

BACTERIAL DIVERSITY IN VADOSE CAVE POOLS: EVIDENCE FOR ISOLATED ECOSYSTEMS

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Abstract

Microbial diversity of cave pools, especially vadose pools, has received relatively little attention. To help fill this gap, this study reports on the bacterial diversity of 17 pools in three New Mexican arid land caves: Carlsbad Cavern, Lechuguilla Cave, and Hell Below Cave. These pools are spread throughout the caves and, with two exceptions, are not connected. The pools share a basic water chemistry, with fresh water of the calcium-magnesium-bicarbonate type. These 17 pools have Chao1 values between 40 and 1738; the Shannon diversity averages 4.6 ± 1.1 , ranging from 2.6 to 6.4; and the Simpson averages 0.881 ± 0.099 , ranging from 0.622 to 0.981. No two pools had the same communities, even at the phylum level. *Nitrospirae*, *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were found >5% abundance in nine or more cave pools. *Actinobacteria*, *Chloroflexi*, *Fibrobacteres*, *Firmicutes* and *Plantomycetes* were at >5 % in four to six pools. Of the top ten widespread bacterial genera, *Nitrospira* was found in all pools, with >5 % in eleven pools. Other common genera include *Polyclovans*, *Propionibacterium*, *Polaromonas*, *Haliangium*, *Bacillus*, Subgroup 6 uncultured Acidobacteria, *Candidatus* Omnitrphica, and uncultured Nitrosomonadaceae. Presence of several potential nitrogen cycling bacteria (e.g., *Nitrospira*) in the study pools suggests that nitrogen cycling may be an important bacterial role. There is some evidence of human contamination, particularly in the heavily visited Big Room, Carlsbad Cavern, but it is not the dominant control. Rather than a single stable cave pool community, adapted to the cave pool ecosystem, the data show 17 different communities, despite relatively similar conditions. The data support the hypothesis that each pool is a unique, isolated ecosystem, with differences likely caused more by the isolation of each pool than by variable chemistry. Thus, the common habit of grouping samples, while useful for some questions, may not capture the diversity present in cave ecosystems.

INTRODUCTION

Karst caves, those found in limestone or dolomite, are recognized as potential windows into subsurface ecosystems (Engel, 2010). Microorganisms are an important part of these ecosystems, but understanding of their diversity is incomplete (Engel, 2010). The bacterial diversity of karst caves is a function of the physicochemical and nutrient setting, which varies widely between and within caves (e.g., Barton and Northup, 2007; Engel, 2010). These local conditions are, in turn, a function of the larger geological and ecological setting of the caves (Jones and Bennett, 2017; Brewer and Fierer, 2018; Alonso, et al., 2019). Because of interest in their unusual ecosystems, some of the best-studied caves are those with chemolithoautotrophy based on sulfur and/or methane (e.g., Movel Cave, Sarbu et al., 1996; Chen et al., 2009; Porter et al., 2009; Kumaresan et al., 2018; Cueva de Villa Luz, Hose et al., 2000; Hose and Rosales-Lagarde, 2017; Lower Kane Cave, Engel et al., 2004a, 2004b; Frasassi Cave, Macalady et al., 2006, 2007, 2008; Jones et al., 2016; and a semi-artificial mineral spring cave in Germany, Karwautz et al., 2018). Other caves also have ecosystems based on oxidation of iron and manganese, resulting in the formation of ferromanganese deposits (FMDs) (Northup et al., 2003; Spilde et al., 2005; Carmichael et al., 2013a, 2013b; Carmichael and Bräuer, 2015; Estes et al., 2017). The Nullarbor caves, Australia, have been shown to have a chemolithoautotrophic system based on nitrite oxidation (Holmes et al., 2001). The majority of karst caves, however, lack an obvious source of chemolithoautotrophy and are generally considered dependent on organic matter from the surface via water, air, or macrofauna transport (Goldscheider et al., 2006; Simon et al., 2007; Griebler and Lueders, 2009). However, karst caves in arid to semiarid regions are more isolated from surface inputs and are more likely to have chemolithoautotrophy (Ortiz et al., 2013, 2014).

Even with this limitation, karst caves are increasingly recognized as containing diverse microbial ecosystems. As a result, there are a growing number of studies in caves looking at the microbial communities on speleothems (Barton et al., 2007; Ikner et al., 2007; Legatzki et al., 2011, 2012; Engel et al., 2013; Yun et al., 2016a, Leuko et al., 2017, Thompson et al., 2019), wall rock (Porca et al., 2012; Carmichael et al., 2013a, 2013b; Carmichael and Bräuer, 2015; Wu et al., 2015; Lavoie et al., 2017; Sauro et al., 2018), cave sediment (Rusterholz and Mallory, 1994; Chelius and Moore, 2004; Adetutu et al., 2012; Wu et al., 2015; Brannen-Donnelly and Engel, 2015; Sauro et al., 2018; Thompson et al., 2019), and cave streams (Brannen-Donnelly and Engel, 2015; Plese et al., 2016). There are fewer studies on cave pools, which are

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areas of standing water in caves (Shabarova and Pernthaler, 2010; Shabrova et al., 2013, 2014; Hershey et al., 2018; Sauro et al., 2018).

Studies of microorganisms in groundwater and cave streams have shown that the attached community is different from the planktonic community (Goldscheider et al., 2006; Brannen-Donnelly and Engel, 2015; Savio et al., 2018). A number of studies looking specifically at the planktonic community in karst aquifers through sampling of springs have identified an autochthonous microbial endokarst community (or AMEC) that is stable over time, particularly in aquifers with longer residence times (Farnleitner et al., 2005; Pronk et al., 2009; Savio et al., 2018). This community is disrupted during recharge with transient microorganisms from the surface, which then decrease over time (Savio et al., 2018, 2019; Wegner et al., 2019). Shabarova et al. (2013; 2014) looked at epiphreatic karst cave pools where seasonal rise of the water table completely flushed the pools. They found that each flood reintroduced the AMEC from the groundwater, which then rapidly lost diversity and abundance within weeks of residence time, such that the full AMEC was no longer present (Shabarova et al., 2013, 2014). The results from these AMEC studies suggest three possible microbial communities with different conditions: a surface community adapted to soil conditions, the AMEC adapted to groundwater conditions, and a cave pool community adapted to the conditions of the cave pool.

Cave pools in the vadose (unsaturated) zone are separate from the karst aquifer as they are perched above the water table. These pools collect infiltrated water, often within days to weeks of rainfall events (Williams, 1983; Oster et al., 2012). However, based on oxygen isotopic and tritium studies (Even et al., 1986; Chapman et al., 1992; Turin and Plummer, 1995, 2001; Williams and Fowler, 2002), residence time for water in the vadose zone is generally much longer. In temperate climates, residence times range from <1 year up to several years (Spötl et al., 2005; Genty et al., 2014; Mischal et al., 2015). In arid climates, residence times are longer, up to several decades (Chapman et al., 1992; Turin and Plummer, 1995, 2001; Kaufman et al., 2003). Depth of the cave is also a factor, with shallow caves often having shorter residence times of weeks to months (Oster et al., 2012). One of the longest residence times (17-36 years) documented is for Carlsbad Cavern, which is both deep and in an arid climate (Chapman et al., 1992). Thus, water entering most karst caves is not usually from the current rain event, but rather from the rain infiltrating from the surface pushing water out into the cave from a reservoir of water in the vadose zone (Fairchild and Baker, 2012; Genty et al., 2014).

This idea of a reservoir within the vadose zone has implications for the microbial community entering the cave with the water. Particularly for caves where drip water has a long residence time within the vadose zone, the soil microbial community from the surface is unlikely to survive unchanged. Even within karst aquifers, the soil community does not persist (Savio et al., 2019; Wegner et al., 2019). Most studies of karst habitats have indeed found cave microbial communities as distinct from the surface communities (e.g., Engel, 2010; Ortiz et al., 2013; Lavoie et al., 2017). Cave pools, however, are almost unstudied. Data on the bacterial diversity of vadose pools is limited to one study that sampled the planktonic community in two deep (~700 m below the entrance) vadose pools in a Swiss Alpine cave (Shabarova and Pernthaler, 2010). These two pools contained very diverse bacterial communities based on comparisons of the 16S rRNA gene sequencing. These pools were dominated by *Betaproteobacteria* (*Oxalobacteraceae*), and *Actinobacteria*. One of the pools also had abundant *Candidatus Omnitrophica* (originally named *OP3*) (Rivas-Marí and Devos, 2018; Kirs et al., 2020). Out of 109 operational taxonomic units (OTUs) identified in the two pools, only five were shared (two affiliated with *Oxalobacteraceae*; one affiliated with *Acinetobacter*, one with *Rhodoferrax*, and one with *Nitrosomonadaceae*). Shabarova and Pernthaler (2010) suggest the large differences between the two pools relatively close to each other is related to differences in chemistry, since one pool has higher SO_4^{2-} and Mg^{2+} , and possibly to different microbial inputs with incoming drips.

Thus, questions remain for the microbial diversity of cave pools, particularly vadose cave pools isolated from the local aquifer. This study reports on the diversity of planktonic bacteria from 17 vadose pools from three karst caves in the Guadalupe Mountains of New Mexico and Texas, including nine pools from Carlsbad Cavern, six pools in Lechuguilla Cave, and two pools from Hell Below Cave. This study uses this larger, more diverse dataset to better explore the suggestion raised by Shabarova et al. (2010) that vadose pools each contain unique communities.

METHODS

Cave Setting and Geologic History

The three karst caves sampled, Carlsbad Cavern, Lechuguilla Cave, and Hell Below Cave, are located in the Guadalupe Mountains of New Mexico and Texas, U.S.A., at the northern edge of the Chihuahuan Desert (Fig. 1). These caves are in the Upper Permian Capitan Reef Complex, mainly in the reef and forereef facies of the Capitan Limestone, but they also extend into the time-equivalent backreef facies of the Seven Rivers, Yates, and Tansill Formations (Hill, 1987; Jagnow, 1999). The backreef facies are dolomite with interbedded sandstones, while the reef and forereef are partly dolomitized limestone (Dunham, 1972; Jagnow, 1979; Melim and Scholle, 2002; Budd et al., 2013; Frost et al., 2013).

The caves of the Guadalupe Mountains are hypogene caves dissolved by reactions with rising waters producing H_2SO_4 at the water table (Hill, 1987; Jagnow et al., 2000; Engel et al., 2004; Hose and Macalady, 2006; Palmer, 2006,

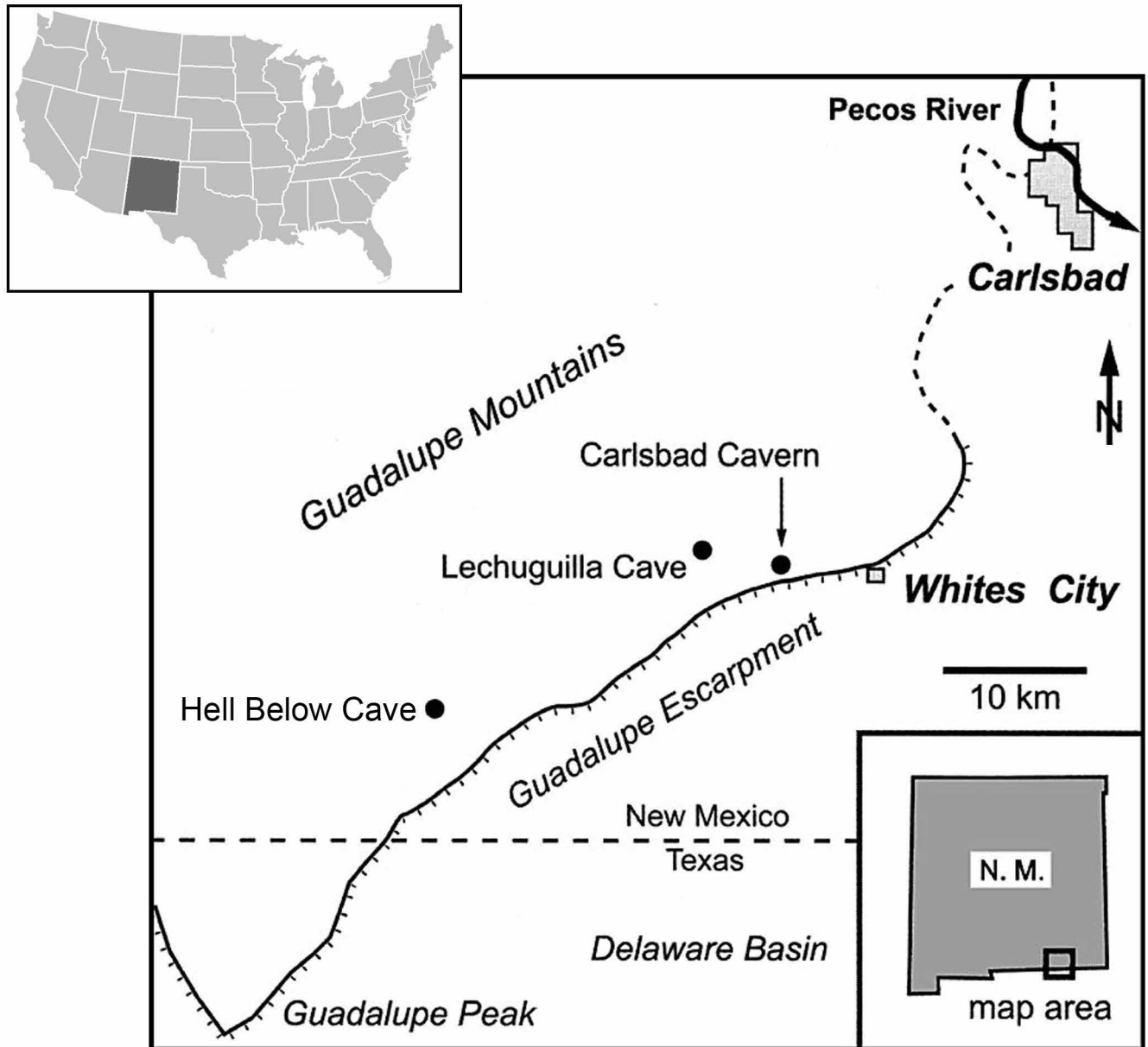


Figure 1. Location map of the three sampled caves, New Mexico, U.S.A. (After Palmer and Palmer, 2000).

2007; Kirkland, 2014). Speleogenesis started in the higher elevation caves (including Hell Below Cave) at ~11.3 Ma and continued during uplift through the upper reaches of Lechuguilla Cave at 6.0 to 5.7 Ma, and finally the lowest dated level, the Big Room in Carlsbad Cavern at 4.0 Ma to 3.9 Ma (Polyak et al., 1998). Since H_2SO_4 is a very strong acid, these karst caves contain unusually large rooms (such as the Big Room at over 3.3 hectares, 8.2 acres), often with flat floors that contain perched cave pools (Hill, 1987; Jagnow et al., 2000). Carlsbad Cavern and Lechuguilla Cave are very large and very deep caves (~64 km long and ~315 m deep; ~242 km long and ~484 m deep respectively, (Gulden 2020). Hell Below Cave is much smaller and more shallow (<400 m long; ~60 m deep) than the other two caves.

The water table in the region is ~960-970 m elevation, well below the elevation of the sampled pools (Hill, 1990; Ingraham et al., 1990; Turin and Plummer, 2000; Land and Burger, 2008; Palmer and Palmer, 2012). Each pool is fed by ceiling drips and loses water by a combination of leaking and evaporation (Ingraham et al., 1990; Forbes, 2000; Turin and Plummer, 2000). With a few exceptions, the pools are not full to the spill point under current climate conditions, but show paleo-water lines that indicate wetter conditions in the past (Melim et al., 2006; Polyak et al., 2012). Dating of shelfstone in the Big Room, Carlsbad Cavern, suggests drying started by 13.5 ka during the transition from cool, wet glacial conditions to the warm, dry Holocene (Polyak et al., 2012). In the current arid climate, water infiltrates during infrequent heavy rains (Williams, 1983; Van der Heidje et al., 1997) and spends considerable time in the vadose zone. For Carlsbad Cavern, the residence time of water in the vadose zone is 17–36 years (Chapman et al., 1992), while for

parts of Lechuguilla Cave it exceeds 50 years (Turin and Plummer, 1995, 2001; Turin et al., 2001). Considering the long residence times and the thin desert soils, the seepage water is expected to have very low TOC (Shen et al., 2015; Blyth et al., 2016; Lechleitner et al., 2017); which has been confirmed for seepage into Lechuguilla Cave (Turin and Plummer, 2000; Levy, 2007b).

Geochemical studies on pools in Lechuguilla Cave (Turin and Plummer, 2000; Levy, 2007a) and Carlsbad Cavern (Chapman et al., 1992; Forbes, 2000) have identified a 'typical pool water' that is fresh (Total dissolved solids, 200-500 mg/L) and of the calcium-magnesium-bicarbonate type, suggesting that leakage balances inflow, and evaporation is less important. Exceptions occur near gypsum deposits (where sulfate exceeds bicarbonate) and in a few pools with minimal leakage where evaporation has concentrated the water to a brine (Forbes, 2000; Turin and Plummer, 2000; Levy and Amrhein, 2011). However, there is considerable chemical variation among pools, even nearby pools, attributed to the complexity of infiltration, evaporation, and rock interaction for each pool (Forbes, 2000; Turin and Plummer, 2000; Levy, 2007a). Combining the chemical variation with the fact that pools do not currently connect via outflow suggests that these pools are completely isolated systems. How long they have been isolated is difficult to determine, but for some it could date back to the time when Big Room pools dried up at 13.5 ka during the terminal Pleistocene drought (Polyak et al., 2012).

Data on total organic carbon (TOC) and nitrate are available for drips and pools in Carlsbad Cavern (Brooke, 1996; Van der Heidje et al., 1997) and Lechuguilla (Turin and Plummer, 2000; Levy, 2007a, 2007b), albeit not for the specific pools sampled in this study. The highest TOC values (20–40 mg/L) were found in the eastern portions of Carlsbad and attributed to a combination of bat guano and a leaky sewage line (Brooke, 1996; Van der Heidje et al., 1997). Big Room and New Mexico Room pools have values between <1 (detection limit) and 15 mg/L; Lower Cave pools are similar except for one pool with 18 mg/L (Van der Heidje et al., 1997). Higher values are near trails used by visitors and Van der Heidje et al., (1997) suggest trail maintenance, including washing, is the source of contamination. TOC values from Lechuguilla are uniformly low (<1.7 mg/L, with the exception of Briny Pool at 11 mg/L; Turin and Plummer, 2000; Levy, 2007a, 2007b). Briny Pool is a very unusual pool that is highly concentrated by evaporation (Levy and Amrhein, 2011).

Nitrate values in Carlsbad are similar to TOC values: higher in eastern portions of the cave (36–238 mg/L), and low in the Big Room, New Mexico Room and Lower Cave (2–22 mg/L), except for one value of 30 mg/L from the Rookery (Brooke, 1996; Van der Heidje et al., 1997). Nitrate values collected by Turin and Plummer (2000) for Lechuguilla Cave are more variable, with 82% below 10 mg/L and 5 % over 30 mg/L. The average for the 95 % of pools with <30 mg/L is 6 mg/L. Levy (2007a, 2007b) reported lower average values for Lechuguilla Cave (<1.7 mg/L).

Sample Sites

A total of 17 pools were analyzed including nine pools from Carlsbad Cavern, six pools in Lechuguilla Cave and two pools in Hell Below Cave. The diversity of sample sites is greater than suggested by just three caves as Carlsbad Cavern and Lechuguilla Cave are very large caves and the sample sites are widely spaced (Figs. 2 and 3).

Three rooms from Carlsbad Cavern were sampled, the Big Room, Lower Cave and the New Mexico Room (Fig. 2). These are all in Hydrologic Domain 2 of Van der Heidje et al., (1997), which they characterized as having diffuse infiltration with recharge in Bat Cave Draw and have no significant nitrate concentrations. The Big Room is over 3.3 ha and ~200 m beneath the surface (Hill, 1987). It is the most developed area in the cave with asphalt paved trails and ~500,000 visitors/year. It is also the most impacted historically with extensive off-trail damage dating back to private ownership (pre-1923). The floor is very irregular with abundant stalagmites and many pool basins (mostly dry). Four widely spaced pools were sampled in the Big Room: BR17, BR25, BR30 and BR44 (Fig. 2). Pool BR17 is ~0.4 m deep pool and is located ~3 m from the public trail (Fig. 4A). Although not visited today, the area around the pool was heavily trampled in the past and loose debris from the surrounding area covers the pool spar on the bottom of the pool. Pool BR25 is also 2–3 m from the public trail, but is separated from the trail by several large stalagmites. BR25 is currently ~0.6 m deep, but has a well-developed shelfstone water line indicating a paleo depth >2 m. This area shows little evidence of past traffic. Pool BR30 is <2 m from the public trail and is the only sampled Big Room pool that is nearly full (Fig. 4B). The maximum depth is ~0.8 m, which is <5 cm from the overflow level. Pool BR44 is under the edge of Crystal Springs Dome, a rare (for Carlsbad) active stalagmite located adjacent to the public trail. The pool is currently up to 1 m in depth, but the paleo-water line suggests a maximum depth of >2 m in the past. Although the closest of the Big Room pools to the public trail (<1 m), this pool sits under an overhang that protects it from direct contact. However, water entering the pool runs off of Crystal Springs Dome that is in reach of visitors.

Lower Cave sits underneath the Big Room at a depth of ~240 m beneath the entrance (Hill, 1987). Visitation to Lower Cave is restricted to ranger-guided tours (12 person limit) on marked trails but not paved as in the Big Room. Three pools were sampled in Lower Cave: LC5, CC2-LC, and CC7-LC (Fig. 2). LC5 is in The Rookery, an area of active drips and many cave pearl nests (Hill, 1987; Melim and Spilde, 2018). This pool is ~4 cm deep in a very flat area of the cave. The guided trail is immediately adjacent to this pool but is elevated on plastic platforms to keep visitors from walking in the water. Pool CC2-LC is the largest pool in Lower Cave (~1 m deep), and the guided trail crosses it on a low bridge.

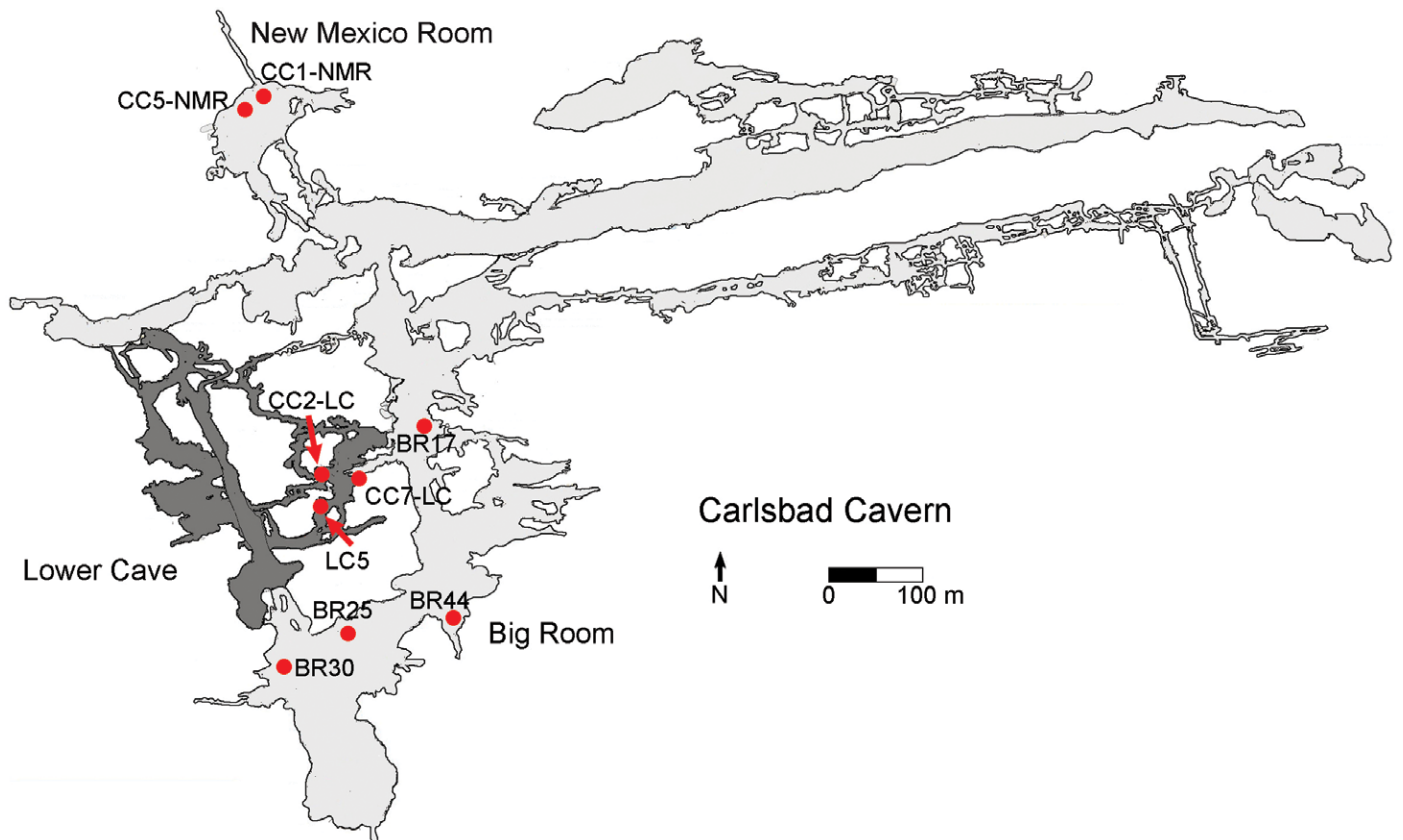


Figure 2. Map of Carlsbad Cavern showing the location of the sampled pools. Map courtesy of the Cave Research Foundation.

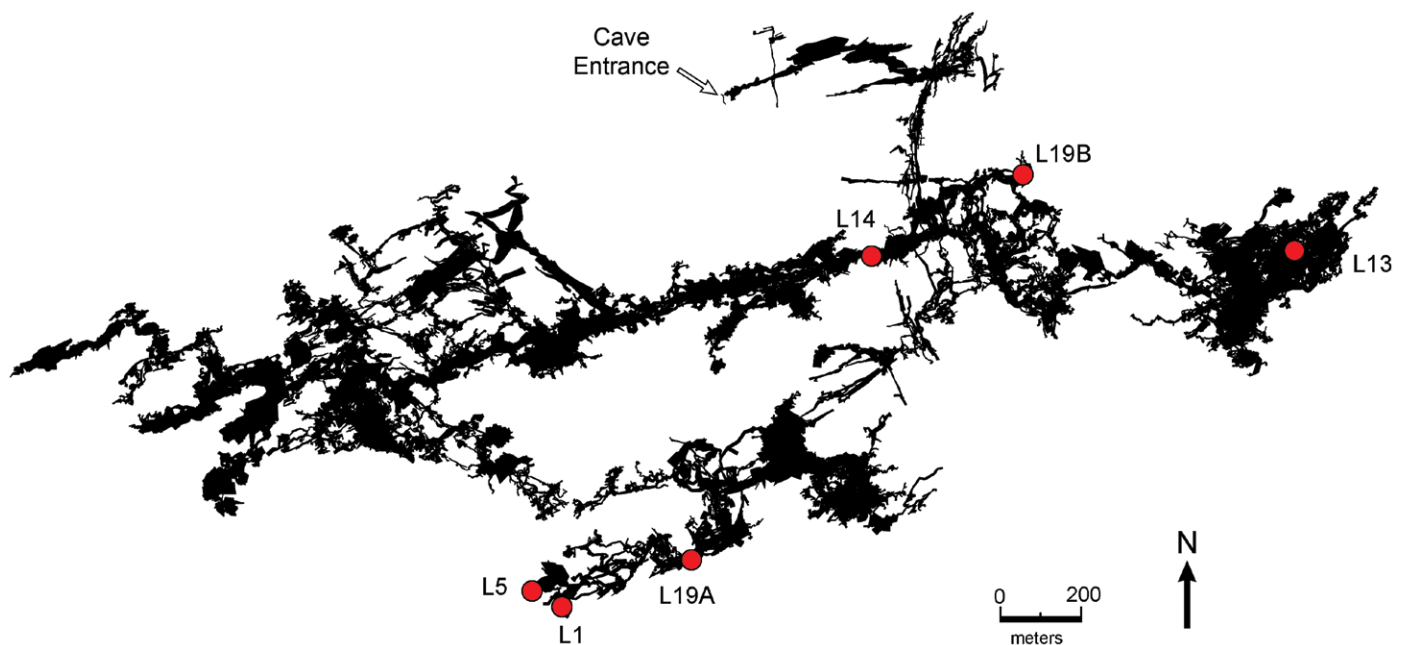


Figure 3. Map of Lechuguilla Cave showing the location of the sampled pools. Map courtesy of the National Park Service.

Like the nearby Rookery, this area has many active drips and the pool is full, or nearly so. CC7-LC, in contrast, is a small pool in an offshoot passage ~50 m from the active trail in an area almost never visited.

The final sample area in Carlsbad Cavern is the New Mexico Room, an offshoot of the main cave at approximately the same elevation as the Big Room (Hill, 1987). It was never developed for visitors and is rarely visited today. Private visits were more common in the past, but never in large numbers like in the Big Room or Lower Cave. Two adjacent

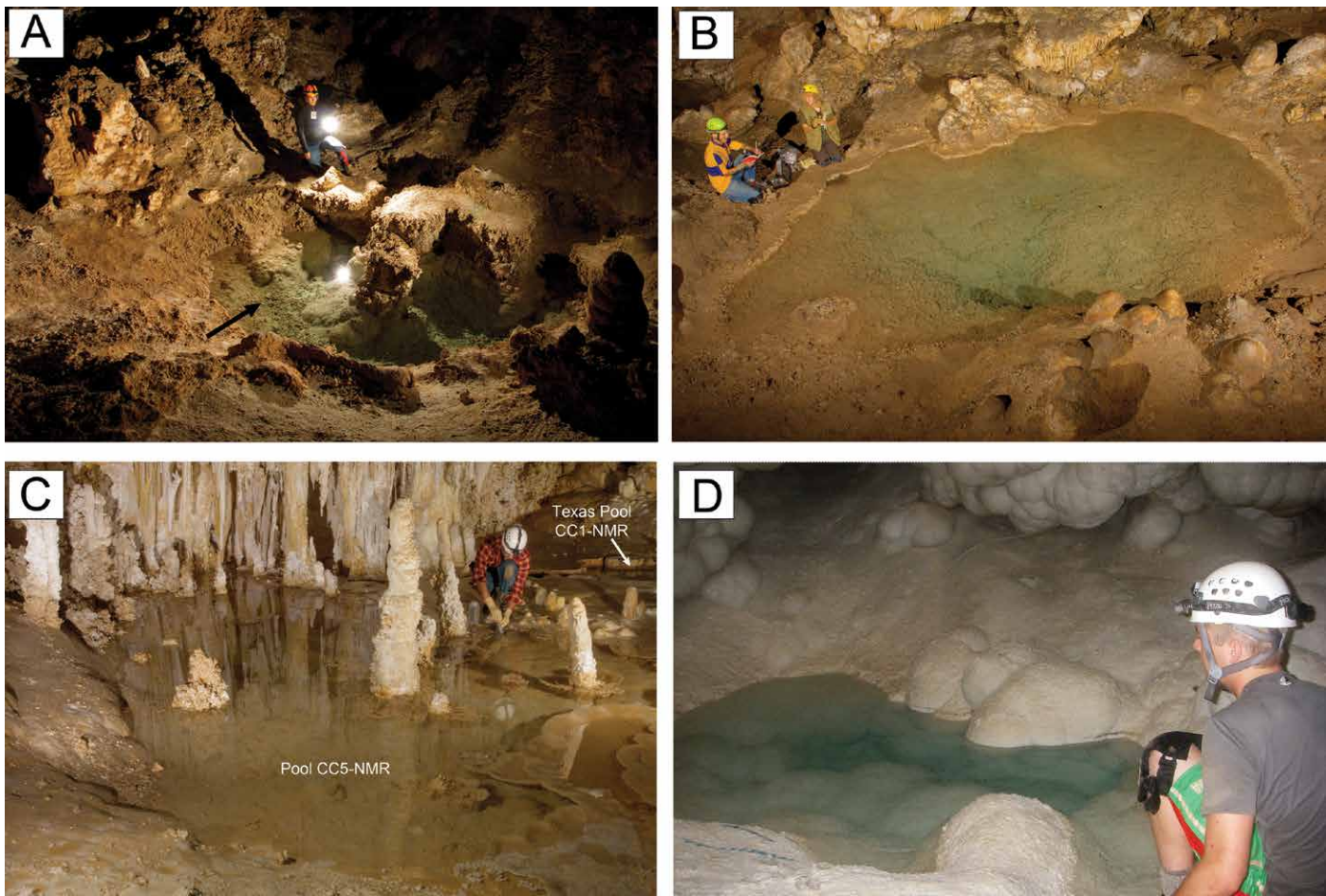


Figure 4. Representative cave pools in this study. A) Carlsbad Cavern, Big Room pool BR17. Photo taken from trail. Note loose debris in the bottom of the pool (arrow), which includes debris from the trail. B) Carlsbad Cavern, Big Room pool BR30, one of the few pools in Carlsbad that is nearly full. C) Carlsbad Cavern, New Mexico Room pools CC5-NMR and CC1-NMR. Note that CC5-NMR drains into CC1-NMR when it is full. D) Lechuguilla Cave pool L14. Photo credits: A-C, Kenneth Ingham; D, Pat Cicero.

pools were sampled, CC1-NMR and CC5-NMR (Figs. 2 and 4C). CC1-NMR is Texas Pool, a shallow (~0.2 m) pool outlined in overhanging shelfstone, which defines a paleo-depth of ~0.4 m. CC5-NMR is a larger pool that is nearly full today. When it overflows, it drains into CC1-NMR. Currently, however, CC1-NMR only collects water from drips and flowstone. The marked trail goes by both pools.

Lechuguilla Cave is also located in the Carlsbad Caverns National Park, about 5.8 km northwest of Carlsbad Cavern (Fig. 1). Access to Lechuguilla Cave is limited by permit, with 50–100 research/exploration visitors per year for the whole cave. Six pools were sampled in Lechuguilla Cave (Fig. 3). Pool L1 is a drinking water source (so more visited than some) near an underground campsite. It is 17 m × 14 m, ~12 m deep, and full. The pool is coated in pool spar and fed by several areas of flowstone on one side. Pool L5 is an irregular pool coated in yellowish pool spar, instead of the more common white, in the Vesuvius area of Lechuguilla Cave. It is shallow (~20 cm) with maximum dimensions of 4 m × 6.5 m in a T-shape. The pool is full to the water line marked by minor areas of shelfstone. However, most of the pool edge is flowstone coming in from surrounding stalagmites. Pool L13 is ~2 m × 5 m and ~1.5 m deep, but is the remnant of a larger pool up to 11 m deep. The larger pool is completely covered in pool spar and pool fingers. There is no evidence of a mineral water line at the current depth. Pool L14 is in the Deep Secrets area of Lechuguilla (Fig. 4D). The pool is a small (2 m × 4 m, <0.5 m deep) remnant of a much larger pool (>3 m deep) coated in pool spar clouds. There is a faint mineral water line near the current water line, suggesting Pool L14 has been stable at this depth for some time. Pool L19A is a small (0.5 × 1 m; ~10 cm deep) oval pool perched on a shelf in an area of continuous flowstone in the Tower Place area. The pool floor is coated in pool spar and the pool is full. L19B is a pool in the Nirvana area of Lechuguilla. No description is available for this pool.

Hell Below Cave is located in southeastern New Mexico in the Guadalupe Mountains, about 60 km southwest of the town of Carlsbad (Fig. 1). The two adjacent (<1 m apart) pools sampled are in an area with minimal visitation (<10 visitors/year), approximately 60 m below the surface. Hell Below pool HB1 is larger, much deeper (>2 m), and was near

the spill level when sampled. HB2, in contrast, is small and shallow (<10 cm), was nearly full when sampled, and is fed by an active drip. HB2 likely drains into HB1 when full.

Sampling and DNA Extraction

Samples were collected under Permit #CAVE-2008-SCI-0004 (Carlsbad Caverns National Park, 2008-2013), issued to Northup, and Forest Service Permit #FS-2700-4 (10/09) (Hell Below Cave, 2011-2030) issued to Melim. Approximately one liter of water from pools was filtered through a sterile Sterivex syringe (Millipore, Billerica, MA) via a micropore 0.2µm filter, to capture microbial cells on the filter. Sucrose lysis buffer (0.5 mL) (Giovannoni et al., 1990) was added aseptically to break open cells and stabilize the DNA. Sterivex filters were capped with the syringe and a pipette tip and were then transported to the lab on ice where they were stored in a -80 °C freezer until DNA extraction. DNA released into the sucrose lysis buffer was extracted using MoBio Power Water DNA extraction kit using the manufacturer's protocol (MoBio, Carlsbad, CA) with bead beating instead of vortexing and elution in 30 µL rather than 50 µL of Buffer EB. Purified, extracted DNA was amplified with the polymerase chain reaction (PCR), using 46F (5'-GCTAAYACATGCAAGTCG-3') as the forward primer and 1409R 5'-GTGACGGGCRGTGTGTRCAA-3' (Northup et al. 2010) as the reverse primer and AmpliTaq LD (Applied Biosystems) with an MJ thermal cycler as follows: 4 min denaturation at 94 °C, followed by 35 cycles of 45 s annealing at 55 °C, 2 min at 72 °C (extension), and 30 s at 94 °C (denaturation), with a final 45 s 55 °C annealing and 20 min 72 °C extension step after cycling was complete.

Sequencing and Quality Control

Samples were analyzed with next-generation sequencing of the 16S SSU gene bacterial V1-3 region (primer 27F, Lane, 1991) using Roche FLX and Titanium 454 technology conducted by MR DNA, Shallowater, TX (<http://www.mrdnab.com/>). All 454 data were processed in QIIME 1.9.1 (Caporaso et al., 2010). Quality control and trimming of the 454 dataset were done using the `split_libraries.py` command with a lower length (-L) of 100 bp and an upper length (-L) of 500. A quality score (-s) of 30 was chosen. Removal of erroneous sequences (denoising) and OTU clustering were done using `pick_de_novo_otus.py` pipeline with the `sumacust` option (Mercier et al., 2013). The `sumacust` algorithm is mainly useful to detect the 'erroneous' sequences created during amplification and sequencing protocols. OTUs were clustered at the 97% similarity level using `sumacust`. The `pick_de_novo` command also picks the representative set and assigns taxonomy using `uclust` (Edgar, 2006) against the *Silva* 1.28 database (McDonald et al., 2012). Chimera checking was done using USEARCH to detect artifacts created during sequencing. Good's coverage showed that we were successful in getting nearly all of the diversity from our samples (Table 1). Values ranged from 87.13 % to 99.11 % with an average value of 94.98 %. Archaea were not analyzed.

Taxonomic Analysis

Bacterial taxonomies were assigned from the phylum to genera levels. OTUs unassigned to a phylogenetic level that passed chimera checking were consolidated into an *Unassigned* category. The ten most abundant bacterial phyla or *Proteobacteria* class by percentage were displayed; the remaining assigned phyla/proteobacterial classes were condensed into an *Other* category. The same method was used to categorize bacterial genera.

Diversity Analysis

Community dissimilarity was visualized using the `phyloseq` package (McMurdie and Holmes, 2013) and `ggplot2` (Wickham, 2009) in R (R Development Core Team, 2012). Alpha diversity was analyzed using observed OTUs, Shannon, Simpson's Index of Diversity (1-D), and Chao1 indices in the `phyloseq` package. Alpha diversity measures were carried out on the raw data as recommended (McMurdie and Holmes, 2013). Observed OTUs and Simpson will scale with increasing library size; however both Chao1 and Shannon are robust measures of richness and diversity. Observed OTUs is the raw number of species OTUs present in each sample of quality controlled and clustered sequences as described above. Shannon index assumes even distribution of all OTUs, while Simpson's index is more influenced by dominated OTUs in a sample (Kim et al., 2017).

To assist in visualizing the diversity, plots and tables illustrate phyla and genera abundant in individual pools. Selection of which phyla (and genera) to illustrate is complicated by the large number present (38 phyla, 509 genera). Simply sorting by number of OTUs over-weighted the resulting lists for pools with large numbers of OTUs (mainly the Carlsbad Big Room pools). To better represent all pools, raw OTU counts were changed to Percent OTUs in each pool. Selecting only those phyla present at >5 % in any pool, 16 total phyla were plotted with less abundant phyla included as "other minor phyla" (0 % to 5 % of total) (Fig. 5). In addition, these 16 phyla are also listed in Table 2, with the more abundant phyla for each pool noted. Table 3 shows the top ten genera across all pools, selecting for those genera present in the most pools. While useful, this table leaves 23 % to 83 % of the genera in the "other genera" category, and thus misses much of the diversity. Trying the top ten genera in each pool included 88 genera, too many to plot. The final genera plot with 25 genera (Fig. 6), includes only the top two genera in each pool (by percent abundance). Between 15 % to 64 % (average 35 %) of the genera in any single pool are not detailed, but included as "other minor genera." To more fully

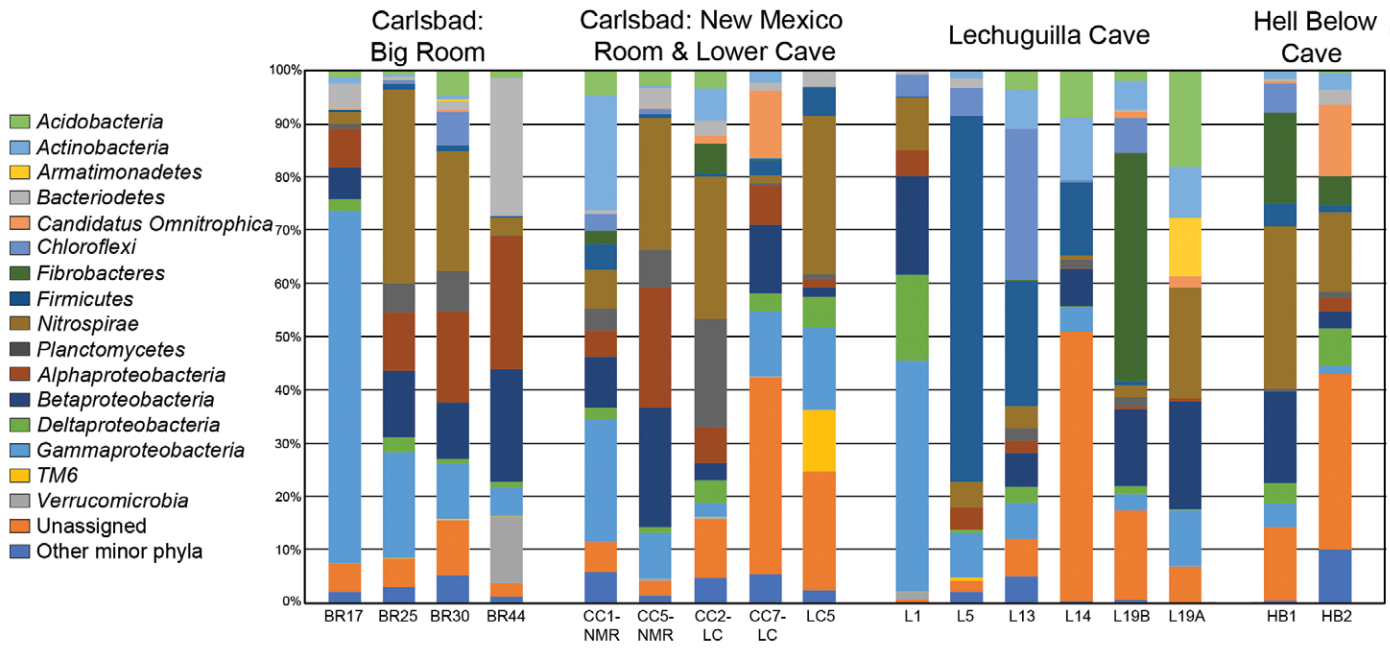


Figure 5. Top phyla/proteobacterial class distribution across pools in Carlsbad Cavern (Big Room and New Mexico Room-Lower Cave), Lechuguilla Cave, and Hell Below Cave.

Top Two Genera: All Pools

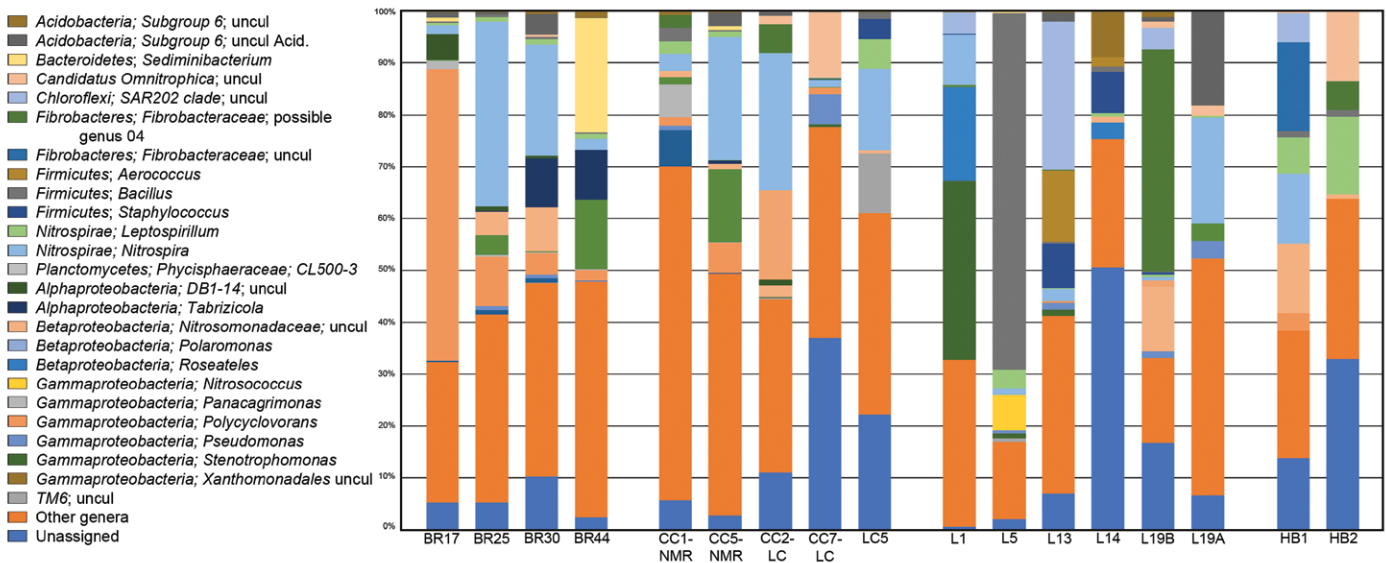


Figure 6. Distribution of the top two genera in pools in Carlsbad Cavern (Big Room and New Mexico Room-Lower Cave), Lechuguilla Cave, and Hell Below Cave.

illustrate the diversity at the genus level, all genera (total of 40) present at 5 % or more in any pool are listed in Table 4. In addition, three pairs of pools were plotted showing all genera above two percent abundance. These include one relatively low-diversity pair (BR17 – L19A, Fig. 7), and two pairs of pools that are adjacent to each other (CC1-NMR – CC5-NMR and HB1 – HB2; Figs. 8 and 9).

RESULTS

Calculated Alpha Diversity of Pools

Pools found in the Big Room of Carlsbad Cavern have the highest number of observed OTUs per pool, ranging from 231 to 925 (Table 1). The remaining pools all have a similar range of observed OTUs: pools in Carlsbad Cavern's Lower Cave and New Mexico Room have between 82 and 226; Hell Below Cave's two pools have 81 and 108, and Lechuguilla

Genera List: Low Diversity Pools BR17 vs. L19A

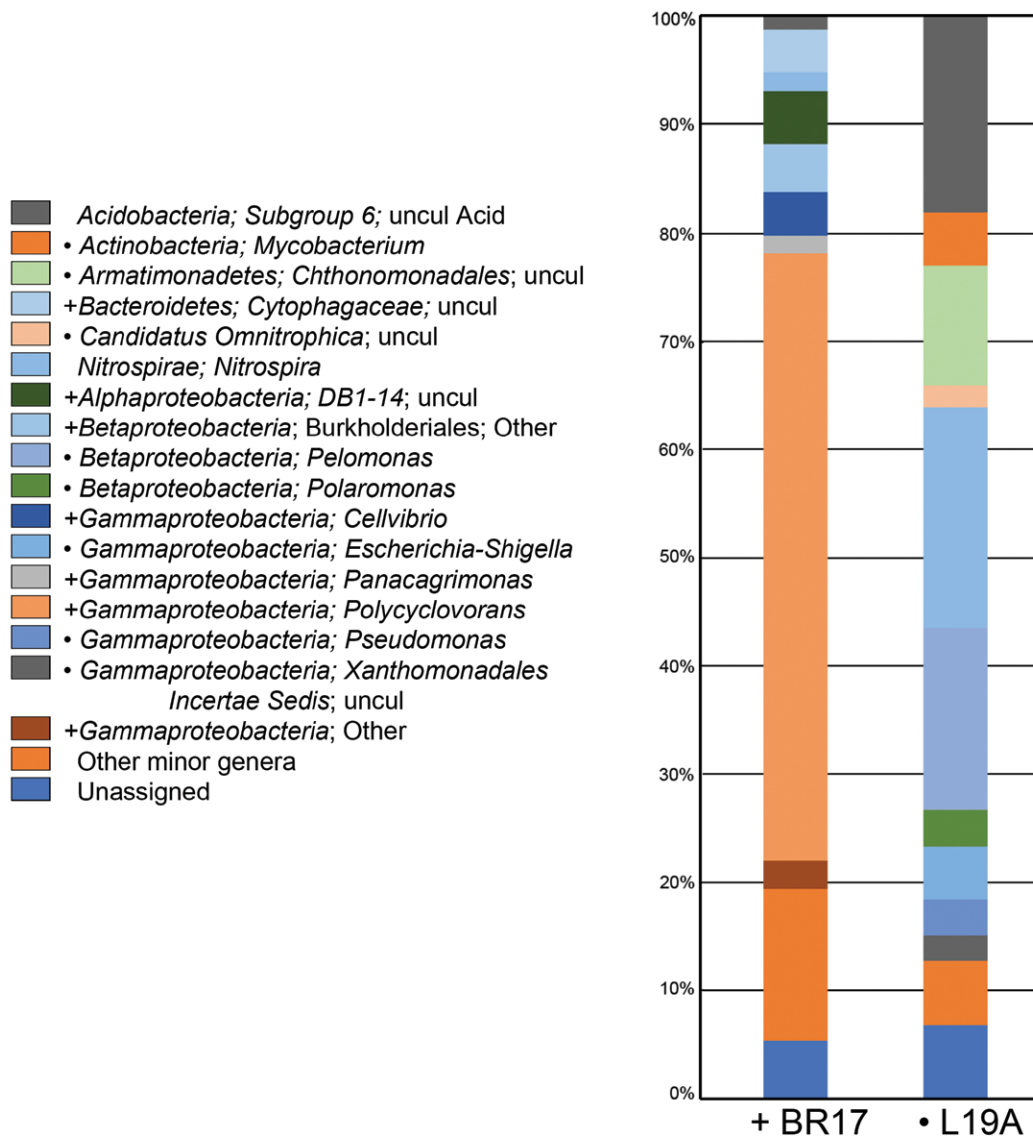


Figure 7. Comparison of genera at >2 % abundance in two relatively low diversity pools, BR17 (Simpson 0.677) and L19A (Simpson 0.881). Code: + genera present only in BR17; • genera present only in L19A.

Table 1). No cave has exclusively high or low diversity values; all caves have Shannon and Simpson values that span the range of total diversity values. Lechuguilla pools are on average less diverse (Shannon of 3.7 ± 0.9 ; Simpson of 0.831 ± 0.112) and include the two pools with lowest Shannon values (L1 and L5). The Big Room has higher diversity except for BR17 (Shannon of 3.5; Simpson of 0.677) that is dominated (56 %) by a single genus, *Polycyclovorans*. Without BR17, the rest of the Big Room has Shannon values of 5.2 ± 0.7 and Simpson values of 0.913 ± 0.036 . New Mexico Room and Lower Cave pools show slightly higher diversity values than the Big Room, with Shannon values of 5.3 ± 0.9 and Simpson values of 0.941 ± 0.042 . Hell Below Cave pools are slightly lower than the Big Room, with Shannon values of 4.8 ± 0.7 and Simpson values of 0.939 ± 0.034 .

Phylum Diversity

Major differences in the bacterial phyla/proteobacterial classes can be seen within caves and between caves (Fig. 4, Table 2); no two pools have the same distribution of bacteria, even those adjacent to each other in a cave. *Nitrospirae*, *Alpha*-, *Beta*-, and *Gammaproteobacteria* are the only phyla/proteobacterial classes found >5% abundance in nine or more cave pools. *Actinobacteria*, *Chloroflexi*, *Fibrobacteres*, *Firmicutes*, and *Plantomycetes* are at >5 % in 4–6 pools.

Cave pools have the lowest value of 24 OTUs and range up to 151. Chao1 diversity scales proportionally with observed OTUs (Table 1; Fig. 10). BR25 has both the highest number of OTUs and the highest Chao1 value (1738). Lechuguilla Cave pool L1 has the lowest number of observed species and the lowest Chao1 value (31).

Shannon diversity averages 4.6 ± 1.1 and ranges from 2.6 in pool L5, Lechuguilla Cave, to 6.4 in CC1-NMR, Carlsbad Cavern. Simpson averages 0.881 ± 0.099 and ranges from 0.622 in pool L5, Lechuguilla Cave, to 0.981 in CC1-NMR, Carlsbad Cavern. Even though Simpson weighs abundant OTUs more, the two indices generally agree on the diversity (Ta-

Table 1. Alpha diversity indices and Good's Coverage for the study pools.

Study Pool	Species OTUs	Chao1	Shannon	Inverse Simpson	Good's Coverage, %	Unassigned, %
Carlsbad, Big Room						
BR17	231	343	3.5	0.677	98.0	5
BR25	925	1738	5.3	0.885	97.6	5
BR30	680	1185	5.8	0.953	97.6	10
BR44	522	1065	4.5	0.899	97.4	2
Carlsbad Cavern, New Mexico Room & Lower Cave						
CC1-NMR	236	335	6.4	0.981	98.7	6
CC5-NMR	151	278	4.7	0.907	97.0	3
CC2-LC	113	141	4.5	0.888	98.6	11
CC7-LC	170	213	6.0	0.976	98.7	37
LC5	82	231	4.9	0.953	98.8	22
Lechuguilla Cave						
L1	24	31	2.9	0.809	99.3	1
L5	89	171	2.6	0.622	99.3	2
L13	71	88	5.0	0.945	96.9	7
L14	102	250	4.0	0.886	99.4	51
L19B	151	249	4.3	0.842	99.0	17
L19A	33	40	3.5	0.881	99.7	7
Hell Below Cave						
HB1	81	147	4.4	0.916	98.0	14
HB2	108	166	5.3	0.963	99.4	33

Acidobacteria, *Armatimonadetes*, *Bacteroidetes*, *Candidatus Omnitrophica*, *Deltaproteobacteria*, *TM6*, and *Verrucomicrobia* are found in less than four pools at >5 % abundance.

The most common phylum is *Proteobacteria*, which is common in karst caves and often dominant (Tomczyk-Żak and Zielenkiewicz, 2016). *Beta*- and *Gammaproteobacteria* are the most widespread. *Betaproteobacteria* show up in all pools except L5 in Lechuguilla Cave and vary from 2 % to 22 %. All pools have *Gammaproteobacteria*, which is the second highest phylum/class overall with 66 % in BR17, and varies across all pools from 2 % to 66 %, but with generally higher percentages in Carlsbad Cavern pools (Table 2). In six pools, *Gammaproteobacteria* are either the most abundant (BR17, CC1-NMR, L1) or the second most abundant (BR25, LC5, L5). *Alphaproteobacteria* are low in Lechuguilla Cave and Hell Below Cave pools (0–5 %, absent in two), and more dominant in Carlsbad Cavern pools (2–25 %, with four pools over 11 %). *Deltaproteobacteria* are present across all pools, but only three pools have more than 5 %.

The bacterial phylum *Nitrospirae* is also noted as common in karst caves (Tomczyk-Żak and Zielenkiewicz, 2016), but not usually in the abundance found in these cave pools. *Nitrospirae* is widespread, being found in every pool water sample, but varying from <1 % to 36 %. It is the most abundant phylum in eight pools. The highest percentage of *Nitrospirae* was observed in Carlsbad Cavern's BR25 (36 %) and it is >20 % in six pools, and >10 % in two pools (Table 2).

Of the five phyla found in 4–6 pools at >5 %, *Actinobacteria*, *Chloroflexi*, and *Firmicutes*, and *Plantomycetes* are also found in some caves worldwide, but *Fibrobacteres* is rare (Tomczyk-Żak and Zielenkiewicz, 2016). *Actinobacteria* is noted as abundant in caves, second only to *Proteobacteria*, but most commonly on surfaces (Tomczyk-Żak and Zielenkiewicz, 2016). *Actinobacteria* is present in 11 pools at >1 %, but at <5 % in five of those. In the other six, it ranges from 7 % to 22 % (Table 2). *Actinobacteria* is not the dominant phylum in any pool. *Chloroflexi* are minor constituents in several pools (1 % to 7 %, Table 2), except in Lechuguilla Cave pool L13, where it is the dominant phylum at 28 % abundance. However, five of the nine Carlsbad Cavern pools had no *Chloroflexi*, nor did one Lechuguilla pool and one Hell Below pool. *Firmicutes*, another phylum commonly found in caves (Tomczyk-Żak and Zielenkiewicz, 2016), showed the highest percentage, 69 % in Lechuguilla pool L5 (Vesuvius), of any phylum observed in any pool (Table 2). In addi-

Table 2. Distribution of phyla/Proteobacteria class in study cave pools with more common phyla in each pool highlighted. Orange, >35 %; dark blue, 20–35 %; light blue, 10–20 %.

Phyla/ Proteobacterial class	Carlsbad Cavern, Big Room, %						Carlsbad Cavern, New Mexico Room						Lower Cave						Lechuguilla Cave, %						Hell Below Cave, %	
	BR17	BR25	BR30	BR44	CC1-NMR	CC5-NMR	CC2-LC	CC7-LC	LC5	L1	L5	L13	L14	L19B	L19A	HB1	HB2									
	1	5	1	0	5	3	3	0	0	0	0	4	9	2	18	0	0									
<i>Acidobacteria</i>	1	5	1	0	5	3	3	0	0	0	0	4	9	2	18	0	0									
<i>Actinobacteria</i>	1	0	1	0	22	0	6	2	0	0	1	7	12	5	10	2	3									
<i>Armatimonadetes</i>	0	0	0	0	0	0	0	0	0	0	0	0	0	0	11	0	0									
<i>Bacteroidetes</i>	5	1	2	26	1	4	3	1	3	1	2	0	0	0	0	0	3									
<i>Candidatus Omnitrophica</i>	0	0	0	0	0	0	2	13	0	0	0	0	0	1	2	0	13									
<i>Chloroflexi</i>	0	1	6	0	3	1	0	0	0	4	5	28	1	7	0	6	0									
<i>Fibrobacteres</i>	0	0	0	0	3	0	6	0	0	0	0	0	0	43	0	17	5									
<i>Firmicutes</i>	0	1	1	0	5	1	1	3	5	0	69	24	14	1	0	4	1									
<i>Nitrospirae</i>	2	36	22	3	7	25	27	2	30	10	5	4	1	2	21	30	15									
<i>Planctomycetes</i>	1	5	8	0	4	7	20	0	1	0	0	2	2	2	0	1	1									
<i>Alphaproteobacteria</i>	7	11	17	25	5	23	7	7	2	5	4	2	0	1	1	0	3									
<i>Betaproteobacteria</i>	6	13	11	21	10	22	3	13	2	18	0	6	7	14	20	17	3									
<i>Deltaproteobacteria</i>	2	3	1	1	2	1	4	3	6	16	1	3	0	1	0	4	7									
<i>Gammaproteobacteria</i>	66	20	10	5	23	9	3	12	15	43	8	7	5	3	10	4	2									
<i>TM6</i>	0	0	0	0	0	0	0	0	12	0	1	0	0	0	0	0	0									
<i>Verrucomicrobia</i>	0	0	0	13	0	0	0	0	0	2	0	0	0	0	0	0	0									
Other minor phyla	2	3	5	1	6	1	5	5	2	0	2	5	0	1	0	0	10									
Unassigned	5	5	10	2	6	3	11	37	22	1	2	7	51	17	7	14	33									

tion, *Firmicutes* are abundant in two other Lechuguilla pools (L13 at 24 % and L14 at 14 %). Elsewhere, *Firmicutes* are absent in four pools (BR17, BR44, L1, and L19A) and present at 1 % to 5 % in the remaining pools. *Planctomycetes* is absent to low (0 % to 2 %) in Lechuguilla Cave and Hell Below Cave pools and four Carlsbad Cavern pools, but are more present (4 % to 8 %) in the four other Carlsbad pools, reaching, 20 % in CC2-LC in Lower Cave. The *Fibrobacteres* phylum is absent from 12 pools across all three caves, present but minor (3% to 6 %) in two Carlsbad Cavern pools and one Hell Below pool, but high in two pools (43 % in Lechuguilla Cave pool L19B and 17% in Hell Below Cave pool HB1).

Acidobacteria, *Bacteroidetes* and *Verrucomicrobia* are widespread in caves (Tomczyk-Żak and Zielenkiewicz, 2016), but less so in the study cave pools (Table 2). *Acidobacteria* are present in 10 pools but at low levels (<5 %) except in two Lechuguilla Cave pools (18 % in L19A and 9 % in L14). *Bacteroidetes* is the most abundant phylum in one Carlsbad Cavern pool, with 26 % abundance in BR44. Other pools in Carlsbad Cavern contained 1 % to 5 % abundance of *Bacteroidetes*. Four of the six Lechuguilla Cave pools and one Hell Below Cave pool contained no *Bacteroidetes*. The other three pools in these two caves contained 1 % to 3 % *Bacteroidetes*. *Verrucomicrobia* is only found in two pools (at a relative abundance greater than 1 %), but makes up 13% of the microbiota in BR44.

Bacterial phyla *Armatimonadetes*, *Candidatus Omnitrophica*, and *TM6* are not known from many caves (Tomczyk-Żak and Zielenkiewicz, 2016), but each is found at >10 % in one or two pools. *Armatimonadetes* is 11% in L19A; *Candidatus Omnitrophica* is 13 % in CC7-LC and 13 % in HB2; *TM6* is 12 % in L5 (Table 2). Other minor phyla (Table 2) include phyla present at <5 % in any pool. Five of those are present at 2-5% including *Cyanobacteria*, *Gemmatimonadetes*, *Saccharibactia*, *SM2F11*, and Other Proteobacteria.

Genera Diversity

Of the top 10 most widespread bacterial genera present within these phyla/proteobacterial classes (Table 3), none were

Table 3. Distribution of top ten most widespread genera in study pools with abundances highlighted. Orange >35 %; dark blue 20–35 %; light blue 10–20 %; peach 2–10 %.

No. OTU ID	Carlsbad Cavern, Big Room, %				Carlsbad Cavern, Lower Cave				Lechuguilla Cave, %					Hell Below Cave, %			
	BR17	BR25	BR30	BR44	CC1-NMR	CC5-NMR	CC2-LC	CC7-LC	LC5	L1	L5	L13	L14	L19B	L19A	HB1	HB2
	1	0	4	0	0	3	1	0	0	0	0	2	0	1	18	0	0
<i>Acidobacteria</i> ; <i>Subgroup 6</i> ; uncul Acid.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
<i>Actinobacteria</i> ; <i>Propionibacterium</i>	0	0	0	0	5	0	0	0	0	0	1	4	6	5	1	2	1
<i>Candidatus Omnitrophica</i> ; uncul	0	0	0	0	0	0	2	13	0	0	0	0	0	1	2	0	13
<i>Firmicutes</i> ; <i>Bacillus</i>	0	0	0	0	3	0	0	0	1	0	0	0	0	0	0	1	1
<i>Nitrospirae</i> ; <i>Leptospirillum</i>	0	1	1	1	2	1	0	0	6	0	4	0	1	0	0	7	15
<i>Nitrospirae</i> ; <i>Nitrospira</i>	2	36	21	2	3	24	26	1	16	10	1	2	0	1	20	14	0
<i>Betaproteobacteria</i> ; <i>Polaromonas</i>	0	4	0	13	1	14	0	0	0	0	0	0	0	0	3	0	0
<i>Betaproteobacteria</i> ; <i>Nitrosomonadaceae</i> ; uncul	0	4	8	0	1	1	2	0	1	0	0	0	1	12	0	13	1
<i>Deltaproteobacteria</i> ; <i>Haliangium</i>	0	0	0	0	0	0	1	1	2	16	0	0	0	0	0	1	2
<i>Gammaproteobacteria</i> ; <i>Polyclovorans</i>	56	10	4	2	2	6	0	1	0	0	0	0	0	0	0	3	0
Other genera	34	39	49	79	76	49	57	46	52	73	23	83	40	62	48	44	34
Unassigned	5	5	10	2	6	3	11	37	22	1	2	7	51	17	7	14	33

found in all pools. The most widespread was *Nitrospira*, a genus classified to the *Nitrospirae* phylum that was present in eight pools at >10 % (and most abundant in six of those eight, Table 4) and present above 2% in four more. *Leptospirillum*, another genus in the *Nitrospirae* phylum, was present >2 % in five pools, including two pools that lack *Nitrospira*. The *Gammaproteobacteria Polyclovorans* was the second most widespread genus, found in six of the Carlsbad pools and one pool in Hell Below Cave. In Carlsbad Cavern pool BR17, *Polyclovorans* was 56 % of the OTUs. Other widespread genera include the *Actinobacteria Propionibacterium* (five pools, 2 % to 6 %); *Betaproteobacteria Polaromonas* (4 pools, 3 % to 14 %); and *Deltaproteobacteria Haliangium* (3 pools, 2 % to 16 %). *Bacillus*, a genus in *Firmicutes* was found in only two pools over 2 %, but one of those (Lechuguilla Cave pool L5) had 69 %, the highest abundance of any genus in this study. Completing the list of the top 10 are three uncultured genera: *Acidobacteria, Subgroup 6*; uncultured *Acidobacteria* bacterium (four pools, 2 % to 18 %); *Candidatus Omnitrophica*, uncultured bacteria (four pools, 2 % to 13 %); and *Betaproteobacteria Nitrosomonadaceae*, uncultured (five pools, 2 % to 13 %).

Table 4 lists all genera present at ≥5 % in any pool, which is 40 genera for only 17 pools. 28 of these 40 genera (70 %) were only present in one pool at ≥5 %. Only six of these 40 genera were present at ≥5 % in three or more pools, one of which, *Nitrospira*, was present in eight pools. *Nitrospira* was also the most abundant genus in six pools (five in Carlsbad Cavern: BR25, BR30, CC5-NMR, CC2-L2, LC5, and one in Lechuguilla Cave: L19A). Each of the other 11 pools had a different most abundant genus (Table 4), including four already noted above in the top 10 most widespread genera (Carlsbad Cavern pool CC7-LC, *Candidatus Omnitrophica*, uncultured bacteria; Lechuguilla Cave pool L5, *Bacillus*; Hell Below Cave pool HB2, *Leptospirillum*; and Carlsbad Cavern pool BR17, *Polyclovorans*). Two Lechuguilla Cave pools (L13, *Chloroflexi*; SAR202 clade; uncultured and L19B, *Fibrobacteres*; *Fibrobacteraceae*; possible genus 04) have top genera also found in other pools at ≥5 %, but in the other five pools (Carlsbad Cavern pools BR44 and CC1-NMR, and Lechuguilla Cave pools L1, L5, and L15) the most abundant genus in the pool was only found at ≥5 % in that one pool.

Since five pools have top genera found in only one pool at >2 %, a bar plot of the top two genera in each pool was done to include all of the top genera (total 25 genera; Fig. 5). Genera not included (“Other genera”) range from 15 % (in Lechuguilla Cave pool L5, which has 69 % *Bacillus*) to 64 % (in Carlsbad Cavern pool CC1-NMR, the most diverse pool (Table 1)). Pools with >10 % unassigned OTUs included

Table 4. List of genera present at ≥5% in any pool. Also listed are the number of pools each is found in at that level and which (if any) pools the genus in the most abundant genus.

OTU, Genus	No. of pools >5%	Most abundant genus in pool(s)
<i>Acidobacteria</i> ; Subgroup 6; uncul Acid	1	...
<i>Acidobacteria</i> ; Subgroup 6; uncul	1	L14
<i>Actinobacteria</i> ; <i>Propionibacterium</i>	3	...
<i>Armatimonadetes</i> ; <i>Chthonomonadales</i> ; uncul	1	...
<i>Bacteroidetes</i> ; <i>Sediminibacterium</i>	1	BR44
<i>Candidatus Omnitrophica</i> ; uncul	2	CC7-LC
<i>Chloroflexi</i> ; AKIW781; uncul	1	...
<i>Chloroflexi</i> ; SAR202 clade; uncul	2	L13
<i>Fibrobacteres</i> ; <i>Fibrobacteraceae</i> ; possible genus 04	3	L19B
<i>Fibrobacteres</i> ; <i>Fibrobacteraceae</i> ; uncul	1	HB1
<i>Firmicutes</i> ; <i>Bacillus</i>	1	L5
<i>Firmicutes</i> ; <i>Staphylococcus</i>	2	...
<i>Firmicutes</i> ; <i>Aerococcus</i>	1	...
<i>Nitrospirae</i> ; <i>Nitrospirales</i> ; 0319-6A21; uncul	2	...
<i>Nitrospirae</i> ; <i>Leptospirillum</i>	3	HB2
<i>Nitrospirae</i> ; <i>Nitrospira</i>	8	BR25, BR30, CC5-NMR, CC2-LC, LC5, L19A
<i>Planctomycetes</i> ; OM190; Other	1	...
<i>Planctomycetes</i> ; <i>Phycisphaeraceae</i> ; CL500-3	1	...
<i>Alphaproteobacteria</i> ; <i>Rhodobacter</i>	1	...
<i>Alphaproteobacteria</i> ; <i>Tabrizicola</i>	2	...
<i>Alphaproteobacteria</i> ; <i>Reyranella</i>	1	...
<i>Betaproteobacteria</i> ; <i>Pelomonas</i>	1	...
<i>Betaproteobacteria</i> ; <i>Polaromonas</i>	2	...
<i>Betaproteobacteria</i> ; <i>Roseateles</i>	1	...
<i>Betaproteobacteria</i> ; <i>Comamonadaceae</i> ; Other	1	...
<i>Betaproteobacteria</i> ; <i>Massilia</i>	1	...
<i>Betaproteobacteria</i> ; <i>Neisseriaceae</i> ; uncul	1	...
<i>Betaproteobacteria</i> ; <i>Nitrosomonadaceae</i> ; uncul	3	...
<i>Deltaproteobacteria</i> ; <i>Haliangium</i>	1	...
<i>Gammaproteobacteria</i> ; <i>Nitrosococcus</i>	1	...
<i>Gammaproteobacteria</i> ; <i>Escherichia-Shigella</i>	1	...
<i>Gammaproteobacteria</i> ; <i>Legionella</i>	1	...
<i>Gammaproteobacteria</i> ; <i>Pseudomonas</i>	1	...
<i>Gammaproteobacteria</i> ; <i>Polycyclovorans</i>	3	BR17
<i>Gammaproteobacteria</i> ; <i>Xanthomonadales</i> ; uncul	1	CC1-NMR
<i>Gammaproteobacteria</i> ; <i>Panacagrimonas</i>	1	...
<i>Gammaproteobacteria</i> ; <i>Stenotrophomonas</i>	1	L1
<i>Saccharibacteria</i> ; uncul	1	...
TM6; uncul	1	...
<i>Verrucomicrobia</i> ; <i>Opiritatus</i>	1	...
Present at ≥5% in one pool	28	
Present at ≥5% in two pools	6	
Present at ≥5% in three or more	6	
Total Genera ≥5%	40	

Genera List: Hell Below Cave

- *Actinobacteria*; *Gaiellales*; uncul
- *Bacteroidetes*; *Sporocytophaga*
- *Candidatus Omnitrophica*; uncul
- + *Chloroflexi*; SAR202 clade; uncul
- *Fibrobacteres*; *Fibrobacteraceae*; possible genus 04
- + *Fibrobacteres*; *Fibrobacteraceae*; uncul
- *Nitrospirae*; *Leptospirillum*
- + *Nitrospirae*; *Nitrospira*
- + *Nitrospirae*; Nitrospirales; 0319-6A21; uncul
- *Alphaproteobacteria*; *Rhodospirillaceae*; uncul
- + *Betaproteobacteria*; *Nitrosomonadaceae*; uncul
- *Gammaproteobacteria*; *Coxiella*
- + *Gammaproteobacteria*; *Polycyclovorans*
- + *Deltaproteobacteria*; *Candidatus Entotheonella*
- *Deltaproteobacteria*; *Myxococcales*; *Blrii41*; uncul
- *Saccharibacteria*; uncul
- Other minor genera
- Unassigned

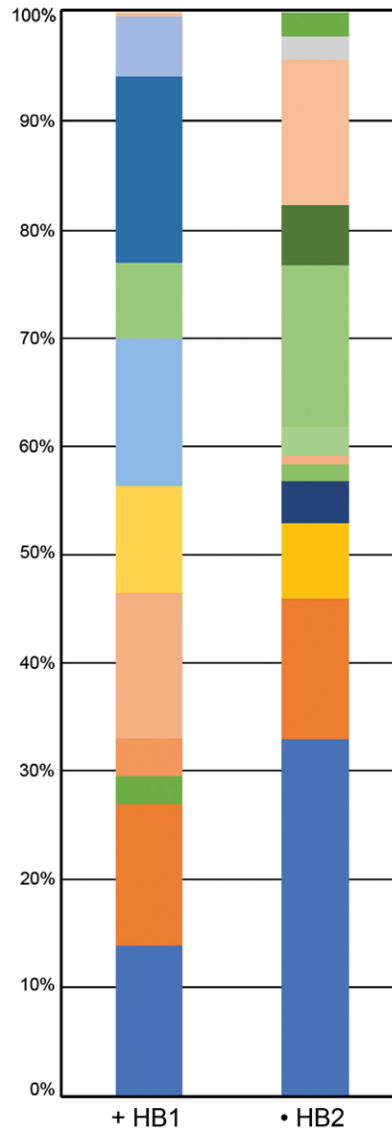


Figure 8. Comparison of genera at >2 % abundance in two relatively high diversity pools, HB1 (Simpson 0.916) and HB2 (Simpson 0.963). Pool HB2 drains into HB1 when full. Code: + genera present only in HB1; • genera present only in HB2.

adjacent pools with higher diversity, Hell Below Cave pools HB1 and HB2 (Fig. 8, Table 1), out of 16 genera only one, *Leptospirillum*, was found in both pools. Of the other 15 genera, seven were in HB1 and eight were in HB2. Based on the setting, HB2 likely drains into HB1 during high flow conditions. Comparing again two adjacent Carlsbad Cavern pools CC1-NMR and CC5-NMR, including the highest diversity pool, CC1-NMR (Fig. 9, Table 1), out of 31 genera only two, *Nitrospira* and *Polycyclovorans* were present in both pools. Of the other 29 genera, 19 were in CC1-NMR and 10 were in CC5-NMR. As for the Hell Below pair, pool CC5-NMR drains into pool CC1-NMR when full (Fig. 4C), although there are no historical reports of this happening. CC1-NMR also has 33 % other minor genera, so even this plot does not fully show the diversity.

DISCUSSION

Introduction

Cave pools in an active epiphreatic zone are replenished with water and groundwater microbial assemblages on an annual basis (Shabarova et al., 2013; 2014), while pools in the vadose zone are thought to function more as “static collectors of microbial diversity” (Shabarova and Pernthaler, 2010). An alternative hypothesis, particularly for isolated vadose pools as the study pools, is that vadose pools act as incubators of diversity. Thus, the microbial community of each pool

four in Carlsbad Cavern (BR30, CC2-LC, CC7-LC, LC5), two in Lechuguilla Cave (L14, L19B) and both pools in Hell Below Cave (HB1, HB2). Besides the widespread genera already noted above, *Betaproteobacteria* and *Nitrosomonadaceae*, uncultured were present in five pools, including two pools at 13 % of genera (L19B and HB1). In addition, 15 of these 25 genera were only found in one or two pools.

To illustrate the microbial diversity better, three pairs of pools were selected to show all genera above 2 % (Figs. 7, 8, and 9). Starting with relatively low diversity pools, Carlsbad Cavern pool BR17 and Lechuguilla Cave pool L19A (Fig. 7; Table 1), out of 17 genera only *Nitrospira* and *Acidobacteria*, Subgroup 6, uncultured *Acidobacteria* bacterium occurred in both pools (Fig. 7). Of the other 15 genera, seven were only in BR17 and eight were only in L19A. Comparing two

Genera List: Carlsbad Cavern; New Mexico Room

- *Acidobacteria; Subgroup 6; uncul Acid*
- + *Actinobacteria; Agrococcus*
- + *Actinobacteria; Gaiella*
- + *Actinobacteria; Janibacter*
- + *Actinobacteria; Knoellia*
- + *Actinobacteria; Lechevalieria*
- + *Actinobacteria; Propionibacterium*
- *Bacteroidetes; Cytophagaceae; uncul*
- + *Fibrobacteres; Fibrobacteraceae; possible genus 04*
- + *Firmicutes; Bacillus*
- + *Firmicutes; Paenibacillus*
- + *Nitrospirae; Leptospirillum*
- *Nitrospirae; Nitrospira*
- + *Planctomycetes; Phycisphaeraceae; SM1A02*
- *Planctomycetes; Planctomyces*
- *Alphaproteobacteria; Novosphingobium*
- *Alphaproteobacteria; Reyranella*
- *Alphaproteobacteria; Rhodobacter*
- + *Alphaproteobacteria; Rhodospirillales; KCM-B-15*
- *Betaproteobacteria; Burkholderiales; Other*
- + *Betaproteobacteria; Comamonadaceae; uncul*
- *Betaproteobacteria; Comamonadaceae; Other*
- *Betaproteobacteria; Polaromonas*
- *Betaproteobacteria; Undibacterium*
- + *Betaproteobacteria; Other*
- + *Gammaproteobacteria; Acinetobacter*
- + *Gammaproteobacteria; Panacagrimonas*
- *Gammaproteobacteria; Polycyclovorans*
- + *Gammaproteobacteria; Xanthomonadales uncul*
- + *Gammaproteobacteria; Xanthomonadales uncul, other*
- + *Proteobacteria; Other*
- Other minor genera
- Unassigned

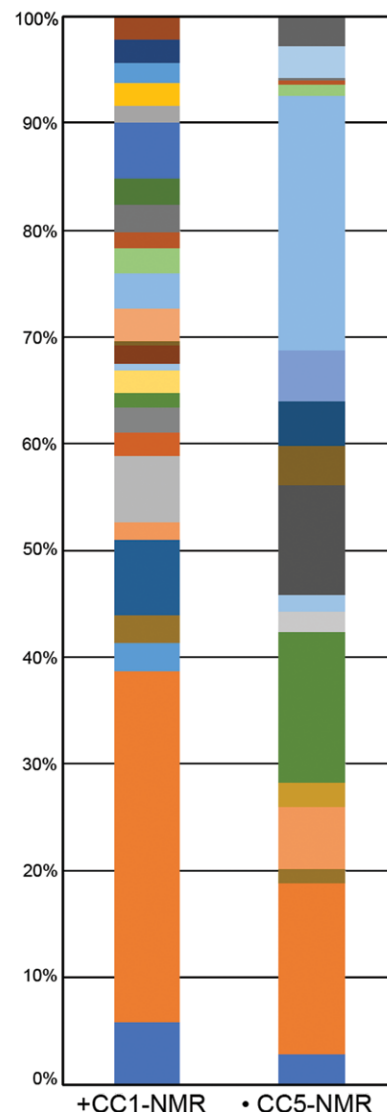


Figure 9. Comparison of genera >2 % abundance in two relatively high diversity pools, CC1-NMR (Simpson 0.981) and CC1-NMR (Simpson 0.907). CC5-NMR drains into CC1-NMR when full. Code: + genera present only in CC1-NMR; • genera present only in CC1-NMR.

decades appear to be long enough for unique communities to evolve, even without significantly different chemistry. With this idea in mind, a number of topics can be addressed with this data set, including potential roles for the bacterial genera found, a comparison between examples of previously-studied cave microbial diversity and these pool results, and evaluating the possible human impact, particularly in the heavily-visited Big Room.

Potential Roles of Bacteria in Study Pools

The diversity of bacterial genera present in the study pools provides the opportunity to identify possible roles that these bacteria may play in cave pools. A predominant potential ecosystem function in the study pools is the presence of putative nitrogen cyclers. Nitrogen is often limited in caves due to their oligotrophic nature, especially in arid land caves (Levy, 2007; Ortiz et al., 2014). Research in caves over the last two decades (e.g., Holmes et al., 2001; Chen et al., 2009; Tetu et al., 2013; De Mandel et al., 2017) revealed possible key roles that microorganisms play in the cycling of nitrogen, including nitrification (ammonia and nitrite oxidation), dissimilatory nitrate reduction, and denitrification. The presence of several potential nitrogen cycling bacteria in the study pools suggests that nitrogen cycling may also be an important microbial role in cave pool ecosystems.

should reflect not only what can survive in the pool, but also how that community has evolved over time.

Based on estimates of the last time pools in Carlsbad were full (13.5 ka; Polyak et al., 2012), most of these individual pool ecosystems have likely been isolated for thousands of years, perhaps even longer. However, the Hell Below pools and Carlsbad Cavern New Mexico Room pools (HB1 – HB2, CC1-NMR – CC2-NMR; Figs. 8 and 9), have probably been isolated from each other for a much shorter time period, perhaps even decades since a large rain event overflowed the upper pools and drained into the lower pools. Since these pools share water, large differences in water chemistry are unlikely. Even so, the bacterial community differences between these paired pools are as great as be-

tween any two pools in this study (Figs. 8 and 9), so mere decades

The most abundant genus across these pools (Fig. 6; Table 4) is the bacterial genus *Nitrospira*, a well-known nitrite oxidizer (Koch et al., 2015). *Nitrospira* is highest in Carlsbad Cavern's Big Room pool BR25 pool with 36 %, and there are four other pools in Carlsbad Cavern with high *Nitrospira* varying between 20 % to 26 %. Only one other study pool exhibited a greater than 20 % abundance (L19A in Lechuguilla Cave). Across all study pools, eight pools ranged from 10 % to 36 % and only two study pools had no *Nitrospira*. Earlier studies (Holmes et al., 2001; Tetu et al., 2013) of an unusual formation in the flooded cave passages of Weebubbie Cave located in the Nullarbor region of Australia, documented the presence of *Nitrospira*, plus some other potential nitrification and denitrification archaea and bacteria.

In addition to *Nitrospira*, *Pseudomonas*, which is the most abundant genus in CC7-LC, is known to play a role in nitrification (Daum et al., 1998). Other abundant genera in Lechuguilla Cave and Hell Below Cave also include genera known to play roles in the nitrogen cycle (Tables 3 and 4). These include *Bacillus* (highest in L5), which can be a denitrifier (Pichinoty et al., 1983), and *Leptospirillum*, (highest in HB2), which can fix nitrogen (Parro and Moreno-Paz, 2004; Tyson et al., 2005) and is an iron-oxidizing chemolithoautotroph (Tyson et al., 2005). A few of the other abundant genera also play roles in the nitrification phase of the nitrogen cycle. These include the *Nitrosomonadaceae*, a family that includes *Nitrosomonas*, which oxidizes ammonia (Zhou et al., 2007; Chen et al., 2009, Movile Cave). Additionally, about half of *Reyanella* species are able to reduce nitrate (Pagnier et al., 2011), and *Polaromonas*, present in five study pools at >1%, has been documented as a denitrifier (Wang, et al., 2014; Jang et al., 2019).

Two of the study genera are common human skin bacteria: *Propionibacterium* and *Staphylococcus* (found in three and two study pools respectively at >5 %), but can also have roles in the nitrogen cycle. Besides being known as a common human skin bacterium (Stackebrandt et al., 2006), *Propionibacterium* is also known to reduce nitrate (Allison et al., 1989; Swart et al., 1998). While *Staphylococcus* is known to be a common member of the human skin microbiome (Grice and Segre, 2011), some species of *Staphylococcus* have recently been documented to have the ability to fix nitrogen (Yousuf et al., 2020).

An interesting uncultured organism found at 13% abundance in both CC7-LC (Carlsbad Cavern) and in HB2 (Hell Below Cave), belongs to the *Candidatus Omnitrophica*, previously known as *OP3*. Sequences classified in this group were recovered from one of three cave pools studied by Shabarova and Pernthaler (2010). The pool in which *Candidatus Omnitrophica* was found by Shabarova and Pernthaler (2010) contained significantly higher NO_3^- levels and *Nitrospira*, a nitrite-oxidizing bacterium. A more recent paper by Momper et al. (2017), who studied the microbiome of deep terrestrial fluids, differentiates *OP3* from *Candidatus Omnitrophica*, describing them as two candidate phyla. However, the NCBI Taxonomy database (NCBI, 2020) only recognizes the *Omnitrophica* candidate phylum, and more recent papers utilize the *Candidatus Omnitrophica* nomenclature (Rivas-Mari and Devos, 2018; Kirs et al., 2020).

Bacillus, as previously noted, is extremely high in Lechuguilla pool L5 (69 %), but is not present in Lechuguilla Cave pool L1, 30 m from L5, which poses a mystery of why it's there and not also in pool L1. *Bacillus* is very common in soils and natural waters (Logan and De Vos, 2015). In caves, it is the most common cultured bacteria associated with calcite precipitation (Cacchio et al., 2003; Ferris et al., 2003; Dhimi et al., 2018; Tredici et al., 2018). This versatile genus is often involved in the nitrogen cycle (Pichinoty et al. 1983) but can also oxidize Mn and Fe (De Vrind et al., 1986; Logan and De Vos, 2015). The oddity, therefore, is not in its presence but in its unusual abundance in Lechuguilla Cave pool L5. Although L5 is more yellow in color than most, which might suggest slightly higher iron, other pools in this study (that do not have the high *Bacillus*) are equally yellow. Lavoie and Northup (2005) found greater presence of high-temperature *Bacillus* (putatively from cavers' boots that hiked across hot desert soils) in areas of Lechuguilla Cave with high human impact, which could suggest that cavers brought *Bacillus*, or its spores, into the cave on their boots. However, because pool L5 is so far from the entrance, this seems less likely.

Polaromonas was first isolated from Antarctica (Irgens et al., 1996) and it remains best-known from glacial and periglacial settings (Vimercati et al., 2019). However, it has also been found in a number of cave settings (Northup et al., 2003; Shabarova and Pernthaler, 2010; Ortiz et al., 2013; Yun et al., 2016b). *Polaromonas* is present in five pools (>1 %) and abundant in two Carlsbad Cavern pools (BR44, 13 %, CC5-NMR 14 %), but it is not the dominant genus in any pool (Table 4). It is a metabolically diverse heterotroph, able to use a wide variety of energy sources (Vimercati et al., 2019) and survive in oligotrophic conditions (Loy et al., 2005; Ortiz et al., 2013). Its ability to denitrify nitrogen suggests it could play a role in the nitrogen cycle in Carlsbad Cavern, or its heterotrophic nature could suggest it is taking advantage of areas with enrichment from human visitation.

Haliangium is only found in higher abundance (16%) in one study pool, Lechuguilla Cave pool L1. Although the only cultured members of this genus are obligate halophiles (Ivanova et al., 2010; Albatineh and Stevens, 2018; Mohr, 2018), *Haliangium* has been found in several low-salt cave samples, including moonmilk (Engel et al., 2013) and cave sediments (de Mandel et al., 2017; Yasir, 2018). Mohr (2018) suggested that the diversity of this hard-to-culture group is larger than currently known, so it may not be as halophilic in caves as currently seen in other habitats.

Acidobacteria Subgroup 6 are uncultured isolates that are present in seven pools, ranging from 1 % to 4 % in six of the pools, and with the highest occurrence being 18 % in Lechuguilla Cave pool L19A (Table 3, Figs. 6, 7, 9). This *Acidobacteria*

dobacteria subgroup has been characterized from a variety of soils (Barns et al., 2007; Li et al., 2019), including forest and grassland soils, with it being dominant in the latter, which are less oligotrophic (Naether et al., 2012). This subgroup has also been documented as common in Altamira Cave in Spain and Roman Catacombs (Zimmermann et al., 2005, 2006) and Lower Kane Cave (Meisinger et al., 2007).

Microbial Diversity of Caves vs. Study Cave Pools

In this study, no two pools contain the exact same set of phyla/Proteobacterial classes and no phylum/Proteobacterial class is present in all pools. (Fig. 5). Additionally, there are large variations in three commonly used diversity indices (Chao1, Shannon, and Simpson; Table 1; Fig. 10). The Chao1 values are an estimate of richness in the sample, or how many OTUs may be present in each pool. Shannon and Simpson indices are both estimates of evenness, or whether each OTU is present in an equal quantity. The Shannon index is more influenced by rare OTUs, while the Simpson is a probability value that is influenced by dominant OTUs. We chose to use both, because of the variability in evenness of the pools.

A comparison of the diversity indices from the study pools with other vadose cave pool bacteria studies is hindered by the general lack of such studies. Shabarova et al. (2010) looked at a pair of vadose pools, but did not present diversity indices. Sauro et al. (2018) included one vadose pool in their study of an orthoquartzite cave and reported higher Shannon (7.854) and Simpson (0.9916) than the carbonate pools in this study. Interestingly, despite the higher Shannon index, they detected a lower number of bacterial phyla (11 total phyla present, but only 3 phyla >2 %, Sauro et al., 2018, their Table S5; versus this study with an average of 14 phyla present, and an average of 5.3 phyla at >2 %). In addition, they have fewer genera in their one pool (6 at >2 %; Sauro et al., 2018, their Table S5) than most of our study pools (8 at >2 %, range 5–16), even though they have a higher Simpson. The dominant groups include *Alphaproteobacteria Rhizobiales* unclassified (40 %), and *Acidobacteria* (27 %), mainly *Acidobacteria Gp13* (18 %; Sauro et al., 2018, Table S5), neither of which are present in our study pools. However, this is not surprising as a silica system is very different from a carbonate system.

Results from other carbonate cave habitats are available (Ortiz et al., 2013; Alonso et al., 2019; Thompson et al., 2019). However, there are several challenges in such a comparison. These studies sampled surfaces of speleothems or cave sediment, whereas the study pool samples are planktonic. Surfaces are known to have more microbes than planktonic communities (Savio et al., 2018). In addition, some studies group samples together from similar settings, but different caves (Lavoie et al., 2017; Alonso et al., 2019; Thompson et al., 2019), whereas this study has not grouped the data, preferring to look at each pool separately. Different sequencing methods between studies also limit some comparisons.

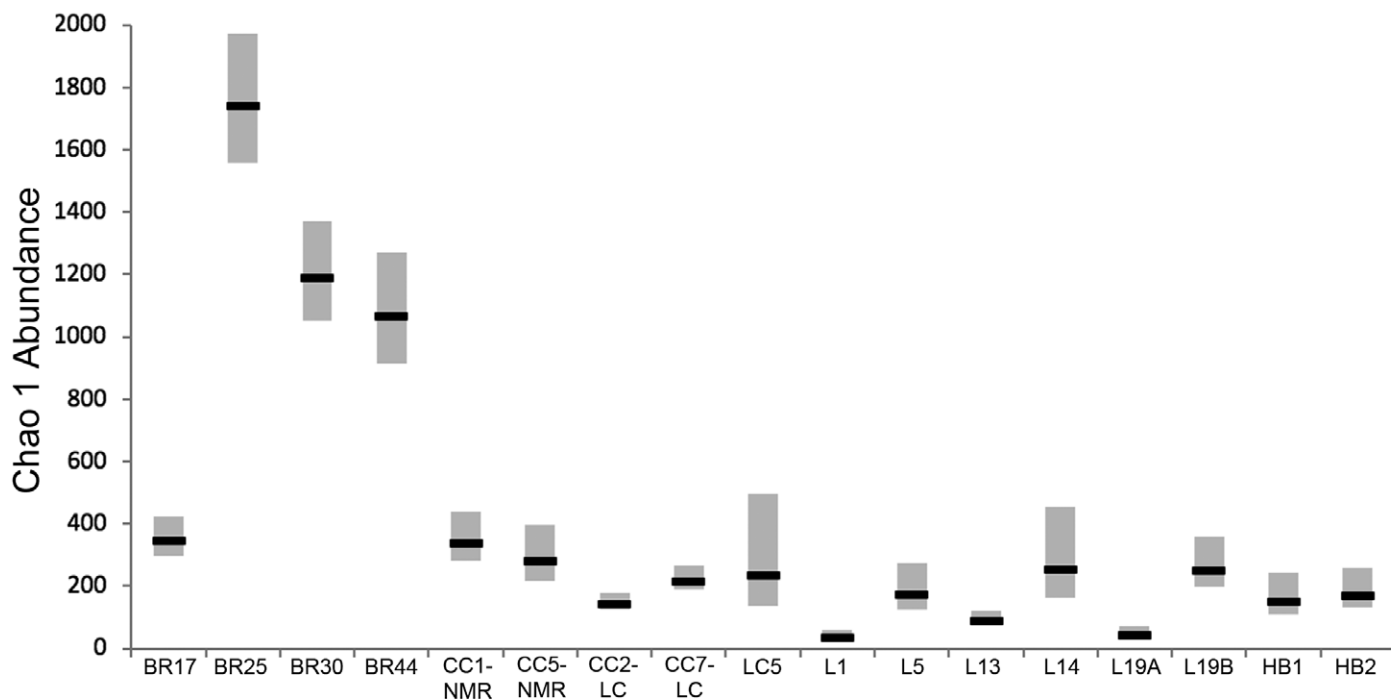


Figure 10. Chao 1 values for the sampled pools. The black line is the Chao value. The gray bars represent the upper and lower bounds of the diversity calculation.

Ortiz et al. (2013) compared speleothem surfaces with surface soil in Kartchner Caverns, an arid land cave in Arizona. The speleothems had much higher Chao1 values (2067–3693) than the study pools, but comparable Shannon (4.3–6.1; Ortiz et al., 2013, their Table 1). Interestingly, they found three separate communities based on variations in *Actinobacteria*, *Proteobacteria*, and *Acidobacteria* and interpreted this as variation in the chemical profile of the speleothems. In comparison to these studies, the study pools have much less *Actinobacteria* and *Acidobacteria*, and much more *Nitrospirae* (Fig. 5, Table 2),

Sampling different microbial mats in lava caves, Lavoie et al. (2017) report much lower diversity in both Shannon (~1.4 to 2.6, with some outliers) and Simpson (~0.40 to 0.75; Lavoie et al., 2017; their Fig. 3 and Supplemental S2) for grouped data. Their top cave groups were *Actinobacteria* (37 % to 44 %), *Gammaproteobacteria* (19 % to 24 %), *Alphaproteobacteria* (10 %), and *Nitrospirae* (4 % to 11 %). Although these groups are also found in these cave pool samples, notable differences include *Actinobacteria* and *Nitrospirae*: *Actinobacteria* are much less common (>5 % in six pools, maximum 21 % in Carlsbad Cavern pool CC1-NMR) and *Nitrospirae* is much more common (>20 % in seven pools; Fig. 5, Table 2).

Alonso et al. (2019) used cave wall samples from nine karst caves in the Dordogne region of France to compare more or less anthropized caves. Looking just at the bacterial results, their Chao1 results are higher (using a different method), their Shannon values are similar (~4.1–5.2), and their Simpson values are higher (above 0.95 except for one cave with values of ~0.82–0.95; Alonso et al., 2019, Fig. 2). The *Proteobacteria* (14 % to 56 %, undifferentiated) and *Actinobacteria* (11 % to 49 %) were their most abundant phyla, as is common on carbonate cave surfaces (Tomczyk-Żak and Zielenkiewicz, 2016).

Thompson et al. (2019) found that speleothems had lower Chao1, Shannon, and Simpson diversity than did the cave sediment and surface soils. All of their Chao1 values were much higher than the study pools. Their much higher Chao1 values may be a reflection of their different methods. Looking at Shannon and Simpson, speleothems are closest to the cave pools (Shannon ~4.7, Simpson ~0.989, Thompson et al., 2019, Fig. 1; compare to Table 1), while both cave sediment and surface soils are more diverse. The most abundant cave phyla are similar to the other studies with the top three being *Actinobacteria* (speleothems 19.47 %, cave sediment 21.91 %), *Proteobacteria* (speleothems 14.21 %, cave sediment 16.05 %), and *Acidobacteria* (speleothems 9.85 %, cave sediment 2.15 %, Thompson et al., 2019, Table 1). *Nitrospirae* were <1 % of any sample.

Thus, the diversity values of cave samples are variable, and the study cave pool samples fall within that variability. The dominant phyla, however, are notably different. Although *Proteobacteria* are abundant in most cave samples, *Actinobacteria* and *Acidobacteria* are more common in surface samples and *Nitrospirae* are more common in the cave pool samples. *Actinobacteria* are very common in caves but are known mainly from surfaces (Tomczyk-Żak and Zielenkiewicz, 2016), which likely explains their greater abundance in other studies than the cave pools. *Nitrospirae* are known to inhabit aquatic environments (Tomczyk-Żak and Zielenkiewicz, 2016), which may help explain the higher pool abundances.

The means of diversity indices, however, do not tell the whole story. Although the cave pool average values are similar to other cave samples, the range of values is much higher. Looking at the carbonate cave samples, the Kartchner speleothems have a Shannon range of values of ~1.7 (4.37 to 6.06; Ortiz et al., 2013, Table 1), the Appalachian cave speleothems range ~2.4 (~3.5 to ~5.9; Thompson et al., 2019, Fig. 1); and the French caves of ~2.2 (~3 to ~5.2, Alonso et al., 2019, Fig. 2). The study cave pools, in contrast, have a range in Shannon values of 3.9 (2.5 to 6.4, Table 1). The range in Simpson values is also higher for our cave pools (Table 1), suggesting there is a greater variation among pools than is represented just by looking at the diversity indices.

A few of the cave pools are strongly dominated by single OTUs. When looking at the distribution of the phyla/proteobacterial classes of the two pools with the lowest Simpson values, Carlsbad Cavern pool BR17 and Lechuguilla Cave pool L5, (Fig. 5), it is apparent there is not an even distribution, as both pools are dominated by a single phylum/proteobacterial class. Looking closer at these two pools, BR17's low Simpson index value is likely due to the dominance of the *Gammaproteobacteria*, *Polycyclovorans* (56 %), and L5 is dominated by *Fimicutes*, *Bacillus* (69 %; Fig. 6 and Table 3). Additional pools with relatively low Simpson values include Lechuguilla Cave pools L1 (0.809) and L19B (0.842), both also dominated by single genera, but not as strongly. Other pools have Shannon and Simpson values comparable to the speleothem data of Thompson et al. (2019). For example, Carlsbad Cavern pools CC1-NMR and CC7-LC (Shannon 6.4 and 6.0; Simpson 0.981 and 0.976, respectively) have higher Shannon values, but slightly lower Simpson values than the Appalachian speleothems. Thus, the differences of the alpha diversity index values in the seventeen pools across three cave systems indicates that sample evenness is variable, even within the same cave system.

Paired comparisons of distant vs. adjacent pools

To examine this variability, the genera from three paired pools were compared (Figs. 7, 8, and 9). Pools Carlsbad Cavern BR17 and Lechuguilla Cave L19A both have lower diversity and may be impacted by human activity. As dis-

cussed previously, BR17 is adjacent to the primary trail in the Big Room of Carlsbad Cavern. L19A was a common water and rest stop for expeditions through Lechuguilla Cave. BR17 has a moderate Chao1 value (343), but a low Simpson index value (0.677) (Table 1). L19A has a low Chao1 value (40), but a moderate Simpson index value (0.881). The two pools have the same Shannon index value (3.5). Despite the similar Shannon, only two genera are shared between the two pools at >2 %, *Nitrospira* and an uncultured *Acidobacteria* (Fig. 7). The abundance of the two genera is different, with both more common in L19A. Of the remaining genera found in greater than 2% abundance, none are shared between the two cave pools. L19A has *Pseudomonas* and *Escherichia-Shigella* present in the pool; both are known human contaminants. BR17 has 56 % *Polycyclovorans*, a hydrocarbon degrader (Gutierrez et al., 2013), which may be related to asphalt from the nearby trail. Although both pools have been impacted by human visitation, they have been impacted in different ways, which can be seen in both the alpha diversity index values and the presence of various OTUs.

The two pools in Hell Below Cave are adjacent to each other, and HB1 has likely drained into HB2 during major rainfall events within the last few decades. However, results from Shabarova et al. (2013, 2014), indicate that the distribution of bacteria within a cave pool can change within a few months after a perturbation. In Hell Below Cave, the two pools have similar Chao1, Simpson, and Shannon values, but have very few shared OTUs. These two pools are adjacent to each other and likely share water, suggesting they receive similar inputs from the vadose zone and experience similar climates. The distribution of phyla in each pool (Table 1) is not dominated by a single phylum. *Nitrospirae* is the highest at 30 % in HB1. There are 16 genera found at greater than 2 % in these pools (Fig. 8). Of these 16 genera, only three are shared between the two pools, the rest are only found in one of the pools.

In the New Mexico Room of Carlsbad Cavern, pool CC5-NMR flows into CC1-NMR when it overflows, and it was nearly full at the time of sample collection (Fig. 4C). These two pools have likely shared water during a major rainfall event within the last several decades, similar to the two pools in Hell Below Cave. However, unlike the Hell Below Cave pools, the pools in the New Mexico Room have different alpha diversity index values and genera present (Table 1, Fig. 9). CC1-NMR has the highest Shannon and Simpson index values of the seventeen pools in this study, 6.4 and 0.981 respectively (Table 1). CC5-NMR has moderate Shannon and Simpson index values of 4.7 and 0.907. There are 30 genera found between the two pools, but only six are found in both pools.

Thus, although the diversity index values of individual pools are similar to that of other cave systems (see above), each pool has its own community, indicated by the low overlap between pool genera (Figs. 6–9, Tables 3 and 4). Studies on pool water chemistry in both Carlsbad Cavern and Lechuguilla Cave have shown the pools are broadly similar, albeit with considerable minor chemical variation among pools (Chapman et al., 1992; Turin and Plummer, 2000; Levy, 2007a; Forbes, 2000). These chemical differences are likely minimal between the adjacent pools that share water in Carlsbad Cavern's New Mexico Room and Hell Below Cave, yet these pools share only a few genera (Figs. 8 and 9). Rather than a single stable cave pool community, adapted to the cave pool system, the study data show 17 different communities despite relatively similar conditions. These data support the hypothesis that each pool is a unique, isolated ecosystem, with differences likely caused as much by the isolation of each pool as by variable water chemistry. Within some of the study cave pools, however, this isolation has been affected by human impact, which can have an impact on microbial communities (see discussion below).

Effects of human visitation on pool communities

The study pools vary in terms of alpha diversity and show significant differences in microbial community structure. They also vary in terms of human visitation with the Big Room (Carlsbad Cavern) having by far the most visitors per year (300,000/year to 500,000/year), and Lechuguilla Cave and Hell Below Cave having the least (<100/year to 200/year). In a previous study of the impact of human visitation in Carlsbad Cavern, Griffin et al. (2014) reported significant human impacts of visitors on the microbiota. Collecting samples from the cave air, they reported that *Staphylococcus* spp., noted above as a common human skin inhabitant (Stackebrandt et al., 2006), were the dominant bacteria cultured along tourist trails. *Knoellia* sp. was most common off trail. *Knoellia* is a novel genus of *Actinobacteria*, first isolated from a cave in China (Groth et al., 2002), which was also found in this study in one of the Carlsbad Cavern New Mexico Room pools (CC1-NMR, Fig. 9). Griffin et al. (2014) also utilized genetic sequencing to show that *Enterobacteriaceae* dominated along the descent trail railing and in the LUNCHROOM, which is not unexpected given the number of fecal deposits from visitors that rangers clean up. They concluded that humans were important sources of non-indigenous microorganisms in Carlsbad Cavern.

The four pools in the Big Room of Carlsbad Cavern have the highest number of OTUs and the highest Chao values of any pools. The larger numbers of OTUs in the Big Room could be driven by a variety of factors, such as increased energy sources from the illumination, shed skin cells, or construction materials. This last item is supported by the presence of the genus *Polycyclovorans*, particularly in Big Room pools, which can facultatively use hydrocarbons for energy (Gutierrez et al., 2013; Thompson et al., 2018). The trail system in the Big Room is asphalt (Brooke, 1996, Van

der Heijde et al., 1997) and BR17, which has 56 % *Polycyclovorans*, is directly below the trail and contains debris from the trail (Fig. 4A). However, *Polycyclovorans* has been documented as a marine genus (Berry and Gutierrez, 2017); other genera isolated from marine environments are found in pools in each cave system. *Polycyclovorans* has also been documented in soils (Lu, 2018), and will probably be discovered in other environments as the knowledge of microbial communities expands. In addition, the *Polycyclovorans* genus is also found in Carlsbad Cavern's New Mexico Room pools and one pool from Hell Below Cave (Table 3), which do not have asphalt trails.

In a study by Lavoie et al. (2017) in Lava Beds National Monument (LBE) volcanic caves, there was only one *Staphylococcaeae* identified, and only 25 OTUs of *Enterbacteriaceae* identified to the level of family detected at any LBE cave or surface sample. Lavoie et al. (2017) hypothesized that there is a threshold of visitors before the effects of human visitation are observed, which is greater than the 30,000/y seen in LBE. The Carlsbad Cavern Big Room pools greatly exceed this threshold with 300,000 visitors/year to 500,000 visitors/year over the last decades. However, the presence of human-associated genera such as *Staphylococcus* and *Pseudomonas* (Table 4) does not correlate with visitation numbers. *Staphylococcus* is most common in Lechuguilla Cave and absent in the Big Room. *Pseudomonas* is present in some, but not all Big Room pools, but also in four Lechuguilla Cave pools. These genera are also known to play roles in the nitrogen cycle, so perhaps they are not simply indicators of human impact in these pools.

Another recent study by Alonso et al. (2019) examined the differences in microbial and micro-eukaryotic diversity in four pristine and four anthropized caves, plus two sections of the highly visited Lascaux Cave (France). Of particular interest was differences noted in the bacterial phylum *Bacteroidetes*, which were higher in anthropized caves, and *Nitrospirae*, which were lower in anthropized caves versus pristine caves. In this study, *Bacteroidetes* were present in all Carlsbad Cavern pools, varying from 1 % to 26 %, but in only two of the Lechuguilla Cave (L1 and L5, 1 % to 2 %), and one of the Hell Below Cave pools (HB2, 3 %) (Table 2).

This study observed considerable variation in *Nitrospirae* across the study pools (Fig. 5). *Nitrospirae* were present in all Carlsbad Cavern pools, varying from 2 % to 36 %, with five of the pools having >20 % occurrence, and the highest percentage of 36 % being found in the Big Room. In Lechuguilla Cave pools, *Nitrospirae* varied from 1 % to 21 %, with only two pools at 10 % and 21 % across pools with varying visitation (but still low in comparison to Carlsbad Cavern). Hell Below Cave had 15 % and 30 % in its pools, with low visitation. Thus, both high and low impact settings can have high *Nitrospirae*, in contrast with Alonso et al. (2019) who found that *Nitrospirae* were more abundant on the walls in less anthropized caves (5 % to 10 %). The different pattern of *Nitrospirae* occurrence in the cave pools suggests further investigation into how the unique bacterial communities utilize the nitrogen cycle and what drives the community make-up of each pool.

Broader Implications

Studies of microbial diversity in caves are often trying to characterize either different caves or different sample types in the context of larger questions like human impact (Adetutu et al., 2012; Leuko et al., 2017; Alonso et al., 2019), soil contributions to cave ecosystems (Ortiz et al., 2013; Lavoie et al., 2017; Thompson et al., 2019), or variation between microbial mat types (Lavoie et al., 2017). It is common to group samples from different locations (Lavoie et al., 2017; Alonso et al., 2019; Thompson et al., 2019). Ortiz et al. (2013) instead looked at individual speleothems and found different microbial communities for adjacent stalactites. They attributed the differences to varying water and speleothem chemistry. This study of vadose pools also found unique ecosystems, even for adjacent pools that likely share water occasionally. Thus, we suggest an additional variable in the cave ecosystem may be time; communities that are isolated from each other will evolve independently over time. This contrasts with groundwater ecosystems that vary less over larger distances (Wegner et al., 2019), perhaps because of more interaction in the connected aquifer. Thus, the common habit of grouping samples, while useful for some questions, misses some of the differences in diversity present in cave ecosystems. Rather, caves may harbor a wide variety of isolated ecosystems, each one potentially containing a unique community impacted by both the local conditions and the amount of time it has been isolated. Further exploration of this hypothesis in different kinds of cave ecosystems would be useful.

Future Directions

Future studies will focus on elucidating the geochemistry of the pools, expanding the sequencing to include the Archaea and expand the Bacteria, and conducting metagenomic and transcriptomic analyses of pool water. The extensiveness of potential nitrogen cycling bacteria in the study samples points to the need to expand future studies to sequencing of the Archaea present, using Archaea-specific primers for more comprehensive recovery (Bahram et al. 2019). Metagenomic sequencing will confirm what nitrogen cycle genes, if any, are present in the pool inhabitants and transcriptomics, if successful, will confirm what nitrogen cycling is taking place. Simultaneous sampling for water chemistry is required to better test the controls on diversity. Expanding the analysis of pool water microbial analyses to other cave types and climate settings would be very beneficial as the number of cave water pool studies is very low. Also, long-term studies are needed to determine the stability of the communities. We also suggest that additional test-

ing of the impact of human activities and built environments that are installed to facilitate tourists in caves with different visitation levels is essential to understanding how to mitigate such impacts. Such studies would expand the knowledge of microbial diversity in caves substantially.

CONCLUSIONS

The 17 study pools across three caves represent unique bacterial communities, suggesting they are isolated systems. *Nitrospirae*, *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria* were the most abundant phyla/proteobacterial classes in over half the cave pools. The most widespread genus observed was *Nitrospira*, while nine other top genera included *Polyclovans*, *Propionibacterium*, *Polaromonas*, *Haliangium*, *Bacillus*, *Subgroup 6 uncultured Acidobacteria*, *Candidatus Omnitrphica*, and uncultured *Nitrosomonadaceae*. Prior studies of several of these genera suggest roles in the nitrogen cycle for pool bacteria. These data suggest there is some evidence of human contamination, but do not clearly support it as a major factor in the diversity observed. Although *Proteobacteria* are abundant in both settings, the dominant bacterial phyla on speleothems and other surfaces (*Actinobacteria* and *Acidobacteria*), are different than that found in the study cave pools (*Nitrospirae*). Rather than a single stable cave pool community, adapted to the cave pool system, these data show 17 different communities despite relatively similar conditions. These data support the hypothesis that each pool is a unique, isolated ecosystem, with differences likely arising from the isolation of each pool and not just from different water chemistry. These data also suggest that to accurately assess diversity present in cave ecosystems future cave studies should not group samples.

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