and on calcite speleothems at all four field locations. Geochemical analyses show that the concentration of total Mn in these crusts and biofilms ranges from 10.9 ppm – 327.5 ppm (Table 1). The Mn:Fe ratios in cave ferromanganese deposits vary between cave systems and within cave deposits, ranging from 0.06 -1.07 (Table 1). However, no obvious trends have emerged that indicate a possible effect of substrate mineralogy or water content on Mn:Fe ratios in these deposits. For example, samples taken from different locations with similar substrate compositions in Carter Salt Peter Cave show both the second highest (0.91) and lowest (0.06) Mn:Fe ratios. Interestingly, the Mn:Fe ratio at all sample sites, with the exception of CSPC Watermark (Table 1), was enriched relative to the average Mn:Fe ratio for Knox Dolomite (ca. 0.1) as reported by Lumsden and Caudle (2001). At sites where the

Mn:Fe ratio approaches 1 (Worley's Ribbon Rock and CSPC Mn Falls), this would represent an order of magnitude increase above the bulk bedrock ratio.

Quantification of microbial abundance using real-time quantitative PCR and fluorescence direct counts

Our qPCR results (Fig. 2) demonstrated that total bacterial SSU rRNA genes in biofilm samples from Mn Falls and Mud Trap Falls (CSPC) represented approximately $9x10^9$ cells/g wet weight, a number that was confirmed via fluorescence direct cell counts (Fig. S2). Total bacterial SSU rRNA genes from ferromanganese crusts (Fig. 2) were up to two orders of magnitude lower, containing from $7x10^7$ (Weathered Ribbon Rock, Worley's Cave)-$2x10^9$ (Hang Out, Rockhouse Cave) cells/g wet weight ($3x10^8$-$4x10^{10}$ SSU rRNA gene copies/g wet weight). Archaeal SSU rRNA gene sequences from cave samples were estimated to represent from $5x10^6$-$1x10^8$ cells/g wet weight ($8x10^6$-$2x10^8$ SSU rRNA gene copies/g wet weight), with an average of $5.6x10^7$ cells/g wet weight in biofilm samples. Archaeal percentages within the total microbial population were highest at several sample sites within the CSPC and Worley's cave systems: Dino Cove (ca. 6%), Ribbon Rock (ca. 6%), Watermark (ca. 7%), and Weathered Ribbon Rock (ca. 11%). The total estimated number of fungal ITS gene sequences from cave samples ranged from below detection limit (257-4,945 cells/g wet weight depending on the DNA extraction efficiency of individual samples) to $6.5x10^7$ cells/g wet weight ($1x10^{10}$ SSU rRNA gene copies/g wet weight) in biofilm samples, with an average of $1.32x10^7$ cells/g wet weight across all sample sites above detection limit. Overall, bacterial SSU rRNA gene copies represented 95% (89-99%) of the total estimated microbial cell numbers at each site, while those of archaea represented 5% (0.2%-11%), and those of fungi represented 0.3% (undetected-0.6%).

Analysis of bacterial and archaeal community structure through SSU rRNA clone library construction

Molecular based characterization of microbial community structure has been a staple of microbial ecology since the recognition that culture-based techniques vastly underestimate prokaryotic diversity within the environment (Staley and Konopka, 1985). PCR and cloning techniques are inherently biased in

all steps of the procedure, from DNA extraction (Klein, 2011), to PCR primer choice and reaction conditions (v. Wintzingerode *et al.*, 1997), to the statistical techniques used to analyze data (Kuczynski *et al.*, 2010; Kuczynski and Knight, 2011). In addition, concern also exists regarding sequencing depth of coverage when using small clone libraries, a factor that would lead to an underestimation of true environmental diversity and the exclusion of rare taxa in sequencing data (Fierer and Lennon, 2011). With the advent of second generation sequencing technologies, this concern is becoming less of an issue (Wooley *et al.*, 2010; Jones *et al.*, 2011). Despite the issues outlined above, 16S rRNA gene sequencing has been a reliable and accurate way of characterizing microbial community structure since its incorporation into the field of microbial ecology (Pace, 1997). In this study, a SSU rRNA clone library survey was conducted on two ferromanganese-rich biofilms (Table 1) located in close physical proximity within the CSPC system, Mn Falls (Fig. S1A, OTUs from this sample are prefaced by a *F* in Fig. 3 and Fig. 4) and Mud Trap Falls (Fig.S1B, OTUs from this sample are prefaced by a *T* in Fig. 3 and Fig. 4). The primary goal of the molecular survey was to characterize the microbes in cave ferromanganese deposits and to infer which microorganisms may be playing a role in Mn oxidation *in situ*, based on phylogenetic association and/or culturing analyses.

Using 98% sequence similarity to define archaeal operational taxonomic units (OTUs), analysis of archaeal libraries revealed 26 unique OTUs out of 65 total sequences. Rarefaction analysis of sequencing data indicated the development of an asymptotic trend (data not shown); therefore, sampling efforts were sufficient in capturing the archaeal diversity within the microbial community. Crenarchaeal sequences were binned into 3 operational taxonomic units (OTUs/species) (Fig. 3), representing ca. 40% of the total archaeal sequences in cave biofilm clone libraries. According to top BLAST hits, these OTUs were related to clone sequences from a variety of environments, from sediment, to the deep subsurface, freshwater systems, and other ferromanganese deposits. Crenarchaeal OTUs represented members of the Marine Group 1 Crenarchaea and SAGMA Groups 1 and 2. The dominant Crenarchaeal sequence in the present study was OTU TDO2, sharing 98% identity over a 765 bp alignment to a clone sequence isolated from freshwater ferromanganous micronodules and sediments (Stein *et al.*, 2001). Euryarchaeal

sequences from clone libraries were binned into 23 OTUs (Fig. 4), representing ca. 60% of the total archaeal sequences in the clone library data. OTUs represented members of the Deep Sea Hydrothermal Vent Group 6, Rice Cluster V, and *Methanobacteriales* and were closely related to other clones isolated from soil, freshwater and marine systems, and low-temperature environments. The majority of Euryarchaeal OTUs in this study represented members of Rice Cluster V. Members of the Deep Sea Hydrothermal Vent Group 6 (DSHV6) represented 29% of the total archaeal sequence types in cave biofilm clone libraries, and 45% of the Euryarchaeal sequences. OTU FG01, a member of the *Methanobacteriales* and representing 6% of the total archaeal sequences, shared 100% identity over a 765 bp alignment to *Methanobrevibacter acididurans*, a novel acid-tolerant, hydrogenotrophic methanogen isolated from an anaerobic digester (Savant *et al.*, 2002).

Bacterial community composition in the CSPC system was much more diverse than archaeal community composition, a pattern that is consistent with most environmental surveys, including caves (Northup *et al.*, 2003; Chelius and Moore, 2004; Macalady *et al.*, 2006; Macalady *et al.*, 2007). Using a 97% identity cutoff, there were 114 unique OTUs out of ca. 180 total sequences. Rarefaction analysis revealed no evidence of the development of asymptotic trend (data not shown), indicating that sampling efforts were not sufficient to measure the full extent of diversity within the biofilm communities. This type of depth of coverage issue is not uncommon in 16S rRNA sequence-based environmental studies using small clone libraries (Fierer and Lennon, 2011). For phylogenetic analysis, data was further reduced into 34 dominant OTUs representing ca. 100 sequences (Fig. 5, Fig. 6, and Fig. S3). A dominant OTU was defined as representing two or more sequences in the clone library, and it is important to note that this approach would result in an underestimation of diversity in the clone library as ca. 80 singleton OTUs were eliminated from downstream phylogenetic analysis.

Dominant bacterial OTUs from cave biofilms represented a diverse taxonomic array, with sequences from the library representing members of the *Bacteroidetes* (26%), *Betaproteobacteria* (20%), *Alphaproteobacteria* (15%), *Acidobacteria* (12%), *Gammaproteobacteria* (10%), *Verrucomicrobia* (7%), *Planctomycetes* (5%), *Chlorobi* (2%), and *Deltaproteobacteria* (2%). The three dominant classes of

bacteria, based on the percentage of sequences represented by dominant OTUs in the library, were members of the *Proteobacteria* (47%), *Bacteroidetes* (26%), and *Acidobacteria* (12%). Combined, these three phyla account for 85% of the dominant OTUs.

Several dominant bacterial OTUs identified in this study were related to clones and environmental isolates from freshwater and marine systems, sediment, contaminated ecosystems, other cave systems, and ferromanganese deposits. Some (ca. 10%) of the 34 dominant OTUs were related to known Mn-oxidizers such as *Leptothrix* (OTU BF2AO7, Fig. 5, 100% identical over a 1,485 bp alignment to *Leptothrix discophora* SP-6 (Emerson and Ghiorse, 1992)) and *Pseudomonas* (OTUs BF2B07 and BF2E03, Fig. 5). In addition, some (ca. 11%) of the 34 dominant OTUs were related to known Fe-oxidizers (*Leptothrix* spp.) or clones isolated from oxidized iron deposits (OTUs BF2C07 and BF2C10, Fig. S3). Combined, 21% of the sequences represented by dominant OTUs were related to known metal-oxidizers or clones isolated from oxidized metal environments.

Isolation of Mn(II)-oxidizing microorganisms

Six Mn(II)-oxidizing isolates obtained from CSPC ferromanganese deposits clustered within the *Gammaproteobacterial* subphylum near members of the genus *Pseudomonas*, a common group of known Mn(II)-oxidizing microorganisms (Fig. 5). Cultures N4, T4, T2, and N3 were isolated on a modified version of Burk's N-free media (Mohandas, 1988), although we have not yet established if these isolates are capable of N_2 fixation. Isolate N3 is 97% identical over a 1,502 bp alignment to OTU BF2E03, and isolates N4 and T4 are 97% identical (over a 1,181 and 1,473 bp alignment respectively) to OTU BF2B07. Isolate Mn Falls 11 is a close relative (99% identity over a 1,128 bp sequence alignment) of *Pseudomonas putida*, a model organism used in the study of the molecular mechanisms involved in Mn(II)-oxidation (Geszvain and Tebo, 2010).

Two Mn(II)-oxidizing isolates obtained from ferromanganese deposits within CSPC fall within the *Betaproteobacteria* (Fig. 5): *Janthinobacterium sp.* A6 and *Leptothrix sp.* G6. *Janthinobacterium sp.* A6 oxidizes Mn(II) in liquid culture, and appears to oxidize Mn extracellularly, since clumps of tissue-

paper like Mn oxides were loosely associated with cells (Fig 7). The closest cultured strain, *Janthinobacterium sp.* Acj215 (Fig. 5), isolated from the gut of a Japanese honeybee, is 99% identical to isolate A6 across a 1,123 base pair alignment. Isolate A6 was obtained from a serial dilution culture containing $2.5x10^{-8}$g wet weight biofilm material. Cave isolate *Leptothrix sp.* G6 oxidizes Mn along the sheath (Fig 7). This organism is 99% identical over a 1,485 bp alignment to the Mn-oxidizing, sheath-forming *Leptothrix discophora* SP-6 (Emerson and Ghiorse, 1992), and it was obtained from a serial dilution culture containing $2.5x10^{-8}$ g wet weight biofilm material.

Two Mn(II)-oxidizing isolates obtained from ferromanganese deposits within CSPC fall within the *Bacteroidetes* (Fig. 6): *Flavobacterium sp.* E8 and *Flavobacterium sp.* MTFA, which oxidize Mn(II) in liquid culture. *Flavobacterium sp.* E8 is 99% identical over a 1,469 bp alignment to its' closest cultivate relative, *Flavobacterium* sp. WB3.4.6, and *Flavobacterium sp.* MTFA is 98% identical over a 1,476 bp alignment to its' closest cultivate relative, *Flavobacterium* sp. PR4-11. Members of this genus have previously been reported to oxidize Mn(II) (Nealson, 1978; Ford and Mitchell, 1990; Santelli *et al.*, 2010). Isolate E8 was obtained from a serial dilution culture containing $2.5x10^{-10}$g wet weight biofilm material.

A single isolate falling within the *Actinobacteria*, *Arthrobacter sp.* L (Fig. 6), was obtained from a ferromanganese deposit located within Daniel Boone Caverns. *Arthrobacter* isolate L oxidizes Mn(II) in liquid culture; members of this genus have been reported to demonstrate this capability (Schweisfurth *et al.*, 1978). *Arthrobacter* sp. L is 98% identical over a 1,457 bp alignment to its' closest cultivated relative, *Arthrobacter methylotrophus*, a facultative methylotroph isolated from an enrichment culture containing dimethylsulfone as the sole source of carbon and energy (Borodina *et al.*, 2000; Borodina *et al.*, 2002). Interestingly, in addition to Mn(II)-oxidizing microorganisms, two putative methylotrophic Mn(II)-oxidizers were isolated in this study: a *Gammaproteobacterium*, *Acinetobacter sp.* V1 (Fig. 5), and a *Bacteroidete*, *Flavobacterium sp.* V2 (Fig. 6). Both isolates were obtained using NMS media, which was designed to target methylotrophic Mn oxidizers by using methane as a sole carbon source. However, methylotrophy has not yet been confirmed in these strains.

Discussion

The cave geochemical environment

Iron and manganese are the fourth and fifth most abundant elements in the Earth's crust, respectively (Edwards *et al.*, 2004; Tebo et al., 2007), where Fe outweighs Mn by a ratio of ca. 58:1 in the upper continental crust (Turekian and Wedelpohl, 1961; Wedelpohl, 1995). The predominance of Fe over Mn in a variety of natural systems is well documented in studies of marine (Edwards *et al.*, 2004; Nitahara *et al.*, 2011) and freshwater (Stein *et al.*, 2001; Johnson *et al.*, 2012) systems. However, biomineralization processes are invoked as a causal factor in the formation of a Mn-enriched geochemical environments in cases where the concentration of Mn is equal to or outweighs the concentration of Fe (e.g., Mn flowstones in caves (Gradziński *et al.*, 1995) and desert rock varnish (Krumbein and Jens, 1981)) or where secondary mineral deposits are enriched in metal concentration relative to substrate geochemistry as is common in many caves throughout the world (Cunningham *et al.*, 1995; Northup *et al.*, 2003; Spilde *et al.*, 2005; Spilde *et al.*, 2006).

Microbial biomineralization processes clearly contribute to ferromanganese oxide accretion in this study, where Mn concentrations were enriched relative to bedrock concentrations and where the Mn:Fe ratio approached 1:1, such as Worley's Ribbon Rock and CSPC Mn Falls (Table 1). A Mn:Fe ratio of ca. 1:1 in cave ferromanganese deposits is a common ratio found in oligotrophic systems in the southwest United States such as Lechuguilla and Spider Caves (Northup *et al.*, 2003; Spilde *et al.*, 2005), whereby darker deposits tend to show an enrichment in Mn relative to lighter deposits (Northup *et al.*, 2003; Spilde *et al.*, 2006). In the present study, geochemical analyses indicated that niches, with varying degrees of Fe and Mn enrichment, were forming within cave systems of the upper Tennessee River Basin. Previous research demonstrates the role of microsite geochemistry in establishing environmental niches (Macalady *et al.*, 2008; Engel *et al.*, 2010; Rossmassler *et al.*, 2012), structuring microbial communities (Goldscheider *et al.*, 2006; Barton *et al.*, 2007; Shabarova and Pernthaler, 2010), and influencing mineral

precipitation (Frierdich *et al.*, 2011) and composition (Post, 1999; White *et al.*, 2009) in subsurface karst systems. Enrichment of Mn relative to Fe at some sample locations, such as CSPC Mn Falls (Table 1, Fig. S1A), emphasizes the importance of understanding the role of Mn-oxidizing and Mn-reducing microorganisms at these sites, as microbial biomineralization and dissolution processes contribute to the formation and transformation of the cave mineral environment. However, the effects of agricultural and soil geochemical inputs on biomineralization processes in the shallow, epigenic cave systems in the upper Tennessee River Basin is not yet known.

Ferromanganous biofilm community composition

In the present study, qPCR data indicated that bacterial cells represented on average 95% of the total microbial cells at sample sites, a finding that is consistent with general thought regarding the abundance of bacteria (90% of the total cell count including archaea and eukarya) in environmental samples. Interestingly, the range of detection of bacterial cell numbers in our study ($7.5x10^7$ to $9.8x10^9$ cells/mL) was two to four orders of magnitude higher than numbers typically reported within similar, pH neutral cave systems (Northup *et al.*, 2000; Barton *et al.*, 2006). In addition, biofilm samples were estimated to contain $9.85x10^9$ total microbial cells/g wet weight, a quantity that is two orders of magnitude greater than studies of ferromanganese deposits in Spider and Lechuguilla caves (Spilde *et al.*, 2005). Deep cave systems with limited energy supply and minimal human impact are considered to be oligotrophic environments, and recent research indicates that they are low-biomass environments with high levels of microbial diversity (Northup and Lavoie, 2001; Barton *et al.*, 2004; Hunter *et al.*, 2004; Boston *et al.*, 2006). Although the total cell count reported in the present study is higher than that found in oligotrophic caves, it is not outside the range of numbers reported in studies of other environmental systems. Microbial cell abundance has been estimated to range from $1.29x10^9$ to $7.6x10^{10}$ cells/mL in marine sediments (Nitahara *et al.*, 2011), cold seep microbial mats (Grünke *et al.*, 2011), arable soil (Torsvik *et al.*, 2002), and filamentous microbial mats in sulfidic springs (Engel *et al.*, 2004b). At the time of sampling, the CSPC Mn Falls site was thought to be heavily impacted by organic input from sewage effluent. Bacterial

biomass in cave pools has been shown to increase proportionally with organic carbon input (Simon and Buikema Jr., 1997), and cell concentrations in the range of $1x10^9$ cells/mL have been reported in sewage (Fierer and Lennon, 2011). Therefore, high cell counts at sites within the CSPC system could be indicative of high levels of anthropogenic impact (e.g., nutrient loading), due in part to the shallow, surface-influenced depths of these caves. In fact, clone library data corroborate this idea as many of the clones obtained here were related to sequences obtained from degraded environments (see Fig. 5, OTUs BF2B07, BF2E04, BF2F03, BLD10, and BLB01).

In general, a high degree of diversity within systems is supported by microbial metabolic plasticity (Whitman *et al.*, 1998) and the development of mutualistic associations in biofilm communities leading to the interdependency of organisms within the community (Fierer and Lennon, 2011). The SSU rRNA data from the present study is suggestive of the presence of a variety of microbial metabolic strategies within CSPC biofilms, as clone sequences obtained in this study are closely related to methanogens (OTU G01, Fig. 4), hydrocarbon degraders (OTU BF2E03, Fig. 5), ammonia-oxidizers (OTU BF2F03, Fig. 5), denitrifiers (OTU BLB01, Fig. 5), and iron or manganese-oxidizers. Several lines of evidence from the SSU rRNA molecular-based survey of CSPC suggest that metal-oxidation plays an important role in the formation of ferromanganous biofilms within the cave system.

Members of the *Proteobacteria* (47% of the sequences represented by dominant OTUs in the present study) are common constituents of cave clone libraries and have been detected in RNA-based surveys as metabolically active members of cave microbial consortia (Portillo *et al.*, 2008). Members of the *Bacteroidetes* (26% of the sequences represented by dominant OTUs in the present study) have been detected in both pH neutral (Schabereiter-Gurtner *et al.*, 2002b; Chelius and Moore, 2004) and sulfidic (Engel *et al.*, 2001; Barton and Luiszer, 2005) cave systems. Molecular work from the present study indicates that *Leptothrix* are dominant members of the CSPC Mn Falls biofilm community, with 6% of the total library sequences represented by OTU BF2A07 (Fig. 5), a close relative (99% identity over a 1,489 bp sequence alignment) of the *Leptothrix* strain isolated in this study suggesting that these organisms play an important role in the biomineralization of Mn in southern Appalachian cave systems.

Leptothrix sp. are known to be capable of both Fe and Mn oxidation (van Veen *et al.* 1978; Spring, 2006) therefore we cannot rule out the role of microbially induced Fe oxidation by *Leptothrix* and other related organisms in the formation of cave ferromanganese biofilms and crusts.

Of particular interest in the present study, is the relatively high proportion of *Acidobacteria* (12% of the sequences represented by dominant OTUs in the present study) in CSPC biofilms. Members of this class have been detected in molecular-based studies of both pH neutral (Schabereiter-Gurtner *et al.*, 2002a, b; Chelius and Moore, 2004; Schabereiter-Gurtner *et al.*, 2004; Northup *et al.*, 2011) and sulfidic (Engel *et al.*, 2004b) cave systems. *Acidobacteria* are known to be recalcitrant to cultivation (Stevenson *et al.*, 2004), therefore information concerning their physiological role in the environment is lacking. Evidence from several studies indicates that *Acidobacteria* may be important metabolically active members of cave microbial consortia (Meisinger *et al.*, 2007; Portillo *et al.*, 2008). However, further research is needed to determine if members of the *Acidobacteria* play a role in cave biomineralization processes. One of the few isolated *Acidobacteria* cultivars, *Geothrix fermentans* (Coates *et al.*, 1999), is a known Fe(III)-reducer, suggesting the potential for these organisms to be involved in the reductive dissolution of cave ferromanganese deposits. Interestingly, OTU BF2C10 (Fig. S3), closely related to other clones isolated from freshwater ferromanganese deposits (Stein *et al.*, 2001) and iron-oxidizing biofilms (Duckworth *et al.*, 2007), shares 100% identity over a ca. 1500 base pair alignment with *Geothrix fermentans*. Overall, results suggest that many of these organisms (among the *Acidobacteria*, *Proteobacteria* and *Bacteroides*) may be responsible for, or at least involved in the transformation (precipitation or dissolution) of cave ferromanganese deposits.

The identification of clones related to organismal or clonal sequences from a variety of oxidized metal environments corroborates studies of ferromanganese deposits in caves and other systems (Stein *et al.*, 2001; Northup *et al.*, 2003; Spilde *et al.*, 2005). In the present study, these sequences represented 21% of the total dominant OTUs, suggesting that metal-oxidizers are abundant members of the CSPC biofilm communities and contribute to the formation of ferromanganese deposits. Horner-Devine *et al.* (2007) demonstrated that a certain degree of structure occurs within microbial communities, as species

assemblages exist in non-random patterns that can be dictated by environmental conditions. Therefore, the observed enrichment of metal-oxidizing organisms in the present study is unlikely to represent a stochastic fluctuation in community structure, and is likely related to the Fe and Mn geochemistry at these sites.

Archaea, like bacteria, are ubiquitous within the environment (DeLong, 1992), and play a key role in the maintenance of biogeochemical cycles (Goldscheider *et al.*, 2006). Despite the widespread distribution of archaea in the environment (Chaban *et al.*, 2006) and the known metabolic diversity of cultured representatives, archaea remain understudied members of microbial communities, particularly in caves. The role of archaea in the formation and transformation of cave ferromanganese deposits is debatable, although molecular evidence of archaea in clone libraries generated from cave ferromanganese deposits (Northup *et al.*, 2003) has led some investigators (Tebo *et al.*, 2005) to speculate regarding the possible existence of a new class of archaea capable of Mn-biomineralization.

In the present study, archaea represented ca. 5.0% of the total microbial population in cave biofilms, a finding that is consistent with the general thought regarding the abundance of archaea in environmental microbial consortia, typically about 10.0% of the total microbial population. Archaeal community diversity was relatively low in comparison to bacterial community diversity; however, this trend is consistent with literature reports in caves (Macalady *et al.*, 2007), marine ferromanganese crusts (Nitahara *et al.*, 2011), and other environments (DeLong, 1992).

Members of the Crenarchaea (Northup *et al.*, 2003; Chelius and Moore, 2004; Spear *et al.*, 2007; Llirós *et al.*, 2008; Chen *et al.*, 2009) and the Euryarchaea (Northup *et al.*, 2003; Llirós *et al.*, 2008; Chen *et al.*, 2009) have been detected in molecular surveys from caves, subterranean environments, and karst systems. The archaeal community in this study was dominated by members of the Marine Group 1 Crenarchaea. OTU TDO2 accounted for 37% of the total archaeal diversity in the library, and 92% of the Crenarchaeal diversity. Members of the Rice Cluster V and Deep Sea Hydrothermal Vent Group 6 (DSHV6) Euryarchaea were the second and third most dominant archaeal groups, representing 32% and 29% respectively of the total archaeal diversity captured in this study. Of the six DSHV6 OTUs identified

in this study, four (FD02, FF04, TD04, and TD06) were less than 95% similar to their closest relatives as determined by BLAST analysis. This high degree of divergence (Amann *et al.*, 1995) indicates that these sequences represent novel lineages unique to CSPC or other similar systems. Several findings emphasize the need to ascertain the functional role of archaea in cave biogeochemical cycling: 1) the dominance of sequences related to Crenarchaeal OTU TD02 in the CSPC Mn(II)-oxidizing biofilm community, 2) recent evidence from other studies of metabolically active Crenarchaea in similar cave systems (Gonzalez *et al.*, 2006) , and 3) the recovery of several novel Euryarchaeal lineages in CSPC.

Fungi, like archaea, are understudied components of cave ecosystems, and little is known about the abundance or environmental relevance of fungi in caves (Bastian *et al.*, 2009). By using 169 copies/fungal genome for our qPCR standardization, it is likely that qPCR measurements of fungal cell abundance in the present study underestimate actual abundance within sample sites. Nevertheless, our results are generally consistent with what is known about soil microbial communities in that eukaryotic cells represent ca. 1.0 % of the total cell population in environmental samples; therefore, fungal:bacterial ratios are typically well below 1 (Fierer *et al.*, 2005). However, it is important to note that abundance within an environment does not necessarily correlate with environmental relevance. Rare taxa, such as fungi, often comprise the majority of microbial diversity in the environment and are thought to be important components of the functional diversity of a system (Curtis and Sloan, 2005; Sogin *et al.*, 2006; Huber *et al.*, 2007). Further, the maintenance of such diversity is a key component contributing to the maintenance of ecosystem stability (Lennon and Jones, 2011).

Fungal diversity in cave systems is thought to be high (Roble *et al.*, 2011; Vaughan *et al.*, 2011), and the existence of tightly coupled fungal-bacterial interactions (e.g., chemolithoautotrophic bacteria providing the organic base to support heterotrophic bacteria and fungi) have been demonstrated in cave ferromanganese deposits (Cunningham *et al.*, 1995). Though fungi mediate key biogeochemical transformations within the environment regularly (Sterflinger, 2000; Fomina *et al.*, 2005; Dupont *et al.*, 2007), the extent to which fungi are involved in the formation of ferromanganese deposits is the subject of long-standing debate (Taylor-George *et al.*, 1983; Grote and Krumbein, 1992; Schelble *et al.*, 2005). Mn-

oxidizing fungi represent a phylogenetically diverse group of organisms, yet little is known about the role of these organisms in the biogeochemical cycling of manganese (Miyata *et al.*, 2006). The importance of fungi as unrecognized constituents of metal-oxidizing communities has recently been highlighted in the work of Santelli *et al.* (2010). The role of fungi in the formation and transformation of the cave mineral environment is a relevant and important future line of study within the broader field of cave geomicrobiology, and is currently being investigated at these sites.

Microbial Mn-biomineralization in cave ferromanganese deposits

The present study is significant due to the broad taxonomic array of bacteria isolated from cave ferromanganese deposits that demonstrate the ability to oxidize Mn(II) in culture. To our knowledge, the isolation of a *Janthinobacterium* sp. capable Mn(II)-oxidation represents the first report of a Mn(II)-oxidizing member of this genus. This finding expands the diversity of known Mn-oxidizers and provides further support for the importance of this process to the microbial cell. In addition, this study represents the first report of *Flavobacterium* and *Arthrobacter* strains isolated from a cave that demonstrate Mn-biomineralization capacity *in vitro*. *Flavobacterium* (Ikner *et al.*, 2007) and *Arthrobacter* (Laiz *et al.*, 2000; Ikner *et al.*, 2007) species have been isolated from cave systems in prior studies, although the Mn-biomineralization capacity of these isolates was not established.

In addition to the above strains, the isolation of a Mn(II)-oxidizing *Leptothrix* from CSPC represents the first reported isolation of this organism from a cave in over twenty years (Moore, 1981; Peck, 1986), although recent work has demonstrated the presence of *Leptothrix* in caves using scanning electron microscopy (de los Ríos *et al.*, 2011; Florea *et al.*, 2011; Frierdich *et al.*, 2011; S.K. Carmichael, unpublished data). Iron and manganese oxidizing *Leptothrix sp.* are commonly isolated from freshwater systems and are particularly abundant at redox interfaces (Spring, 2006). However, *Leptothrix sp.* typically lose their sheath-forming capacity in culture, so isolation of a close relative of the sheath-forming strain SP-6 from a high dilution is unusual and noteworthy.

Over 30 different metal oxide and hydroxide minerals can be found in caves (Hill and Forti, 1997), though it is important to note that not all black or dark brown deposits in caves are Mn oxides (Hill, 1982; Gázquez *et al.*, 2012) and it is unlikely that all are formed by microbes. However, Mn-oxidizing microbes have been shown to produce several of the Mn oxide minerals (such as birnessite and todorokite) commonly found in caves (Noskin, 2001; Spilde *et al.*, 2005; Santelli *et al.*, 2010; Frierdich *et al.*, 2011). The isolation of three Mn(II)-biomineralizing organisms from high dilutions ($2.5x10^{-8}$ g wet weight and $2.5x10^{-10}$ g wet weight) is suggestive of the environmental relevance of these genera (*Janthinobacterium*, *Leptothrix*, and *Flavobacterium*) in the formation of cave ferromanganese deposits in the upper Tennessee River Basin. The diversity of Mn(II)-oxidizers isolated in this study, combined with the evidence of biomineralization capacity within cave isolates demonstrates the importance of these organisms in cave biogeochemical cycles.

Conclusions

The present study represents the first geomicrobiological analysis of ferromanganese deposits within the cave-rich, yet vastly understudied southern Appalachian karst system. Experimental results better clarify the role of microbes in 1) biogeochemical transformations within the Carter Salt Peter Cave system and 2) the formation and transformation of the cave mineral environment. Elemental analysis indicates the presence of Mn-enriched geochemical environments within some ferromanganese deposits in the CSPC system. SSU rRNA based molecular surveys at Mn-enriched sites reveal the genetic potential for Mn-biomineralization within these deposits. Biomineralization capacity was inferred via culture-dependent surveys, which resulted in the isolation of a broad taxonomic array of Mn(II)-oxidizing bacteria from cave ferromanganese deposits. Overall, molecular evidence and cultivation-based techniques demonstrate that Mn(II)-oxidizing bacteria are abundant and environmentally relevant species in cave ferromanganese deposits. Although archaea are also present in these deposits, their contribution to the formation of the metal-oxide minerals is not conclusive as cultivation attempts did not succeed in isolating these microbes. We cannot rule out the possibility that archaea do not participate in the biogeochemical cycling of metals

in this cave environment. The Mn-oxidizing bacteria identified here likely play a role in mediating cave biogeochemical cycles, forming and transforming the cave mineral environment, and are vital contributors (via functional diversity) to the maintenance of cave microbial consortia (Warren and Kauffman, 2003) within these fragile and unique cave systems. However, more work is needed to better elucidate the full cycle of metal transformation within the cave environment.

Experimental Procedures

Field Description

The study area (Fig. 1, inset) is comprised of several epigenic caves, all located in the Ordovician Knox Dolomite unit (Oder, 1934) within the upper Tennessee River Basin (spanning northeast Tennessee and southwest Virginia). The primary study site, Carter Salt Peter Cave (Carter County, Tennessee, Fig. 1), is an epigenic cave system typical of those found within the Appalachian region. Carter Salt Peter Cave, herein referred to as CSPC, is relatively shallow cave system occurring at a depth of approximately 30 m. Evidence of anthropogenic impact is widespread throughout the system. Within the dark zone of the cave, ambient temperature remains around 13°C year-round and humidity levels approach 100%. These conditions are typical of cave systems where temperature in the deep zone annually hovers around MAST (Mean Annual Surface Temperature) for a given region (Northup and Lavoie, 2001). Rockhouse Cave is located less than 2 km east of CSPC, and the two systems are hydrologically connected (Gao *et al.*, 2006b; Gao *et al.*, 2006a). Worley's cave, located 24 km northeast of CSPC in Sullivan County, TN, is frequently visited by humans and contains a substantial subterranean creek system that exits the cave to a surface water system. Recent work (Y. Gao, unpublished data) has demonstrated that Worley's cave is hydrologically connected to sinkholes in nearby farm fields and therefore may be susceptible to agricultural runoff. In contrast to these three anthropogenically-impacted caves, Daniel Boone Caverns (herein referred to as DBC) in Scott County, VA is rarely visited and is located in an isolated forest location on the top of a ridge. Therefore, DBC is not subject to agricultural or municipal runoff. It

contains several pools and drip networks, but does not have an extensive subsurface hydrologic system at present.

Sample collection

Samples were collected in roughly three month intervals from July 2009 to September 2011 in four cave systems (Fig. 1) located in eastern Tennessee and southwest Virginia: Carter Salt Peter Cave, Daniel Boone Caverns, Rockhouse Cave, and Worley's Cave. Ferromanganese deposits were identified within cave systems as black/chocolate brown patinas coating cave rocks and walls. Deposits were screened for the presence of Mn oxides using 0.04% Leucoberbelin Blue (LBB), a redox indicator that is oxidized by Mn(III) or Mn(IV) to produce a bright blue color change (Krumbein and Altmann, 1973). Deposit morphology was highly variable within systems (Fig. S1), with LBB-positive samples collected from biofilms, ferromanganese coatings and crusts, and ferromanganous micronodules. Samples were collected aseptically by scraping the deposit surface using a sterile 50 mL Falcon tube. Samples were stored on ice, transported to the lab, and immediately processed for downstream use.

Geochemistry and biogeochemical analyses

A subset of cave ferromanganese samples were analyzed at Appalachian State University to determine substrate mineralogy (Table 1) using a Shimadzu 6000 powder X-ray diffractometer with a Cu X-ray source and measured from 5-80° 2θ. Species were identified using the PDF/4+ Minerals Database. A subset of samples were collected for metal analyses in December 2009 (Worley's Cave) and January 2010 (Carter Salt Peter Cave), lyophilized over a 48 hour period, then following USEPA SW846 Method 3051A: Microwave Assisted Acid Digestion of Sediments, Sludges, Soils, and Oils (Link *et al.*, 1998; USEPA, 2007). Elemental analysis was performed in duplicate on several rock and biofilm samples (Table 1) to determine total Mn and Fe content using a Varian 710-ES Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES).

Real-time quantitative PCR

Ten sample sites across three cave systems were analyzed using real-time quantitative PCR to quantify the relative abundance of archaea, bacteria, and fungi in the Carter Salt Peter, Rockhouse, and Worley's cave systems (Fig. 2). Primers were selected for each domain that demonstrated broad coverage over the 16S rRNA (archaea and bacteria) and fungal ITS1 regions. The forward primer 338F 5'TCC TACGGGAGGCAGCAGT (Nadkarni *et al.*, 2002) was paired with the reverse primer 518R 5'ATTACCGCGGCTGCTGG (Einen *et al.*, 2008) to target the bacterial 16S rRNA gene sequence. The primer pair 967F 5'AATTGGCGGGGGAGCAC/1060R 5'GGCCATGCACCWCCTCTC (Cadillo-Quiroz *et al.*, 2006) was selected to target the archaeal 16S rRNA gene sequence. The fungal ITS1 gene sequence was targeted using the ITS1F 5'TCCGTAGGTGAACCTGCGG/5.8sR 5'CGCTGCGTTCTTCATCG primer pair (Fierer *et al.*, 2005). Amplifications were performed in triplicate on an Applied Biosystems™ 7300 Real-Time PCR System (Carlsbad, CA) using Maxima™ SYBR Green/ROX qPCR Master Mix (Fermentas, Glen Burnie, MA) with 2 ng sample DNA/well. Calibration curves for quantification were generated using one of the following standards: 1) plasmid DNA containing the SSU rRNA gene from *Rhodobacter* sp. CR07-74 (bacteria, range of 10^2–10^9 target copies/µL), 2) genomic DNA extracted using the Qiagen DNeasy Blood and Tissue Kit (Valencia, CA) from *Methanoregula boonei* 6A8 (archaea, range of 10^2-10^7 target copies/µL), and 3) plasmid DNA containing the fungal ITS1 gene from *Saccharomyces cerevisiae* (fungi, range of 10^1-10^8 target copies/µL). Circular plasmid DNA standards have been reported to cause overestimation of sample cell number using quantitative PCR (Hou *et al.*, 2010). To circumvent this potential issue, plasmid DNA standards were lineraized by restriction digest using BssHII (bacteria) and NCOI-HF™ (fungi) (New England BioLabs, Ipswich, MA). rRNA operon copy numbers in microbial cells are variable (Fogel *et al.*, 1999; Warner, 1999) and change based on environmental conditions (Klappenbach *et al.*, 2000; Anderson and Cairney, 2004). Therefore, SSU rRNA gene copy number was normalized in experimental results using the average copy number for archaea (1.07 copies/cell) and bacteria (4.08 copies/cell) as reported by the Ribosomal RNA Operon Copy Number Database (Klappenbach *et al.*, 2001) in March, 2010. A

conservative normalization of fungal copy number was made by standardizing results using the average rRNA gene operon copy number (169 copies/cell) for the chytrid *Batrachochytrium dendrobatidis* (Lefèvre *et al.*, 2010), as chytrids are known members of the CSPC microbial consortia (B. Zorn, unpublished data).

Fluorescence direct counts

Fluorescence direct counts were performed on samples from Mn Falls and Mud Trap Falls (CSPC) to confirm the validity of real-time quantitative PCR results (Fig. S2). Cave biofilm samples were collected for fluorescence direct counts in Fall 2009. Upon receipt in the lab, 0.1 g (wet weight) of biofilm material was mixed 1:10 w/v with 0.1% (final concentration) sodium pyrophosphate ($Na_4P_2O_7.10\ H_2O$) and vortexed for ten minutes to disrupt cell clumps and homogenize the material. Samples were fixed in a 4% paraformaldehyde solution and stored at 4°C overnight. The following morning, samples were re-suspended by vortexing, and a 5 µL sample was then applied to a slide and evenly spread over 484 mm^2 surface area. Samples were stained with 1 µg/mL (final concentration) DAPI (4,6-diamino-2-phenylindole), and Citiflour Antifadent Mounting Medium AF1 (Electron Microscopy Sciences, Hatfield, PA) was applied to prevent bleaching of the DAPI fluorescent signal. Fluorescence direct counts were conducted at 100X magnification on an Olympus Bx51 fluorescence microscope. Fields of view were randomly selected and counted until a minimum of 300 cells/sample were visualized and recorded.

DNA extraction, cloning, sequencing, and phylogenetic analysis

DNA was extracted from cave samples using a bead beating protocol with the Fast DNA Spin Kit for Soil (MP Biomedicals, Solon, OH). The concentration of extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Extracted DNA was used to create a total of four clone libraries. For bacteria, one library each was created from DNA extracted from light and dark material at the Mn Falls site. For archaea, one library was created from DNA extracted from each cave biofilm (Mn Falls and Mud Trap Falls). PCR amplification of bacterial and archaeal SSU

rRNA gene sequences was conducted using the primer 27F 5'AGAGTTTGATCMTGGCTCAG (Lane, 1991) combined with a modified version of 1492R primer 5'RGYTACCTTGTTACGACTT (for bacteria) (Emerson and Moyer, 2002) and the 109F 5'ACKGCTCAGTAACACGT and 912R 5'CTCCCCCGCCAATTCCTTTA primer pair for archaea (Lueders and Friedrich, 2000). PCR amplifications were conducted in triplicate, and amplified PCR products were pooled before purification using either the Montage® PCR Purification Kit (Millipore, Billerica, MA) or the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). PCR products were cloned into TOPO TA *pcr*®2.1 vectors (Invitrogen, Carlsbad, CA), and plasmid DNA extracted from transformants using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was screened by sequencing using the M13F(-20) primer. 96 well plates of glycerol stocks were prepared for each sample site using each primer set and sequenced using M13F(-20) and M13R(-27) primers. Sequencing was conducted at Beckman-Coulter Genomics (Danvers, MA). Chimeric sequences were eliminated from analysis prior to consensus sequence construction. Sequences from both libraries were pooled (creating a ca. 180 sequence bacterial library and 65 sequence archaeal library) for DOTUR analysis in order to make OTU determinations (Schloss and Handelsman, 2005). Representative sequences for each OTU for archaea, or each dominant OTU (as defined by two or more sequence representatives) for bacteria were chosen based on sequence length and quality. For the bacterial dominant OTUs, additional sequencing of transformant plasmid DNA was conducted using primers 357F 5'CCTACGGGAGGCAGCAG, 926R 5'CCGYCWATTCMTTTRGTTT, and 1098R 5'GGGTYKCGCTCGTTGC to obtain a full-length SSU rRNA gene sequence. Contigs were assembled using Sequencer sequence analysis software (Gene Codes Corporation, Ann Arbor, MI). For phylogenetic analysis, additional sequences of interest were selected using ARB (Ludwig *et al.*, 2004) and the NCBI taxonomic database (Johnson *et al.*, 2008). OTU and additional sequences of interest were aligned using the on-line SILVA aligner (Pruesse *et al.*, 2007). Phylogenetic trees (Fig. 3-6 and Fig. S3) were constructed using the PHYLIP software package (Felsenstein, 2004) by conducting both neighbour-joining and maximum likelihood analysis. Clone sequences were deposited in GenBank under the accession numbers JN820160-JN820219.

Isolation of Mn(II)-oxidizing cave microorganisms on agar media

Mn oxide rich samples from ferromanganese biofilms, micronodules, and coatings on rock walls and speleothems were collected in an attempt to cultivate Mn(II)-oxidizing cave microorganisms. Samples were transported to the lab on ice and immediately plated on a variety of media designed to target the phylogenetically diverse array of Mn-oxidizers. Some plates were incubated under full oxygen conditions, while others were incubated microaerophilically, with the recognition that oxygen limited environments constitute important niches in cave systems (Portillo and Gonzalez, 2009). More than ten media types were utilized over the course of the study. A modified version of AY media (Santelli *et al.*, 2011) was created by supplementing the media post-autoclaving with 100 µM $MnCl_2$. A modified version of Burk's nitrogen-free medium (Mohandas, 1988) was created to target putative nitrogen-fixing, Mn(II)-oxidizing microorganisms by substituting an equimolar concentration of succinic acid, disodium salt for sucrose as a carbon source and amending the media (post-autoclaving) with 100 µM $MnCl_2$ and 3.7 mM $FeCl_3$. A novel media, Nitrate Mineral Salts (NMS), was designed for this study by T. Craig to target methylotrophic Mn(II)-oxidizers. NMS contains (in g L^{-1})1 $MgSO_4$•$7H_20$, 0.14 $CaCl_2$•$2H_20$, 1 KNO_3, 0.27 KH_2PO_4, 0.3 NaH_2PO_4, 1 mL trace element solution (containing in mg L^{-1} 1000 EDTA, 400 $FeSO_4$•$7H_20$, 250 $CuSO_4$•$5H_20$, 20 $ZnSO_4$•$7H_20$, 6 $MnCl_2$•$4H_20$, 60 H_3BO_3, 40 $CoCl_2$•$6H_20$, 2$CaCl_2$•$2H_20$, 4 $NiCl_2$•$6H_20$, 6 Na_2Mo_4•$2H_20$). pH of the media was adjusted ca. 7.1-7.2 before autoclaving, and 15 g agar was added for plates. NMS media was supplemented post-autoclaving with sterile 0.02 M Hepes buffer pH 7.2, 5 µM ferrous ammonium citrate, 0.2% v/v vitamin solution for J medium (Tebo *et al.*, 2007), and 100 µM $MnCl_2$. For agar plates, a 0.05% v/v methanol was added as a carbon source; a 50:50 CH_4(g):air mix was used as the sole carbon source for liquid media. A new medium, FMO2, was designed for this study by S.L. Bräuer and contains (in g L^{-1}) 10 mL Major Metals 1 solution (containing in g L^{-1} 12NaCl, 1.2 KCl, 5 $MgCl_2$•6H20, 1 KH_2PO_4, 2 NH_4Cl, 1 $CaCl_2$•$2H_20$), 1 mL 1000X Trace Metal 1 Solution with NTA (containing in g L^{-1} 0.15 $CoCl_2$•$6H_20$, 0.15 $ZnCl_2$, 0.05 H_3BO_3, 0.02 $NiCl_2$•$6H_20$, 0.01 Na_2Mo_4•$2H_20$, 0.4 $FeCl_2$•$4H_20$, 0.1 $MnSO_4$•$4H_20$, 3 $MgSO_4$•$7H_20$, 0.1 $CaCl_2$•$2H_20$, 0.01 $CuSO_4$•$5H_20$,

0.18 $AlK(SO_4)_2 \cdot 12H_20$, 1.5 NTA), and 0.05 yeast extract. pH of the media was adjusted to ca. 7.0-7.2 before autoclaving, and either 15 g agar or Gelrite gellan gum (as suggested by Hara *et al.*, 2009) was added for plates. The media was supplemented post-autoclaving with sterile 0.02 M Hepes buffer pH 7.2, 5 µM ferrous ammonium citrate, 0.2% v/v vitamin solution for J medium (Tebo *et al.*, 2007), 100 µM $MnCl_2$, and either 10% 2M arabinose, 10% 2M succinate, or 10% casamino acids as a carbon source. Plates were inoculated by spreading 80 µL of a 1% v/v Mn oxide rich sample in 0.02M Hepes buffer pH 7.2 on agar-solidified media. All cultures were incubated in the dark at 10°C to mimic environmental conditions within caves. Mn(II)-oxidation was confirmed in isolates by LBB testing; LBB-positive isolates were re-streaked for isolation a minimum of three times on the equivalent growth medium.

Isolation of Mn(II)-oxidizing cave microorganisms from serial dilutions

Serial dilutions to extinction were inoculated in Cellstar 96 well culture plates (greiner bio-one, Monroe, NC) using three different media types: FMO2 media with either 10% 2M arabinose, 10% 2M succinate, or 10% casamino acids as a carbon source. Inocula from the most dilute sample that grew and produced dark brown/black precipitates was transferred to the equivalent agar-solidified growth medium and were re-streaked for isolation a minimum of three times. Mn-oxidation was confirmed in isolates by LBB testing. All cultures were incubated in the dark at 10°C, mimicking environmental conditions within caves.

Identification of the isolates

Once a colony was isolated, a colony PCR reaction was used to screen the microorganism for phylogenetic placement using the universal bacterial primer 357F 5'CCTACGGGAGGCAGCAG. Isolates of interest were cloned using TOPO TA *pcr*®2.1 vectors (Invitrogen, Carlsbad, CA), and plasmid DNA containing the SSU rRNA gene sequence was sequenced using M13F(-20), M13R(-27), 357F 5'CCTACGGGAGGCAGCAG, 926R 5'CCGYCWATTCMTTTRAGTTT,and 1098R 5'GGGTYKCGCTCGTTGC primers to obtain a full-length SSU rRNA gene sequence for phylogenetic

placement as described above. Contigs were assembled using Sequencher sequence analysis software (Gene Codes Corporation, Ann Arbor, MI). Isolate sequences were deposited in GenBank under the accession numbers JN820147-JN820159.

TEM microscopy and elemental analysis

Several Mn-oxidizing bacterial cultures were examined using a JEOL JEM-1400 transmission electron microscope (TEM) equipped with an Oxford INCA energy dispersive X-ray detector (EDS) to confirm the presence of Mn oxides associated with microbial cells. Samples were mounted on Formvar Carbon Type-B, 200 mesh Cu TEM grids (Ted Pella, Redding, CA) by diluting liquid cultures 1:5 using sterile deionized water and applying 5 µL dilution to each grid. TEM grids were allowed to air dry in a laminar flow cabinet. This process was repeated a total of three times, with a total volume of 15 µL of diluted culture applied to each grid, and then carbon coated. Samples were initially imaged using transmission electron microscopy and spot analyzed with EDS to confirm the presence of Mn deposits. Several samples were selected for elemental mapping via scanning transmission electron microscopy (STEM) to confirm the locations of Mn within the sample (Fig. 7). In all cases, EDS analysis (data not shown) demonstrated the presence of concentrated Mn deposits associated with microbial cells.

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Figures and Tables

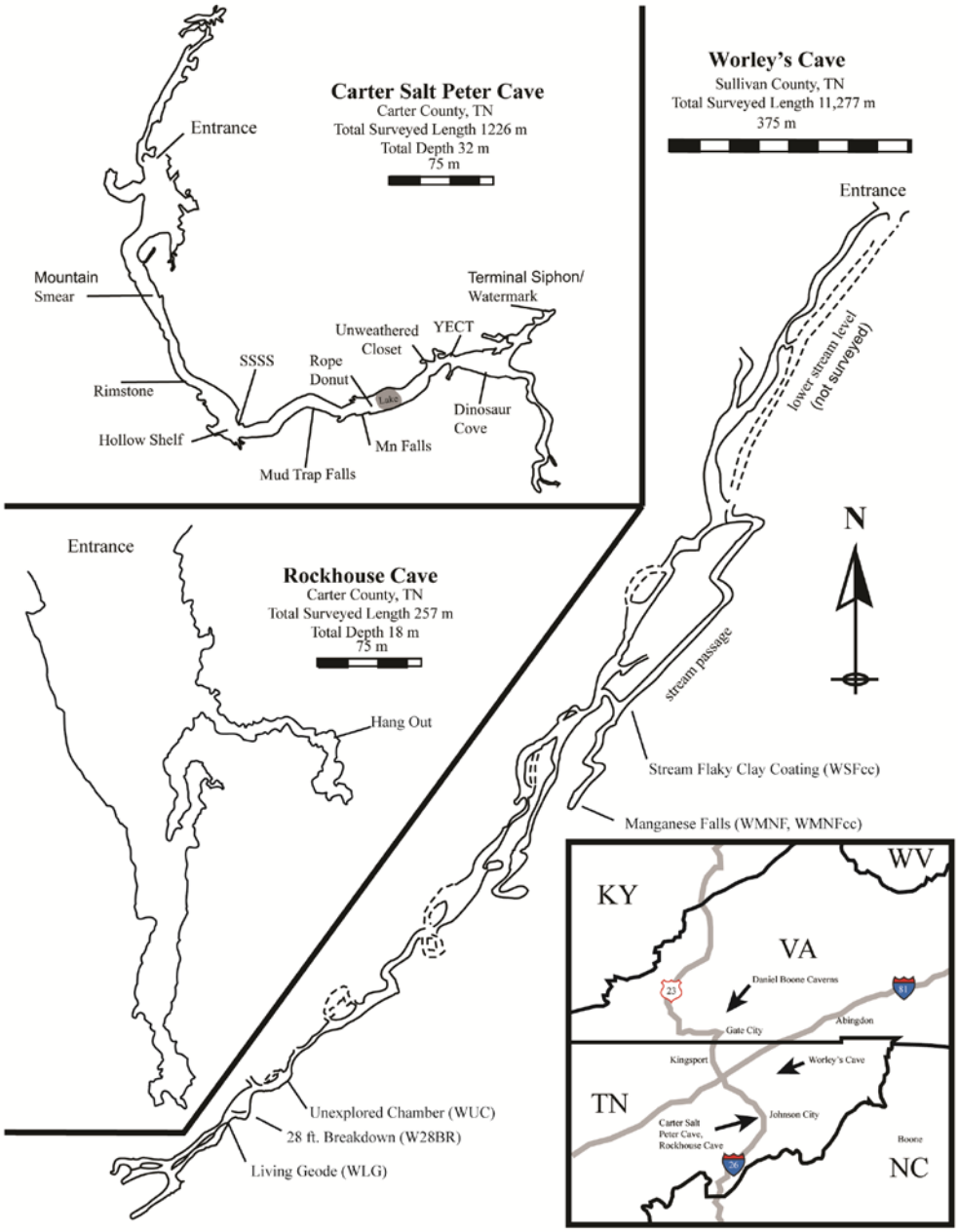
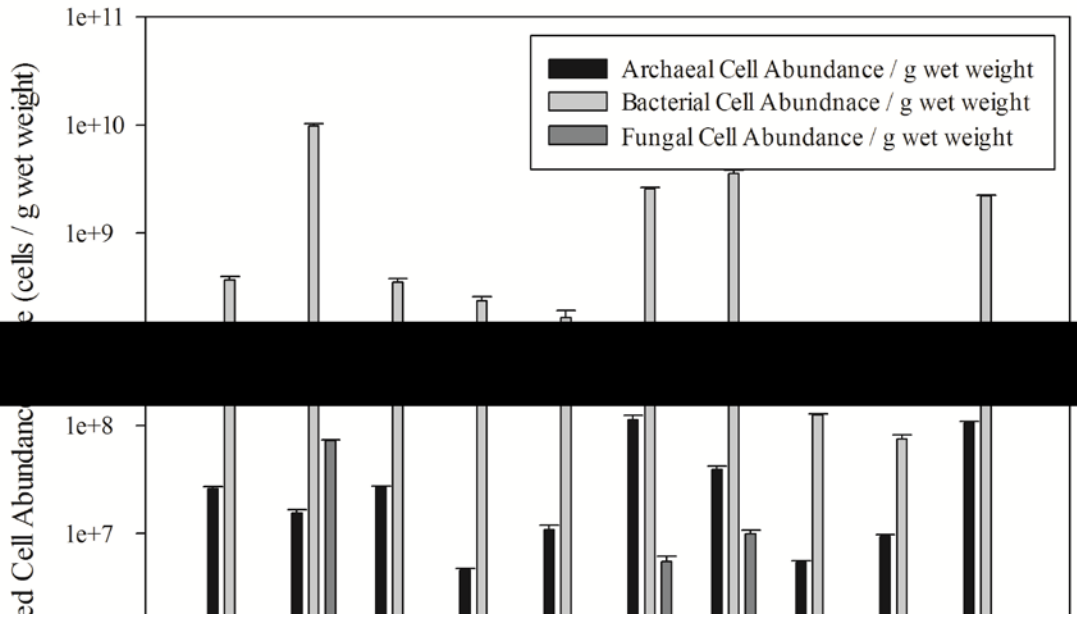


Fig. 1. Maps of the primary study sites: Carter Salt Peter Cave, Rockhouse Cave, and Worley's Cave. Daniel Boone Caverns has not yet been surveyed at the time of this writing. Sampling locations are labeled with arrows. Regional map inset shows the relative location of all four cave systems that are included in this study. Carter Salt Peter Cave survey conducted on February 8, 1981 by L. Adams, R. Knight, R. Page, and T. Wilson. Rockhouse Cave survey conducted on May 6, 1980 by L. Adams, T. Gingrich, and D. Nelms. Worley's Cave survey conducted from December 22, 1971 - August 8, 1973 by M. Adams, T. Anderson, C. Booth, R. Bowery, J. Cox, T. Harrison, D. Mire, A. Powers, D. Powers, and J. Powers. All cave maps adapted by S.K. Carmichael.

Table 1. Biogeochemical analyses of ferromanganese deposits located in cave systems within the upper Tennessee River Basin. Mn:Fe concentrations at sample sites were determined by ICP-OES analysis and are reported as an average. Substrate geochemistry was determined by X-ray diffraction. Location of sample site is abbreviated below as follows: Worley's Cave (W), and Carter Salt Peter Cave (CSPC).

Sample Site	Mn (ppm)	Fe (ppm)	Mn:Fe Concentration	Substrate Geochemistry
Ribbon Rock (W)	284.6	266.8	1.07	Quartz, Illite, Trace Dolomite
River Bank (W)	123.2	275.7	0.45	Quartz, Orthoclase, Illite, Trace Dolomite
Weathered Ribbon Rock (W)	125.4	323.8	0.39	Quartz, Orthoclase, Illite
Mn Chamber (W)	77.4	342.6	0.22	Quartz, Trace Calcite, Minor Orthoclase
Dinosaur Cove Popcorn (CSPC)	22.3	124.8	0.18	Calcite
Dinosaur Cove Mud (CSPC)	205.3	343.8	0.59	Nontronite
Watermark (CSPC)	14.3	254.7	0.06	Nontronite
Mn Falls (CSPC)	327.5	361.4	0.91	Nontronite
Dinosaur Cove (CSPC)	10.9	50.1	0.22	Calcite

[redacted]



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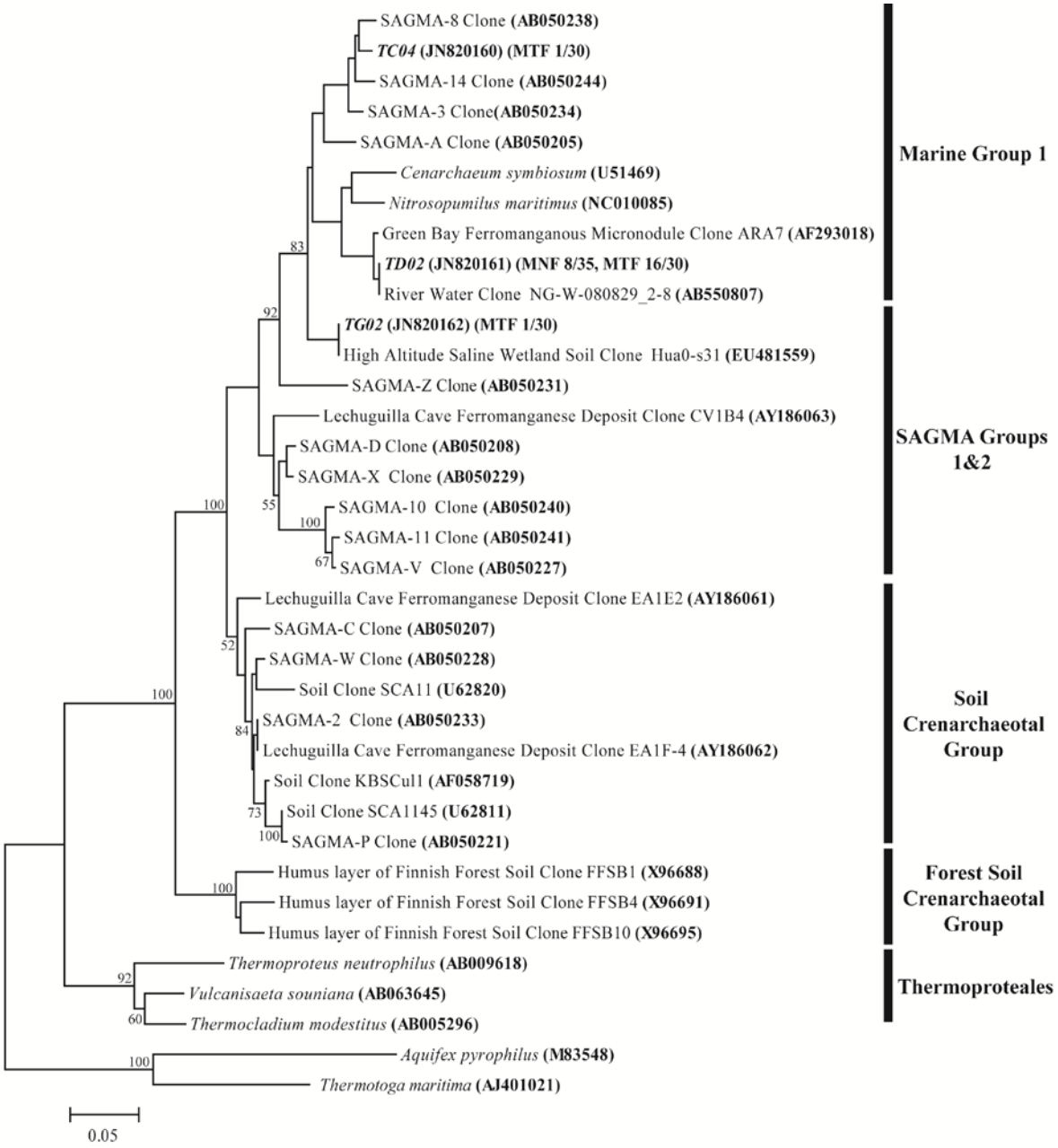

Fig. 3. Neighbour-joining tree based on Crenarchaeal SSU rRNA gene sequences obtained from biofilms found in Carter Salt Peter Cave, Carter County, TN in this study. The number of sequences from each library, Mn Falls (MNF) and Mud Trap Falls (MTF), that are represented by a particular OTU is given in parentheses following the NCBI accession number.Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position.

Fig. 4. Neighbour-joining tree based on Euryarchaeal SSU rRNA gene sequences obtained from biofilms found in Carter Salt Peter Cave, Carter County, TN in this study. The number of sequences from each library, Mn Falls (MNF) and Mud Trap Falls (MTF), that are represented by a particular OTU is given in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position.

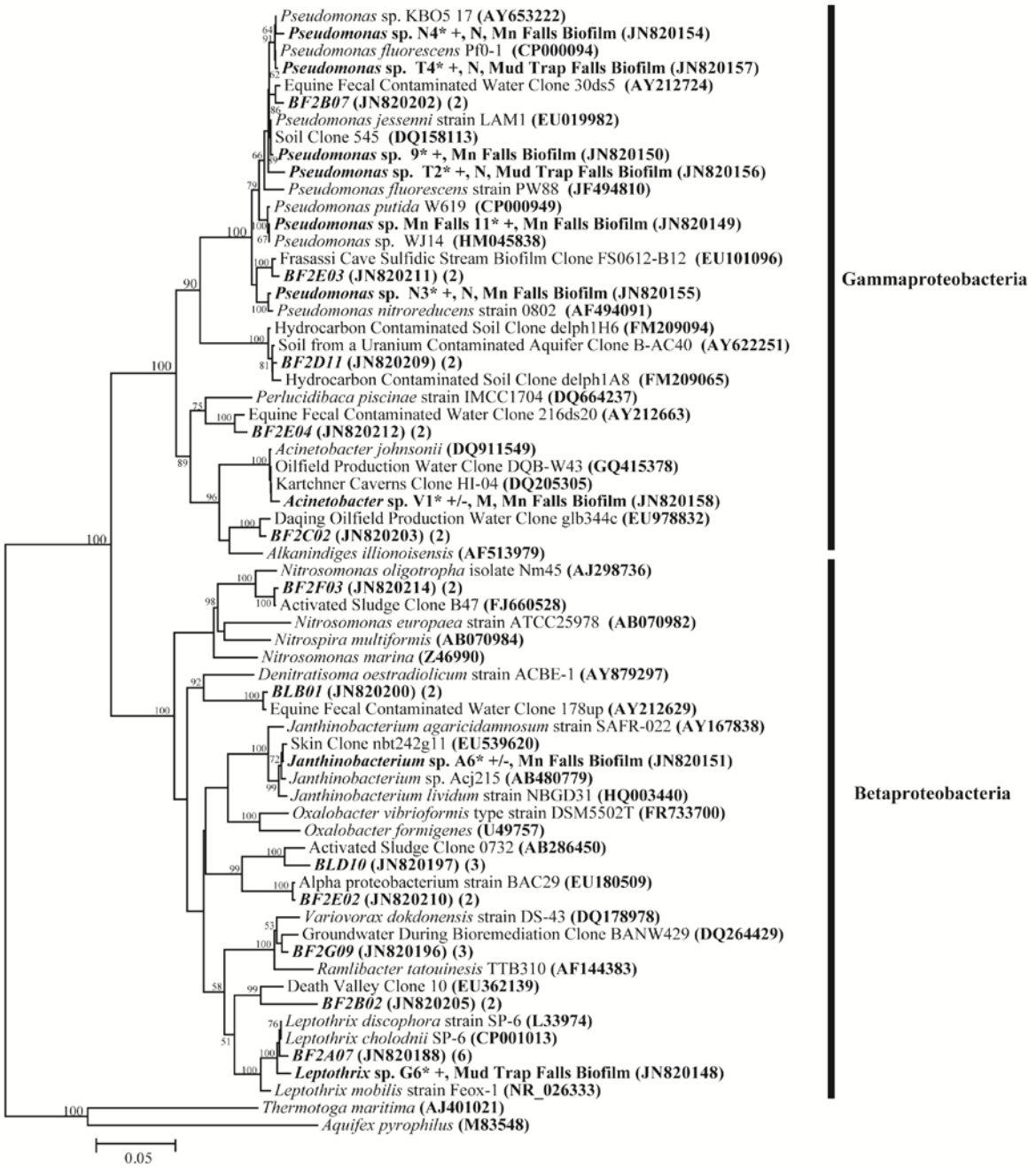

Fig. 5. Neighbour-joining tree inferring the phylogenetic relationship between ***cultured strains**** (asterisks) and those found in ***clone libraries*** in Carter Salt Peter Cave, Carter County, TN in this study. Isolates that oxidize Mn continually are indicated by a +; isolates that oxidize Mn intermittently are indicated by a +/-. Putative Nitrogen-fixing, Mn(II)-oxidizers are indicated by a **N**; putative methylotrophic Mn(II)-oxidizers are indicated by a **M**. Source of isolation is noted immediately before accession number. The number of sequences from the library that represented a particular OTU is given in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicated the expected number of changes per sequence position (see scale bar).

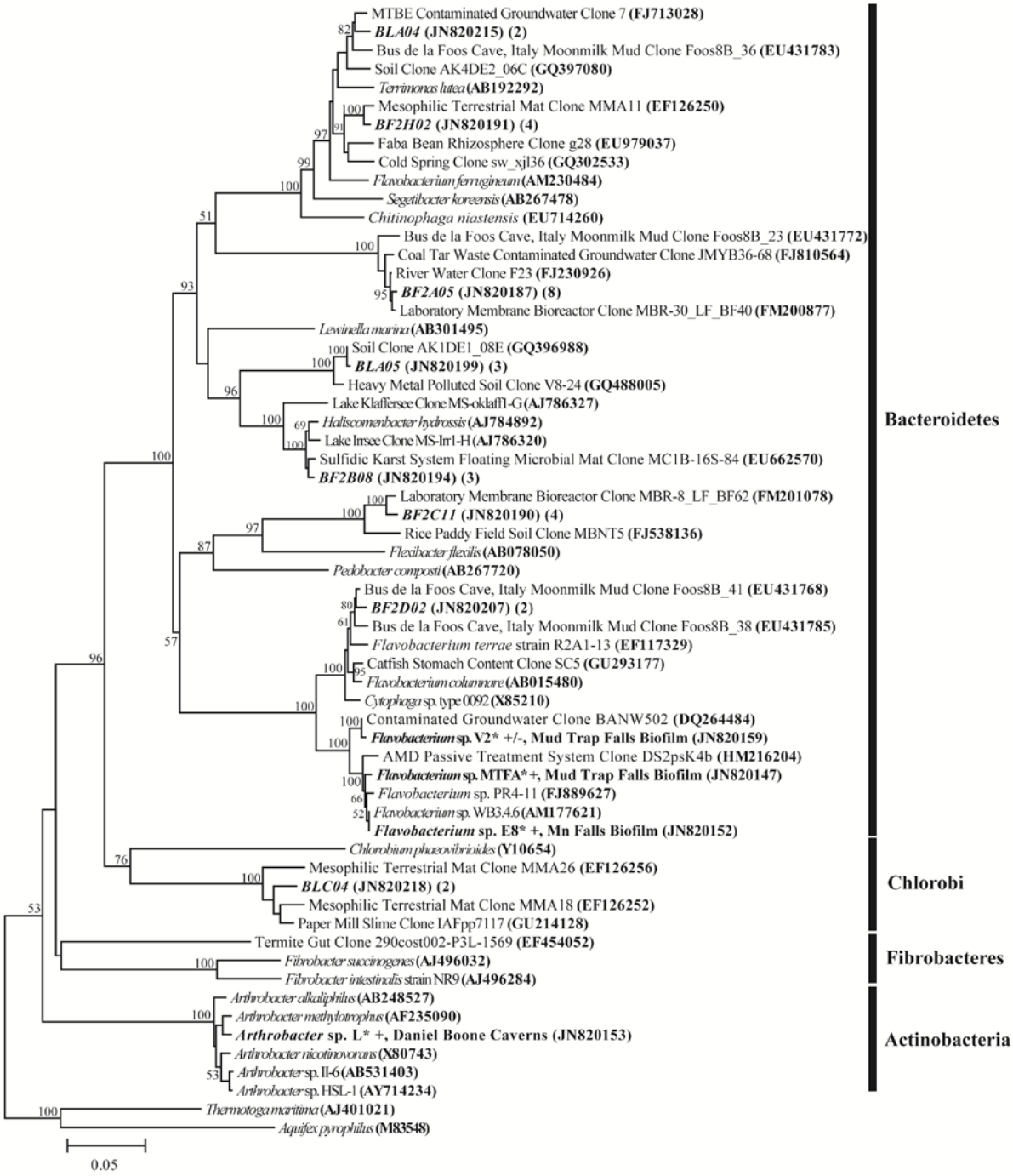

Fig. 6. Neighbour-joining tree inferring the phylogenetic relationship between ***cultured strains**** (asterisks) and those found in ***clone libraries*** in Carter Salt Peter Cave, Carter County, TN in this study. Isolates that oxidize Mn(II) continually are indicated by a +; isolates that oxidize Mn(II) intermittently are indicated by a +/-. Source of isolation is noted immediately before accession number. The number of sequences from the library that represented a particular OTU is given in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position (see scale bar).

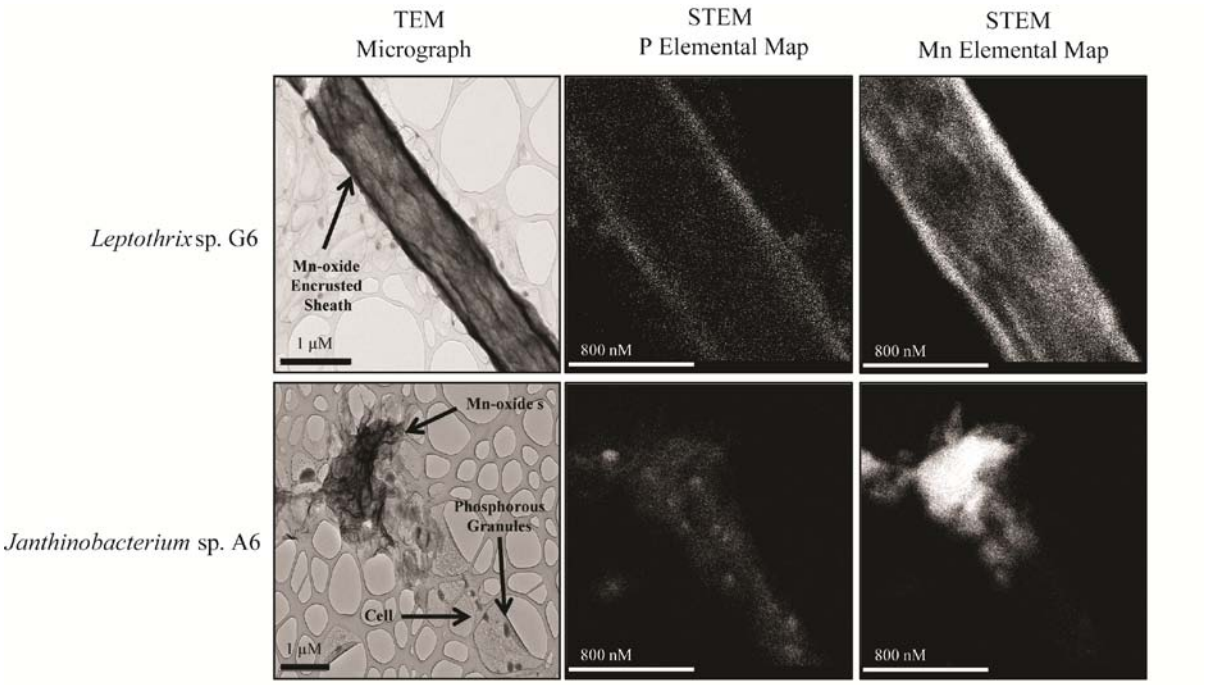

Fig. 7. TEM microscopy of the sheath-forming isolate *Leptothrix* sp. G6 and *Janthinobacterium* sp. A6, a genus not previously recognized to contain members capable of Mn(II)-oxidation. TEM micrographs (scale bars represent 1 µm) demonstrate the presence of electron-dense Mn deposits associated with bacterial cells. STEM P and Mn elemental maps (scale bars represent 800 nm) reveal the location of phosphorous and manganese within bacterial cells. Image Credit: S.K. Carmichael.

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Supporting Information

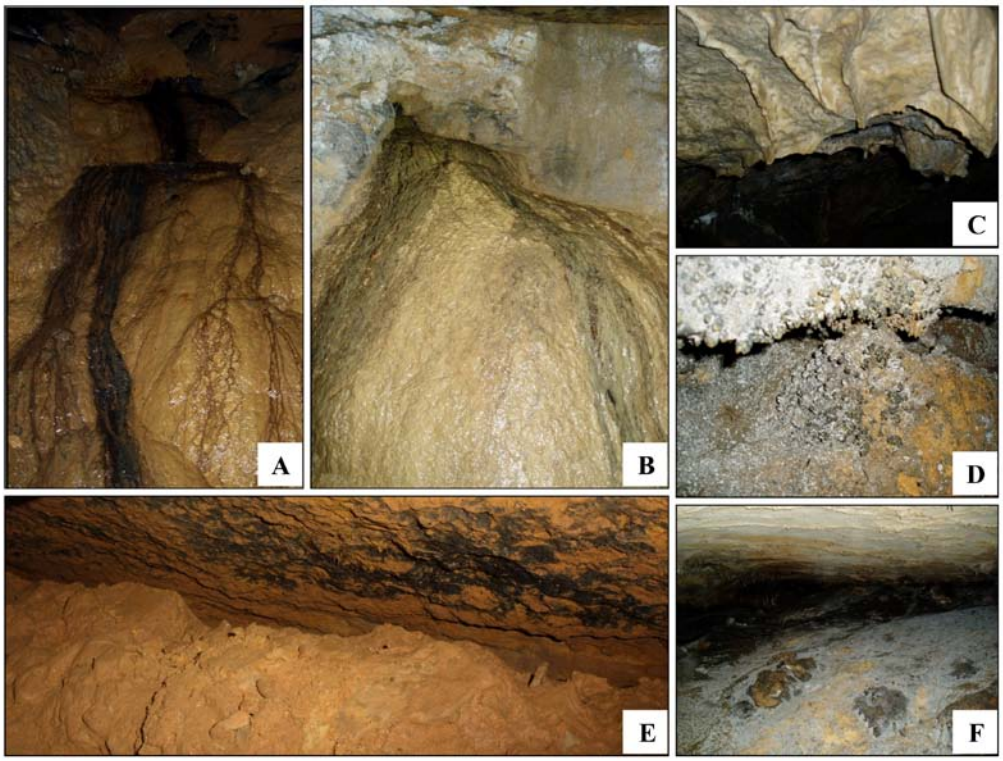

Fig. S1. Variation in ferromanganese deposit morphologies found in caves of the upper Tennessee River Basin: A) Mn Falls biofilm (CSPC), B) Mud Trap Falls biofilm (CSPC), C) Biovermiculations at Hang Out (R), D) Micronodules at Rimstone (CSPC), E) Ferromanganese Coating at Lower Ledge (DBC), and F) Ferromanganese Crust at Hollow Shelf (CSPC). Cave systems are abbreviated as follows: Carter Salt Peter Cave (CSPC), Daniel Boone Caverns (DBC), and Rockhouse Cave (R).

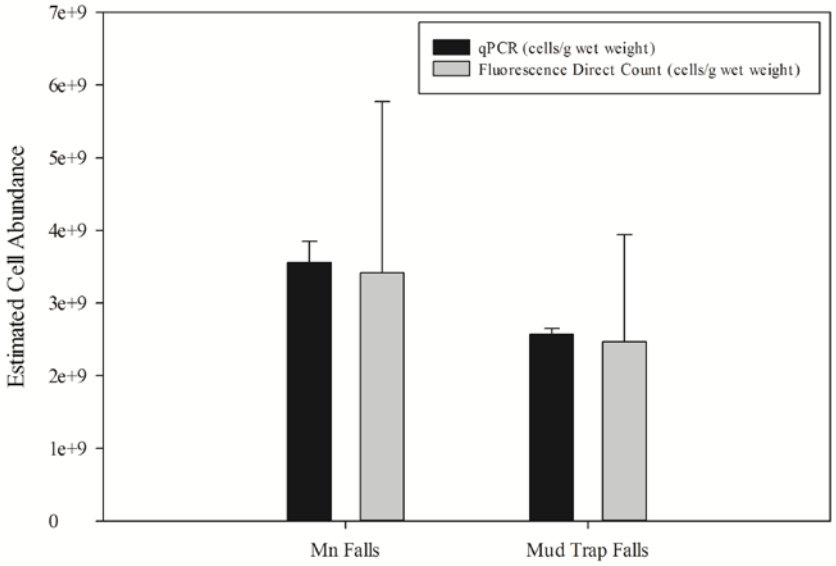

Fig. S2. A comparison of estimated microbial cell number in cave biofilms using real-time quantitative PCR and fluorescence direct counts.

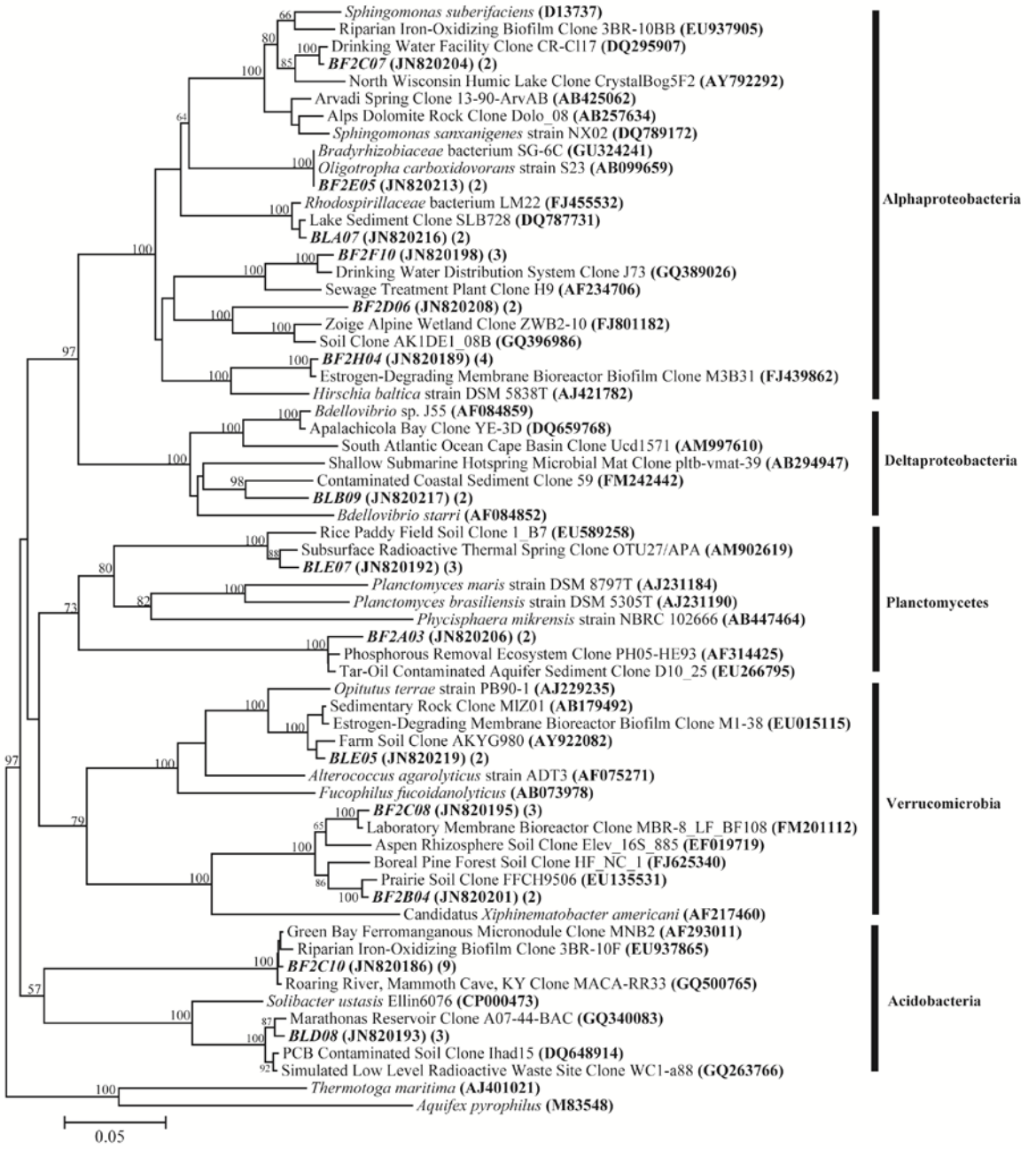

Fig. S3. Neighbour-joining tree inferring the phylogenetic placement of SSU rRNA gene sequences obtained from biofilms found in Carter Salt Peter Cave, Carter County, TN in this study. The number of sequences from the library that represented a particular OTU is given in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time and with maximum-likelihood analysis (data not shown). *Aquifex pyrophilus* and *Thermotoga maritima* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position (see scale bar).

CHAPTER 3: EVIDENCE OF SUSTAINED ANTHROPOGENIC IMPACT IN CARTER SALT PETER CAVE, CARTER COUNTY, TENNESSEE

Summary

Carter Salt Peter Cave, Carter County, Tennessee, is an epigenic cave system typical of those found within the Appalachian region. Evidence of anthropogenic impact is widespread throughout the cave system, and sites within the cave were evaluated for signs of contamination by human sewage material. Molecular-based analyses of DNA extracted from a seep biofilm, Mn Falls, demonstrated the presence of a *Bacteroides-Prevotella* human-signature in DNA extracted from the biofilm in both July 2009 and July 2011. Culture-based enumeration of estimated cell number at the Mn Falls biofilm seep site revealed a consistently higher count of culturable heterotrophic bacteria and cultivable percentage of the total bacterial population in comparison to a nearby seep biofilm, Mud Trap Falls, with less water flow. Both findings are consistent with a hypothesis of nutrient loading via sewage contamination at the Mn Falls site and provide evidence for sustained anthropogenic impact within the Carter Salt Peter Cave system. Cave and karst systems contain unique and highly-adapted macro- and microflora that are intrinsically vulnerable to pollution. Therefore, continued monitoring of the Carter Salt Peter Cave system is imperative to provide baseline data for the development of a management plan to protect this unique cave ecosystem.

Introduction

Karst terrain is a heterogeneous landscape that covers 20% of the Earth's dry land surface and 40% of the land in the United States east of Oklahoma (White *et al.*, 1995; Ford, 2006). Characteristic topographic features of karst regions include sinkholes, subsidence zones, and swallow holes, while subsurface features include complex, dendritic internal drainage patterns (conduits) that widen to form spaces accessible to humans, such as caves and caverns (Palmer, 1991). The evolution of conduit systems within a karst region leads to the development of complex subterranean flow patterns and a high degree of interconnectivity between karst drainage basins (Poulson and White, 1969; Green *et al.*, 2006). When water enters a conduit system, a variety of internal factors determine its ultimate fate, and rapid fluctuations in water level and velocity via surface input render flow within conduits highly variable. Water can move at rates of 100-1000+ feet/day (Taylor and Nelson Jr., 2008), and peak flow rates during storm events can be up to two orders of magnitude above the base flow rate (Vesper *et al.*, 2001).

The unique geomorphologic and hydrologic features of karst terrain render the landscape highly vulnerable to pollution. If overlying soil filtration capacity is poor, contaminants can enter karst drainage basins quickly (Vesper *et al.*, 2001; Wong *et al.*, 2011). High hydrologic conductivity permits the evolution of a complex conduit system in which contaminants can 1) easily penetrate the bedrock, 2) quickly move through these conduit systems (Boyer and Pasquarell, 1999; Field, 2002; Boyer and Kuczynska, 2003; Graening and Brown, 2003; Pronk *et al.*, 2006), and 3) be dispersed across a broad spatial scale (Field, 2002; Simon *et al.*, 2007). Once contaminants enter they can either be flushed quickly, or persist in the environment for lengthy durations (Mahler *et al.*, 2000; Green *et al.*, 2006; Goeppert and Goldscheider, 2011).

Contamination of karst groundwater resources poses a significant human and environmental threat: karst groundwater resources are the primary source of drinking water for a significant portion of the Earth's population (Ford and Williams, 1989; Brosig *et al.*, 2008), and karst systems are home to fragile and unique communities of micro- and macro-organisms that are highly adapted to the

subterranean environment (Porter, 2007). Common contaminants within karst systems have been grouped into six broad categories (Vesper *et al.*, 2001). (1) Water soluble compounds easily infiltrate karst systems, are dispersed with groundwater flow, and are ultimately discharged from the system. (2) Lightly, slightly insoluble, organic compounds (light nonaqueous phase liquids, or LNAPLs) are less dense than groundwater and rise to the surface. These compounds are known to persist in systems for lengthy durations. Heavy, insoluble, organic compounds (dense nonaqueous phase liquids, or DNAPLs) (3) are denser than groundwater and also have long residence times in karst systems because they sink into sediment/pore water. (4) Metal contamination may either be naturally occurring through host rock dissolution or be introduced by humans due to land use practices. Bioavailability of metal contaminants is primarily controlled through adsorption to metal-oxides in karst systems in pH neutral waters, thus this type of contaminant is typically flushed from the groundwater during pulse events that expel sediment and particulate matter from of karst systems (Post, 1999). (5) Pathogens and exogenous microbial consortia enter karst aquifers easily due to the extreme porosity of the bedrock. (6) In heavily visited caves, trash from irresponsible recreational cavers litters cave systems. Trash can also be introduced from nearby landowners who may use sinkholes as dumps for home and farm refuse (White *et al.*, 1995). If transported deep within karst systems, trash can become a persistent leaky source of groundwater pollution. Impacts of the aforementioned categories of chemical and biological contamination have been extensively documented in literature (Simon and Buikema Jr., 1997; Kozar, 2002; Graening and Brown, 2003; Wood *et al.*, 2008; Iker *et al.*, 2010; Goeppert and Goldscheider, 2011; Hu *et al.*, 2011). These studies demonstrate the importance of developing and utilizing novel methods of monitoring karst groundwater resources for indicators of high levels of surface impact (Pronk *et al.*, 2006; Pronk *et al.*, 2007; Pronk *et al.*, 2009).

The primary impediment to research in karst systems is the inaccessibility of the closed conduit system to researchers (Goldscheider *et al.*, 2006). As a result, indirect methods of water quality monitoring are frequently employed by researchers: culture based studies to detect coliform bacteria (Rusterholz and Mallory, 1994; Mikell Jr. *et al.*, 1996), molecular techniques to identify the

presence/absence of fecal indicators and endemic species in karst systems (Porter, 2007; Ahmed *et al.*, 2008a; Roslev and Bukh, 2011), and the use of fluorescent dye-tracers and microspheres to model water flow and pathogen dispersal in conduit systems (Gao, 2011; Goeppert and Goldscheider, 2011). Direct study of karst environments is restricted to open conduits that are large enough to allow for human movement and environmental manipulation. For this reason, cave research has become a focal point in delineating the effects of anthropogenic impact on karst terrain.

As is the case with karst systems in general, anthropogenic impact in cave systems is a phenomenon that has been documented worldwide (Watson *et al.*, 1997; Ciferri, 1999; Chelius and Moore, 2004; Zhong *et al.*, 2011). The cave interior environment can be altered by vandalism, speleothem removal, and graffiti. Changes in hydrological flow regimes, with a downstream impact on speleogenic processes, have been linked to human activity: even something as simple as touching a speleothem without a glove could deposit oil on the surface of a rock and alter drip patterns and speleothem formation (Barton, 2006). The cave microclimate and geochemical environment is easily altered by human presence, which can disrupt the delicate balance of cave ecosystems by impacting nutrient cycling and biomineralization processes (Hoyos *et al.*, 1998; Schabereiter-Gurtner *et al.*, 2002; Barton *et al.*, 2007; Bastian and Alabouvette, 2009; Cuezva *et al.*, 2009; Portillo *et al.*, 2009; Faimon *et al.*, 2011; Fernandez-Cortes *et al.*, 2011). Human foot traffic in caves has been linked to sediment compaction/erosion and the introduction of allocthonous nutrients and organisms (Simon and Buikema Jr., 1997; Hunter *et al.*, 2004; Lavoie and Northup, 2005; van Beynen and Townsend, 2005). These changes have been shown to destroy microhabitats (Northup, 2011), alter cave biogeochemical cycles, and impact sensitive cave fauna such as bats (Blehert *et al.*, 2011). Finally, surface impacts such as land use changes and alterations in vegetation can affect soil and epikarst filtration of drip water and have the potential to introduce nutrient loads and pathogenic microorganisms into cave systems through groundwater percolation (Watson *et al.*, 1997). Anthropogenic impacts can be localized at a single site within a cave (Shapiro and Pringle, 2010; Gillieson, 2011), or spread throughout an entire cave system (Watson *et al.*, 1997). Any type of impact within caves is primarily manifest at the lower trophic levels, particularly in cave microbial communities,

which have the potential to exert powerful bottom-up controls on ecosystem health and stability within a cave (Horner-Devine *et al.*, 2003). Due to the constancy of the cave environment, impact is real, quickly detrimental, and hard to reverse.

In 2009 a study was initiated to characterize the geomicrobiology of ferromanganese deposits in Carter Salt Peter Cave (herein CSPC), Carter County, Tennessee. Early on, impact within the CSPC system was evident. During an initial exploratory visit to the cave in July 2009, researchers noted 1) an abundance of graffiti covering cave rocks and walls, 2) a prevalence of litter throughout the cave system, and 3) a distinct sewage odor was present in portions of the cave with active water flow. At one of these sites, a thick, dark black, microbial biofilm (Mn Falls), full of microcrystalline Mn oxide minerals, flowed down the cave wall and onto the cave floor. One of the water sources for CSPC is known to be contaminated by a variety of inputs, including fecal coliforms (Gao *et al.*, 2006), and many streams in this region are listed as impaired bodies of water (Gao, 2011). A member of the Mountain Empire Grotto in Johnson City, Tennessee, noted that the appearance of the Mn Falls biofilm in 2008 coincided with a time in which a local septic tank company had been reportedly dumping raw sewage into a sinkhole that is hydrologically linked to CSPC (John Matthews, personal communication). We began monitoring the site in 2009. From 2009-2011, the appearance of the Mn Falls biofilm changed drastically, losing its dark black color, and exhibiting a dramatic visual reduction in Mn(IV)-oxide production, though estimated cells/g wet weight biofilm material remained relatively constant and field tests continued to demonstrate the presence of Mn oxides, as detected using Leucoberbelin Blue.

Early water quality monitoring efforts at the Mn Falls site documented fluorine concentrations that were an order of magnitude greater than background concentrations (S.K. Carmichael, unpublished data). Because there are no natural sources of fluorine in the region, this fluorine likely comes from the local, fluoridated municipal water source. In addition, high nitrogen and/or phosphorous loads were detected in cave water, which can potentially be linked to agricultural impacts and/or sewage infiltration within the cave system. Microbiological studies documented the presence of several dominant OTUs (as defined by two or more sequences represented by an OTU) in clone libraries constructed from Mn Falls

biofilm material that provide further circumstantial evidence of nutrient loading/sewage contamination at this site (Chapter Two). Five OTUs (ca. 11% of sequences represented by dominant OTUs) were closely related (≥97% identical) to environmental clones isolated from fecal contaminated water and activated sludge. A sixth OTU (6% of sequences represented by dominant OTUs) shared 99% identity over a 1,488 bp alignment to *Leptothrix chlodnii*, a species that has been isolated from organic-rich polluted water and activated sludge (van Veen *et al.*, 1978; Spring, 2006). In addition, we were able to isolate a Mn(II)-oxidizing *Leptothrix* sp. (isolate G6) from the Mn Falls biofilm that shared 99% identity over a 1,489 bp alignment to this OTU and 99% identity over a 1,488 bp alignment with *Leptothrix chlodnii*. *Leptothrix* sp. G6 was isolated from a serial dilution culture containing $2.5x10^{-8}$ g wet weight biofilm material, indicating that *Leptothrix* sp. are abundant and environmentally relevant species at the Mn Falls site (Chapter Two).

Mn(II)-oxidizing bacteria are known to grow and oxidize Mn using a variety carbon sources (van Veen *et al.*, 1978), from glucose, peptone, and yeast extract (Siering and Ghiorse, 1996; Tebo *et al.*, 2007) to succinate (Chapter Two), acetate, propionate, and butyrate (van Veen *et al.*, 1978). These last three carbon sources are known byproducts of fermentation in both ruminant and human guts (Cummings, 1981; Topping and Clifton, 2001) and are excreted in feces. This provides further circumstantial evidence to support a hypothesis linking nutrient/sewage infiltration at the Mn Falls site and a link between blooms of Mn(II)-oxidizing biofilms in this shallow cave system. Given the dramatic change in the appearance of the Mn Falls biofilm over the duration of the study and the possibility that this change may have somehow been linked to exogenous nutrient input to the cave system, an effort was initiated to document the extent of human impact within the CSPC system, and to either validate or alleviate concern over potential fecal contamination at the site.

Controlled experimentation of limestone inoculated with a known microbial consortia has demonstrated that culture-dependent and culture-independent techniques yield contrasting snapshots of true community diversity in karst systems (Laiz *et al.*, 2003). Due to this phenomenon, comprehensive analyses of microbial ecology are best completed by pairing both types of methodologies (Donachie *et*

al., 2007; Ritz, 2007; Vartoukian *et al.*, 2010). Therefore, a polyphasic approach using water chemistry analyses, molecular markers, and culture-dependent cell enumeration assays was utilized to determine the presence of anthropogenic impact via nutrient loading. Data collected over a three-year extended study has provided evidence of sustained anthropogenic impact within the Carter Salt Peter Cave system.

Results

Description of sample sites within Carter Salt Peter Cave

Carter Salt Peter Cave contains abundant ferromanganese deposits, which are visible in the form of black or chocolate brown biofilms and patinas that coat cave walls and speleothems. The source of reduced Fe(II) and Mn(II) necessary for the formation of these deposits is likely the Knox Dolomite bedrock as studies in similar systems indicate that these reduced compounds may enter a cave via seepage, groundwater percolation, or as a result of bedrock geochemistry (Moore, 1981; Levy, 2007; Rossi *et al.*, 2010). Samples from this study were obtained from two Mn(II)-oxidizing biofilms located in physical proximity to one another (Fig. 1). Mn Falls, the site of alleged sewage contamination, experienced a previously described dramatic change in appearance (Fig. 2A and 2B) over the duration of one year. Mud Trap Falls (Fig. 2C), an adjacent site that is not hydrologically linked to Mn Falls (Y. Gao, personal communication), did not undergo the dramatic change in appearance that was seen at Mn Falls. Therefore, Mud Trap Falls was selected as a comparison site.

Detection of human-specific Bacteroides-Prevotella 16S rRNA gene sequences in DNA extracted from the Mn Falls biofilm site

A molecular-based survey of the Mn Falls site was initiated to detect the presence or absence of human fecal indicators in DNA extracted from the biofilm from the time of the alleged pollution and at regular intervals post impact. A primer set designed by Bernhard and Field (2000a, b) targeting human-specific *Bacteroides-Prevotella* was chosen due to its sensitivity and reliability according to previous reports in

the literature (Ahmed *et al.*, 2009b; Ahmed *et al.*, 2009a). Multiple attempts at amplification of DNA extracted from both Mn Falls and Mud Trap Falls resulted in two positive amplifications, both from Mn Falls: sample *F*, extracted in July 2009 during an initial sampling trip to the cave when the biofilm was in bloom (OTUs from this extraction are prefaced by a *F* in Fig. 3), and sample *4*, extracted in July 2011 during a more recent sampling trip to the cave when the biofilm was drastically reduced in appearance (OTUs from this extraction are prefaced by a *4* in Fig. 3). Multiple attempts at amplification of DNA extracted from Mud Trap Falls were unsuccessful. Due to the high degree of similarity among all cloned sequences, clones were binned into OTUs for phylogenetic analysis. Using a 99% cutoff, analysis of bacterial clones revealed 6 unique OTUs (Fig. 3) out of ca. 15 total sequences.

Three OTUs, *FB12*, *4A08*, and *FB01*, represented sequences that are members of the *Prevotella* spp., a genus that is commonly isolated from the oral cavity, upper respiratory tract, and urogenital tract of humans (Shah and Collins, 1990). Clone *FB12* shared 100% identity over a ca. 700 bp read to a clone isolated from a study of the microbiota of the human intestine (Walker *et al.*, 2011), and 99% identity to clones isolated from the human gut (Hayashi *et al.*, 2002). *FB12*'s closest cultured relative was *Prevotella copri*, the type strain of which was isolated from human feces (Hayashi *et al.*, 2007). Clone *4A08* shared 99% identity to environmental clones isolated from fecal contaminated watersheds (Lamendella *et al.*, 2007; Lamendella *et al.*, 2009), and 97% identity to its' closest cultured relative, *Prevotella paludivivens*, a species isolated from rice-plant residue (Ueki *et al.*, 2007). Clone *FB01* shared 99% identity to environmental clones isolated from equine-fecal contaminated water (Simpson *et al.*, 2004).

The three remaining OTUs, *4A09*, *FB08*, and *FB10*, represented sequences that are members of the *Bacteroides* spp., a genus commonly isolated from the mammalian gastrointestinal tract (Shah and Collins, 1990). Clone *4A09* shared 98% identity over a ca. 700 bp read to environmental clones isolated from river water polluted with feces (Ju-Yong *et al.*, 2010) and human sewage samples (Dorai-Raj *et al.*, 2009). Clone *FB08* shared 100% identity to clones isolated from the human intestine (Walker *et al.*, 2011) and human feces. Clone *FB10* shared 99% identity to clones isolated from the human intestine and human

feces in as study investigating the association of human gut microbial ecology with obesity (Ley *et al.*, 2006).

Molecular evidence from this study indicates the presence of a *Bacteroides-Prevotella* fecal signature in DNA extracted from the Mn Falls biofilm in July 2009 and July 2011. Phylogenetic analysis from clone sequences suggests a stronger human-specific signature in July 2009, with 50% of the sequences clustering with sequences from human feces, a conclusion that is consistent with the hypothesis of contamination localized at this site within the cave. A weaker human signature, as defined by a lower percentage of sequences (20%) clustering with sequences from human feces, was detected in the July 2011 sample of Mn Falls, providing evidence of sustained anthropogenic impact at this site within the Carter Salt Peter Cave system.

Enumeration of culturable heterotrophic bacteria in cave biofilms using most probable number assays

It is a well established fact that the majority of microbes within the environment are recalcitrant to cultivation, a problem that compounds the lack of knowledge regarding the role that microbes play within the environment (Staley and Konopka, 1985; Rappé and Giovannoni, 2003). A variety of factors contribute to the inability to cultivate the majority of microbial species using traditional media- from dormancy (Jones and Lennon, 2010; Lennon and Jones, 2011) to anaerobic, oligotrophic, or chemoautotrophic lifestyles (Mikell Jr. *et al.*, 1996). In environmental studies, direct cell counts typically exceed viable cell counts by several orders of magnitude (Amann *et al.*, 1995; Goldscheider *et al.*, 2006; Kimura *et al.*, 2011). The issue of non-cultivability is magnified in cave environments where microbes are adapted to the unique geochemical and environmental conditions of a given cave (Barton, 2006; Glausiusz, 2007).

Microbial species within pristine caves (those that have little or minimal human impact) are adapted to leading an oligotrophic lifestyle. Utilization of a typical carbon-rich media in an attempt to cultivate indigenous cave organisms would be unsuccessful, as the slow-growth lifestyle of oligotrophs is disrupted in the presence of rich carbon resources with devastating effects to the microbial cell (Koch,

1997). However, human-related microbiota and opportunistic pathogens, which are generally not endogenous members of the cave microflora, are adapted to high-quality carbon environments and grow well on carbon-rich media. Therefore, it is reasonable to hypothesize that sites within cave systems that experience impact from sewage/nutrient loading would demonstrate higher culturable heterotrophic cell counts than areas without impact. Further, data in the literature support this hypothesis (see Table 1 in Amann *et al.*, 1995). Most probable number (MPN) assays (Table 1) were utilized to test this hypothesis through the enumeration of total culturable heterotrophic bacteria and total culturable heterotrophic Mn(II)-oxidizers in two Mn(II)-oxidizing cave biofilms, Mn Falls and Mud Trap Falls.

Samples from cave biofilms for MPN assays were obtained on three different occasions, February 2010 (Mud Trap Falls), May 2010 (Mn Falls), and July 2010 (Mn Falls and Mud Trap Falls) (Table 1). Data from a prior molecular-based experiment (Chapter Two) indicated that total estimated bacterial cell counts remained relatively constant over the duration of the study period. A difference was observed between total estimated bacterial cell counts and total culturable heterotrophic bacteria at the Mud Trap Falls site; however, there was significant overlap between the total estimated bacterial cell counts and total culturable heterotrophic bacteria at the Mn Falls site. Results from cultivation-based enumeration of heterotrophic Mn(II)-oxidizing bacteria revealed no significant differences between population numbers at Mud Trap and Mn Falls, though a slight increase in the percent culturable Mn(II)-oxidizing bacteria was observed at Mud Trap Falls. This observation is likely a result of a decrease in estimated total bacteria at this site as compared to the Mn Falls site.

Results from cultivation-based enumeration of total heterotrophic bacteria revealed an interesting trend. Data from the February 2010 sampling of Mud Trap Falls indicated an average count of 1.1×10^{8} cells/g wet weight; data from the May 2010 sampling of Mn Falls indicated an average count of 2.5×10^{10} cells/g wet weight. No overlap in 95% confidence intervals was observed between these two samples, which is indicative of a significant difference in total culturable heterotrophic bacteria between these two sites. July 2010 data from both sites reflects the same pattern, with an average count of 9.6×10^{6} cells/g wet weight at Mud Trap Falls and 9.6×10^{9} cells/g wet weight at Mn Falls. The difference between the two

sites in July 2010 data is more pronounced, as total cultivable heterotrophic bacteria at Mn Falls outnumbered Mud Trap falls by three orders of magnitude, with no overlap observed in 95% confidence intervals. An observed increase in the total culturable heterotrophic bacteria at the Mn Falls site is suggestive of greater nutrient loading at this site. A slight overlap was observed between the 95% upper confidence interval at Mud Trap Falls in February 2010 and the 95% lower confidence interval at Mn Falls in July 2010. However, the percent of culturable heterotrophic bacteria within the total population was consistently higher at Mn Falls, approaching 100%, a finding that is supportive of nutrient loading at the site. Interestingly, the percent of culturable heterotrophic bacteria within the total population showed a slight variation between the two sampling dates at Mud Trap Falls. MPN results indicate that culturable counts at this site were significantly higher (roughly a two order of magnitude increase) in February 2010 as compared to July 2010. This trend is reflected in a slight increase in the percent of culturable heterotrophic bacteria within the total population in February 2010 as well. These results mirror what was seen at Mn Falls, making it enticing to speculate that the Mud Trap Falls site may have been impacted by nutrient loading in February 2010.

Discussion

Due to the porous nature of bedrock in karst systems and the poor filtration capacity of the soil and epikarst zone, karst systems are inherently vulnerable to pollution (Caumartin, 1963; Field, 2002). A high degree of interconnectivity is observed in karst conduit systems, therefore surface impacts throughout a karst drainage basin can be dispersed over long distances quickly. Karst aquifers are particularly vulnerable to bacterial contamination and nutrient loading (Drew, 1996; Kozar, 2002; Coxon, 2011; Worthington, 2011) and exogenous input of this type can enter a cave or karst system via atmospheric deposition, percolation through the soil and epikarst, groundwater flow, or via animal/human foot traffic (Mahler *et al.*, 2000; Barton and Jurado, 2007; Pronk *et al.*, 2007). The persistence and/or endogenous nature of human associated microbial pathogens in the karst environment is a subject of debate (Hunter *et al.*, 2004; Personné *et al.*, 2004; Barton and Pace, 2005; Scott *et al.*, 2005; Goldscheider *et al.*, 2006;

Ahmed *et al.*, 2008b). However, it is recognized that autochthonous microbial communities in cave and karst systems represent diverse, highly adapted, stable consortia (Farnleitner *et al.*, 2005; Goldscheider *et al.*, 2006; Pronk *et al.*, 2009) residing within environments that are fragile and highly vulnerable to disturbance (Roth, 1993; Watson *et al.*, 1997; van Beynen and Townsend, 2005).

The occurrence of human and ruminant-related fecal indicators in karst groundwater and subsurface systems is well documented in the literature (Simon and Buikema Jr., 1997; Boyer and Pasquarell, 1999; Kozar, 2002; Boyer and Kuczynska, 2003; Hunter *et al.*, 2004; Personné *et al.*, 2004; Pronk *et al.*, 2007; Goeppert and Goldscheider, 2011). Molecular-based methods have been successfully employed in the past to detect the presence of human fecal indicators in karst aquifers (Reischer *et al.*, 2007). *Bacteroides* spp. represent a prominent new alternative indicator for the detection of fecal pollution in environmental samples due to an inability to survive in a non-host environment for lengthy periods of time, a strict association with warm-blooded animals, and a relative abundance of members of this genus in fecal samples as compared to traditional indicators (Ahmed *et al.*, 2008a). In the present study, molecular-based characterization of the Mn Falls microbial community demonstrated the presence of a *Bacteroides-Prevotella* fecal signature in DNA extracted from the Mn Falls biofilm in July 2009 and July 2011, providing evidence of sustained anthropogenic impact at this site.

Karst aquifers and deep cave systems with minimal human impact are considered to be oligotrophic environments, defined by less than 2 mg total organic carbon per liter (Barton and Jurado, 2007), and several nutrients such as nitrogen, sulfur, phosphorous, and iron are considered to be additional limiting factors in these systems (Goldscheider *et al.*, 2006). Organisms that thrive in rich carbon environments, like those associated with feces, would not thrive in these types of systems without nutrient loading from exogenous sources. Several prior studies establish a link between carbon amendment/nutrient loading and an increase in culturable heterotrophic bacteria at impacted sites within cave systems. A recent study by Ikner *et al.* (2007) investigating the culturable microbial diversity of Kartchner Caverns, Arizona revealed that areas of high human impact were associated with cultivable counts of bacteria that were two orders of magnitude higher than similar counts in low impact zones

within the cave. In addition, localized sites associated with the input of rich carbon sources (e.g., guano, animal feces, human traffic) in several European cave systems demonstrated higher culturable cell counts than sites not affected by nutrient loading (Mulec *et al.*, 2012). In other impacted cave systems, bacterial biomass was shown to increase by an order of magnitude in cave pools impacted by septic system effluent as compared to reference pools within the same system (Simon and Buikema Jr., 1997). In Wind Cave, South Dakota, experimental manipulations of soil plots revealed an increase in bacterial biomass within plots fertilized by feces or a combination of lint and feces relative to plots fertilized with lint alone or control plots, findings that confirm the nutrient-limited status of cave environments (Chelius *et al.*, 2009). Significantly higher average cultivable cell counts at the Mn Falls site and consistently higher percentage of culturable bacteria support the hypothesis of localized nutrient loading/sewage contamination at the Mn Falls site in CSPC. However, it is important to note that cultivation-based experiments are inherently biased by media design and inoculation/incubation techniques (Rusterholz and Mallory, 1994), and that dilution of fecal matter, which would occur within karst conduit systems, does affect the ability to culture and detect fecal indicator bacteria in environmental samples (Ahmed *et al.*, 2008b).

Results from the present study demonstrate the presence of human-specific fecal indicators within the CSPC system and provide compelling support for impact via nutrient loading at the Mn Falls site. Due to the inherent stability of the cave environment and the highly adapted cave macro- and microfauna, this type of impact has the potential to disrupt the delicate balance of life within a cave and exert a strong negative effect on ecosystem function (Fernandez-Cortes *et al.*, 2011). Eutrophication of the cave environment has an immediate and cumulative effect (Gillieson, 2011) on cave microbial communities and rich carbon input is known to drive successional change within microbial communities (Fierer *et al.*, 2010). In many cave systems, microbial communities are an important source of the primary productivity that sustains the ecosystem; therefore, changes within cave microbial communities have the potential to exert powerful bottom-up controls on ecosystems structure and function (Torsvik *et al.*, 2002; Simon *et al.*, 2003; Simon *et al.*, 2007). Localized variations in geochemistry have been shown to impact microbial community structure (Barton *et al.*, 2007; Shabarova and Pernthaler, 2010; Sonnleitner *et al.*, 2011),

which can impact biogeochemical cycling and mineralization processes within an environment (Frierdich *et al.*, 2011; Santelli *et al.*, 2011). This is especially true in the case of nutrient loading within oligotrophic systems, as changes in resource availability have the potential to alter the biochemistry of individual cells (Klappenbach *et al.*, 2000; Grünke *et al.*, 2011), which would in turn impact ecosystem processes (Lennon and Cottingham, 2008).

This latter point is best demonstrated in the case of European show caves that have been ravaged by human activity (Cañaveras *et al.*, 2001; Bastian *et al.*, 2010; Saiz-Jimenez *et al.*, 2011). Similar conclusions are reached by Iker *et al.* (2010) in research that linked changes in nitrogen-cycling within a cave system to a change in cave microfloral composition as a result of pesticide contamination within the system, and by Bastian *et al.* (2009) in research that linked changes in endogenous cave microflora with biocide treatments in Lascaux Cave. The effect of nutrient enrichment on microbial community diversity is less clear; however, it is clear that nutrient availability in cave systems is linked to niche diversity (Macalady *et al.*, 2008; Engel *et al.*, 2010), which plays a role in determining microbial community structure and species richness. The composition of cave microbial communities directly impacts the cave environment through mediating biogeochemical processes (Horner-Devine *et al.*, 2003), shaping the cave environment (Portillo *et al.*, 2009; Stomeo *et al.*, 2009; Portillo and Gonzalez, 2010), and providing the functional diversity that is necessary for the maintenance of ecosystem stability (Torsvik *et al.*, 2002).

Cave microbial consortia and the environments in which they reside are highly vulnerable to an array of impacts as a result of anthropogenic disturbance (Bastian and Alabouvette, 2009; Northup, 2011). Karst management is a hot topic in research today, as anthropogenic impact in karst systems continues to be a widespread problem (van Beynen, 2011). Legal protection for cave and karst systems has been extended at the Federal and State levels (Huppert, 1995); but, enacted legislation varies in the degree to which it protects these vulnerable systems and is too often wrought with loopholes (Carpenter and Busch, 1993; van Beynen and Townsend, 2005). There are several non-profit and community organizations (such as grottos) that actively work through outreach efforts to educate the public regarding the effects of human impact in karst regions, the importance of sustainable practices in karst regions, and the utilization

of minimal impact techniques when caving. These types of efforts have been called for by the caving community for a long time (Barton, 2006). National and international organizations, such as the National Speleological Society and the IUCN, have issued recommendations for the protection of cave and karst systems (Watson *et al.*, 1997). However, the effectiveness of these guidelines, recommendations, and management plans is contingent on the accumulation of baseline data that delineates the sources of contamination within a system and the documentation of specific impacts within a cave or karst system (Northup, 2011). This conclusion demonstrates the continued need for research, especially in regions such as the Appalachians, where cave density is high and research in cave systems is lacking. Our hope is that the studies already undertaken within the CSPC system, when paired with continued monitoring of cave health, provide the requisite baseline evidence to develop an informed management plan for the protection and conservation of this fragile and unique subterranean system.

Experimental Procedures

Field description

Carter Salt Peter Cave (Carter County, Tennessee, Fig. 1), located in the Ordovician Knox Dolomite unit (Oder, 1934), is an epigenic cave system typical of those found within the Appalachian region. At a depth of approximately 30 m, Carter Salt Peter Cave (herein CSPC) represents a relatively shallow system. Environmental conditions within the dark zone of the cave are typical of those found in other cave systems (Northup and Lavoie, 2001), as ambient air temperature remains around 13°C year-round (mean annual surface temperature for the region) and humidity approaches 100%. A variety of carbonate speleothem formations occur throughout the cave system (flowstone, dripstone, soda straws, corrosion residue), and the cave is particularly enriched in ferromanganese deposits. The cave is located in close proximity to agricultural land and residential areas, and the cave entrance is neither gated nor protected from human traffic. As a result, evidence of anthropogenic impact is widespread throughout the cave system.

Sample collection

Samples were collected from two Mn-oxidizing cave biofilms, Mn Falls (Figs. 2A and 2B), the site of alleged impact, and Mud Trap Falls (Fig. 2C), and a second biofilm community located in physical proximity to Mn Falls, in roughly three month intervals from July 2009 to June 2011. Deposits were screened for the presence of Mn oxides using 0.04% Leucoberbelin Blue (LBB), a redox indicator that is oxidized by Mn(III) or Mn(IV) to produce a bright blue color change (Krumbein and Altmann, 1973). Samples were collected aseptically by scraping the deposit surface using a sterile 50 mL Falcon tube. Care was taken to sample at locations within a deposit that tested LBB-positive for Mn(II)-oxidation and to maximize the sampling of black/chocolate brown coatings in these locations. Samples were stored on ice, transported to the lab, and immediately processed for downstream use.

DNA extraction, and PCR amplification, cloning, and phylogenetic analysis of human-specific Bacteroides-Prevotella 16S rRNA gene sequences

DNA was extracted from cave samples using a bead beating protocol with the Fast DNA Spink Kit for Soil (MP Biomedicals, Solon, OH). The concentration of extracted DNA was determined using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Human-specific *Bacteroides-Prevotella* 16S rRNA gene sequences were amplified from Mn Falls biofilm material using a primers designed by Bernhard and Field (Bernhard and Field, 2000a) with a demonstrated detection limit of 1.4×10^{-6} g dry feces/liter. A nested PCR approach was utilized in an attempt to amplify the region of interest in DNA extracted from cave biofilms in early July 2009 (*F*), late July 2009 (*F2*), January 2010 (*F3*-*W* and *T*), July 2011 (*4*). Approximately 3 ng of environmental DNA was used as a template for the first round of PCR amplification using the universal *Bacteroides-Prevotella* primers 32F (5'-AACGCTAGCTACAGGCTT) and 708R (5'-CAATCGGAGTTCTTCGTG) (Bernhard and Field, 2000b). Each 50 µL reaction contained 1.25 U AmpliTaq Gold (Applied Biosystems, Carlsbad, CA), 50 µM each primer, 1X PCR Gold Buffer (Applied Biosystems, Carlsbad, CA), 2 mM $MgCl_2$ Solution

(Applied Biosystems, Carlsbad, CA), 200 µM each dNTP, and 2X BSA (New England Biolabs, Ipswich, MA). A MJ Mini Personal Thermal Cycler (Bio-Rad, Hercules, CA) was used for all PCR-amplification reactions. The amplification protocol for the first round of PCR is as follows: an initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min, followed by a final extension of 72°C for 6 min. PCR amplifications were conducted in triplicate and visualized on a 1% agarose gel stained with GelRed Nucleic Acid Stain (Phenix Research, Candler, NC). A single positive band of approximately 700 bp was visualized in DNA samples *F* (July 2009) and *4* (July 2011) obtained from the Mn Falls biofilm. Amplifications of each DNA template were pooled for downstream use in the next round of PCR.

1 µL of PCR product from the pooled amplifications of *F* (July 2009) and *4* (July 2011) DNA was used as a template for PCR amplification of human-specific *Bacteroides-Prevotella* 16S rRNA gene sequences in the second round of the nested protocol. PCR amplification was conducted using the *Bacteroides-Prevotella* human-specific forward primer HF183 (5'- ATCATGAGTTCACATGTCCG) paired with the *Bacteroides-Prevotella* universal reverse primer 708R (5'-CAATCGGAGTTCTTCGTG) (Bernhard and Field, 2000a). Reaction conditions mimicked those given for the first round of the nested protocol, with the following adjustment in the amplification protocol: an initial denaturation of 94°C for 5 min, followed by 25 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 2 min, followed by a final extension of 72°C for 6 min. Amplifications were conducted in triplicate, and 5 µL of PCR product for each template was visualized on a 1.5% agarose gel stained with GelRed Nucleic Acid Stain (Phenix Research, Candler, NC) to verify the presence of a ca. 600 bp band. Amplifications of each PCR template were pooled and concentrated by rotary evaporation to a volume of approximately 10 µL. The concentrated PCR product for each template was run on a 1.5% agarose gel stained with GelRed Nucleic Acid Stain (Phenix Research, Candler, NC). A ca. 600 bp band for each template was manually excised from the gel and purified using an UltraClean GelSpin DNA Extraction Kit (Mo-Bio Laboratories, Carlsbad, California). Purified PCR products of each DNA template were cloned into TOPO TA *pcr*®2.1 vectors (Invitrogen, Carlsbad, CA), and plasmid DNA extracted from transformants using the QIAprep

Spin Miniprep Kit (Qiagen, Valencia, CA) was screened using the M13F(-20) primer. A small clone library consisting of ca. 15 clones for each DNA template was prepared and glycerol stocks were sequenced using the M13F(-20) primer. Sequencing was conducted at Beckman-Coulter Genomics (Danvers, MA). OTUs were determined by DOTUR analysis (Schloss and Handelsman, 2005), and representative sequences for each OTU were chosen based on sequence length and quality. For phylogenetic analysis, additional sequences of interest were selected using ARB (Ludwig *et al.*, 2004) and the NCBI taxonomic database (Johnson *et al.*, 2008). OTU and additional sequences of interest were aligned using the on-line SILVA aligner (Pruesse *et al.*, 2007). A phylogenetic tree (Fig. 3) was constructed using the PHYLIP software package (Felsenstein, 2004) by conducting both neighbour-joining and maximum likelihood analysis. Clone sequences were deposited in GenBank under the accession numbers JN820135-JN820146.

Most probable number assays

Most probable number (MPN) assays were employed to determine the total number of culturable heterotrophic microorganisms and heterotrophic Mn(II)-oxidizing microorganisms in CSPC biofilms (Table 1). Biofilm samples were collected from Mn Falls and Mud Trap Falls in February, May, and July of 2010 and stored overnight at 4°C. FMO2 growth medium (Chapter Two), was used for MPN assays. Biofilm samples were centrifuged to concentrate the wet biomass and the supernatant was removed. Samples were weighed and diluted 1:10 with medium. Serial dilutions of 1:10 diluted biofilm samples were made, ranging from 10^{-2} to 10^{-11} and inoculated in Cellstar 96 well culture plates (greiner bio-one, Monroe, NC). Each well contained 250 µL sterile media and was inoculated with 25 µL of either biofilm material, a positive control (*Leptothrix sp.*), or a negative control in eight replicates per sample. A fifth plate for each sample was inoculated and tested immediately for Mn(II)-oxidation and heterotrophic metabolism using 50 µL 0.04% LBB (rows 1-4) and 50 µL 0.3% iodonitrotetrazolium chloride (INT) (Sigma Aldrich) (rows 5-8) respectively. Colorometric results from the fifth-plate test were used to ascertain background levels for comparison after incubation. Plates were incubated in the dark at 10°C for

4 weeks (to mimic cave conditions) and scored immediately using LBB and INT as described above. Plates were then returned to the dark at 10°C and allowed to incubate overnight to note any color change. MPN assays were scored again after 24 hours, with no notable change in results being observed. Results were applied to a downloadable MPN calculator (Curiale, 2004) to determine the total number of culturable heterotrophic microorganisms and culturable heterotrophic Mn(II)-oxidizing microorganisms in cave biofilms.

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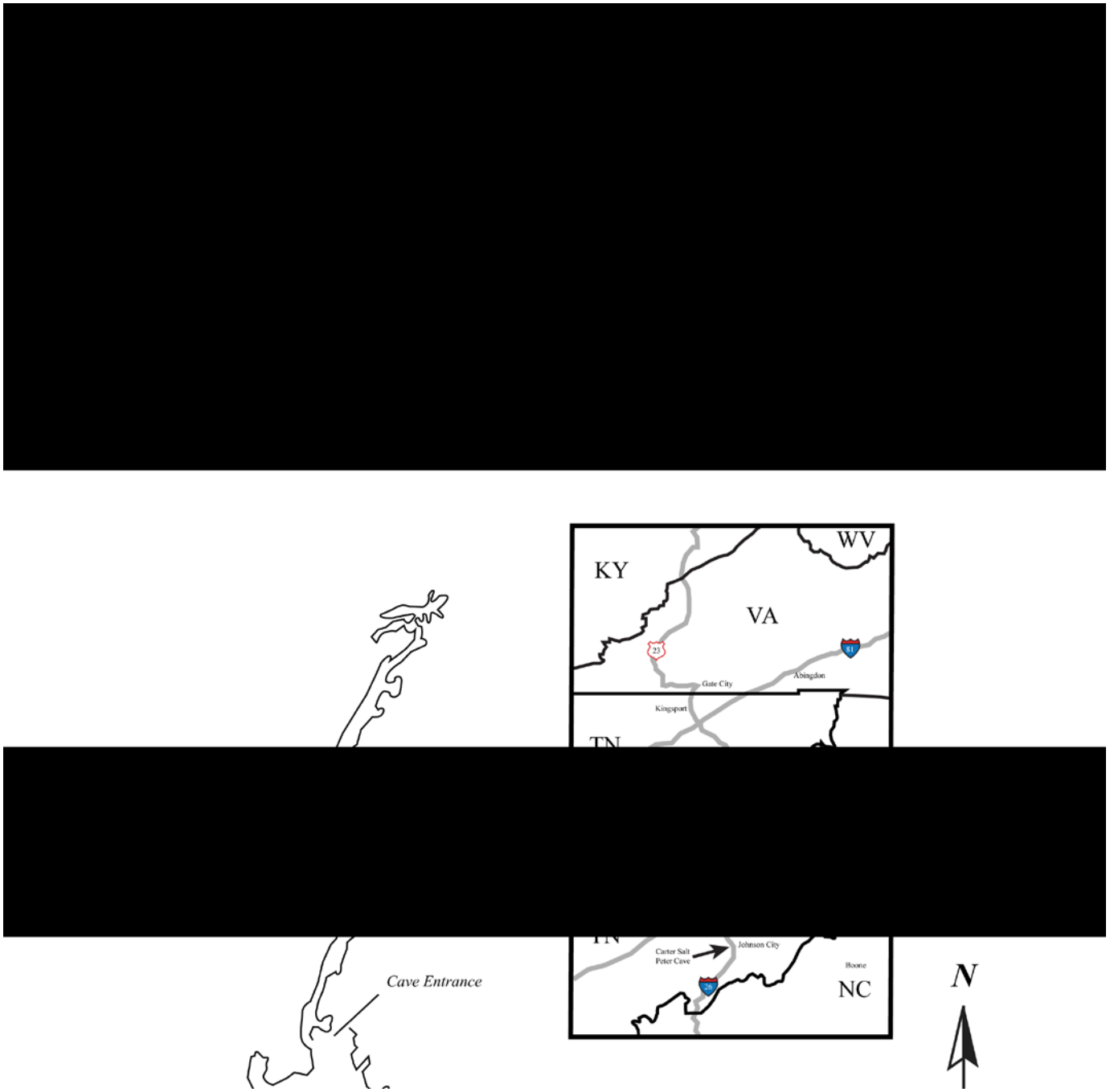
Figures and Tables



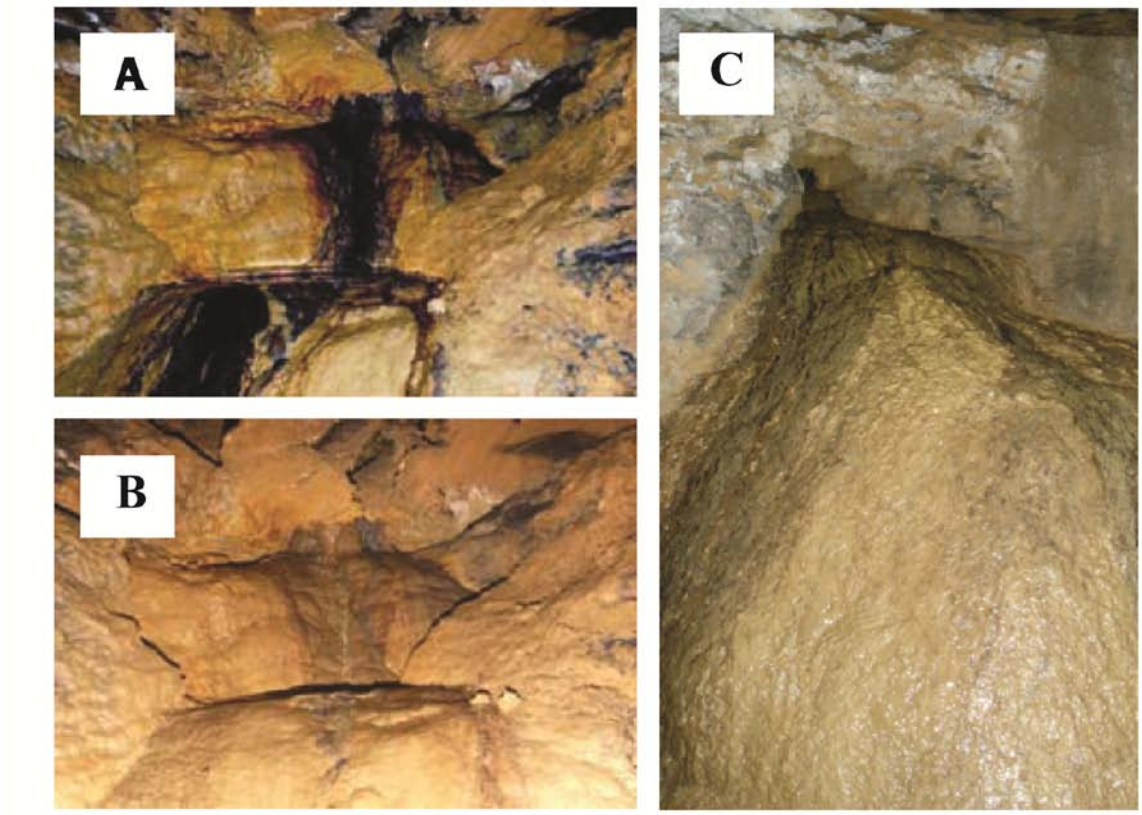


Fig. 2. (A) Carter Salt Peter Cave, Mn Falls site, July 2008, photo taken by Mountain Empire Grotto member John Matthews months after an alleged septic tanker truck released sewage into a local sinkhole. Image is published with permission. (B) Carter Salt Peter Cave, Mn Falls site, July 2010, Mn oxides reduced. Mn oxides have steadily decreased with time at the site since sampling began in July 2009. (C) Carter Salt Peter Cave, Mud Trap Falls site, July 2009.

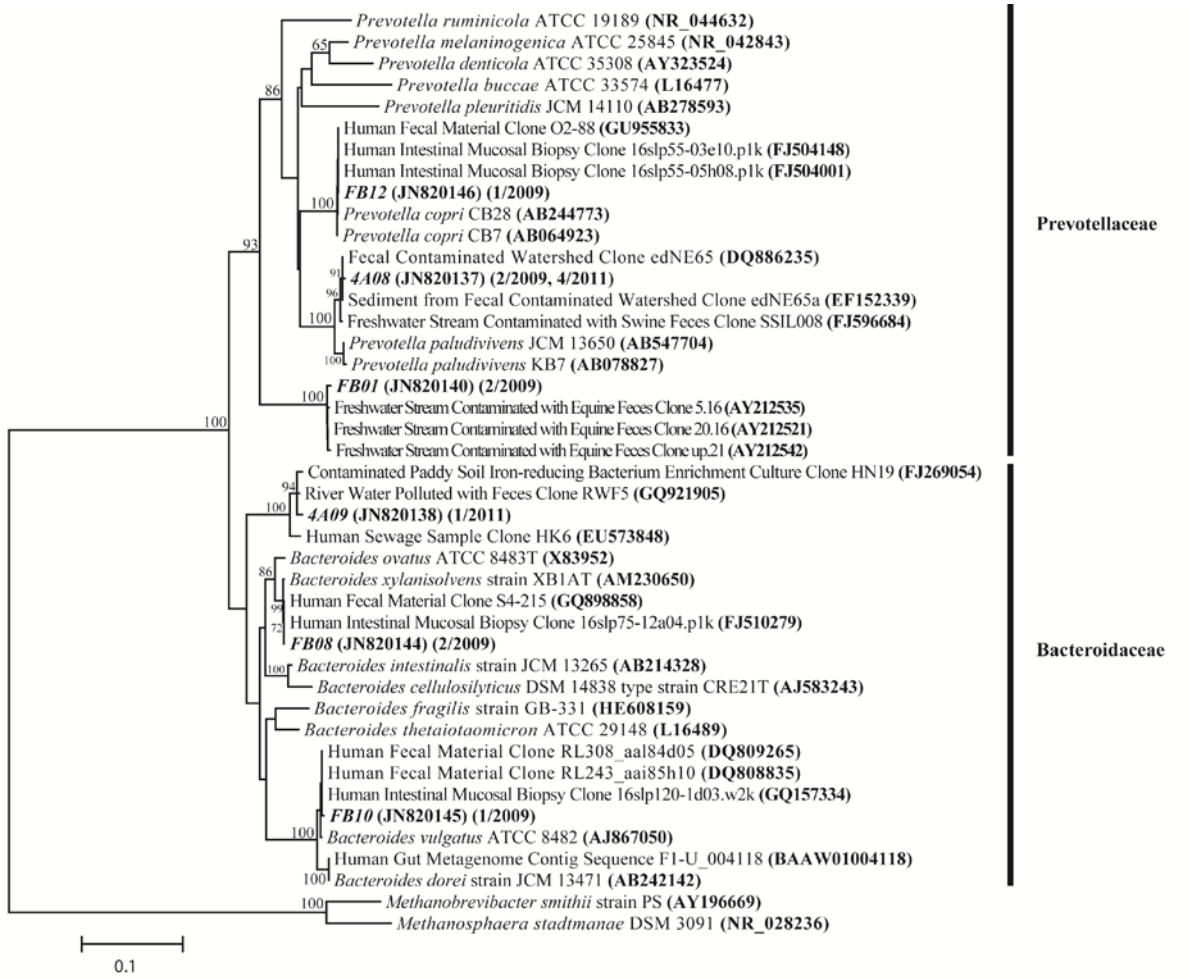


Fig. 3. Neighbour-joining tree inferring the phylogenetic placement of SSU rRNA gene sequences obtained from the Mn Falls biofilm found in Carter Salt Peter Cave, Carter County, TN in this study in either July 2009 (sequences beginning with F) or July 2011 (sequences beginning with 4). The number of seqeunces represented by each OTU are given along with the year DNA was extracted in parentheses following the NCBI accession number. Alignments were created using the on-line SILVA aligner. Dendogram was created using PHYLIP. Bootstrapping values are shown for nodes that were supported >50% of the time with maximum-likelihood analysis (data not shown). *Methanobrevibacter smithii* and *Methanosphaera stadtmanae* were used as outgroups. Branch lengths indicate the expected number of changes per sequence position (see scale bar).

Table 1. Total culturable heterotrophic bacteria and culturable heterotrophic Mn(II)-oxidizing bacteria present in Carter Salt Peter Cave biofilms.

Sample (Sample Date)	Heterotrophic Bacteria			Heterotrophic Mn(II)-oxidizing Bacteria			Total Bacteria[a] (cells/g wet weight)	Culturable Bacteria (%)	Culturable Mn(II)-oxidizing Bacteria (%)
	95% CI lower	MPN (cells/g wet weight)	95% CI upper	95% CI lower	MPN (cells/g wet weight)	95% CI upper			
Mud Trap Falls (Feb. 2010)	3.6×10^7	1.1×10^8	3.2×10^8	4.5×10^7	1.4×10^8	4.6×10^8	2.6×10^9	4.2	5.4
Mn Falls (May 2010)	6.9×10^9	2.5×10^{10}	8.9×10^{10}	9.3×10^7	3.2×10^8	1.1×10^9	9.8×10^9	≥100.0[b]	3.3
Mud Trap Falls (July 2010)	3.2×10^6	9.6×10^6	2.9×10^7	…	…	…	2.6×10^9	0.4	…
Mn Falls (July 2010)	3.2×10^8	9.6×10^9	2.9×10^{10}	…	…	…	9.8×10^9	97.9	…

[a]Total bacterial cell number was estimated in a previous study by real-time quantitative PCR. SSU rRNA gene copy number was normalized in experimental results using the average copy number for bacteria (4.08 copies/cell) as reported by the Ribosomal RNA Operon Copy Number Database (Klappenbach *et al.*, 2001) in March 2010.

[b]The 95% CI lower bound for heterotrophic bacteria lies within the error range of total bacterial cell number as estimated by real-time quantitative PCR.

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CHAPTER 4: SUMMARY, CONCLUSIONS, AND BROADER IMPACTS

Introduction

My research attempts to answer questions regarding the geomicrobiology of ferromanganese deposits in the cave-rich, but vastly understudied upper Tennessee River Basin. Specifically, I set out to answer questions regarding the role of Mn-biomineralization in the formation and transformation of the cave mineral environment (Chapter Two). Empirical observations during the study period led to the evolution of a second line of inquiry (addressed in Chapter Three), which specifically focused on the documentation of evidence of sustained anthropogenic impact within the Carter Salt Peter Cave (CSPC) system in Carter County, Tennessee. Here I present a summary of these results, conclusions from the work in its entirety, and potential lines of future investigations. I then will frame the emerging field of cave geomicrobiology in a broader perspective.

Summary

Chapter Two: Mn-biomineralization in ferromanganese deposits in caves of the upper Tennessee River karst Basin

Chapter Two of this thesis explores the role of Mn-biomineralization in the formation of ferromanganese deposits in the caves of the upper Tennessee River Basin. Results from this study document a Mn-rich geochemistry at many sites within the region's caves. One such site was the ferromanganous biofilm, Mn Falls located within CSPC, where Mn:Fe ratios (as measured by ICP-OES) were an order of magnitude greater than the bulk bedrock ratio for the Knox Dolomite as reported by Lumsden and Caudle (2001). Molecular evidence from the Mn Falls site revealed a microbial community that was enriched in

organisms related to environmental clones and isolates from metal-rich environments. Culture-based evidence demonstrated *in vivo* Mn(II)-biomineralization capacity in a phylogenetically diverse group of organisms. Some of these isolates (e.g., *Flavobacterium*, *Janthinobacterium*, *Leptothrix*) were obtained from high dilutions (10^{-8} to 10^{-10}) of cave biofilm material, providing further support for the environmental relevance of Mn(II)-oxidizing biofilms in cave ferromanganese deposit formation. Microbial biomineralization processes are known to increase the rate of Mn(II)-oxidation up to five orders of magnitude above abiotic oxidation rates (Nealson *et al.*, 1988; Dixon and Skinner, 1992), which are especially slow in low temperature environments such as caves (Moore, 1981). Therefore, rapid depositional rates, especially those which exceed oxidation rates within an environment, are indicative of microbial involvement in deposit formation (Nealson *et al.*, 1988). Overall, experimental evidence indicates that Mn(II)-oxidizing organisms play an important role in ferromanganese deposit accretion in caves of the upper Tennessee River Basin.

Because the present study represents the first investigation of geomicrobiology in southern Appalachian epigenic caves, several questions remain regarding the role of microbes in 1) cave biogeochemical cycling and 2) the formation and transformation of secondary mineral deposits within these systems. Herein, I include some suggestions for future work aimed at elucidating the comprehensive role of microbes in the formation and dissolution of cave ferromanganese deposits. First, oxygen-limited environments are recognized as important niches in cave environments (Portillo and Gonzalez, 2009), yet we know very little about the role of anaerobes, specifically Mn(IV)-reducers, in the dissolution of cave ferromanganese deposits. Microbial Mn(IV)-reduction is a widespread phylogenetic trait among bacteria, archaea, and fungi (Lovely, 1991; Lovely *et al.*, 2004; Gadd, 2007). Dissimilatory Mn(IV)-reduction is a known mode of chemolithoautotrophic growth in bacteria (Lovely, 1991), and leads to the mobilization of Mn within an environment (Gadd, 2004). Thus, microbial Mn(IV)-reduction represents a potentially important process in the dissolution of cave ferromanganese deposits that warrants future investigation.

Second, fungi are known to mediate key biogeochemical transformations (Sterflinger, 2000), yet the role of these organisms in the formation of ferromanganese deposits has long been debated (Taylor-

George *et al.*, 1983; Grote and Krumbein, 1992; Schelble *et al.*, 2005). Recent research indicates that Mn(II)-oxidizing fungi (Miyata *et al.*, 2003; Miyata *et al.*, 2006; Cahyani *et al.*, 2009; Santelli *et al.*, 2010; Santelli *et al.*, 2011) and Mn(IV)-reducing fungi (Gadd, 2007) are found in a variety of environments. Fungal diversity in caves is thought to be high (Roble *et al.*, 2011; Vaughan *et al.*, 2011), but the role of fungi in cave biogeochemical cycles is virtually unknown (Cunningham *et al.*, 1995; Bastian *et al.*, 2009). Evidence from Chapter Two indicates that fungi represent on average 0.3% of the total microbial population in cave ferromanganese deposits. Because fungal species can be capable of Mn-oxidation and reduction, processes that would lead to the formation and dissolution of cave ferromanganese deposits, understanding the role of fungi cave biogeochemical cycling is a relevant and important future line of study.

Finally, continued cultivation-based efforts are needed to continue to isolate cave organisms involved in Mn-biomineralization. These efforts should be focused on the development of new *in situ* and lab based culture techniques (Stevenson *et al.*, 2004; Nichols *et al.*, 2008; Nichols *et al.*, 2010; Vartoukian *et al.*, 2010), adapted to the cave environment, that target the isolation of novel species involved in the biogeochemical transformation of Mn, thus shedding light on the functional role and environmental relevance of these organisms within the cave environment. In addition, techniques such as RNA-based community surveys (Gonzalez *et al.*, 2006; Portillo *et al.*, 2008) and FISH (Meisinger *et al.*, 2007; Macalady *et al.*, 2008) should be developed and utilized as a supplement to cultivation efforts. These molecular-based methodologies would be useful in determining the metabolically active portion of the microbial population in cave ferromanganese deposits, as these organisms would be most influential in the formation/transformation of the cave mineral environment. However, it is important to note that Mn-biomineralization is not necessarily linked to metabolic activity, as Mn(II)-oxidation has been associated with spores of metabolically dormant *Bacillus* spp. (de Vrind *et al.*, 1986; Francis and Tebo, 2002; Dick *et al.*, 2006).

Chapter Three: Evidence of sustained anthropogenic impact in the Carter Salt Peter Cave (Carter County, Tennessee) system

Over the duration of the study period, we observed a striking change in the appearance of the Mn Falls biofilm (documented in Chapter Three), which suggested there was a link between the bloom of the Mn Falls biofilm and nutrient loading at this site within CSPC. Therefore, we initiated a second study within the CSPC system to detect the presence or absence of fecal signatures in DNA extracted from the Mn Falls biofilm at the time of alleged eutrophication (July 2009) and two years post-impact (July 2011). Results from molecular-based surveys (Chapter Three) detected the presence of *Bacteroides-Prevotella* fecal signature in DNA extracted from the Mn Falls biofilm at both sampling points; however, a stronger human-specific signature was detected in the July 2009 sample. DNA amplification attempts at a second Mn(II)-oxidizing biofilm located in CSPC, Mud Trap Falls, were unsuccessful; therefore a *Bacteroides-Prevotella* fecal signature was not detected at this second site where environmental eutrophication was not suspected. Supplementary cultivation-based MPN analyses supported the hypothesis of nutrient-loading at Mn Falls, as a significant increase in total culturable heterotrophic bacteria was observed at this site when compared to Mud Trap Falls. Results from the present study indicate that nutrient loading in shallow cave systems may alter microbial community composition and function, which could exert a downstream impact on nutrient cycling within the cave system.

Results also suggested that monitoring microbial community structure and function can be used as a way to trace contamination in karst systems. However, additional work is needed to test this hypothesis. First, continued monitoring of the CSPC system is required to document changes in microbial communities associated with fluctuations in source water quality measurements (e.g., TOC/DOC, nitrogen and phosphorous content, heavy metals, and fecal indicators). In addition, it would be useful to use cultivation-based methodologies to test which nutrient (e.g., carbon, nitrogen, or phosphorous) is a limiting factor in these shallow cave systems. In addition, a GIS overlay should be created to show the location of potential diffuse and point pollution sources in relation to CSPC. Several layers of information could be condensed into one GIS overlay, which would allow for manipulation and visualization of

watershed drainage patterns, sources of pollution, and the location of the cave. Pertinent GIS layers may include bedrock type, faults/fractures, overburden, hydrology, elevation, water and sewer lines, septic tank locations, and cave location/declination. GIS technology has been previously used in the development of cave management strategies to protect Wind Cave, South Dakota (Horrocks and Szukalski, 2002; Ohms and Reece, 2002), one of the oldest known cave systems in the world. Finally, baseline studies need to be conducted in pristine analogous cave systems within the upper Tennessee River Basin to provide data for comparison purposes.

Conclusions

Historically, research within the field of cave geomicrobiology in the United States has been constrained to 1) active, sulfidic cave systems that are hypogene in origin (Engel *et al.*, 2003; Engel *et al.*, 2004b; Engel, 2007; Spear *et al.*, 2007; Porter *et al.*, 2009; Engel *et al.*, 2010), 2) deep, inactive hypogene systems located in the southwestern United States (Cunningham *et al.*, 1995; Northup *et al.*, 1997b; Northup *et al.*, 2000; Provencio and Polyak, 2001; Northup *et al.*, 2003; Spilde *et al.*, 2005; Levy, 2007b, a), and 3) cave systems with high levels of economic/tourist impact such as Wind Cave, South Dakota (Chelius and Moore, 2004; Chelius *et al.*, 2009) and Kartchner Caverns, Arizona (Buecher and Sidner, 1999; Hill, 1999; Ikner *et al.*, 2007; Vaughan *et al.*, 2011). Only a handful of studies (Angert *et al.*, 1998; Engel *et al.*, 2001; Simon *et al.*, 2003; Shapiro and Pringle, 2010; Campbell *et al.*, 2011) exist documenting the microbial ecology of caves in the southern Appalachians, one of the five major cave regions in the United States (Christman and Culver, 2001).

In addition, none of the aforementioned studies within the Appalachian region address the microbial ecology of epigenic cave systems, which reflect the most common form of speleogenic process worldwide (Palmer, 1991). Therefore, the body of work contained within this thesis represents the first study of the geomicrobiology of epigenic caves in the southeastern United States. For a long time, it has been obvious that the microbial communities of acidic, hypogenic cave systems (Angert *et al.*, 1998;

Vlasceanu *et al.*, 2000; Engel *et al.*, 2001; Engel *et al.*, 2003; Engel *et al.*, 2004b) and pH-neutral, epigenic systems (Schabereiter-Gurtner *et al.*, 2002; Northup *et al.*, 2003; Barton *et al.*, 2004; Chelius and Moore, 2004; Barton and Jurado, 2007) are strikingly different in composition. This pattern is evident on a regional scale, where the microbial community structure of Cesspool Cave in Allegheny County, Virginia (Engel *et al.*, 2001) shares little overlap with the dominant communities found in the present study (Chapter Two) in Carter Salt Peter Cave. However, in many ways the vastly different community structures found in sulfidic and carbonic cave systems display properties of functional redundancy, acting as agents of speleogenesis (Northup *et al.*, 2000; Engel *et al.*, 2004a; Taboroši, 2006) and providing the basis of energy for a system that is deprived of photosynthetic input (Sarbu *et al.*, 1996; Simon *et al.*, 2003; Simon *et al.*, 2007).

As we learn more about the geomicrobiology of cave systems within the upper Tennessee River Basin, it may become increasingly apparent that these relatively shallow cave systems share little similarity with their deeper analogous counterparts in the southwestern United States. Preliminary evidence from the present study (Chapter Two) suggests that this may be the case. Lechuguilla Cave (New Mexico) was formed over 12 million years ago by hypogenic processes; however, the cave is no longer actively forming and now represents a pH neutral system with similar environmental parameters (Boston *et al.*, 2006) to those found in our primary study site, CSPC. The primary difference between the two systems is the depth of the caves: Lechuguilla is the deepest known cave system in the United States (depth ca. 489m) (Boston *et al.*, 2006), whereas CSPC is a relatively shallow system (depth ca. 30m). Ferromanganese deposits are widespread throughout both cave systems and microbial biomineralization processes appear to be contributing to the accretion of these deposits. However, the microbial community structure in each cave appears to be markedly different. Ferromanganese deposits in Lechuguilla Cave are dominated by members of the *Firmicutes*, *Nitrospira*, the *α-Proteobacteria* (*Mesorhizobium* spp.), and the *β-Proteobacteria* (*Aquaspirillum* spp.) (Northup *et al.*, 2003). None of these groups were dominant members of a ferromanganous biofilm in CSPC (Chapter Two). In addition, the archaeal community

composition in Northup *et al.*'s study was composed of several novel lineages, which do not resemble those found in CSPC (Chapter Two).

So, the question arises, what might be causing the differences in microbial community structure observed in ferromanganese deposits between these analogous systems? One possible explanation for the observed differences is the role of surface impact in deep vs. shallow cave systems. The degree of surface impact within a cave system depends on 1) the depth of the system, as shallow cave systems experience greater impact due to the lessened effect of soil/bedrock filtration (van Beynen and Townsend, 2005), 2) the occurrence of flowing water within a system, as water flushes nutrients and exogenous material into cave systems (Northup, 2011), and 3) the level of animal/human traffic within the system, as heavily visited caves receive higher levels of exogenous nutrient and material input (Hunter *et al.*, 2004; Ikner *et al.*, 2007; Chelius *et al.*, 2009; Chroňáková *et al.*, 2009). Shallow and deep cave systems would be on opposite ends of the impact spectrum based on these criteria. Because shallow cave systems would receive high levels of surface impact, it may be more effective to compare shallow caves to those impacted by high levels of human traffic (e.g., recreational caving activity, tourism) (Chelius and Moore, 2004; Ikner *et al.*, 2007; Chelius *et al.*, 2009; Vaughan *et al.*, 2011). However, more research is needed to determine if this comparison is valid.

The effects of anthropogenic/surface impacts on cave microbial communities have been well documented in prior research (Kozar, 2002; Graening and Brown, 2003; Iker *et al.*, 2010; Hu *et al.*, 2011). Organic supplementation of karst systems via leached human and animal waste is known to affect nutrient cycling in oligotrophic karst environments by increasing both the quality and quantity of carbon that is available to microorganisms. The impact of carbon-rich leachate is readily observed in karst microbial communities, as carbon supplementation through organic contamination events has been shown to increase bacterial biomass in polluted subterranean pools and sediments (Simon and Buikema Jr., 1997). In addition, experimental manipulations in Wind Cave, South Dakota provided direct evidence of a shift in microbial community composition, exerting a negative impact on the endemic microflora, as a result of alterations in organic inputs in cave soil (Chelius *et al.*, 2009). Evidence of shifts in microbial

community composition as a result of organic supplementation/nutrient loading is particularly intriguing in light of the observations in Chapter Three of this thesis, and with the realization that little information exists regarding the role of anthropogenic/surface impacts (e.g., organic supplementation) on biomineralization processes in caves.

One possible explanation for the differences in community structure observed within Lechuguilla and CSPC may be the immediate impact of nutrient enrichment on microbial community composition. In the case of the Mn Falls biofilm, nutrient enrichment appeared to alter microbial function by increasing Mn-biomineralization. This, in turn was visible as a thick, hairy, black biofilm coating on the surface of the cave wall, which steadily decreased in appearance following the initial pulse period of nutrient influx. Findings from the present study suggest that blooms of Mn-oxidation within cave systems may be linked to exogenous nutrient input and therefore may provide a method to gage ecosystem health and levels of anthropogenic disturbance in caves.

The idea of using microbial communities to monitor ecosystem health in karst terrain has previously been asserted by cave and karst researchers (Lavoie and Northup, 2005; Pronk *et al.*, 2009; Campbell *et al.*, 2011). If this hypothesis proves to be true, monitoring of Mn oxide deposits would be viewed as an efficient and cost-effective method of tracing nutrient loading/anthropogenic impact in karst terrain. This idea could then be extended to include monitoring of Mn oxide bulk elemental composition using ICP-OES, as Mn oxides are known to be efficient scavengers of heavy metals (Post, 1999; White *et al.*, 2009), which could enter a karst system as a result of industrial contamination (Vesper *et al.*, 2001; van Beynen and Townsend, 2005).

Broader Impacts

Strong selective pressures (Porter, 2007) have led to the development of a unique cave macroflora (Poulson and White, 1969; Fong, 2011) and diverse microflora (Northup and Lavoie, 2001; Barton, 2006; Engel, 2007) that has traditionally been the focus of research efforts within cave systems. These

endeavors have led to the discovery of several new stygo- and troglobitic species that are uniquely adapted to an underground lifestyle (Poulson and White, 1969; Porter, 2007; Fong, 2011), the first report of a freshwater or terrestrial microbe-invertebrate symbiosis (Dattagupta *et al.*, 2009), and evolving thoughts on the role of microbial activity in 1) catalyzing speleogenic processes (Northup *et al.*, 1997a; Cañaveras *et al.*, 1999; Barton *et al.*, 2001; Jones, 2001; Engel *et al.*, 2004a; Barton and Luiszer, 2005; Cañaveras *et al.*, 2006) and 2) controlling biogeochemical cycling within subterranean systems (Sarbu *et al.*, 1996; Chaban *et al.*, 2006; Gorbushina, 2007; Simon *et al.*, 2007; Portillo *et al.*, 2009; Engel *et al.*, 2010; Akob and Küsel, 2011). This latter point is one of critical understanding in the face of climate change, as microbes exert a keystone control on ecosystem processes through the maintenance of Earth's biogeochemical cycles (Postgate, 1992).

Research within the emerging field of cave ecology has begun to move beyond basic exploratory efforts, with the recognition of a variety of niches through which the cave environment contributes to applied science. The most prominent emerging application of cave research within a broader context deals with astrobiology and the search for life in extraterrestrial environments. As addressed in Appendix A herein, caves, as an access point to the deep subsurface, are recognized as an astrobiological analog as they contain biogeochemical cycles similar to those hypothesize to occur on Mars (Boston *et al.*, 2001; Boynton *et al.*, 2009), the current focal point of NASA's search for extraterrestrial life (Baross, 2007). Research in the cave environment concerning microbes, biomineralization, and microbial biosignatures plays an integral role in the development of criteria and analytical techniques to assess and detect extraterrestrial biosignatures and provides a focal point for NASA's search for extraterrestrial life.

In addition to applications within the biological and planetary sciences, caves are recognized as a novel source of bioprospecting (Onaga, 2001), the search for novel therapeutic or pharmaceutical compounds in the environment (Schmidt, 2004; Bhullar *et. al.*, 2012). Efforts in this realm are often concentrated on the study of rare and extreme environments, as these systems are seen as unexplored caches of novel compounds. Several promising initial results have been identified in cave systems within North America (Rule *et al.*, 2011; Sadoway and Cheeptham, 2011) and Europe (Yücel and Yamaç, 2010).

In the latter study, a group of researchers from Turkey have isolated an antibiotic-producing strain of *Streptomyces* sp. that demonstrates efficacy against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococcus faecium*, and *Acinetobacter baumannii* (Yücel and Yamaç, 2010). Isolation of novel antibiotic compounds has profound implications for human medicine in the face of rising concerns over the compounding problem of an increase in multi-drug resistant organisms (Dooley *et al.*, 1992; Perl, 1999; Noskin, 2001; Podnos *et al.*, 2001; Weigel *et al.*, 2003; Dias and Caniça, 2004; Sebaihia *et al.*, 2006; Flatow, 2011; IDSA, 2011) paired with a decrease in drug development efforts by the pharmaceutical industry (Austin *et al.*, 1999; Schmidt, 2004; Peleg and Hooper, 2010; IDSA, 2011).

As points of access to the deep subsurface, caves offer us a window into an unexplored territory. Despite the progress that has been made within the complementary fields of cave ecology and geomicrobiology, we are only scratching the surface of what there is yet to discover. We are still in the beginning stages of understanding the true value of the biodiversity contained within cave systems and the critical role of cave organisms in the maintenance of ecosystem processes. These conclusions place further emphasis on the need to protect and preserve these unique and fragile ecosystems.

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APPENDIX A: MICROBIAL BIOSIGNATURES IN EASTERN TENNESSEE CAVES

Preface

In 1988 the National Space Grant College and Fellowship program was established by the United States Congress to support research in science, technology, engineering, and mathematics with aerospace and/or NASA applications. The program is implemented under the auspices of NASA and consists of 52 Space Grant Consortia located throughout the United States. The North Carolina (NC) Space Grant was established in 1991 and has provided over $14 million in support through thirteen member institutions and seven industry, government, and non-profit partners since its inception. In the summer of 2011 my research documenting microbial biosignatures in an eastern Tennessee cave was supported by a NC Space Grant Graduate Research Fellowship, a merit based competitive fellowship program that provides up to $6,000 in annual support for a Master's level student. The manuscript that follows provides a concise summary of my summer work

Summary

Biogeochemical cycling within cave systems and the physical transition from a surface to subsurface based ecosystem are often cited as reasons that astrobiologists have recognized caves as an ideal Earth-based analog for the study of Mars. Media was designed to target the cultivation of cave microorganisms involved in the cycling of methane, a compound that is abundant on Mars and the metabolism of which is known to be a form of chemoautotrophic growth for microbial cells. Fourteen putative methanotrophs were isolated from Carter Salt Peter Cave (Carter County, Tennessee), and evidence of methane production was detected in culture media targeting

methanogens. In addition, the morphologies and chemical signatures of biogenic Mn oxides were characterized using TEM-EDS analysis. The presence of electron-dense Mn deposits associated with microbial cells was confirmed in five actively-oxidizing cultures isolated in a prior study from caves in the upper Tennessee River Basin. However, Synchotron based structural studies are needed to identify the mineral phases produced by these bacteria. Further, using terrestrial analogues of Martian subsurface environments will allow for the development of analytical techniques to detect and assess extraterrestrial biosignatures, both living and extinct.

Introduction

Astrobiology integrates knowledge gained from a variety of scientific disciplines to answer fundamental questions concerning the origin, limits, and future of both terrestrial and extraterrestrial life. Mars is a current focal point of NASA's search for extraterrestrial life (Baross, 2007), as several recent lines of evidence indicate that 1) hydrologic cycles may have once been present on the planet (Komatsu *et al.*, 2009; Hurowitz *et al.*, 2010), 2) the geochemistry of the planet is conducive to the evolution of chemolithoautotrophic microbial life , and 3) strong recent emissions of gases (such as methane) that may not be explained solely by abiotic processes (Mumma *et al.*, 2009; Etiope *et al.*, 2010). It is widely recognized within the scientific community that the search for extraterrestrial life starts on Earth in systems that are analogous to planetary systems that may be hospitable to life (Catling, 2004; Szynkiewicz *et al.*, 2012). Our knowledge of the requirements for life adopts a terran-centric approach. At a basal level all terran life needs to exist is water and a source of energy (Baross, 2007). The remaining environmental parameters can be fairly benign or toxic, and microbial life has found ways to thrive and evolve in both types of conditions (Brock, 1981; Brock, 1985; Gold, 1992; Whitman *et al.*, 1998; Pedersen, 2000; Baross, 2007; Staley *et al.*, 2007; Northup *et al.*, 2011; Stivaletta, 2011).

Prokaryotes arguably represent the most diverse kingdom of life (Curtis and Sloan, 2005). A primary driving force in the evolution of this diversity is microbial metabolic plasticity (Woese, 1987; Torsvik *et al.*, 2002), a key contributing factor towards the ability of bacteria and archaea to inhabit a wide variety of ecosystems, some of which represent the most extreme environments found on the Earth. One microbial metabolic strategy that is targeted in astrobiological research is chemolithoautotrophy, the ability of an organism to synthesize ATP and fix carbon by harnessing the energy contained in naturally occurring redox couples within an environment (e.g., sulfur oxidation/reduction, iron oxidation, nitrification, methane oxidation, and methanogenesis). Chemolithoautotrophic communities are abundant in the Earth's deep subsurface (Stevens, 1997), making this an ideal environment for the study of astrobiology and microbial biosignatures (Boston *et al.*, 2001; Gorbushina *et al.*, 2002; Boston *et al.*, 2006).

Caves are a point of access to the subsurface and are an ideal astrobiological analog, as they represent a transition zone from surface to subsurface based ecosystems (Pedersen, 2000) and contain biogeochemical cycles similar to those hypothesized to occur on Mars (Boston *et al.*, 2001; Boston *et al.*, 2006). Recent evidence suggests that the deep Martian bedrock may be partially composed of Mn- and Fe-rich calcium carbonates (Boynton *et al.*, 2009; Glotch, 2010; Michalski and Niles, 2010), similar in gross composition to the dolomite that underlies a large portion of the upper Tennessee River Basin (Montañez, 1994), a region has been a focus of my thesis research for the past three years. The research presented in the present study focused on the identification of cave microorganisms involved in the cycling of methane and the signatures of biogenically produced Mn-mineral deposits. Ultimately, these types of studies will provide insight into the current limits of life on Earth, and aid in the development of criteria and analytical techniques to detect and assess extraterrestrial biosignatures.

Results

Cultivation of cave organisms involved in methane cycling

Four different media types designed to target the cultivation of methanotrophs were utilized in this study. A total of fourteen isolates were obtained from the four media types (Table 1). Based on BLAST analysis (Johnson *et al.*, 2008), isolates were closely related (≥98% shared identity over a ca. 500 bp sequence) to clone sequences from a variety of environments, including soil, freshwater, the deep subsurface (e.g., caves and mines), hydrocarbon-rich environments (e.g., oil fields), sewage sludge, and low temperature systems (e.g., Alpine soil). In addition, several isolates (e.g., AA1, NN3) are members of a genus (*Flavobacterium*) that contains known methylotrophs (Madhaiyan *et al.*, 2010). Three sequences (isolates AS, NS, 2T) generated low quality (PHRED ≤ 20) reads with substantial amounts of contamination; therefore, these isolates were eliminated from bioinformatic analysis. All media was supplemented with Mn(II) in an attempt to isolate a methanotrophic Mn(II)-oxidizer, however this effort was unsuccessful as all isolates tested LBB-negative for Mn(II)-oxidation.

Two different media (Slurry and TMM) were designed to target methanogens in the cave. Methanogens, a group of methane-producing anaerobic archaea, are notorious slow-growers, with incubation times on the order of a month or more required to detect methane production in a culture. A total of 48 attempts were made at inoculation (8 on 5/16/2011 and 40 on 6/16/2011), of those 17 cultures maintained anaerobic conditions. After four weeks of incubation, the four cultures inoculated on 5/16/2011 that maintained anaerobic conditions were producing small amounts of methane, measured below 0.01% headspace volume. At the six week measurement point, these cultures all had a grey tint. This coloration is an indication of the presence of sulfate-reducing bacteria, which typically out-compete methanogens for H_2 in media due to their faster doubling time (A. Hawkins, personal communication). From this point on, methane was undetectable in the headspace. Unfortunately, all cultures inoculated on 6/16/2011 suffered the same fate as putative sulfate-reducing species were evident after the initial 2 weeks of incubation. Future attempts at cultivation of cave methanogens will include an addition of 10

mg/L rifampicin to each culture tube as a bactericidal agent (Bräuer *et al.*, 2004) to eliminate competition for H_2 by sulfate-reducing bacteria.

Mineralogy of biogenic Mn oxides

TEM-EDS analysis of actively-oxidizing cave isolates confirmed the presence of electron-dense Mn deposits associated with microbial cells in culture (Fig. 2): *Janthinobacterium* sp. A6, *Flavobacterium* sp. E8, *Leptothrix* sp. G6, *Arthrobacter* sp. L, and *Pseudomonas* sp. Mn Falls 11. TEM-EDS analysis (Fig. 3) revealed the presence of intracellular phosphorous storage granules in *Pseudomonas* sp. Mn Falls 11.

Discussion

Cultivation of cave organisms involved in methane cycling

Results from preliminary sequencing (ca. 500 bp of the 1500 bp 16S rRNA gene sequence) suggested the isolation of one *Arthrobacter* sp. (Table 1, isolate 2S) and four *Pseudomonas* sp. (Table 1, isolates 1N, 1S, 1T, and 2N) from media containing both citrate and methane as carbon sources. *Arthrobacter* spp. have been isolated from methane-rich environments previously (Kageyama *et al.*, 2008), can be capable of hydrocarbon degradation (Keuth and Rehm, 1991), and have also been associated with methylotrophic metabolisms (Levering *et al.*, 1981). *Pseudomonas* spp. have been identified in molecular-based surveys of methane-rich gas hydrate sediments (Marchesi *et al.*, 2001) and have been isolated from methane-rich seafloor sediments (Kobayashi *et al.*, 2008) and laboratory bioreactors (Wilkinson *et al.*, 1974; Cardinali-Rezende *et al.*, 2011). In addition, some *Pseudomonas* spp. are known methanotrophs (Davis *et al.*, 1964). Although the isolates from the present study were not growing on media containing methane as the sole carbon source, they are clearly capable of growth in a methane-enriched (e.g., 50% CH_4) environment. Therefore, it is possible that these isolates are capable of methane-based (e.g methanotrophic or methylotrophic) metabolisms.

Six isolates were obtained from media containing methane as the sole carbon source. Four of these isolates (Table 1, isolates AA2, AA3, NT1, and NT3) were phylogenetically affiliated with the genus *Acinetobacter*, which contains species whose genomes encode several genes involved in methane-based metabolisms (Kanehisa *et al.*, 2002). In addition, members of the genus *Acinetobacter* have been isolated from methane-rich sediments (Kobayashi *et al.*, 2008) and organic-waste degrading bioreactors (Cardinali-Rezende *et al.*, 2011). The remaining two isolates (Table 1, isolates AA1 and NN3) were phylogenetically affiliated with the genus *Flavobacterium*, members of which have been detected in molecular-based surveys of methane-rich environments, such as rice paddy soil (Henckel *et al.*, 1999) and petroleum reservoirs (Orphan *et al.*, 2000), and have been isolated from methane-rich laboratory bioreactors (Wilkinson *et al.*, 1974). In addition, some members of the *Flavobacterium* genus have been identified as facultative methylotrophs (Madhaiyan *et al.*, 2010) and are known to contain genes that are involved in methane-based metabolic pathways (Kanehisa *et al.*, 2002). These six isolates, obtained from media containing methane as the sole carbon source, show the most promise as being associated with a methanotrophic lifestyle.

Active methanotrophic bacteria have been identified using stable isotope probing in active, sulfidic cave systems (e.g., Movile Cave, Romania) (Hutchens *et al.*, 2004). In addition, several members of the genera isolated in this study have been detected in culture and molecular-based surveys of Carter Salt Peter Cave ferromanganous biofilms (Chapter Two). Therefore, it is possible that some or all of the isolates from the present study are associated with methane-based metabolisms. However, additional molecular-based work is needed to 1) provide definitive phylogenetic placement of these isolates by obtaining full-length SSU rRNA gene sequences (Amann *et al.*, 1995), 2) assess the potential for methanotrophic metabolisms by using molecular-based screening for the functional genes associated with methane-oxidation (Murrell *et al.*, 1998), and 3) identify active methanotrophic populations by the use of stable isotope probing (Morris *et al.*, 2002; McDonald *et al.*, 2005) or through RNA-based community analysis (Portillo *et al.*, 2008).

Organisms closely related (100% identical over a ca. 795 bp sequence alignment) to

methanogenic archaea (Savant *et al.*, 2002) have been identified via molecular-based methods in molecular-based surveys of Carter Salt Peter Cave ferromanganese deposits (Chapter Two). At this time, cultivation attempts in the present study towards the isolation of a cave methanogen appear to have been unsuccessful. However, in the months following the completion of the present study, methane measurements of the lab GC were found to be unreliable due to column leakage. Therefore, it would be worthwhile to replicate the cultivation techniques utilized in this study on new cave samples, as low amounts of methane production were detected in some cultures early on during the study period. Expanding the scientific community's knowledge of microbes involved in the cycling of methane will better elucidate the groups of organisms which are likely to be present in the current environmental conditions of Mars that are favorable for the microbial metabolic transformation of methane.

Mineralogy of biogenic Mn oxides

In the present study, TEM-EDS analysis confirmed the presence of Mn oxides associate with microbial cells in all actively-oxidizing cultures. Initial TEM-EDS analysis of the Mn Falls 11 *Pseudomonas* isolate produced some noteworthy findings, revealing the presence of intracellular phosphorous storage granules in *Pseudomonas* sp. Mn Falls 11. This finding is particularly intriguing given the oligotrophic nature of many cave systems (Northup and Lavoie, 2001; Barton *et al.*, 2004; Hunter *et al.*, 2004; Boston *et al.*, 2006), though the extent of surface impact within shallow cave systems such as Carter Salt Peter Cave is currently unknown.

Although the oxidation of Mn(II) produces over 30 known Mn(IV) minerals (Hill and Forti, 1997; Post, 1999), several of which are found in caves (White *et al.*, 2009; Onac and Forti, 2011), at this time biogenic Mn oxides appear limited to todorokite and birnessite (Tebo *et al.*, 2004; Tebo *et al.*, 2005; Toner *et al.*, 2005; Miyata *et al.*, 2006; Northup *et al.*, 2010; Rossi *et al.*, 2010; Learman *et al.*, 2011). It is still not fully understood how many of these microbes are oxidizing manganese (Tebo *et al.*, 1997), so electron microscopy is necessary to map where the Mn oxides are being formed in or on each cell. Due to the angstrom scale and disordered and delicate crystal structure of biogenic Mn(IV) oxides (Nealson *et*

al., 1988; Gradziński *et al.*, 1995; Tebo *et al.*, 1997; Nelson *et al.*, 1999b; Northup *et al.*, 2004; Frierdich *et al.*, 2011; Santelli *et al.*, 2011), it is not possible to use transmission electron microscopy or standard powder X-ray diffraction to successfully identify mineral phases (White *et al.*, 2009). Instead, less energy-intensive techniques such as Synchroton-based x-ray absorption fine-structure (XAFS) spectroscopy must be used. Limited information on Mn oxide crystal structure can be attained from single crystal micro-X-ray diffraction and Fourier Transform Infrared Spectroscopy (FT-IR) as well.

Future work will be conducted in conjunction with Dr. Sarah Carmichael and Dr. Jeffrey Post (Smithsonian Institute) using single crystal micro-XRD and FT-IR to definitively determine which Mn-minerals are produced by *Janthinobacterium* sp. A6 and *Flavobacterium* sp. E8, as these isolates are representative of genera that were recently described to have members capable of Mn-oxidation (Santelli *et al.*, 2010; Chapter Two). In addition, future work will include microtome thin sectioning of cultures for TEM analysis to better determine the locations in the cell where Mn oxidation is occurring, and will also include *in situ* culturing of these strains for scanning electron microscopy analysis to better visualize surface morphologies. Mineralogical studies of actively-oxidizing cave isolates will allow for the identification of *in vivo*, biogenically produced Mn minerals, thus increasing our understanding of which Mn minerals in the fossil record or in extraterrestrial samples may be attributed to biological activity (Boston *et al.*, 2001; Gorbushina *et al.*, 2002). If these minerals are identified in samples from Mars, it would provide further evidence for the potential existence of life on the red planet.

Experimental Procedures

Field description

Carter Salt Peter Cave (Carter County, Tennessee, Fig. 1), located in the Ordovician Knox Dolomite unit of the upper Tennessee River Basin, is a relatively shallow, epigenic cave system. The cave is particularly enriched in ferromanganese (mixed Fe and Mn) deposits, which are visually identified as black/chocolate

brown deposits that coat the surfaces of cave rocks, walls, and speleothems (e.g., flowstone, dripstone, soda straws, corrosion residue). Environmental conditions within the dark zone of the cave are typical of those found in other similar cave systems (Northup and Lavoie, 2001): ambient air temperature hovers around MAST (Mean Annual Surface Temperature) for the region (13°C) and humidity approaches 100%.

Sample collection

Samples were collected for cultivation-based experimentation from two Mn-oxidizing cave biofilms in Carter Salt Peter Cave (herein CSPC), Mn Falls and Mud Trap Falls (Fig. 1), in May 2011 and June 2011. Biofilms were screened *in situ* for the presence of Mn oxides using 0.04% Leucoberbelin Blue (LBB), a redox indicator that is oxidized by Mn(III) or Mn(IV) to produce a bright blue color (Krumbein and Altmann, 1973). Care was taken to sample at locations within the biofilm matrix that tested LBB-positive for Mn(II)-oxidation.

Cultivation of cave methanotrophs: Bacteria that utilize methane as a sole source of carbon and energy

Samples that were designated for the cultivation of cave methanotrophs were stored on ice, transported to the lab, and immediately inoculated on media by spreading 100 µL of a 1% v/v biofilm sample in 0.02M HEPES buffer (pH 7.2) on agar-solidified media. Four different media types targeting organisms involved in the catabolism of methane were developed and utilized in cultivation efforts. NMS (Nitrate Mineral Salts) (Chapter Two) and AMS (Ammonium Mineral Salts), designed by Dr. Trevor Craig, were designed to target cave methanotrophs and contained methane as the sole carbon source. AMS Media contained (in g L^{-1}) 0.7 K_2HPO_4, 0.54 KH_2PO_4. 1.0 $MgSO_4$•$7H_2O$, 0.2 $CaCl_2$•$2H_2O$, 0.004 $FeSO_4$•$7H_2O$, 0.5 NH_4Cl, 1 mL Trace Element Solution (containing in mg L^{-1} 100 $ZnSO_4$•$7H_2O$, 30 $MnCl_2$•$4H_2O$, 300 H_3BO_3, 200 $CoCl_2$•$6H_2O$, 250 $CuSO_4$•$5H_2O$, 20 $NiCl_2$•$6H_2O$, 60 6 Na_2Mo_4•$2H_2O$). pH of the media was adjusted to ca. 7.1-7.2 before autoclaving, and 15 g agar was added for plates. AMS media was supplemented post-autoclaving with sterile 0.02 M Hepes buffer pH 7.2, 5 µM ferrous ammonium citrate, 0.2% v/v vitamin

solution for J medium (Tebo *et al.*, 2007), and 100 µM $MnCl_2$. AMS and NMS agar plates were incubated at 10°C in the dark (to mimic cave conditions) in a sealed chamber with a 50:50 CH_4(g):air mix.

CM1 (Cave Methanotroph Media 1) and CM2 (Cave Methanotroph Media 2) , modified versions of Gerretsen Medium and Beijerinck Medium (Bromfield and Skerman, 1950) respectively, were also designed to target cave methanotrophs. However, CM1 and CM2 media contained both methane and citrate as carbon sources. CM1 media contained (in g L^{-1}) 20 calcium citrate tetrahydrate, 2 $(NH_4)_2SO_4$, 0.01 NH_4MgPO_4. CM2 media contained (in g L^{-1}) 20 calcium citrate tetrahydrate, 0.5 NH_4Cl, 0.5 K_2HPO_4. pH of each media was adjusted to ca. 7-7.2 before autoclaving, and 20 g agar was added for plates. CM1 and CM2 media were supplemented post-autoclaving with sterile 0.02M Hepes buffer pH 7.2, 100 µM $MnCl_2$, 4 µM $FeCl_2 \cdot 4H_2O$, and 0.04 µM $CuSO_4$ and incubated at 10°C in the dark (to mimic cave conditions) in a sealed chamber with a 50:50 CH_4(g):air mix.

Mn(II)-oxidation in isolates was assessed via LBB colorimetric determination as previously described. Individual colonies of interest were re-streaked for isolation a minimum of three times. Once a colony was isolated, a colony PCR reaction was initiated to screen the microorganism for phylogenetic placement (Table 1) using the universal bacterial primer 357F 5'CCTACGGGAGGCAGCAG (Chapter Two), which targets a region within the 16S rRNA gene sequence. Phylogenetic affiliation of isolates was determined by BLAST analysis (Johnson *et al.*, 2008).

Cultivation of cave methanogens: Anaerobic archaea that generate methane through chemolithoautotrophic processes

Samples that were designated for the cultivation of cave methanogens were inoculated *in situ* using anaerobic techniques. Briefly, a 5 mL syringe with a 16G, 11/2″ needle (BD Biosciences, Franklin Lakes, NJ) attached was thoroughly flushed five times with sterile N_2 (g). The syringe was then plunged deep into the base of the biofilm and material was drawn up into the syringe. Prepared media tubes containing either 10 mL Slurry or Tennessee Methanogen Media (TMM) were then inoculated *in situ* with approximately 1 mL of biofilm material. This procedure was repeated for samples obtained from each

biofilm. TMM media, a modified version of a basal medium targeting *Methanobrevibacter* spp. (Asakawa *et al.*, 1993), a known member of the CSPC ferromanganese deposit microbial consortia (Chapter Two), contained in g L^{-1} 0.75 KH_2PO_4, 0.75 K_2HPO_4, 1 NH_4Cl, 0.36 $MgCl_2$•$6H_2O$, 1 yeast extract, 9 mL trace metal solution (Morii *et al.*, 1983). pH of the media was adjusted to ca. 7-7.2, and the media was moved into an anaerobic chamber. Once in the chamber, 10 mL of media was aliquoted into each culture tube, and the tubes were sealed with stoppers and crimped. Tubes were then moved out of the chamber, flushed with 80/20% v/v N_2/CO_2 headspace, and autoclaved. Each individual tube was supplemented post-autoclaving with the following sterile, anaerobic additions to a final concentration of 0.5X Balch vitamins (Balch *et al.*, 1979), 0.03M $NaHCO_3$, 1 mM Na_2S, 1.5 mM L-cysteine hydrochloride, 0.0005% resazurin solution. Slurry media was prepared using the same methodology as described above and consisted of sterile, anaerobic water, with an 80/20% v/v N_2/CO_2 headspace. 0.0005% (final concentration) sterile, anaerobic resazurin solution was added to each individual tube post autoclaving.

Post inoculation, cultures were transported back to the lab and immediately checked to ensure the maintenance of anaerobic conditions by colorimetric screening via resazurin indicator. Any cultures with a pink tint, indicating aerobic conditions, were killed with 10% bleach and discarded. Cultures that maintained anaerobic conditions were flushed with an overpressure of 12 psi H_2. Cultures were incubated horizontally (to allow for maximum contact with the H_2/CO_2 headspace in the tubes) and at 10°C in the dark (to mimic cave conditions). Methane production by cultures was assessed every two weeks by gas chromatography, using a Shimadzu GC-2014 (Shimadzu Corporation, Kyoto, Japan) and external standards of 0.1%, 0.45%, 1%, 2%, 5%, and 10% CH_4.

Mineralogy of biogenic Mn oxides: TEM microscopy and elemental analysis

Five actively-oxidizing (as confirmed via LBB colorimetric screening) cultures isolated from a prior study (Chapter Two) were examined using a JEOL JEM-400 transmission electron microscope (TEM) equipped with an Oxford INCA energy dispersive X-ray detector (EDS) to confirm the presence of precipitated manganese associated with microbial cells (Fig. 2): *Janthinobacterium* sp. A6,

Flavobacterium sp. E8, *Leptothrix* sp. G6, *Arthrobacter* sp. L, and *Pseudomonas* sp. Mn Falls 11. Samples were prepared and imaged as previously described (Chapter Two).

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Figures and Tables

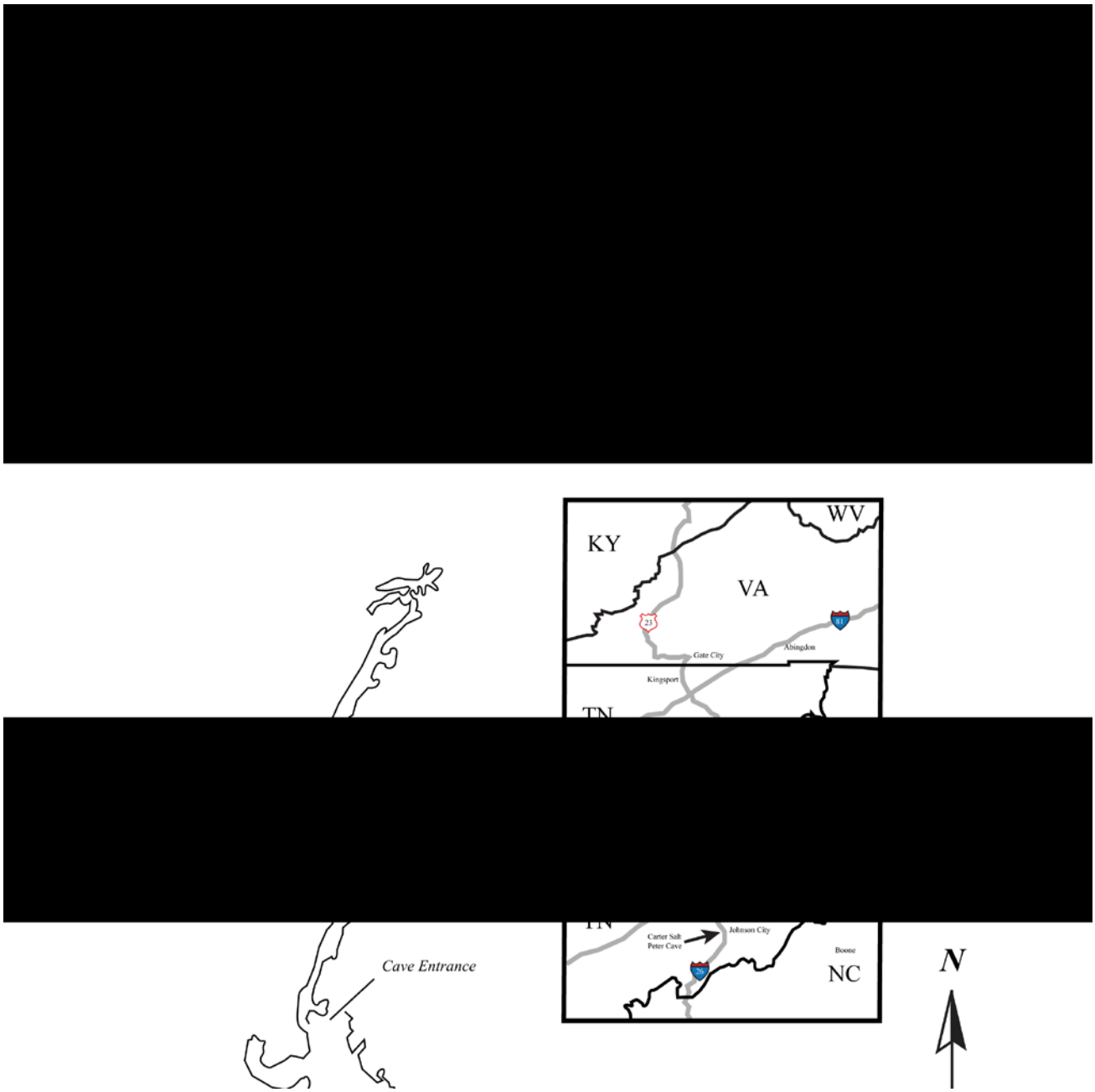

Table 1. Phylogenetic affiliation of cultivated isolates from Carter Salt Peter Cave, Carter County, TN.

Classification	Isolate[a]	Media	Closest Identified Relative[b]	% Sequence Identity[c]	Closest Cultivated Relative[d]	% Sequence Identity[c]
Actinobacteria	2S	CM2	*Arthrobacter humicola* strain JL9 (HQ857769)	100	-	-
CFB	AA1	AMS	*Flavobacterium* sp. PR4-11 (FJ889627)	99	-	-
	NN3	NMS	*Flavobacterium* sp. WB2.3-15 (AM934646)	99	-	-
γ-Proteobacteria	AA2	AMS	Soil Clone Bacterium MCF42 (HQ179087)	100	*Acinetobacter* sp. bk_13 (HQ538659)	100
	AA3	AMS	Soil Clone Bacterium MCF42 (HQ179087)	100	*Acinetobacter* sp. bk_13 (HQ538659)	100
	NT1	NMS	Soil Clone Bacterium DC38 (HQ178965)	99	*Acinetobacter* sp. HL-13 (JF693989)	99
	NT3	NMS	Soil Clone Bacterium MCF42 (HQ179087)	99	*Acinetobacter* sp. bk_13 (HQ538659)	99
	1N	CM1	*Pseudomonas* sp. Clone HP4F5 (JF968472)	98	*Pseudomonas* sp. JH-3-14 (JF820111)	98
	1S	CM1	*Pseudomonas* sp. CL1.82 (AM934700)	99	-	-
	1T	CM1	*Pseudomonas* sp. k2 (JF501095)	100	-	-
	2N	CM2	Rhizosphere Soil Clone 25 (HQ731027)	100	*Pseudomonas jessenii* strain SR3 (JF766371)	100

[a] All isolates tested LBB-negative for Mn(II)-oxidation.

[b] Data obtained via BLAST analysis (Johnson *et al.*, 2008). NCBI accession number in parentheses.

[c] Percent identity over a ca. 500 bp sequence alignment.

[d] If closest identified relative was an environmental clone.

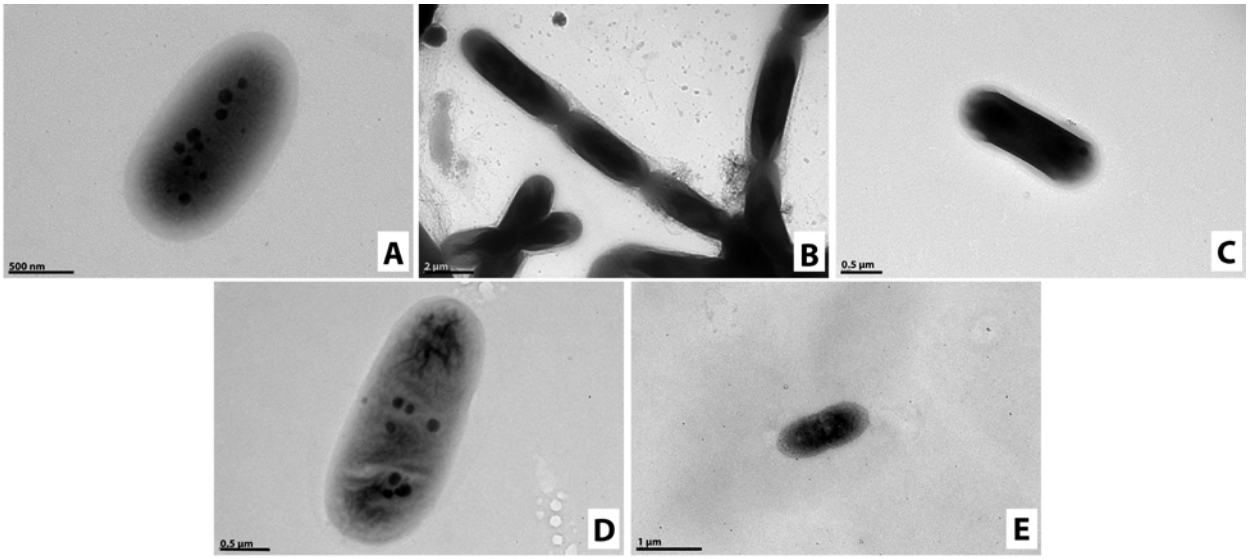

Fig. 2. TEM micrographs of biogenic Mn-oxides associated with bacterial cells: (A) *Pseudomonas* sp. Mn Falls 11, (B) Sheath-forming *Leptothrix* sp. G6, (C) *Arthrobacter* sp. L, (D) *Janthinobacterium* sp. A6, and (E) *Flavobacterium* sp. E8. Scale bars shown in each panel. All cultures were LBB-positive, indicating the presence of biomineralized Mn(IV)-oxides. EDS analysis (data not shown) confirmed the presence of Mn deposits associated with microbial cells

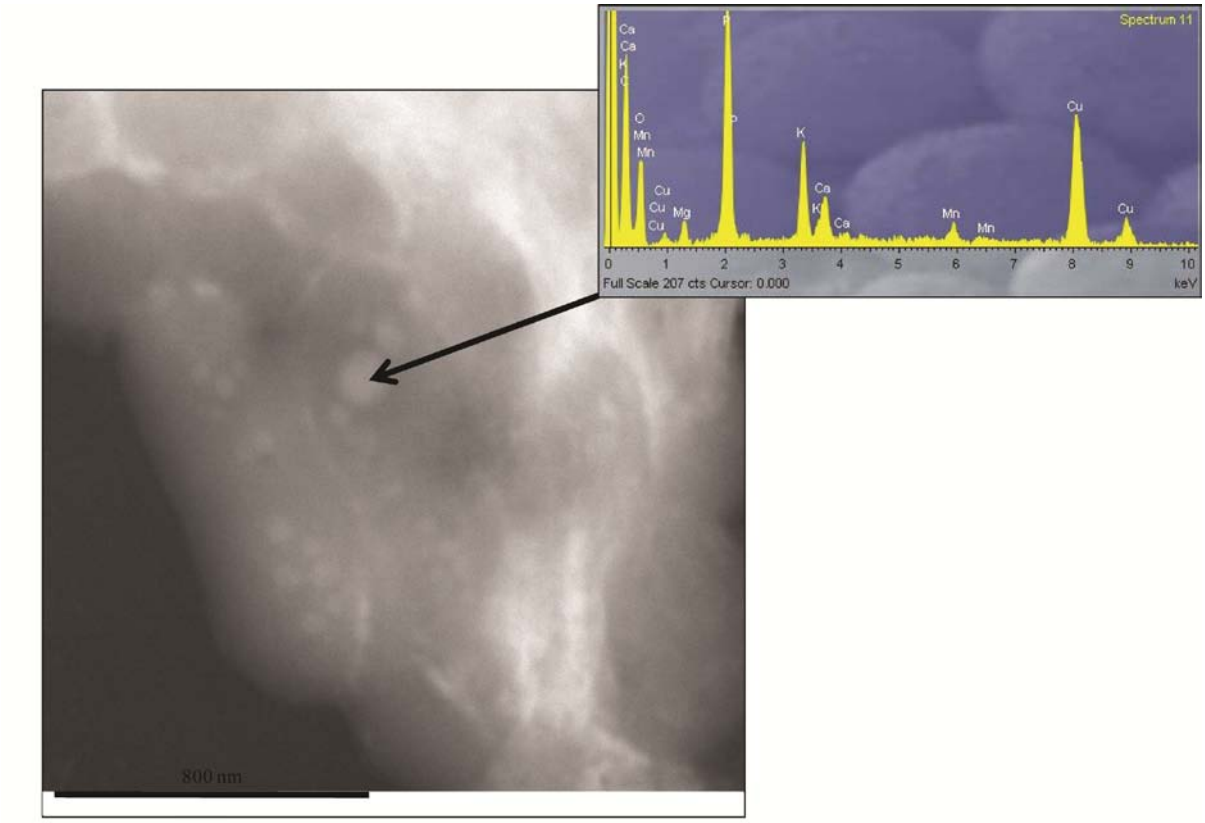

Fig. 3. TEM micrograph of LBB-positive *Pseudomonas* sp. Mn Falls 11 reveals the presence of intracellular phosphorous storage granules. Scale bar represents 800 nm. Image Credit: S.K. Carmichael.

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