Carbon Quantity And Quality Drives Variation In Cave Microbial Communities And Regulates Mn(II) Oxidation

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Abstract

Cave ecosystems are carbon limited and thus are particularly susceptible to anthropogenic pollution. Yet, how carbon quality and quantity that can modulate the pathways and amount of Mn cycling in caves remains largely unknown. To explore Mn cycling, baseline bacterial, archaeal, and fungal com-munities associated with Mn(III/IV) oxide deposits were assessed in both relatively 'pristine' and anthropogenically impacted caves in the Appalachian Mountains (USA). Cave sites were then amended with various carbon sources that are commonly associated with anthropogenic input to determine whether they stimulate biotic Mn(II) oxidation in situ. Results revealed patterns between sites that had long-term exogenous carbon loading compared to sites that were relatively 'pristine,' particularly among Bacteria and Archaea. Carbon treatments that stimulated Mn(II) oxidation at several sites resulted in significant changes to the microbial communities, indicating that anthropogenic input can not only enhance biotic Mn(II) oxidation, but also shape community structure and diversity. Additional carbon sources amended with copper were incubated at various cave sites to test the role that Cu(II) plays in in situ biotic Mn(II) oxidation. Media supplemented with 100 lM Cu(II) inhibited bacterial Mn(II) oxidation but stimulated fungal Mn(II) oxidation, implicating fungal use of multicopper oxidase (MCO) enzymes but bacterial use of superoxide to oxidize Mn(II). In sites with low C:N ratios, the activity of the Mn(II) oxidizing enzyme manganese peroxidase (MnP) appears to be limited (particularly by MnP-utilizing Basidiomycetes and/or Zygomycetes).

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Abbreviations

LBB	Leucoberbelin blue
MnP	Manganese peroxidase
MCO	Multicopper oxidase
NMDS	Non-metric multidimensional scaling
PCA	Principal component analysis
SOM	Soil organic matter
TC	Total carbon
TN	Total nitrogen
TOC	Total organic carbon

Introduction

Manganese oxides, hydroxides, and oxyhydroxides (herein referred to as Mn(III/IV) oxides) are found in a variety of environments and can be either biological or abiotic in origin. The relatively low/neutral pH of most surface environments does not favor abiotic Mn(III/IV) oxidation (Luther 2010); therefore microbial Mn(III/IV) oxide production is typically dominant in many natural systems. Bacterial Mn(II) oxidation occurs through several mechanisms: via the production of multicopper oxidase enzymes (MCOs) (Dick et al. 2009), siderophores (Duckworth et al. 2009), or superoxide-mediated reactions (Learman et al. 2011). Fungi can likewise oxidize Mn(II) by producing MCO-like enzymes (Hofer and Schlosser 1999) or superoxide–mediated mechanisms (Hansel et al. 2012), as well as secreting manganese peroxidase (MnP) (Kuan et al. 1993) or versatile peroxidase (Ruiz-Duenas et al. 2007). Copper may be used as a method of inferring which mechanism of Mn(II) oxidation is dominant since bacterial Mn(II) oxidation via MCO enzymes is enhanced with the addition of 2–180 μM Cu(II) (Brouwers et al. 1999; El Gheriany et al. 2011; Larsen et al. 1999), but is inhibited with 100 μM Cu(II) when bacterial or fungal superoxide–mediated mechanisms are utilized (Andeer et al. 2015; Hansel et al. 2012; Learman et al. 2011). Care must be taken with result interpretation, however, since microbial communities in situ are complex. Further, in vitro certain microbes can oxidize Mn(II) through multiple enzymatic pathways, which are differentially stimulated under specific external conditions such as high/low pH or solid versus liquid growth media (Bohu et al. 2015; Geszvain et al. 2013). Once formed, biogenic Mn(III/IV) oxides can sequester heavy metals from the environment (Yin et al. 2011), degrade recalcitrant carbon sources into more labile carbon sources (Sunda and Kieber 1994), and can increase the pathogenicity of fungi (Thompson et al. 2006). Despite having identified various microbial pathways that lead to the oxidation of Mn(II), little is known about the environmental parameters that may be affecting biotic Mn(II) oxidation in situ.

Based on our current knowledge, biotic Mn(II) oxidation is not energy yielding. Therefore, the microbes responsible for Mn(II) oxidation need to acquire energy from either chemolithoautotrophic or heterotrophic processes. Several studies have demonstrated that in situ Mn(II) oxidation can be enhanced/stimulated with the addition of exogenous carbon. For example, Carmichael et al. (2013b) found that sewage infiltration into a cave in the southern Appalachian Mountains stimulated bacterial Mn(II) oxidation, but the inferred mechanisms of oxidation were inconsistent between isolates: *Leptothrix* sp. G6 most likely used multicopper oxidase and *Janthinobacter* sp. A6 utilized an extracellular mechanism such as superoxide and/or haem peroxidase (Carmichael et al. 2013a). In addition to Bacteria, Mn(II)-oxidizing fungi were cultured from other waste associated with anthropogenic activity that can act as a nutrient source including electrical tape, batteries, socks, and fireworks (Carmichael et al. 2013b). More recent research by Carmichael et al. (2015) demonstrated that fungal Mn(II) oxidation in a cave can be directly stimulated in situ with exogenous carbon input. Carbon that enters cave systems can either be from local environmental sources such as dissolved organic matter or particulate organic matter, or from human-derived waste materials, such as lint from clothes, skin cells, hair follicles, crumbs from food, and increased CO_2 levels via human respiration that may be subsequently incorporated via autotrophy (Carmichael et al. 2015; Ikner et al. 2007; Simon and Buikema Jr. 1997).

To date, few studies have specifically addressed the bacterial and fungal ecology associated with Mn(III/IV) oxide deposits in cave systems (see review by Carmichael and Bräuer 2015), and each of these studies has been relatively limited in scope. To address many of the unknowns, we designed a study to assess how both abiotic and biotic factors may stimulate or enhance

bacterial and fungal Mn(II) oxidation in situ. These factors included: (a) the effects that humans have on bacterial and fungal communities, (b) the effect that host-rock/sediment geochemistry has on bacterial and fungal communities and Mn(II) oxidation, (c) whether biotic Mn(II) oxidation can be stimulated or inhibited with elements such as Cu, Sr, and N, (d) whether high quality carbon (easily degradable and more energy-yielding carbon) may stimulate different microbial groups than low quality carbon sources (low energy-yielding and/or more recalcitrant), and (e) whether enzymatic Mn(II) oxidation pathways can be inferred from field observations and analyses.

Methods

Study caves and sites

Three caves were chosen for this study: Carter Saltpeter Cave (CSPC) in eastern Tennessee, and Daniel Boone Caverns (DBC) and Rye Cove-Cox Ridge (RCCR) in southwestern Virginia (Fig. 1). There is a documented history of anthropogenic impact throughout CSPC that includes trash, debris, and sewage (Carmichael et al. 2013b). Additionally, CSPC is hydrologically connected to sinkholes and subsurface flow systems. For these reasons, we classified CSPC as an anthropogenically impacted cave. In contrast, DBC and RCCR were classified as near 'pristine' caves, as they receive very few visitors due to accessibility restrictions and are also isolated from subsurface flow (Carmichael et al. 2015). These caves are all hosted within the Ordovician upper Knox Dolomite, and experience similar weather patterns and precipitation. Between five and six sites were chosen within each cave for carbon treatment experiments (Fig. 1). Since the goal was to stimulate/enhance oxidation, sites that were chosen for carbon treatments did not test strongly positive for Mn(II) oxidation via leucoberbelin blue (LBB) tests. Sample sites will be herein referred to first by the cave in which the sample was taken and then by the site name; for example, site MNF (Mn Falls) in CSPC will be referred to as CSPC-MNF.

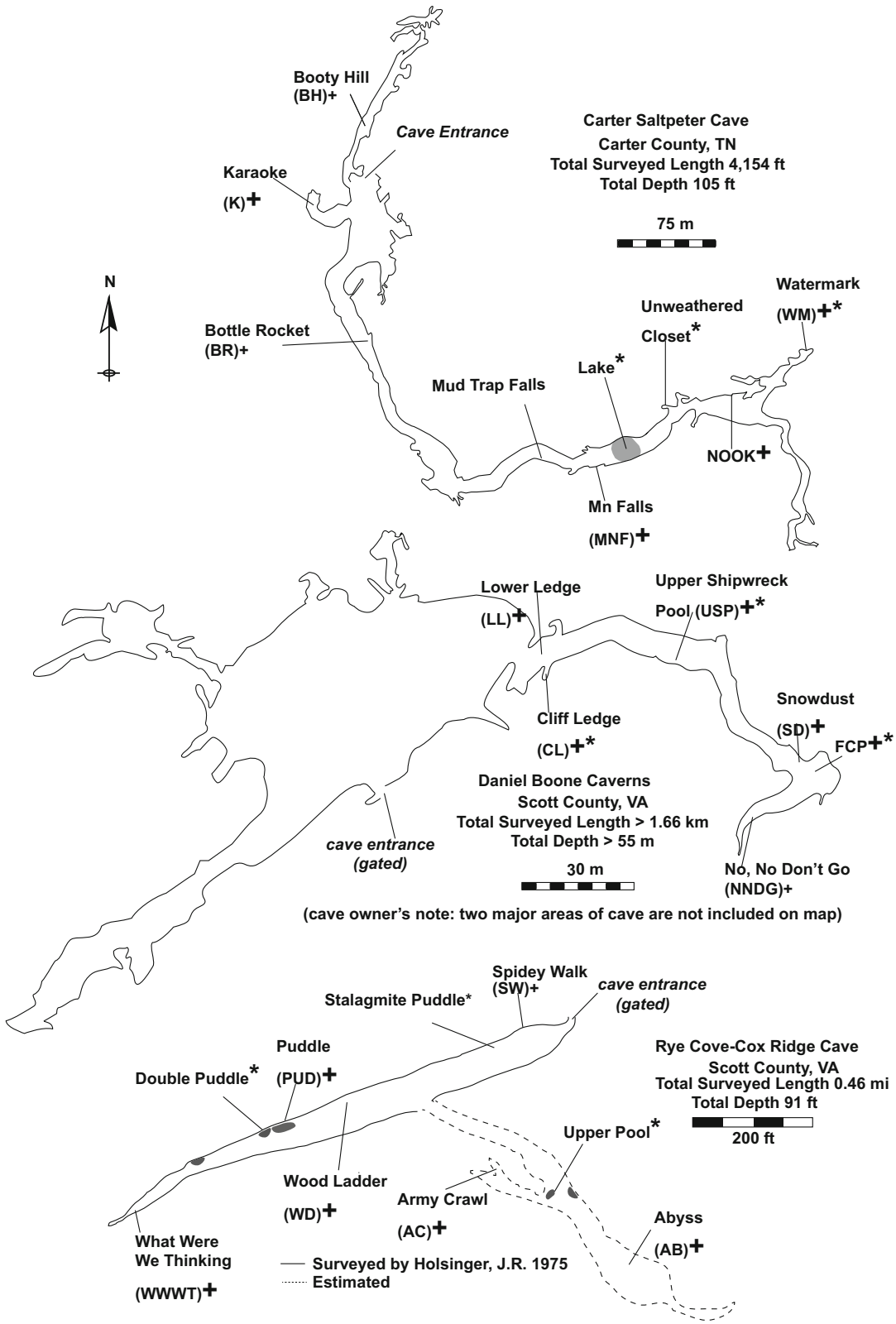
Baseline community sampling

Sediment samples were taken in triplicate from each site before incubations to provide baseline values, transferred on ice and stored at −80°C. DNA was extracted from the sediment samples using the MP Biomedicals Fast DNA™ Spin Kit for Soil, and concentrations were measured using a Fisher Scientific NanoDrop 1000 spectrophotometer. Fungal DNA samples were amplified using the protocol outlined by Zorn (2014) and Bacteria, plus a small subset of Archaea (Raymann et al. 2017), were amplified following the protocol used by Caporaso et al. (2012). Fungal primers ITS1F and ITS2R, along with bacterial primers 515F and 806R were modified with Illumina adapters, a unique Golay barcode, a reverse primer pad and a linker. Following PCR amplification, samples were standardized, pooled, and gel purified. Purified samples were shipped to the West Virginia University's Genomic Core Facility (Morgantown, WV, USA). Paired-end sequencing (2 × 300 bp) was performed on the samples using a MiSeq sequencer (Illumina, Inc., San Diego, CA, USA).

Microbial community analysis

PANDAseq commands (Masella et al. 2012) in QIIME (Caporaso et al. 2010) were used to build contigs from forward and reverse sequence reads and to discard sequences shorter than 245 bp or longer than 335 bp. Sequences were dereplicated with USEARCH (Edgar 2010), while UPARSE was used in conjunction with USEARCH to cluster operational taxonomic units (OTUs) at a 97% identity (Edgar 2010, 2013). For bacterial and archaeal communities, chimeras were filtered via the RDP Gold database (Wang et al. 2007). Taxonomic classifications were performed on the clustered OTUs with the Greengenes database (DeSantis et al. 2006). Singletons were then discarded using a QIIME supported command (Caporaso et al. 2010). PyNAST was used to align sequences and a maximum likelihood phylogenetic tree was built with FastTree (Price et al. 2010). For fungal communities, UCHIME was used in conjunction with the UNITE reference database to discard chimeric sequences (Edgar et al. 2011; Kõljalg et al. 2013). Taxonomy was assigned to OTU clusters using the BLAST method and UNITE 7 database (Altschul et al. 1990; Kõljalg et al. 2013).

Alpha-diversity metrics, Shannon, Simpson, and observed OTUs were calculated in QIIME for fungal and bacterial and archaeal communities (Caporaso et al. 2010). OTU tables were modified to BIOM


Booty Hill
(BH)+
Cave Entrance
Karaoke
(K)+
Carter Saltpeter Cave
Carter County, TN
Total Surveyed Length 4,154 ft
Total Depth 105 ft
75 m
N
Watermark
(WM)+*
Unweathered
Closet*
Bottle Rocket
(BR)+
Lake*
Mud Trap Falls
NOOK+
Mn Falls
(MNF)+
Lower Ledge
(LL)+
Upper Shipwreck
Pool (USP)+*
Snowdust
(SD)+
Cliff Ledge
(CL)+*
FCP+*
Daniel Boone Caverns
Scott County, VA
Total Surveyed Length > 1.66 km
Total Depth > 55 m
cave entrance
(gated)
30 m
No, No Don't Go
(NNDG)+
(cave owner's note: two major areas of cave are not included on map)
Spidey Walk
(SW)+
cave entrance
(gated)
Stalagmite Puddle*
Puddle
(PUD)+
Double Puddle*
Rye Cove-Cox Ridge Cave
Scott County, VA
Total Surveyed Length 0.46 mi
Total Depth 91 ft
200 ft
Upper Pool*
Wood Ladder
(WD)+
Army Crawl
(AC)+
Abyss
(AB)+
What Were
We Thinking
(WWWT)+
Surveyed by Holsinger, J.R. 1975
Estimated

◀**Fig. 1** Maps of Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC), and Rye Cove-Cox Ridge (RCCR). Maps modified with *arrows* to indicate sampling locations, plus signs (+) to indicate carbon incubations sites and *asterisks* (*) to denote sites for water sampling. Regional map inset shows the relative location of the three caves within the upper Tennessee River Basin. Cave survey of CSPC performed on 02/08/81 by L. Adams, R. Knight, R. Page, and T. Wilson. Initial cave survey of DBC conducted in 1969 by M. Starnes, B. Lucas, D. Breeding, C. Stowers, and B. Balfour, and an additional survey was conducted from July-November 1996. Two subsequent passages in DBC have not been surveyed. Cave survey of RCCR performed by Holsinger (1975). Passages in RCCR not previously surveyed but used for this study are represented with dotted lines

formatted tables (McDonald et al. 2012) and were imported into R using the phyloseq package (McMurdie and Holmes 2013). Vegan was used to calculate Bray-Curtis matrices in R (Oksanen et al. 2016; R Core Team 2015). RColorBrewer (Neuwirth 2014), and ggplot2 (Wickham 2009) were used to plot distance matrices in non-metric multidimensional (NMDS) format. A permutational multivariate analysis of variance (PERMANOVA) was completed using the Adonis function within the Vegan package in R (Oksanen et al. 2016). Sequences were deposited into the National Center for Biotechnology Information under Project Identification Number PRJNA325803 with Accession Number SRP079012.

Carbon stimulation

Nutrient agar casts were prepared as agar media in petri dishes that were later removed from the petri dish and left in the field. These nutrient agar casts were designed to either: (1) stimulate bacterial Mn(II) oxidation, (2) stimulate fungal Mn(II) oxidation, (3) test the effect of Cu(II) on bacterial and fungal Mn(II) oxidation, or (4) to test whether Mn(II) oxidation was limited by carbon or Mn(II) (Online Resource 1). To stimulate bacterial Mn(II) oxidation, FMO2 nutrient agar casts were prepared as outlined by Carmichael et al. (2013a) and supplemented with organic acids: arabinose (.A), casamino acids (.C), or succinic acids (.S). To stimulate fungal Mn(II) oxidation, agar casts were supplemented with malt extract (.M), glucose (.G), and yeast extract (.AY) (Santelli et al. 2010). Additional sets of the nutrient media described above were amended with 100 μM $CuCl_2$ to test whether biotic Mn(II) oxidation in situ would be inhibited or stimulated with Cu(II). To test whether Mn(II) oxidation at the chosen incubation sites was limited by Mn(II) or carbon availability, agar casts were not augmented with additional carbon sources (Fig. 1). All nutrient agar casts were augmented with 100 μM $MnCl_2$. Agar casts aimed at stimulating bacterial Mn(II) oxidation were incubated in situ for 10 weeks and fungal carbon sources were incubated for 16 weeks. Complex carbohydrates including sawdust (.SAW), cotton (.COT), and cellulose were also deployed to stimulate fungal oxidation, but Mn was not added to these complex carbon sources. LBB tests were then performed and triplicate DNA samples were taken from each nutrient agar or complex carbohydrate source that tested positive for Mn(II) oxidation.

Culturing

In addition to in situ experiments, samples were collected from each site before carbon incubations for culturing experiments. To enrich for Bacteria and fungi, samples were streaked aseptically on nutrient agar including AY and citrate agar, both supplemented with 100 μM $MnCl_2$. Cultures were continually re-plated using either the stab and swipe transfer method or the streak for isolation method, until they were axenic. Cultures were kept in the dark at 28 °C and were continually monitored for Mn(II) oxidation using LBB as a chemical indicator. To test whether or not Sr or N inhibited Mn(II) oxidation, Mn(II)-oxidizing isolates were cultured on nutrient media that contained 50 or 100 μM $SrCl_2$ or NH_4^+ and were tested for Mn(II) oxidation via LBB. Isolated cultures that tested positive for oxidation were colony PCR amplified using 12.5 μL New England BioLabs One Taq Hot Start 2× Master Mix with Standard Buffer, 0.5 μL 10 mM forward primer (either ITS1F or Edel-Hermann nu-SSU-08 for fungal cultures or 27F for bacterial cultures), 0.5 μL 10 mM reverse primer (either ITS4 or Edel-Hermann nu-SSU-153 for fungal cultures or 1492R for bacterial cultures), and 11.5 μL PCR water. Thermocycler parameters were set to 94°C for 10 min., 30 cycles of 94 °C for 30 s, 54 °C for 45 s, 68 °C for 60 s, followed by 68 °C for 5 min., and 4 °C hold. PCR products were purified, prepped for quick lane sequencing, and sent to Beckman Coulter Genomics (Danvers, MA, USA). Sequence assembly was performed with CodonCode sequence aligner software (Version 6.02, CodonCode Corporation).

Assembled sequences were identified using a BLAST search (Altschul et al. 1990). Isolated sequences were deposited in GenBank under the Accession Numbers KY670787-KY670797.

Electron microscopy

Scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM–EDS) was performed on several Mn(II)-oxidizing isolates. Cultures were preserved with glutaraldehyde and dehydrated with ethanol. Samples were then critically point dried with liquid CO_2 in a Polaron critical point dryer and gold coated with a Denton Vacuum Evaporator and Desk III Sputter Coater. Samples were imaged and analyzed with the FEI Quanta 200 Environmental SEM with an EDAX Genesis XM energy dispersive X-ray spectrometer in the William and Ruth Dewel Microscopy Facility at Appalachian State University.

Geochemical and mineralogical analysis

Whole rock geochemical analyses for major, minor, trace, and rare earth elements was performed on selected sediment samples to determine which, if any, elements may be linked with Mn(II) oxidation. Sediment samples were analyzed at ActLabs, Inc. (Ancaster, Ontario) using the 4E-Research Total Ident package (see Online Resource 2 for data). Sediment samples were collected in sterile vials from sites where agar casts supplemented with Cu(II) were positive for Mn(II) oxidation, sites where carbon sources that were not supplemented with Mn(II) eventually tested positive for biotic Mn(II) oxidation after stimulation, and sites where fungal growth was observed but did not exhibit oxidation (Table 1). Control samples were also collected from calcite flowstones and from Mn(III/IV) oxide deposits that were not manipulated. Principal component analysis (PCA) was performed in R (R Core Team 2015) to determine relationships between sediment geochemistry and caves.

Additional sediment samples were taken for total carbon (TC) and total nitrogen (TN) from several sites chosen for carbon incubations in all three caves. Samples were dried, homogenized, weighed, and analyzed using a FlashEA 1112 Elemental Analyzer (Thermo Fisher Scientific, USA). TC was converted to soil organic matter (SOM) using the conversion TC %*1.724 = SOM proposed by van Bemmelen (1891).

Water analysis

Three sites in each cave were chosen for water collection (Fig. 1). Water samples were collected approximately every six weeks for one year in sterile amber glass bottles, and acidified to a pH < 2. Total organic carbon (Krumholz et al.) measurements were performed by Arizona Laboratory for Emerging Contaminants at the University of Arizona (Tuscon, AZ, USA). TOC analyses were compared to Quality Controlled Local Climatological Data taken by NOAA (http://www.ncdc.noaa.gov/qclcd) and to discharge data collected by USGS of the north fork of the Holston River near Gate City, VA (http://help.waterdata.usgs.gov). Water was unable to be collected from DBC-CL and DBC-FCP from June-November, 2015, as the sites were dry.

Results/discussion

Baseline community analysis of bacteria and archaea

After removing singletons, 20,063 OTUs were recovered through Illumina sequencing, most of which were unidentified at the species level. CSPC and DBC had the highest number of bacterial phyla, with 55 and 49 phyla retrieved, respectively. Approximately 36 bacterial phyla were described at RCCR, which is similar to the number obtained via next-generation sequencing of other caves (Mandal et al. 2015a, b; Ortiz et al. 2012). Proteobacteria had the highest relative read abundance in the baseline communities across all sites (Fig. 2), in contrast to previous studies where Actinobacteria dominated cave samples (Mandal et al. 2015a, b; Ortiz et al. 2012). Differences observed in this study compared to previous studies may be due to the sampling location within caves, since we only sampled Mn(III/IV) oxide deposits, or sites with aberrant geochemical composition relative to other sites. The phylum-level distribution is consistent with previous clone libraries of ferromanganese deposits in CSPC where Bacteroidetes, Betaproteobacteria, Gammaproteobacteria, and Acidobacteria groups dominated while Actinobacteria were not represented (Carmichael et al. 2013a), indicating some degree of temporal stability in the phylum-level diversity of microbial assemblages associated with Mn(III/IV)

Table 1 Sites where carbon sources were incubated

Carbon sources commonly associated with sewage used to stimulate bacterial Mn oxidation							Carbon sources commonly associated with waste from human activity used to stimulate fungal Mn oxidation								
	A	C	S	A + Cu	C + Cu	S + Cu	Cotton	Sawdust	Filter Paper	G + Cu	M + Cu	G	M	AY	Fungal growth observed
CSPC-BH	+									+		+	+		Y
CSPC-BR															Y
CSPC-K															N
CSPC-MNF	+		+					+		+		+	+		Y
CSPC-WM	+		+				+				+	+	+		Y
CSPC-NOOK	+		+												N
DBC-FCP															N
DBC-LL	+	+	+				+	+		+		+	+		Y
DBC-CL		+	+					+	+	+		+	+		Y
BC-NNDG															N
DBC-UPS															N
DBC-SD								+		+		+	+		N
RCCR-AC										+		+	+		Y
RCCR-PUD							+	+		+		+	+		Y
RCCR-SW															N
RCCR-AB	+	+	+									+	+		Y
RCCR-WL															N
RCCR-WWWT										+		+	+		Y

Plus (+) denote that biotic Mn(II) oxidation was stimulated with that carbon source

Organic acid carbon sources included succinic acid (S), casamino acids (C), and arabinose (A)

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

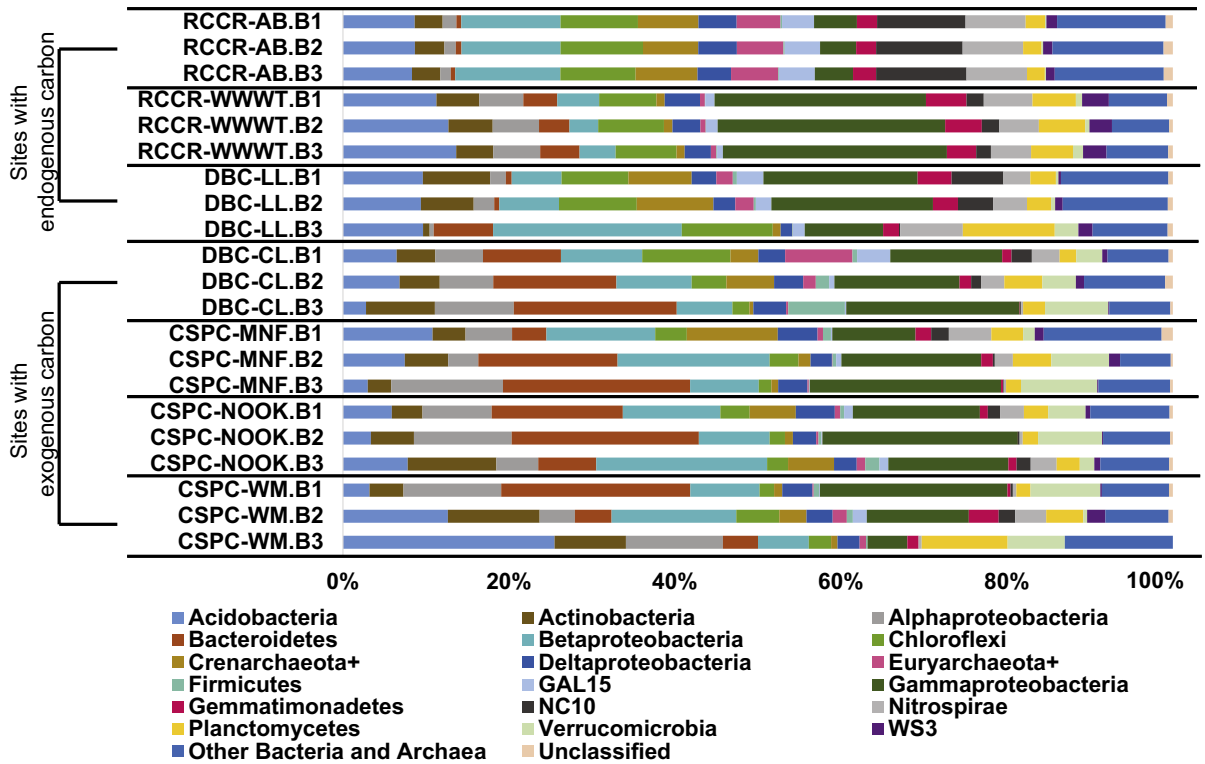


Fig. 2 Basleine phylum-level distribution of Bacteria and Archaea with a 1% abundance cut-off. *Plus signs* (+) denote an archaeal phylum. B indicates baseline values and 1, 2, or 3 indicate the replicate sample number. (Color figure online)

oxide deposits in these caves. Previously, Carmichael et al. (2013a) cultured *Leptothrix* from a 10^{-8} serial dilution and *Flavobacterium* from a 10^{-10} serial dilution from samples taken from CSPC and DBC, indicating that these two genera were relatively abundant. Despite the in-depth sequencing approach used for this study, *Leptothrix* was not detected in these caves at the time of sampling; although *Flavobacterium* did account for up to 3.2% of the total bacterial and archaeal sequence reads (Carmichael et al. 2013a; Online Resource 2). Due to the apparent absence of *Leptothrix* from all of the cave sites in this study and the observation of *Leptothrix* spp. in sewage samples (Mulder 1989; Mulder and Van Veen 1963; Yu and Zhang 2012), it was hypothesized that *Leptothrix* spp. may not be endogenous bacteria within these caves, but may have been introduced into CSPC with sewage contaminated water before the 2009 sampling (Carmichael et al. 2013a). The failure to detect *Leptothrix* at CSPC sites during the 2014 sampling compared to the 2009 sampling demonstrates the temporal variability at the species level that can occur in response to environmental impact or seasonal changes (Lauber et al. 2013).

Analyses of alpha-diversity measurements calculated with Shannon and Simpson indices suggest that all sites had diverse bacterial and archaeal baseline communities with values ranging from 7.43 to 8.31 and 0.97–0.99, respectively (Online Resource 4). There was no observable pattern between caves among alpha-diversity measurements (including observed OTUs, Shannon diversity, and Simpson diversity) across the baseline community samples (Online Resource 4). CSPC, a cave which is anthropogenically impacted, was expected to have decreased diversity compared to sites within DBC or RCCR, which are more 'pristine,' but the diversity values in this study surprisingly show that this was not the case. Our results are inconsistent with studies of alpha-diversity in other cave systems where alpha-diversity decreased as human traffic increased, most likely in response to skin cell shedding, lint, food crumbs, and other inputs from human visitations (Carmichael et al.

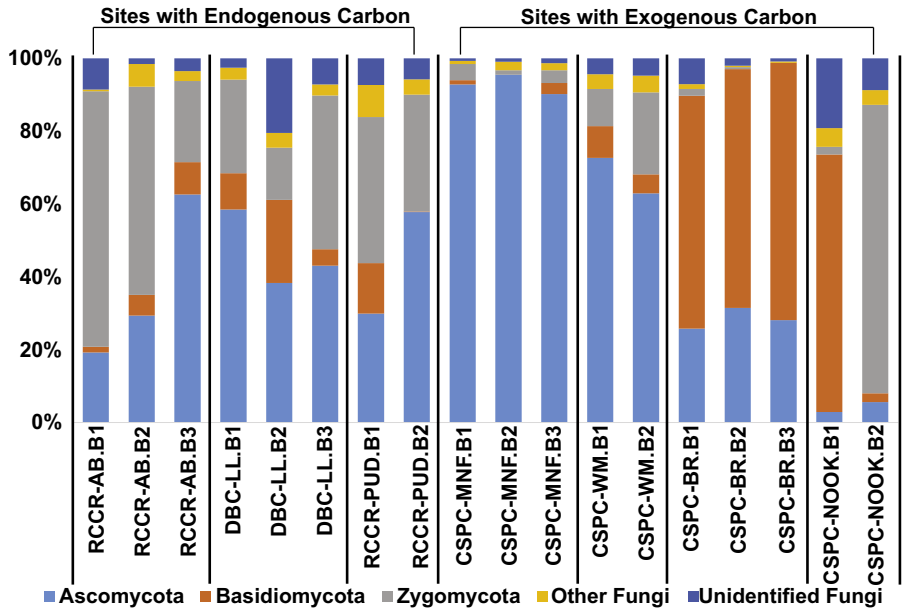


Fig. 3 Baseline fungal phylum-level distribution at sites. Sample names that include either B1, B2, or B3 indicate that the replicate samples reflect baseline values

2015; Ikner et al. 2007). This discrepancy may be explained by the methods used to quantify bacterial communities and diversity; culture based techniques were exclusively applied in other studies, whereas high-throughput sequencing was used in this study.

Baseline fungal community analysis

At the species level, 934 fungal OTUs were assigned, and similar to bacterial and archaeal taxa, most OTUs could not be identified at the species level. Phylum-level abundance of the fungal communities was site dependent and was dominated by Ascomycota (4.3–92.8%), Basidiomycota (1.4–66.7%), and/or Zygomycota (3.1–49.8%; Fig. 3). Similar to our present findings, past research has also demonstrated that Zygomycetes were in relatively low abundance at both CSPC and DBC and represented no more than 10% of the communities (Carmichael et al. 2015). Despite the similar results observed for Zygomycota relative abundances, Carmichael et al. (2015) observed several patterns between fungal dominance that were not identified in this study; Ascomycota dominated CSPC sites and Basidiomycota were relatively more abundant at DBC. Variation in fungal phylum-level abundance was observed between the replicate samples taken from every site, which is also in agreement with the study by (Carmichael et al. 2015). The within site variation of fungal communities observed indicates that there is high spatial variability in fungal communities in these caves.

Alpha-diversity of baseline fungal communities did not vary by cave in which the sample was taken (Online Resource 4). This is, again, in contrast to previous results by Carmichael et al. (2015), where they observed significantly higher alpha-diversity at sites in the impacted cave, CSPC, compared to the 'pristine' cave, DBC. Nonetheless, alpha-diversity metrics measured in this study are within the range of alpha-diversity measurements from our previous study (Carmichael et al. 2015). The disparity between fungal community structure and alpha-diversity patterns described in this study compared to Carmichael et al. (2015) may be due to temporal changes in fungal communities; as most bacterial communities remained relatively constant between studies. In contrast, this discrepancy may be due to the fact that different sampling sites were selected in this study (spatial variation), or may be due to seasonal variation.

Table 2 PERMANOVA pairwise comparisons of baseline microbial communities

	Pairwise comparisons		Bray-Curtis	
			R^2	Pr(>F)
Bacterial and archaeal communities	**RCCR-AB.B**	**DBC-CL.B**	**0.364**	**0.003***
	RCCR-AB.B	DBC-LL.B	0.226	0.097
	RCCR-AB.B	**CSPC-MNF.B**	**0.142**	**0.005***
	RCCR-AB.B	**CSPC-NOOK.B**	**0.146**	**0.006***
	RCCR-AB.B	**CSPC-WM.B**	**0.126**	**0.01***
	RCCR-AB.B	**RCCR-WWWT.B**	**0.149**	**0.004***
	DBC-CL.B	CSPC-MNF.B	0.026	0.894
	DBC-CL.B	CSPC-NOOK.B	0.030	0.826
	DBC-CL.B	CSPC-WM.B	0.041	0.615
	DBC-LL.B	CSPC-MNF.B	0.101	0.055
	DBC-LL.B	**CSPC-NOOK.B**	**0.116**	**0.022***
	DBC-LL.B	CSPC-WM.B	0.092	0.068
	DBC-LL.B	RCCR-WWWT.B	0.087	0.072
	CSPC-MNF.B	CSPC-NOOK.B	0.022	0.932
	CSPC-MNF.B	CSPC-WM.B	0.033	0.773
	CSPC-NOOK.B	CSPC-WM.B	0.038	0.645
	RCCR-WWWT.B	**DBC-CL.B**	**0.137**	**0.006***
	RCCR-WWWT.B	**CSPC-MNF.B**	**0.139**	**0.006***
	RCCR-WWWT.B	**CSPC-NOOK.B**	**0.165**	**0.002***
	RCCR-WWWT.B	**CSPC-WM.B**	**0.133**	**0.008***
Fungal communities	RCCR-AB.B	DBC-LL.B	0.065	0.428
	RCCR-AB.B	CSPC-NOOK.B	0.080	0.166
	RCCR-AB.B	RCCR-PUD.B	0.060	0.54
	RCCR-AB.B	CSPC-WM.B	0.050	0.692
	CSPC-BR.B	**RCCR-AB.B**	**0.184**	**0.002***
	CSPC-BR.B	**DBC-LL.B**	**0.189**	**0.001***
	CSPC-BR.B	**CSPC-MNF.B**	**0.223**	**0.001***
	CSPC-BR.B	**CSPC-NOOK.B**	**0.162**	**0.002***
	CSPC-BR.B	**RCCR-PUD.B**	**0.164**	**0.002***
	CSPC-BR.B	**CSPC-WM.B**	**0.185**	**0.001***
	DBC-LL.B	**CSPC-MNF.B**	**0.170**	**0.003***
	DBC-LL.B	CSPC-NOOK.B	0.095	0.104
	DBC-LL.B	RCCR-PUD.B	0.099	0.073
	DBC-LL.B	CSPC-WM.B	0.060	0.492
	CSPC-MNF.B	**RCCR-AB.B**	**0.111**	**0.024***
	CSPC-MNF.B	**CSPC-BR.B**	**0.223**	**0.001***
	CSPC-MNF.B	**CSPC-NOOK.B**	**0.159**	**0.001***
	CSPC-MNF.B	**RCCR-PUD.B**	**0.162**	**0.006***
	CSPC-MNF.B	**CSPC-WM.B**	**0.147**	**0.006***
	CSPC-NOOK.B	RCCR-PUD.B	0.066	0.416
	CSPC-NOOK.B	CSPC-WM.B	0.101	0.079
	RCCR-PUD.B	CSPC-WM.B	0.058	0.559

Asterisks (*) and bolding denote a significant shift in beta-diversity between two sites/treatments

Sample names shown here represent the cave in which they were sampled, Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC), or Rye Cove-Cox Ridge (RCCR), followed by the site name, and .B represents baseline communities

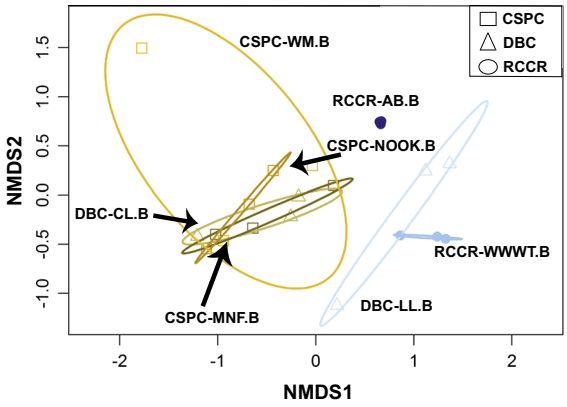


Fig. 4 Beta-diversity analysis using non-metric multidimensional scaling (NMDS) and the Bray-Curtis dissimilarity index of baseline bacterial and archaeal communities (.B1–.B3 samples). *Ellipses* denote 95% confidence intervals. *Blue-shaded colored elipses* and *shapes* represents endogenous carbon sites and *gold-shaded colored elipses* and *shapes* represent exogenous carbon sites. (Color figure online)

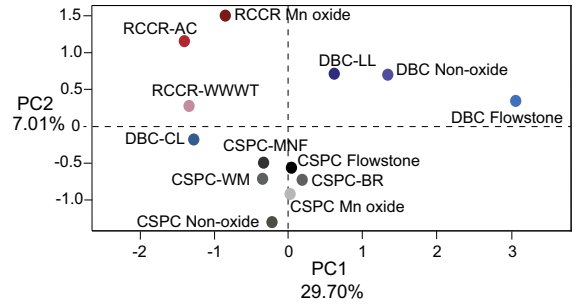


Fig. 5 PCA of whole rock geochemical analyses performed on sediments from sites within Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC), and Rye Cove-Cox Ridge (RCCR)

Beta diversity: geochemical effects on microbial community structure

Pairwise comparisons from the Bray-Curtis dissimilarity matrix demonstrated differences between bacterial and archaeal communities in all sites within the impacted cave, CSPC, compared to the two 'pristine' sites within RCCR, AB and WWWT (PERMANOVA, $P < 0.05$; Table 2; Fig. 4). However, results also demonstrated that the beta-diversity of bacterial and archaeal communities at the anthropogenically impacted cave sites (CSPC) were not different than the two sites within the 'pristine' cave, DBC-CL and DBC-LL (PERMANOVA, $P > 0.05$). An exception to this is the carbon limited site CSPC-NOOK, which was significantly different from site DBC-LL (Table 2) and will be discussed further below (see *Environmental Parameters Affecting Biotic Mn(II) Oxidation* in situ). Principal component analysis of cave host-rock/sediment geochemistry (Fig. 5) primarily shows similarities among cave sites in a single cave (with the exception of DBC-CL). While host-rock/sediment geochemistry can tentatively explain the observed similarities in microbial diversity in CSPC and DBC-CL (which still show very high scatter), it cannot explain the observed similarities between DBC-LL and RCCR-WWWT. These latter sites have similar microbial assemblages (Fig. 4) but very different host-rock/sediment geochemistry (Fig. 5). If host-rock/sediment geochemistry plays any role at all in community structure, it is likely only a minor component for the caves in this study.

A more plausible explanation for the similar bacterial and archaeal communities found at sites within DBC and CSPC sites is reflected in the carbon analyses from water samples (Fig. 6a). Although relatively 'pristine' in regards to anthropogenic impact, site DBC-CL had higher TOC infiltration through drip water sources than other sites during two sampling events ($P < 0.05$) and site DBC-LL had a higher concentration of SOM compared to every other site tested ($P < 0.05$; Fig. 6b). Thus, TOC results from DBC-CL and SOM results from DBC-LL reveal similarities to sites in anthropogenically impacted CSPC, which has experienced pulses of exogenous carbon and/or other nutrients from various sources, including sewage infiltration, bottle rockets, cigarettes, tape, and others (Carmichael et al. 2013b). In addition, sites at CSPC and DBC had noticeably more variation in the phylum-level abundance of bacterial and archaeal communities across the triplicate samples analyzed compared to the sites in RCCR, which may be attributable to increased carbon loading at CSPC and DBC sites providing increased microniches (Fig. 2). These results (specifically for DBC-CL and DBC-LL) suggest that localized carbon and/or nutrient loading can have significant impacts on microbial community composition and on culturable microbes from caves, consistent with previous studies (Carmichael et al. 2013b; Simon and Buikema Jr 1997).

Beta-diversity of baseline fungal communities exhibited some differences according to PERMANOVAs calculated with the Bray-Curtis index ($P < 0.05$;

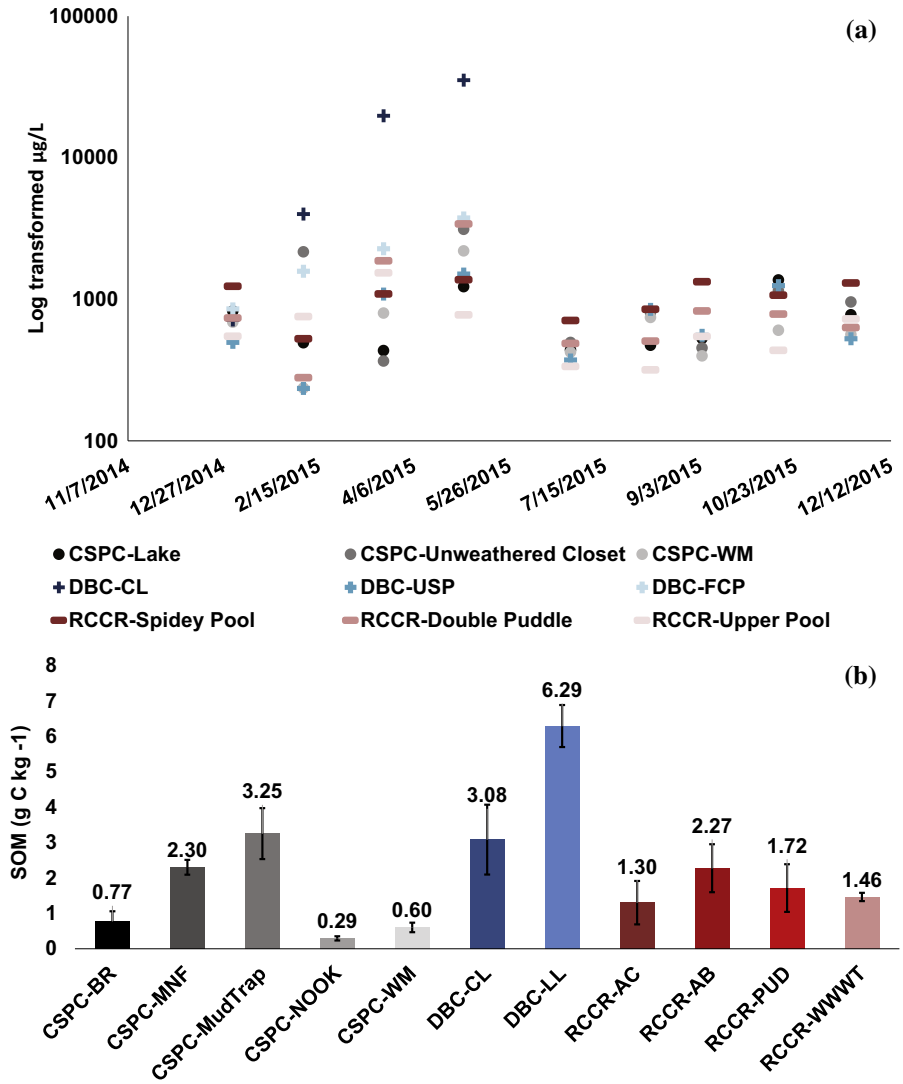


Fig. 6 **a** Log transformed total organic carbon (Krumholz et al.) from water samples taken within Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC), and Rye Cove-Cox Ridge (RCCR). **b** Soil organic matter (SOM) concentrations measured from sediment samples taken within CSPC, DBC, and RCCR (*error bars* represent standard error)

Table 2). Generally, fungal community structure did not correlate with either host-rock geochemistry or carbon analyses (TOC or SOM). Instead, CSPC showed heterogeneity in fungal communities within different sites of the cave, and in particular, fungal communities within CSPC-BR and CSPC-MNF were distinct from those within every other site regardless of cave (Table 2 and Online Resource 5). This result is likely due to the long-term exogenous carbon input documented at CSPC-MNF (Carmichael et al. 2013a, b, 2015), and due to the noticeable impact on the geochemical signature within the sediment due to firework debris at CSPC-BR (see *Environmental Parameters Affecting Biotic Mn(II) Oxidation* in situ). Overall, results suggest that only major or significant disturbance events (such as fireworks and sewage input) significantly impact fungal community assemblages, and contrasts results from bacterial analyses suggesting that bacterial and archaeal communities are more sensitive to carbon input.

Bacterial and archaeal community responses to the stimulation of Mn(II) oxidation

To complement laboratory cultures, bacterial Mn(II) oxidation was stimulated in situ with the addition of organic acids at multiple sites across all three caves, with the exception of organic acids that were supplemented with Cu(II) (Table 1). Generally, phylum-level changes were universal across treatments (i.e.

Gammaproteobacteria increased across all treatments and Actinobacteria decreased with most treatments; Online Resource 6). Treatments at sites that are not receiving exogenous carbon input, or rather are recycling endogenous carbon (RCCR-AB, RCCR-WWWT, and DBC-LL), experienced the most significant increases/decreases in phylum abundances compared to sites with either constant or intermittent exogenous carbon input (Online Resource 6). Increased responses of the bacterial and archaeal communities at sites not receiving exogenous carbon demonstrates that those sites are more sensitive to the direct addition of carbon compared to sites experiencing exogenous carbon input (DBC-CL and CSPC sites). These results corroborate the hypothesis that significant changes in bacterial and archaeal communities may be reflective of impacts such as long-term exogenous carbon input.

Alpha-diversity measurements indicated that the bacterial and archaeal assemblages at treated sites decreased at some sites but the effect was not universal (Online Resource 4). The results herein are in partial agreement with other studies that have found a decreased microbial diversity in response to exogenous carbon input (Carmichael et al. 2015; Ikner et al. 2007; Shapiro and Pringle 2010). Beta-diversity measurements with pairwise comparisons of baseline communities and communities following treatments with either organic acids or simple sugars resulted in significant shifts for ~62% of the treatments (18 out of 29; -PERMANOVA, $P < 0.05$; Table 3 and Online Resource 7). These results are consistent with results presented by Eilers et al. (2010) and (Goldfarb et al. 2011), where exogenous carbon input significantly shifted the beta-diversity of bacterial communities.

Fungal community responses to the stimulation of Mn(II) oxidation

Alpha-diversity for baseline fungal communities was lower than that observed for bacterial communities, which is analogous to fungal and bacterial diversity found in other caves (see review by Vanderwolf et al. 2013; Online Resource 3). Alpha-diversity of fungal communities tended to decrease with the addition of carbon at most sites. The decreased alpha-diversity measurements with carbon additions observed with this study are in contrast to results from a recent study that used beef and yeast extract to stimulate fungal growth (Marques et al. 2016; Online Resource 3) but supports results from other studies (Carmichael et al. 2015; Vanderwolf et al. 2013). However, the decrease in alpha-diversity was statistically significant only for cotton and sawdust additions ($P < 0.05$; Online Resource 4), indicating that alpha-diversity of Mn(II)-oxidizing fungal communities was sensitive to and/or responded to poorer carbon sources and was immutable with higher quality carbon additions.

Carbon incubations resulted in significant shifts to Mn(II)-oxidizing fungal communities in only ~14% of the incubations (2 out of 14). Incubations with sawdust, a poor carbon source, was the only carbon treatment to alter fungal beta-diversity (PERMANOVA, $P < 0.05$, Table 3), while fungal communities were immutable in response to simple sugars, a higher quality carbon source. In contrast, Mn(II)-oxidizing bacterial communities had significant shifts in response to organic acids and simple sugars (Table 3). Consequently, we hypothesize that fungal communities may be sensitive to only poor quality carbon sources (i.e. complex carbon sources like cellulose and/or wood), whereas bacterial and archaeal communities may be more sensitive to higher quality carbon sources (i.e. simple sugars and organic acids). Thus, exogenous carbon quality may differentially affect Mn(II)-oxidizing microbial communities.

Fungal community structure seemed to be strongly associated with the ability to oxidize Mn(II) during carbon incubations. Within sites where Mn(II) oxidation was stimulated via carbon incubations, Ascomycota was the dominant phylum in comparison with sites where Mn(II) oxidation was not stimulated, which were dominated by Basidiomycota and/or Zygomycota (Fig. 3). Results are consistent with the work of Carmichael et al. (2015), who found that Mn(II) oxidation was not stimulated at sites that had lower abundances of Ascomycota during a ten week incubation period. In addition, Ascomycota increased in abundance in response to carbon incubations at sites in DBC and RCCR, nearing baseline values at CSPC sites (Online Resource 8). This positive association between Ascomycota abundance and Mn(II)-oxidizing activity of fungal communities indicates that an increased relative abundance of Ascomycota may lead to a more readily stimulated fungal Mn(II)-oxidizing community.

Table 3 PERMANOVA pairwise comparisons of baseline microbial communities versus after communities after stimulation

	Pairwise comparisons		Bray-Curtis	
			R^2	Pr(>F)
Bacterial and archaeal communities	**RCCR-AB.B**	**RCCR-AB.A**	**0.383**	**0.001***
	RCCR-AB.B	RCCR-AB.C	0.152	0.126
	RCCR-AB.B	**RCCR-AB.S**	**0.247**	**0.013***
	DBC-CL.B	**DBC-CL.C**	**0.434**	**0.007***
	DBC-CL.B	**DBC-CL.S**	**0.379**	**0.022***
	DBC-LL.B	**DBC-LL.A**	**0.164**	**0.005***
	DBC-LL.B	**DBC-LL.C**	**0.191**	**0.001***
	DBC-LL.B	**DBC-LL.S**	**0.187**	**0.003***
	DBC-LL.B	**DBC-LL.GCU**	**0.232**	**0.001***
	DBC-LL.B	**DBC-LL.M**	**0.226**	**0.001***
	CSPC-MNF.B	**CSPC-MNF.A**	**0.124**	**0.011***
	CSPC-MNF.B	CSPC-MNF.S	0.094	0.092
	CSPC-MNF.B	**CSPC-MNF.G**	**0.106**	**0.047***
	CSPC-MNF.B	CSPC-MNF.GCU	0.101	0.053
	CSPC-MNF.B	**CSPC-MNF.M**	**0.108**	**0.044***
	CSPC-MNF.B	**CSPC-MNF.A**	**0.124**	**0.011***
	CSPC-MNF.B	CSPC-MNF.S	0.094	0.092
	CSPC-MNF.B	**CSPC-MNF.G**	**0.106**	**0.047***
	CSPC-MNF.B	CSPC-MNF.GCU	0.101	0.053
	CSPC-MNF.B	**CSPC-MNF.M**	**0.108**	**0.044***
	CSPC-NOOK.B	CSPC-NOOK.A	0.098	0.353
	CSPC-NOOK.B	CSPC-NOOK.C	0.087	0.47
	CSPC-NOOK.B	CSPC-NOOK.S	0.119	0.189
	CSPC-WM.B	CSPC-WM.A	0.116	0.213
	CSPC-WM.B	**CSPC-WM.C**	**0.175**	**0.04***
	CSPC-WM.B	**CSPC-WM.S**	**0.193**	**0.026***
	CSPC-WM.B	**CSPC-WM.G**	**0.195**	**0.003***
	CSPC-WM.B	CSPC-WM.M	0.108	0.251
	CSPC-WM.B	CSPC-WM.MCU	0.140	0.08
Fungal communities	DBC-LL.B	DBC-LL.COT	0.153	0.09
	DBC-LL.B	DBC-LL.S	0.082	0.617
	DBC-LL.B	DBC-LL.GCU	0.110	0.318
	DBC-LL.B	DBC-LL.SAW	0.148	0.11
	CSPC-MNF.B	CSPC-MNF.A	0.008	0.994
	CSPC-MNF.B	CSPC-MNF.C	0.040	0.644
	CSPC-MNF.B	CSPC-MNF.G	0.016	0.968
	CSPC-MNF.B	CSPC-MNF.M	0.106	0.104
	CSPC-MNF.B	**CSPC-MNF.SAW**	**0.255**	**0.002***
	RCCR-PUD.B	RCCR-PUD.GCU	0.062	0.986
	RCCR-PUD.B	RCCR-PUD.M	0.114	0.615
	RCCR-PUD.B	**RCCR-PUD.SAW**	**0.202**	**0.02***
	CSPC-WM.B	CSPC-WM.COT	0.220	0.400
	CSPC-WM.B	CSPC-WM.M	0.374	0.444

Baseline communities (.B) compared to other baseline sites or after stimulation with arabinose (.A), casaminio acids (.C), succinic acid (.S), glucose (.G), glucose + Cu (.GCU), malt extract (.M), malt extract + Cu (.MCU), cotton (.COT), or sawdust (.SAW)

Asterisks (*) and bolding denote a significant shift in beta-diversity between two sites/treatments

Mn(II)-oxidizing isolates cultured from cave samples

Most Mn(II)-oxidizing microbes isolated from all three caves were assigned to genera previously obtained as isolates from either CSPC or DBC including the fungal genera *Acremonium*, *Plectosphaerella*, *Pleosporales*, or *Alternaria,* and bacterial genera *Pseudomonas* and *Flavobacterium* (Online Resource 8; Carmichael et al. 2013a, b, 2015). In addition, one new Mn(II)-oxidizing fungus represents the first Mn(II)-oxidizing Basidiomycete isolated from subterranean habitats (see review by Carmichael and Bräuer 2015), and was isolated on citrate agar from site CSPC-MNF. This new cave isolate shares 99% identity across 524 bp of the ITS region and 99% identity across 747 bp of the SSU rRNA gene of *Trichosporon spp.* within the Tremellales order of the Basidiomycota phylum (Online Resource 9). SEM images of the ascomycotal fungi were taken from Mn(II)-oxidizing isolates and EDS analyses confirmed the presence of Mn(III/IV) oxides associated with the cell wall of the fungal hyphae (Online Resource 10 and 11). Cell wall associated Mn(II) oxidation is proposed to be exclusively performed by MCO enzymes (Santelli et al. 2011; Tang et al. 2013), therefore, it is speculated that the fungal isolates obtained from these caves are also using MCO enzymes.

Environmental parameters affecting biotic Mn(II) oxidation in situ

Carbon was demonstrated to be a limiting nutrient for microbial Mn(II) oxidation, as agar casts containing Mn(II) and no carbon sources did not stimulate Mn(II) oxidation at any site used for incubations. Carbon sources designed to stimulate bacterial Mn oxidation that were supplemented with 100 μM Cu(II) inhibited bacterial Mn(II) oxidation in situ, whereas the same carbon sources without Cu(II) promoted oxidation (Table 1). Previous research indicates that Cu(II) concentrations ≥100 μM inhibit superoxide-mediated Mn(II) oxidation but can stimulate MCO-mediated Mn(II) oxidation (Brouwers et al. 2000; El Gheriany et al. 2011; Hansel et al. 2012; Learman et al. 2013). Results from this study therefore suggest that bacterial Mn(II) oxidation in these caves is occurring via superoxide production and not MCO enzymes. In contrast, fungal Mn(II) oxidation was stimulated with carbon sources supplemented with 100 μM Cu(II), which corroborates microscopy data (Online Resource 10 and 11) indicating that species of Ascomycota isolated from these caves are most likely using MCOs and not superoxide to oxidize Mn(II), corroborating microscopy data above.

Although most sites stimulated with carbon showed Mn oxidation consistent with the enzymatic pathways described above, there were two sites where fungal Mn(II) oxidation was expected to be stimulated, yet LBB tests remained negative: CSPC-NOOK and CSPC- BR. At NOOK, simple sugar agar casts were incubated for 19 months and fungal Mn(II) oxidation was not stimulated, yet when organic acids were incubated at NOOK, bacterial Mn(II) oxidation was stimulated within 10 weeks of incubation. CSPC-BR ("bottle rocket") was originally chosen as a study site due to Mn(II) oxidation on a spent firework. Simple sugar treatments at BR lead to the visible growth of fungal hyphae that did not show Mn(II) oxidation, in contrast to the nine other sites in this study that exhibited Mn(II)-oxidizing fungal growth with the same treatments. Geochemical analyses performed on the underlying bedrock at BR showed increased concentrations of sulfur, strontium, and nitrogen (Online Resource 12), which are common components of bottle rockets. Although these elements may possibly inhibit biotic Mn(II) oxidation in situ, culturing experiments showed that Mn(II)-oxidizing fungal isolates did not lose their ability to oxidize Mn(II) in the presence of Sr or N in vitro.

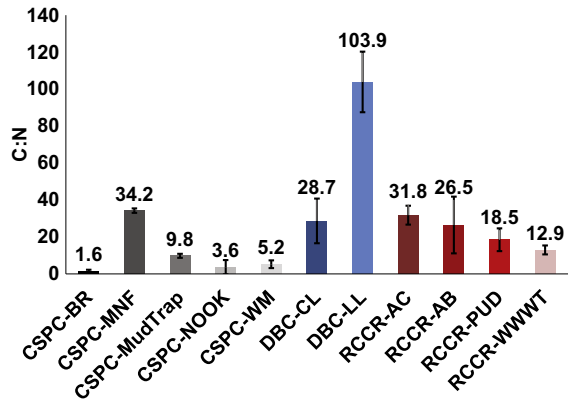


Fig. 7 Carbon to nitrogen ratios measured from sediment samples taken at sites within Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC), and Rye Cove-Cox Ridge (RCCR) (*error bars* represent standard error)

As host-rock/sediment geochemistry could not explain the lack of Mn(II) oxidation at these two sites, a more plausible explanation was revealed through carbon and nitrogen analyses of CSPC-NOOK and CSPC-BR. Fungal communities at CSPC-NOOK and CSPC-BR were dominated by either Basidiomycetes or Zygomycetes, which oxidize Mn(II) through the production of MnP (Bonugli-Santos et al. 2009; Hofrichter 2002). These two sites had sediment with uniquely low C:N ratios compared to other sites examined (Fig. 7), and also had SOM concentrations (CSPC-BR = 0.77 g C kg^{-1} and CSPC-NOOK = 0.29 g C kg^{-1}; Fig. 6b) below those commonly found in soils (1.2–70.5 g C kg^{-1}) (Batjes 1996; Bowles et al. 2014; Chen et al. 2009; Matsuura et al. 2012). Additionally, the C:N ratios found at CSPC-BR and CSPC-NOOK (<3.7:1; Fig. 7) are lower or equal to the C:N ratio requirements predicted for enzymes, ~3.5:1 (Allison 2005). Microbes need to consume products that have a C:N >3.5:1 owing to the relatively low carbon use efficiency (0.3–0.6) suggested for soil microbes (Allison 2014; Moorhead et al. 2013; Sinsabaugh et al. 2013). Thus, the low SOM concentrations and low C:N ratios at those sites may prohibit MnP production and/or activity.

Conclusion

Previous studies have identified changes to microbial community diversity in response to anthropogenic impact in cave systems. Here, we focused on how exogenous carbon inputs can *specifically* alter biogeochemical cycling in cave systems, either directly or indirectly. This study demonstrated that in situ stimulation of biotic Mn(II) oxidation via exogenous carbon sources (organic acids, simple sugars, and complex carbohydrates) can affect the diversity of microbial communities. By testing for Mn(II) oxidation in the presence of Cu(II), the results from this research suggest that bacteria and fungi are likely using different enzymatic pathways to oxidize Mn(II) in situ. Further analysis of SOM and microbial community structure indicated that for some enzymatic reactions (such as MnP), Mn(II) oxidation may be limited by the amount of organic carbon (or ratio of C:N) present at sites. Analysis of our research findings has lead us to propose that Mn(II) oxidation may be used as an indicator of contamination in cave and karst systems, and may be used to trace changes in groundwater quality as well as track ecosystem stresses in show caves and sites open to recreational caving.

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