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Sustained Anthropogenic Impact in Carter Saltpeter Cave, Carter County, Tennessee and the Potential Effects On Manganese Cycling

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ABSTRACT

Anthropogenic impact is a pervasive problem in heavily trafficked cave systems and fecal contamination is equally problematic in many cave and karst waters worldwide. Carter Saltpeter Cave in Carter County, Tennessee exhibits Mn(III/IV) oxide coatings associated with groundwater seeps, as well as manganese oxide growth on litter. Culturing results revealed that Mn(III/IV) oxide production on litter was associated with Mn(II)-oxidizing fungi. Immediately prior to this study, a massive Mn(II)-oxidizing biofilm bloomed at a cave seep. During the course of this study from 2009–2011, the seep exhibited a dramatic visual reduction in Mn(III/IV) oxide production, which was hypothesized to correlate with a decrease in fecal nutrient input. Molecular methods (16S rRNA gene sequencing) confirmed the presence of Bacteroides-Prevotella human fecal indicators in this seep, and most probable number assays and ion chromatography of the associated seep water confirmed nutrient loading at the site. Further, phylogenetic analysis from clone sequences suggested a strong initial human-specific fecal signature (50% of the sequences clustering with human feces sequences) in July 2009, and a weaker human signature (20% clustering) by June 2011. Most Probable Number (MPN) analyses of heterotrophic bacteria at this site suggested that Mn(II) oxidation was correlated with heterotrophic activity, due to point source exogenous nutrient loading.

INTRODUCTION

Karst systems are vital sources of drinking water and support some of the most fragile and diverse ecosystems on Earth (van Beynen and Townsend, 2005). However, a combination of bedrock porosity and high hydrologic conductivity allow contaminants to penetrate limestone bedrock and move through karst conduit systems quickly, especially during periods of high velocity flow (Vesper et al., 2001). For this reason, karst hydrologic systems are often associated with channeled non-point source groundwater pollution (Green et al., 2006; Worthington, 2011).

It is not always possible to visually assess biological contamination in a karst or cave system. As a result, indirect methods of water monitoring for microbial contaminants are frequently employed by researchers: culture-based studies of water to detect coliform bacteria (Mikell et al., 1996; Rusterholtz and Mallory, 1994), molecular techniques to identify the presence/absence of fecal indicators and endemic species in karst systems (Ahmed et al., 2008; Johnson et al., 2011; Johnston et al., 2012; Porter, 2007; Roslev and Bukh, 2011), and the use of fluorescent dye- tracers and microspheres to model water flow and pathogen dispersal in conduit systems (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. Thus, 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 (Gillieson, 2011). Human- induced alterations in the cave environment have been shown to destroy microhabitats (Northup, 2011), alter cave biogeochemical cycles, and impact sensitive cave fauna such as bats (Blehert et al., 2011). Any type of impact within caves is primarily manifested at the lower trophic levels, particularly in cave microbial communities that have the potential to exert powerful bottom-up controls on ecosystem health and stability (Horner-Devine et al., 2003). Due to the constancy of the cave environment, impact is quickly detrimental, and hard to reverse.

In 2009, a study was initiated to characterize the geomicrobiology of ferromanganese deposits in Carter Saltpeter Cave, Carter County, Tennessee (Carmichael et al., 2013). Human impact within the cave system was evident throughout the 2009–2011 study period and researchers often 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 present in some portions of the cave, some of which have active water flow. One of the water sources for the cave 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 (Johnson, 2002). At one of these sites, a thick, dark black, microbial biofilm was present in the water seeping from the cave wall and

flowing onto the cave floor (Carmichael et al., 2013). The dark color of the seep was due to microcrystalline Mn(III/IV) oxide minerals, as detected with a Leucoberbelin Blue field test developed by Krumbein and Altmann (1973). Members of the Mountain Empire Grotto in Johnson City, Tennessee, suggested that the appearance of the biofilm coincided with a time in which a local septic tank company had been seen dumping raw sewage into a sinkhole that is hydrologically linked to the cave (John Matthews, personal communication). Additionally, early molecular microbiological analyses documented the presence of several dominant Operational Taxonomic Units (OTUs) in clone libraries constructed from biofilm material that were closely related ($97% identical) to environmental clones isolated from fecal contaminated water and/or activated sludge (Carmichael et al., 2013), providing further circumstantial evidence of nutrient loading/sewage contamination at this site.

From 2009–2011, the appearance of the biofilm changed drastically, losing its dark black color, and exhibiting a dramatic visual reduction in Mn(III/IV) oxide production, though estimated cells/g wet weight biofilm material remained relatively constant (Carmichael et al., 2013) and field tests continued to demonstrate the presence of microcrystalline Mn oxides, as detected using Leucoberbelin Blue (Krumbein and Altmann, 1973). Given the dramatic change in the appearance of the biofilm over the duration of the study and the molecular evidence of potential contamination, we hypothesized that the appearance and bloom of the biofilm was linked to an acute, point source nutrient loading event in an area hydrologically connected to this shallow cave system. Thus, we initiated this study to document the extent of human impact within the cave system, and to either validate or alleviate concern over potential fecal contamination at the site.

METHODS

FIELD SITES AND SAMPLE COLLECTION

Carter Saltpeter Cave (Fig.1) is located in the Ordovician Knox Dolomite in Carter County, Tennessee. At a depth of approximately 30 m, Carter Saltpeter Cave (CSPC) represents a relatively shallow epigenic cave system typical of those found within the southern Appalachian region. Environmental conditions within the dark zone of the cave are consistent with those found in other cave systems (Northup and Lavoie, 2001). A variety of carbonate speleothem formations occur throughout the cave system (e.g. flowstone, dripstone, soda straws, corrosion residue), and the cave is particularly enriched in ferromanganese deposits. The cave is located in close proximity to both agricultural land and residential areas, and during the time of this study the cave entrance was neither gated nor protected from human traffic. Daniel Boone Caverns (Fig. 1), located 53 km north of CSPC near Ft. Blackmore, Virginia, is also located in the Knox Dolomite, but is on an isolated and forested ridge and

the entrance is gated and locked. While there are also ferromanganese deposits in Daniel Boone Caverns, there is no agricultural runoff infiltrating into the cave, nor is there evidence of significant human impact. For the purposes of this study, Daniel Boone Caverns is considered a geologic analogue of CSPC, as it shares the same host rock and has a similar climate and rainfall patterns; and therefore, is used as an intact control system to compare with the heavily impacted CSPC.

From 2009–2012, deposits from seeps and litter scattered throughout CSPC 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 from seeps were collected aseptically by scraping the deposit coating down to the solid rock base using a sterile 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 DNA extraction, electron microscopy, and/or culturing (Table 1).

DNA samples in this study were obtained from July 2009 to June 2011 in roughly three-six month intervals from two sites containing Mn(III/IV) oxides in CSPC (Fig. 1). The first site, Mn Falls, experienced a dramatic change in appearance over the course of one year (Fig. 2a and 2b). The water coming from the Mn Falls seep is hypothesized to be hydrologically connected to a sinkhole where there was an alleged sewage release from a septic tanker truck in 2008 (John Matthews, personal communication). Mud Trap Falls (Fig. 2c) is a second Mn(II)-oxidizing community located approximately 30 m away from the Mn Falls seep. However, Mud Trap Falls is not hydrologically connected to Mn Falls and did not exhibit massive biofilm streamers, or other similar changes in appearance during this time. Therefore, Mud Trap Falls was selected as a comparison site. Water samples were also obtained from the Upper and Lower Shipwreck drip pools in Daniel Boone Caverns (Fig. 1) for comparison, as these water sources did not appear to have ferromanganese deposits associated with them. The temperature, pH, and conductivity of the water at the Mn Falls seep and in the drip pools in Daniel Boone Caverns was measured using either a VWR SymPHony, Fisher Scientific accumet AP85 Portable Waterproof pH/Conductivity Meter, or a YSI 556 MPS (Multiprobe System), then sampled via syringes and filtered through a 0.45 mm micropore filter into sterilized Nalgene bottles and refrigerated.

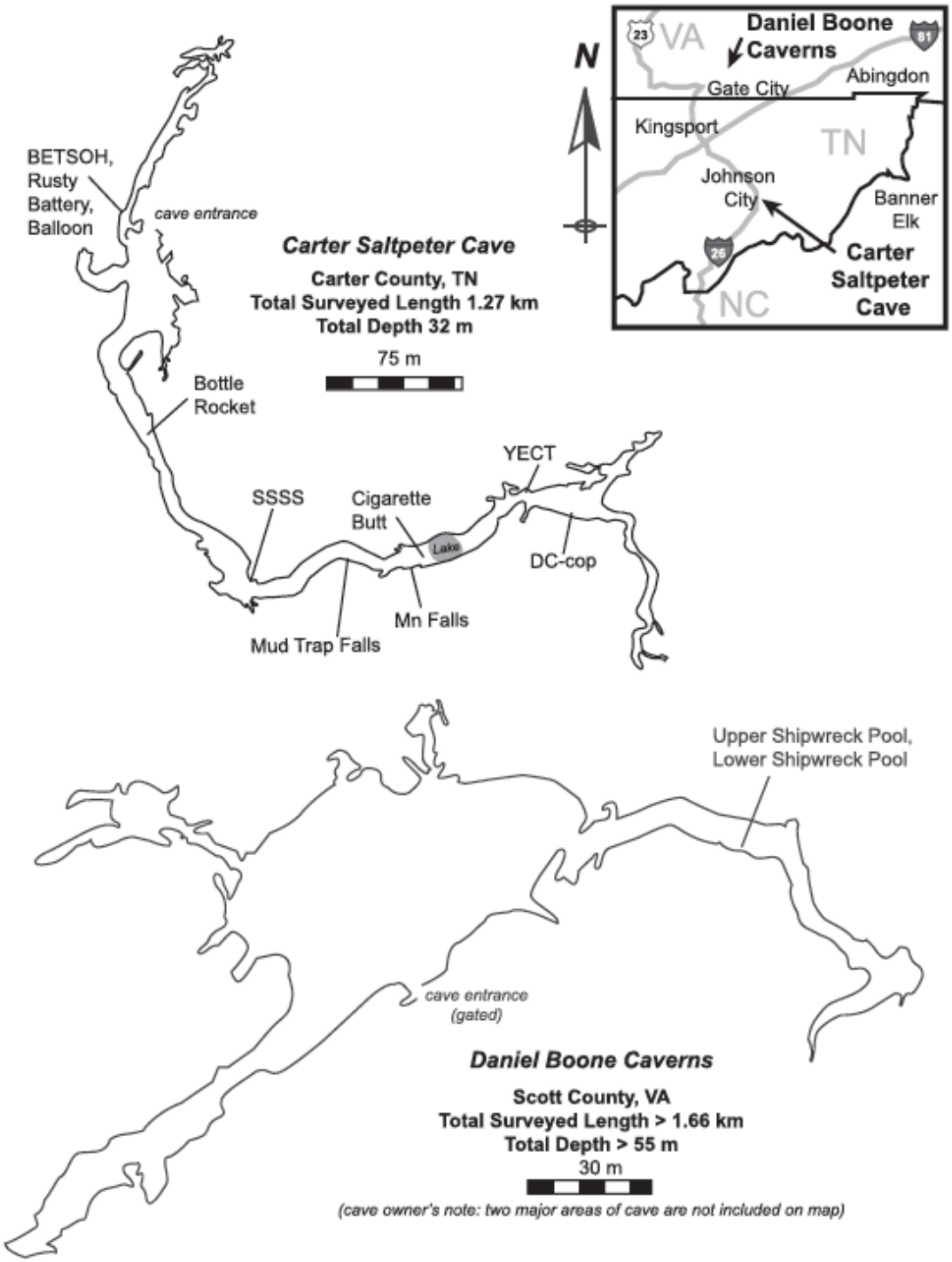

Figure 1. Maps of the Carter Saltpeter Cave (CSPC) and Daniel Boone Caverns, modified with arrows to indicate sampling locations. Regional map inset shows the relative location of the two cave systems within the upper Tennessee River Basin. Cave survey of CSPC conducted on February 8, 1981 by L. Adams, R. Knight, R. Page, and T. Wilson. Cave map drafted by L. Adams and adapted by S. Carmichael. Initial cave survey of Daniel Boone Caverns conducted in 1969 by M. Starnes, B. Lucas, D. Breeding, C. Stowers, and B. Balfour, and an additional survey was conducted from July-November 1996. Two substantial passages in the cave have not yet been surveyed. Cave map adapted by S. K. Carmichael.

Table 1. Descriptive summary of samples obtained from cave Mn(III/IV) oxide deposits in this study. Cave names are abbreviated as follows: Carter Saltpeter Cave (CSPC), Daniel Boone Caverns (DBC). See Figure 1 for locations of the individual samples. Analyses conducted are abbreviated as follows: Most probable number assays (MPNs), polymerase chain reaction (PCR), powder X-ray diffraction (XRD), single crystal micro X-ray diffraction (μ-XRD), Fourier Transform Infrared Spectroscopy (FT-IR), ion chromatography (IC), and electron microscopy (EM).

Sample (Location) and Sample Type	Date Collected (mm/dd/yyyy)	Analyses Conducted
Mn Falls (CSPC)	09/01/2009	μ-XRD (biofilm)
	05/05/2010, 06/30/2010	MPNs
Mn(III/IV) oxide biofilm	07/04/2009, 07/31/2009, 01/15/2010, 05/05/2010 09/14/2010, 10/05/2010 11/09/2010, 05/16/2011, 06/16/2011	PCR with *Bacteroides* primers
water	03/24/2010, 05/05/2010, 06/30/2010, 08/18/2010 10/05/2010, 04/21/2011 09/23/2011, 09/06/2012, 11/15/2012	IC
flowstone, mud substrate	09/01/2009	XRD, EM
Mud Trap Falls (CSPC)		
Mn(III/IV) oxide coating	07/04/2009, 07/31/2009, 01/15/2010, 05/05/2010 06/30/2010, 09/14/2010 10/05/2010, 11/09/2010, 05/16/2011, 06/16/2011	PCR with *Bacteroides* primers
	01/152010, 06/30/2010	MPNs
	09/10/2009	μ-XRD (coating material)
flowstone, mud substrate	09/10/2009	XRD, EM
DC-cop (CSPC)		
animal feces	05/10/2010	Fungal cultures, XRD (substrate)
SSSS (CSPC)		
cotton sock	10/05/2010	Fungal cultures, EM
YECT (CSPC)		
black electrical tape	06/30/2010	Fungal cultures, μ-XRD (coating material, cultures), EM, FT-IR (cultures)
BETSOH (CSPC)		
black electrical tape	09/06/2012	Fungal cultures
Bottle Rocket (CSPC)		
fireworks	09/06/2012	Fungal cultures
Balloon (CSPC)		
balloon	09/06/2012	Fungal cultures
Rusty Battery (CSPC)		
rusty battery	09/06/2012	Fungal cultures
Upper Shipwreck Pool (DBC)		
water	07/18/2010, 09/22/2011, 10/14/2012	IC
Lower Shipwreck Pool (DBC)		
water	07/18/2010, 09/22/2011, 10/14/2012	IC

GEOCHEMICAL AND MINERALOGICAL ANALYSES

The anion concentrations in water from the Mn Falls seep and from drip pools in Daniel Boone Caverns were measured using a DionexICS-1600 Ion Chromatograph at Appalachian State University. Charactertization of the sampled substrate was performed on a Shimadzu 6000 X- ray Diffractometer (XRD) with ICDD PDF-4 Minerals software, and on a FEI Quanta 200 Environmental Scanning Electron Microscope with an EDAX Si-Li energy dispersive spectrometer (ESEM-EDS). Identification of Mn oxide species was performed on a Rigaku D/Max Rapid micro-X-ray Diffractometer at the Smithsonian National Museum of Natural History, and via Fourier Transform Infrared Spectroscopy (FT-IR) using a KBr pellet press method with a Thermo Nicolet Magna 550 IR Spectrometer at Appalachian State University. FT-IR spectra were compared to Mn oxide reference spectra from Potter and Rossman (1979).

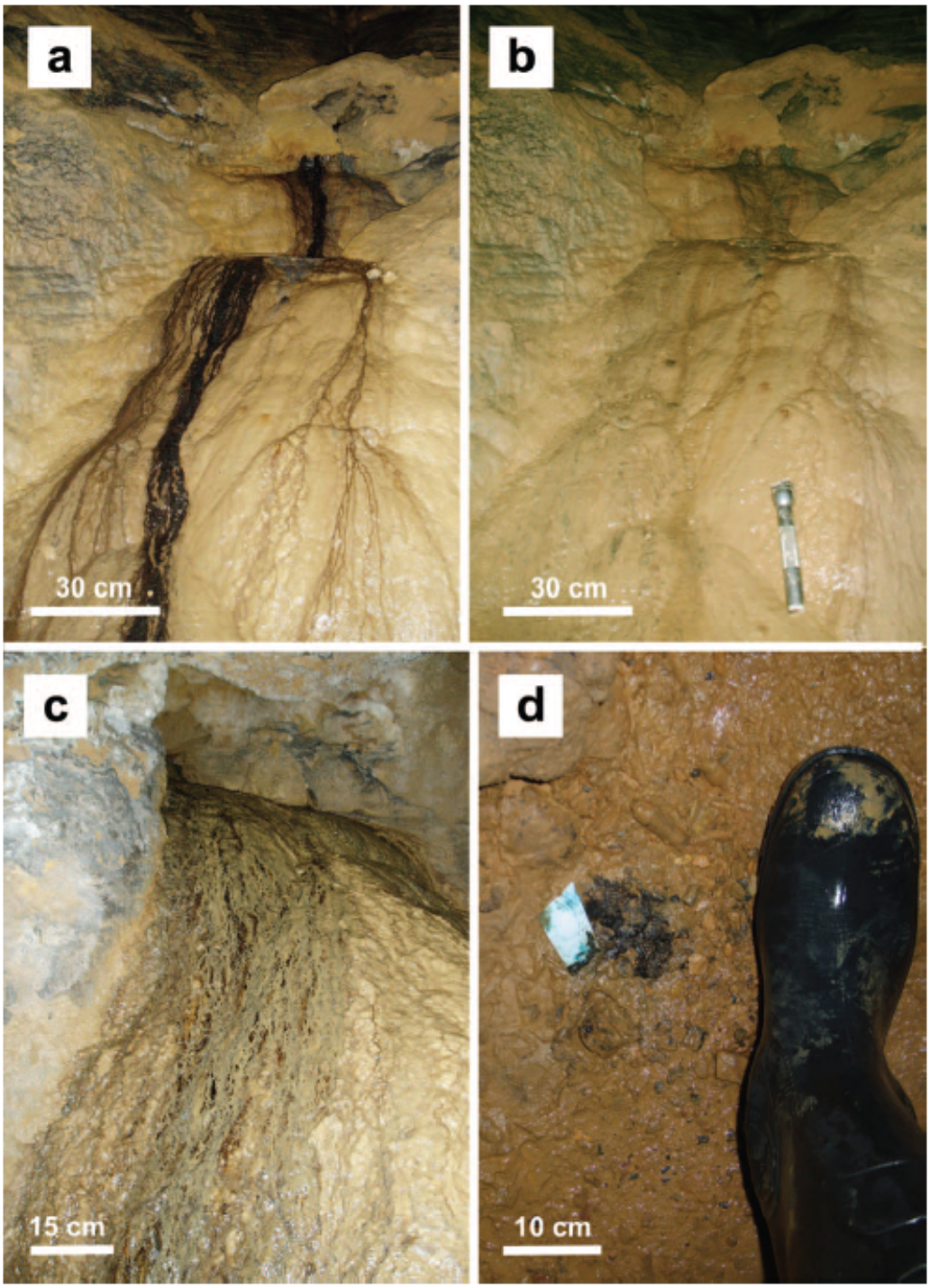

Figure 2. (a) Carter Saltpeter Cave (CSPC), Mn Falls site, July 2008, photo taken by Mountain Empire Grotto member John Matthews several months after a septic tanker truck may have released sewage into a local sinkhole. Image is published with permission. (b) CSPC, Mn Falls site, June 2010, Mn oxides reduced. Mn oxides have steadily decreased with time at the site since sampling began in July 2009. (c) CSPC, Mud Trap Falls site, July 2009. (d) LBB positive test (in bright blue on the filter paper) demonstrating the presence of Mn oxides on feces. Boot for scale. The surrounding clay tested LBB negative.

DETECTION 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 (2000a) with a demonstrated detection limit of 1.4310^{26} g dry feces/liter. A nested PCR approach was utilized in an attempt to amplify the region of interest in DNA extracted from samples collected at Mud Trap Falls and Mn Falls between July 2009 and June 2011. Only two samples from Mn Falls amplified: July 2009 (F) and June 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 (59- AACGCTAGCTACAGGCTT-39) and 708R (59-CAAT- CGGAGTTCTTCGTG-39) (Bernhard and Field, 2000b). Each 50 mL reaction contained 1.25 U AmpliTaq Gold (Applied Biosystems, Carlsbad, CA), 50 mM each primer, 1X PCR Gold Buffer (Applied Biosystems, Carlsbad, CA), 2 mM $MgCl_2$ Solution (Applied Biosystems, Carlsbad, CA), 200 mM each dNTP, and 2X BSA (New England Biolabs, Ipswich, MA). An 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 94uCfor5min,followedby35cyclesof94uCfor30s,60 uC for 1 min, and 72 uC for 2 min, followed by a final extension of 72 uC for 6 min. PCR amplifications were conducted in triplicate to reduce individual PCR bias 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 (June 2011) obtained from the Mn Falls biofilm. Replicate amplifications of each DNA template were pooled for downstream use in the next round of PCR.

One mL of PCR product from the pooled amplifications of either F (July 2009) or 4 (June 2011) DNA was used as a template for PCR amplification of human-specific Bacte- roides-Prevotella16S rRNA gene sequences in the second round of the nested protocol. PCR amplification was conducted using the Bacteroides-Prevotella human-specific forward primer HF183 (59- ATCATGAGTTCACATG-TCCG-39) paired with the Bacteroides-Prevotella universal reverse primer 708R (59-CAATCGGAGTTCTTCGTG-39) (Bernhard and Field, 2000a). Reaction conditions mim- icked those given for the first round of the nested protocol, with the following adjustment in the amplification proto- col: an initial denaturation of 94 uC for 5 min, followed by 25cyclesof94uCfor30s,60uCfor1min,and72uCfor 2 min, followed by a final extension of 72 uC for 6 min. Amplifications were conducted in triplicate to reduce individual PCR bias, and 5 mL 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 mL. 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 UltraCleanGel- Spin DNA Extraction Kit (Mo-Bio Laboratories, Carlsbad, California). Purified PCR products of each DNA template were cloned into TOPO TA pcrH2.1 vectors (Invitrogen, Carlsbad, CA), and plasmid DNA extracted from transformants using the QIAprep Spin Miniprep Kit (Qiagen, Valencia, CA) was screened using the M13F(220) primer. Glycerol stocks were sequenced using the M13F (220) 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 (Figure 3) was constructed using the PHYLIP software package (Felsenstein, 2004) by conducting both neighbor-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 estimate the number of culturable heterotrophic microorganisms and heterotrophic Mn(II)-oxidizing microorganisms in CSPC (Table 3), as described previously (Brauer et al., 2011; Johnson et al., 2012), with some modifications. Samples were collected from Mn Falls and Mud Trap Falls in January, May, and June of 2010 and stored overnight at 4uC. A new media that we designed, FMO2 growth medium (Carmichael et al., 2013), was used for MPN assays. 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 samples were made, ranging from 10^{22} to 10^{211} and inoculated in Cellstar 96 well culture plates (Greiner Bio-One, Monroe, NC). Each well contained 250 mL sterile media and was inoculated with 25 mL of either sample material, a positive control (Leptothrix sp.), or a negative control in eight replicates per sample. A control plate for each sample was inoculated and tested immediately for Mn(II) oxidation and heterotrophic metabolism using 50 mL 0.04% LBB (rows 1–4) and 50 mL 0.3% iodonitrotetrazolium chloride (INT) (Sigma Aldrich) (rows 5–8), respectively. Background level colorimetric results from the control plate test were recorded and used for comparison with the incubated plates. Plates were incubated in the dark at 10uC 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 10uC and allowed to incubate overnight to note additional color change, if any. MPN assays were scored again after 24 hours, with no notable change in results being observed. Results were applied to Curiale's freeware MPN calculator (accessed at http://www.i2workout.com/mcuriale/mpn/index.html) to estimate the total number of culturable heterotrophic microorganisms and culturable heterotrophic Mn(II)-oxidizing microorganisms in cave samples.

CULTURING OF FUNGI FROM LITTER

Fungal culture enrichments were obtained using litter that tested LBB-positive in the cave. Litter was swiped on plates containing an AY (Santelli et al., 2011) agar- solidified media. The plated cultures were tested for Mn(II)-oxidation via LBB colorimetric assay. To discourage contamination by other fungi and bacteria, all Mn(II)- oxidizing fungal samples were recultured on fresh media at least three times using a stab and swipe method. Several samples (YECT Stab 1 and SSSS) were maintained on media containing Ampicillin to prevent bacterial contamination. Samples were incubated in the dark at room temperature for 2–6 weeks.

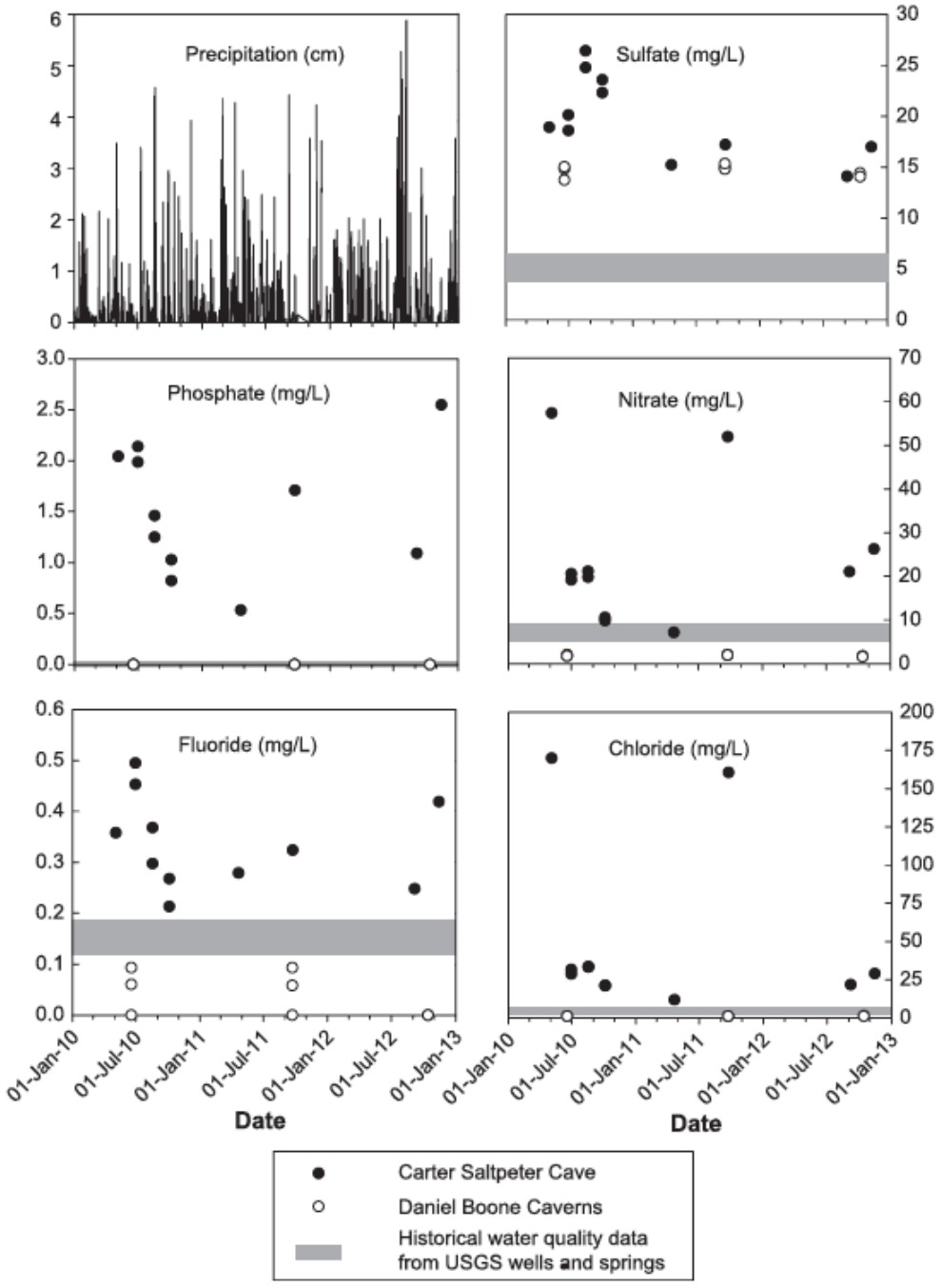

Figure 3. Ion chromatography results for water collected from Carter Saltpeter Cave (CPSC) Mn Falls seep, and from the upper and lower drip pools of Daniel Boone Caverns from May 2010–December 2012. Historical water quality data was collected by the United States Geological Survey in 1997–1998 from wells and springs in the Knox Dolomite located within 25 km of CSPC (data from USGS National Water Information System, http://nwis.waterdata.usgs.gov/usa/nwis/qwdata, accessed August 29, 2012) using sites USGS 03465770, USGS 03486175, and USGS 361726082181801. Precipitation data for Johnson City for the time covering the study were obtained from the National Climatic Data Center (http://www1.ncdc.noaa. gov/pub/orders/118435.csv, accessed January 8, 2013) using gauge GHCND:USC00404666.

Table 2. Field measurements of temperature, pH, conductivity, and dissolved oxygen for Mn Falls in Carter Saltpeter Cave (CSPC) and drip networks in Daniel Boone Caverns (DBC) at various dates from May 2010 to December 2013.

Date	Cave	Temp., °C	pH	Conductivity, μS cm^{-1}	DO, ppm	Field Observations
05/05/2010[a]	CSPC	13.1	7.00	436	8.2	water running clear, sampled from water flowing into pool at base of seep
05/05/2010[a]	CSPC	13.1	6.96	460	7.2	water running clear, sampled from water running across flowstone in middle of seep
06/30/2010[b]	CSPC	13.9	7.44	[d]	…	sampled from water flowing into pool at base of seep; cave floor damp to dry
06/30/2010[b]	CSPC	15.7	7.47	[d]	…	sampled from stagnant pool at base of seep; cave floor damp to dry
04/21/2011[b]	CSPC	13.4	8.51	523	…	sampled from middle of seep, water running over flowstone; cave floor very wet
09/23/2011[b]	CSPC	13.1	8.03	737	…	sampled from water flowing into pool at base of seep
09/06/2012[b]	CSPC	13.4	8.44	677	…	sampled from middle of seep, cave floor damp
11/15/2012[b]	CSPC	13.1	7.64	[d]	…	sampled from top of seep, cave floor dry
07/28/2011[b]	DBC	[d]	8.07	483	…	upper pool
07/28/2011[b]	DBC	[d]	8.06	428	…	lower pool
09/22/2011[b]	DBC	12.2	8.02	354	…	upper pool
09/22/2011[b]	DBC	11.8	8.34	352	…	lower pool
10/14/2012[c]	DBC	11.8	7.87	327	11.5	upper pool
10/14/2012[c]	DBC	11.7	7.97	321	10.2	lower pool

[a] Measurements collected with a Fisher Scientific accumet AP85 Portable Waterproof pH/Conductivity Meter Kit and Hanna Instruments HI 9146 Portable Microprocessor Dissolved Oxygen Meter.
[b] Measurements collected with a VWR SymPHony.
[c] Measurements collected with a YSI 556 MPS (Multiprobe System).
[d] Meter error.

ELECTRON AND LIGHT MICROSCOPY OF FUNGAL CULTURES

Light microscopy of fungal cultures grown on AY media was performed using an Olympus SZX12 Zoom Stereo Microscope with a Sony HDR-HC7 HDV video camcorder. For transmission electron microscopy with energy dispersive X-ray spectroscopy (TEM-EDS) and for element mapping via scanning transmission electron microscopy (STEM), samples were mounted on Carbon Type B formvar-coated copper 200 mesh grids (Ted Pella, Inc.), air dried, carbon coated, and analyzed using a JEOL JEM-1400 TEM with a G-135 Pentafet Sealed Window X-ray detector at 80 kV and/or 120 kV in the Dewel Microscopy Facility at Appalachian State University.

Environmental scanning electron microscopy with energy dispersive X-ray spectroscopy (ESEM-EDS) was performed on a FEI Quanta 200 Environmental SEM with an EDAX Si-Li EDS detector in the Dewel Microscopy Facility at Appalachian State University. Portions of fungal growth from AY stab cultures were transferred onto a piece of carbon tape and mounted on a 13 mm aluminum stub and run under low vacuum conditions between 15 and 20 kV, with a 5 mm beam spot size.

ophic bacteria and culturable heterotrophic Mn(II)-oxidizing bacteria present in Carter Saltpeter Cave biofilms.

phic Bacteria		Heterotrophic Mn(II)-oxidizing Bacteria					
J	95% CI (upper)	95% CI (lower)	MPN	95% CI (upper)	Total Bacteria[a] (cells mL^{-1})	Culturable Bacteria (%)	CulturableMn(II)-oxidizing Bacteria (%)
0^{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
0^{10}	8.9×10^{10}	9.3×10^{7}	3.2×10^{8}	1.1×10^{9}	3.6×10^{9}	≥100.0[b]	8.9
0^{6}	2.9×10^{7}	…	…	…	…	0.4[c]	…
0^{9}	2.9×10^{10}	…	…	…	9.8×10^{9}	98	…

ious study by real-time quantitative PCR (Carmichael et al., 2013).
ıe error range of total bacterial cell number as estimated by real-time quantitative PCR (Carmichael et al., 2013).
er estimated from the qPCR analysis from the winter collection (2.6×10^{9}; Carmichael et al., 2013).

RESULTS

GEOCHEMISTRY AND MINERALOGY

Measurements of temperature, pH, dissolved oxygen, and conductivity were conducted in the field (Table 2). Temperatures ranged from 13–15 uC, pH from 7.4–8.0, and conductivity from 436–737 mS/cm. Higher conductivities were associated with a shallow (5 cm deep) pool at the base of the Mn Falls seep, which contained significant fine sediment. IC analyses of phosphate, nitrate, sulfate, fluoride, and chloride for Mn Falls from 2010–2011 all demonstrated variable but significantly higher values of these anions in comparison to water from Daniel Boone Caverns and water from nearby wells and springs, and these variations did not appear to be correlated with precipitation patterns (Fig. 3).

SEM-EDS and XRD measurements indicate that the substrate for both Mn Falls and Mud Trap Falls consists of a mixture of nontronite clay and calcite. Bacterial Mn(III/IV) oxides at both Mud Trap Falls and Mn Falls were tentatively identified via single crystal micro-XRD as poorly crystalline buserite, a Na-rich layered phase in the birnessite group (Post, 1999) with broad peaks indicating the presence of 9.5-7.3 A$^{\circ}$ sheets. FT-IR analysis of cultured fungi from electrical tape contained a broad absorbance peak from 3700–3200 cm^{21}, indicating O-H bonds, and additional sharper peaks associated with Mn oxide octahedral bonds at approximately 1631, 727, 530, 470 cm^{21}. Peaks associated with amines and polysaccha- rides were also present. This pattern indicates that disordered todorokite, a Ca-rich mineral with a tunnel structure, may be the dominant Mn(III/IV) oxide present, although poorly crystalline todorokite and birnessite group minerals do frequently coexist in biologically produced Mn(III/IV) oxides, as the biological formation of either layer or tunnel structures is dependent on local solution chemistry (Zhu et al., 2010). Regardless, the Mn(III/IV) oxides described in this study are nm scale and poorly crystalline; and therefore, cannot be definitively deter- mined using traditional powder X-ray and electron diffraction techniques. Our findings are consistent with observations of biologically produced Mn(III/IV) oxides in previous studies (Spiro et al., 2010, and references therein).

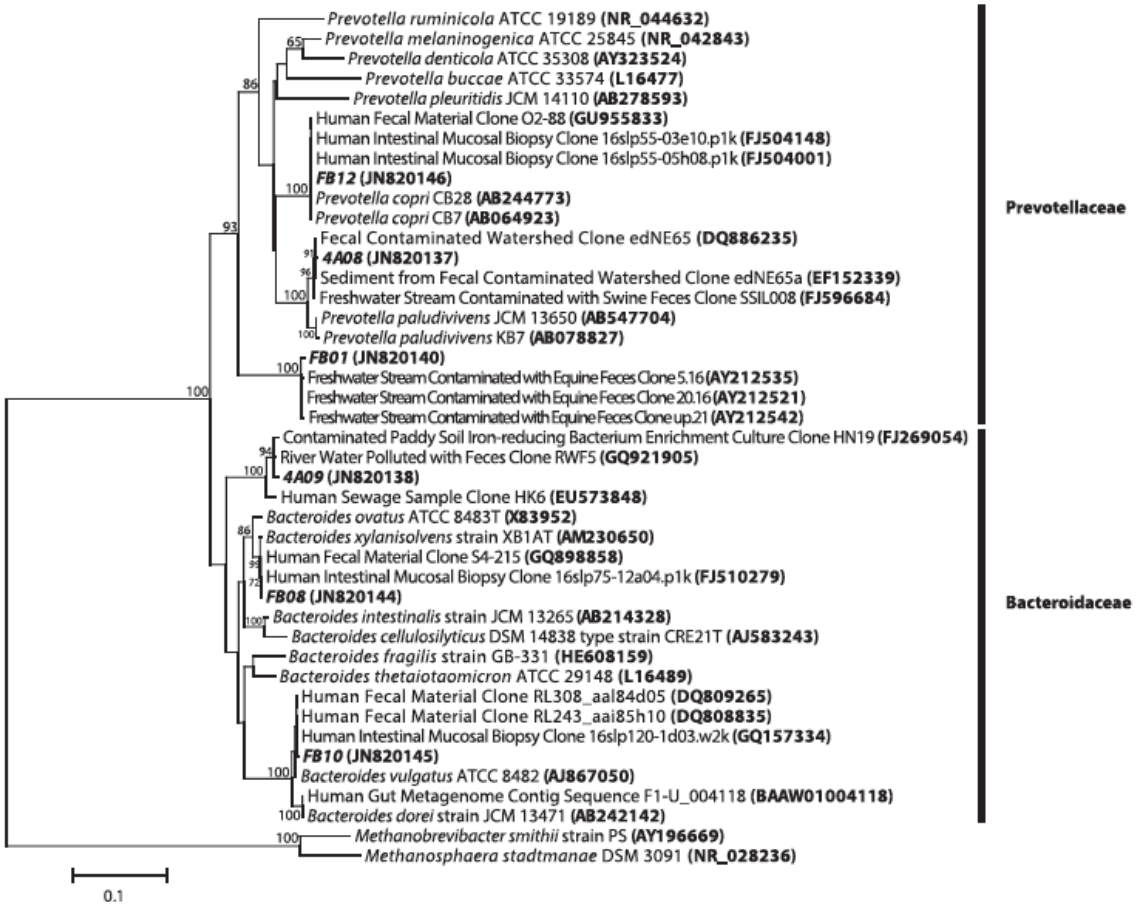

Figure 4. Neighbor-joining tree inferring the phylogenetic placement of SSU rRNA gene sequences obtained from the Mn Falls biofilm found in Carter Saltpeter Cave (CSPC) in this study in either July 2009 (sequences beginning with F) or June 2011 (sequences beginning with 4). Alignments were created using 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).

DETECTION OF HUMAN-SPECIFIC BACTEROIDES- PREVOTELLA 16S rRNA GENE SEQUENCES

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 2009– 2011. A primer set designed by Bernhard and Field (2000a; 2000b) targeting human-specific Bacteroides-Prevotella was chosen due to its sensitivity and reliability according to previous reports in the literature (Ahmed et al., 2009a; 2009b). Multiple attempts at amplification of DNA extracted from both Mn Falls and Mud Trap Falls samples across multiple collection dates approximately every 3– 6 months from 2009–2011 (Table 1) resulted in only two positive amplifications. Both were 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 an F in Figure 4), and sample 4, extracted in June 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 Figure 4). Multiple attempts at amplification of DNA extracted from Mud Trap Falls were unsuccessful. Clones were binned into OTUs for phylogenetic analysis using a 99% cutoff. Results revealed 6 unique OTUs (Fig. 4) out of ca. 15 total sequences.

Three OTUs (FB12, 4A08, and FB01) clustered within the Prevotella spp. (Fig. 4), 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 (La- mendella et al., 2007; 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 (Fig. 4), 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 a study investigating the association of human gut microbial ecology with obesity (Ley et al., 2005).

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 June 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 sewage 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 June 2011 sample of Mn Falls.

MOST PROBABLE NUMBER ASSAYS

Most probable number (MPN) assays of total culturable heterotrophic bacteria and total culturable heterotrophic Mn(II)-oxidizers at Mn Falls and Mud Trap Falls were conducted on samples obtained on three different occasions, January 2010 (Mud Trap Falls), May 2010 (Mn Falls), and June 2010 (Mn Falls and Mud Trap Falls) (Table 3). Cultivation-based enumeration of heterotrophic Mn(II)-oxidizing bacteria revealed no significant differences between population numbers at Mud Trap and Mn Falls.

Results from cultivation-based enumeration of total heterotrophic bacteria revealed an interesting trend (Table 3), however. Data from the January 2010 sampling of Mud Trap Falls indicated an average count of $1.13 10^{8}$ cells/g wet weight; data from the May 2010 sampling of Mn Falls indicated an average count of $2.53 10^{10}$ 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.

June 2010 data from both sites reflects the same pattern, with an average count of $9.63 10^{6}$ cells/g wet weight at Mud Trap Falls and $9.63 10^{9}$ cells/g wet weight at Mn Falls. The difference between the two sites in June 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 January 2010 and the 95% lower confidence interval at Mn Falls in June 2010. However, the percent of culturable heterotrophic bacteria, within the total population estimated using qPCR, was consistently higher (approaching 100%) at Mn Falls (Table 3), a finding that is supportive of nutrient loading at the Mn Falls site.

MN(II) OXIDATION ASSOCIATED WITH LITTER

Field observations indicated distinct increases in microbial Mn(II) oxidation in response to nutrient input. Field samples unintentionally amended with litter or feces became encased in a slimy Mn(III/IV) oxide coating, examples of which included an abandoned sock, a corroding battery, cigarette remains, electrical tape, fireworks, balloons, and feces from a small herbivorous mammal (Fig. 2d). In an effort to better characterize the responsible organisms, culturing experiments on the litter produced a variety of Mn(II)-oxidizing fungal species (Fig. 5), many of which have not yet been described in the literature and whose phylogenetic associations are still uncertain. Scanning and transmission electron microscopy of these fungi have shown that Mn is sequestered in spores, and/or along hyphal junctions and septa (Fig. 5e, 5f).

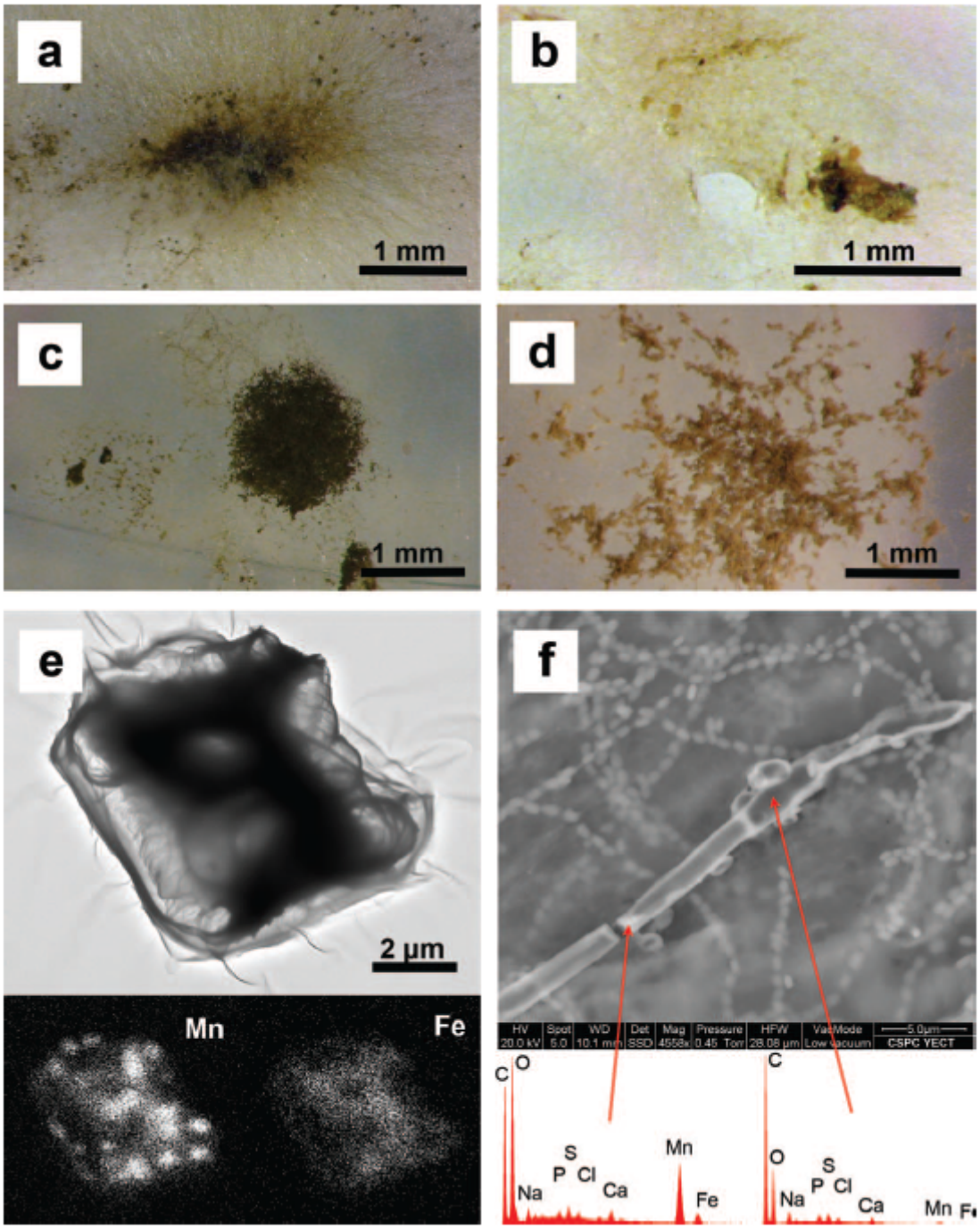

Figure 5. Light microscopy of Mn (II)-oxidizing fungi from Carter Saltpeter Cave on AY agar-solidified media, cultured from electrical tape (a), fireworks (b), an abandoned sock (c), and a rusty battery (d). Scanning transmission electron microscopy of fungi cultured from the abandoned sock indicates the presence of Mn and Fe within fungal spores (e). Backscattered scanning electron microscopy imaging of fungi cultured from electrical tape indicates elevated levels of Mn within hyphal junctions, but minimal Mn content within the hyphal interiors (f).

DISCUSSION

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). Microbial species within pristine caves (those that have little or minimal human impact) are adapted to leading an oligotrophic lifestyle (Northup et al., 2003). However, gut-related microbiota, which are generally not endogenous members of the cave microflora, are adapted to environments containing high levels of carbon and other nutrients (Ley et al. 2006). 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. There was no significant difference between the total bacterial number estimated using qPCR in a separate study (Carmichael et al., 2013) and total culturable heterotrophic bacteria estimated using MPN analyses at the Mn Falls site on the June 2010 sampling date, indicating that a large number of the bacteria present at the site were culturable.

Significantly higher average cultivable cell counts and consistently higher percentages of culturable bacteria at the Mn Falls site relative to Mud Trap Falls support the hypothesis of localized nutrient loading/sewage contamination at the Mn Falls site in CSPC. This hypothesis is bolstered by several field studies investigating nutrient loading in caves via tourism (Ikner et al., 2007), septic effluent (Simon and Buikema, 1997), and input of rich carbon sources such as guano, feces, and human traffic (Mulec et al., 2012) where, in all three studies, cultivable counts of bacteria (either total CFUs, total aerobic bacteria, or total fecal coliform bacteria), were at least two orders of magnitude higher than similar counts in low impact zones within the caves. Experimental manipulations of soil plots have also 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 (Chelius et al., 2009), findings which support our hypothesis that the bloom of biofilm material and increase in culturable numbers of bacteria at Mn Falls were likely due to nutrient-loading at this site.

It is important to note that cultivation-based experiments are inherently biased by media design and inoculation/incubation techniques (Rusterholtz 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 environ- mental samples (Ahmed et al., 2008b). The present study addresses this bias via molecular-based characterization of the Mn Falls microbial community. Molecular surveys demonstrated the presence of a Bacteroides-Prevotella fecal signature in DNA extracted from the Mn Falls biofilm in both July 2009 and June 2011, with a stronger human signature detected in the July 2009 sample. Bacteroides spp. represents 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). Molecular-based methods have also been successfully employed in other studies to detect the presence of human fecal indicators in karst aquifers (Johnson et al., 2011; Reischer et al., 2007) and human impact in caves (Johnston et al., 2012).

Water chemistry from Mn Falls also points to point source nutrient loading via septic effluent. Field tests (pH, conductivity, etc.) revealed values for CSPC that were consistent with those reported from other caves and local springs contaminated with fecal indicators (Johnson et al., 2011; Simon and Buikema, 1997). Elevations in septic- associated chemicals including chloride, fluoride, and phosphate strongly suggest the presence of septic input from 2010 to 2011 (Fig. 3). Chloride and phosphate are commonly associated with septic effluent, and chloride is often used as a tracer for septic plumes (Denver, 1989; Minnesota Pollution Control Agency, 1999; Robertson et al., 1998). Fluoride concentrations, while highly variable, may also be elevated quite significantly (Minnesota Pollution Control Agency, 1999). Naturally occurring elevated fluoride levels are unlikely, as fluorite is not abundant in the surrounding bedrock (Hoagland et al., 1965) and is not soluble at pH values ,8 in the presence of calcite (Miller and Hiskey, 1972). In addition, Johnson City (located less than four miles to the northwest) fluoridates their municipal water to attain values 1 mg/L (Washington County Water and Sewer Department, personal communication), which also points to septic effluent as a likely source of high fluorine levels in the seep. Further, CSPC is located in an active conduit system, which would be consistent with relatively rapid changes in water chemistry. Sulfate and nitrate are also variable yet elevated with respect to pristine cave waters and local well and spring waters. The proximity of the USGS wells and springs to CSPC make these elevations unlikely to be the result of land use differences. The elevated levels of nutrients (particularly fluoride) that remain in the water may be due to continued flushing of the conduit leading to the seep rather than continuous sewage input, which would have been detected via MPN analyses and gene sequencing.

Although it is difficult to visually assess changes in microbial diversity in response to eutrophication or other disturbances directly in the field, changes in function are easily seen among Mn and Fe oxidizing microbial communities, where such changes are visible as a significant alteration in the abundance of metal oxide produced. The Mn Falls bloom, with an associated distinct fecal odor, appeared in 2008, and both have slowly disappeared over the course of the study (Fig. 2). Several mechanisms of heterotrophic Mn(II) oxidation have been proposed. Bacteria such as Pseudomonas putida GB-1 (Geszvain et al. 2013), various species of Bacilli (Francis and Tebo, 2002), and Leptothrix discophora SS-1 (Corstjens et al. 1997) are all thought to use multicopper oxidases for Mn(II) oxidation. Whereas the bacteria Aurantimonas maganoxydans SI85-9A1 and Erythrobacter sp. strain SD- 21 are thought to use a manganese-oxidizing peroxidase (Anderson et al. 2009). Similarly, fungi of the Basidiomy- cetes are known to use a manganese peroxidase enzyme (Liers et al. 2011) to oxidize Mn

(II). MPN analyses in this study, however, suggest that Mn(II)-oxidation at this site was most likely correlated with heterotrophic activity, an idea consistent with one that has recently emerged in the geomicrobiological and geochemical literature: that Mn (II)-oxidation can be affiliated with reactive oxygen species (ROS) production by both heterotrophic bacteria and fungi. For example, Learman et al. (2011) reported that Roseobacter oxidizes Mn(II) indirectly via ROS that are most likely generated as a normal part of electron transport during aerobic, heterotrophic respiration. In addition, Hansel et al. (2012) have shown that Stilbella aciculosa, an Ascomycete fungus, oxidizes Mn(II) via extracellular ROS produced during asexual reproduction. Heterotrophic Mn(II)-oxidation associated with ROS production also makes sense thermodynamically, since the first electron transfer in the oxidation of Mn(II) to Mn(III/IV) was demonstrated to be a rate limiting step, whereas Mn(II)- oxidation by superoxide, hydrogen peroxide or a hydroxyl radical were shown to be favored reactions (Luther, 2010).

Although previous studies have indicated that Mn(III/ IV) oxide production in the CSPC groundwater seeps was primarily associated with Mn(II)-oxidizing bacteria (Carmichael et al., 2013), culturing results in this study revealed that Mn(III/IV) oxide production on litter was associated with Mn(II)-oxidizing fungi (Fig. 5). Rapid fungal Mn(III/IV) oxidation of feces and litter (including a balloon, fireworks, a discarded sock, electrical tape, and a battery) over the course of several weeks also supported heterotrophic growth in a nutrient-limited cave environment.

Although there was a dramatic (albeit gradual) visual reduction in Mn oxide production during sampling trips from 2009–2011 (Fig. 2a, 2b), minor amounts of Mn oxides continued to be produced during this time, as shown by LBB testing. The reasons for this reduction are not entirely clear, but dissolution of birnessite group structures by siderophores (chelating agents) in microbial secretion and/or membranes, as demonstrated for Shewanella oneidensis (Duckworth and Sposito, 2007; Fischer et al., 2008), is a possible mechanism to reduce the presence of birnessite group Mn oxides. Poorly crystalline buserites (Na-rich birnessites) were identified in the biofilm at Mn Falls in this study, and Mn(II)-oxidizing bacteria are known to produce poorly crystalline birnessite group minerals (Spiro et al., 2010 and references therein), which is consistent with siderophore dissolution of Mn oxides in the Mn Falls biofilm. Other chemical factors such as pH, redox potential, or concentrations of nutrients or Mn(II) may also have played a role.

CONCLUSIONS

Multiple techniques including molecular, chemical, and culture-based analyses conducted over a three-year extended study have provided evidence of sustained anthropogenic impact within the Carter Saltpeter Cave system. Our study has provided compelling field data in support of the hypothesis that Mn(II)-oxidation can be associated with both bacterial and fungal heterotrophic activity,

which in this case, was most likely stimulated in response to point source exogenous nutrient loading.

Due to the inherent stability of the cave environment and the highly adapted cave macro- and microfauna, this type of anthropogenic impact has the potential to disrupt the delicate balance of life within a cave and exert a strong negative effect on ecosystem function. Localized variations in geochemistry and nutrient availability have been shown to impact microbial community structure (Johnston et al., 2012; Barton and Jurado, 2007; Shabarova and Pernthaler, 2010) and niche diversification (Engel et al., 2010; Maca- lady et al., 2008). Further, the composition of cave microbial communities mediates and stabilizes biogeo- chemical cycling and mineralization processes within an environment (Portillo and Gonzalez, 2010; Portillo et al., 2009).

Legal protection for cave and karst systems exists at the Federal and State levels, although enacted legislation varies in the degree of protection and is often wrought with

loopholes (van Beynen and Townsend, 2005). The effectiveness of legislation, guidelines, recommendations, and management plans is contingent on the accumulation of high quality baseline data that delineates the sources of contamination within a system and on the documentation of specific impacts within a cave or karst system (Northup, 2011). Clearly, there is a continued need for research, especially in regions such as the Appalachians, where cave density is high and research in cave systems is lacking.

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