BACTERIA AS INDICATORS OF HUMAN IMPACT IN CAVES

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Abstract

This project used select microbes as indicators of human impact in caves by culturing in areas with a range of visitation impacts. Human Indicator Bacteria are those that would not normally be present in a cave unless there has been substantial impact by humans in terms of presence, activities, or pollution. Preliminary study of Human Indicator Bacteria in Lechuguilla Cave compared low impact (alcoves, off-trail sites, non-drinking water pools) with high human impact areas (camps, trade routes, rocks that humans slither over, urine dumps, drinking water sources). Enrichment culture procedures targeted high-temperature *Bacillus* spp., *Escherichia coli*, and *Staphylococcus aureus* sampled from these sites. The study was recently expanded to Mammoth Cave and included fungi, with additional study planned in Carlsbad Caverns and Spider Cave. There is variation by cave, but our results show increased levels of *Staphylococcus aureus*, *E. coli*, and high-temperature *Bacillus* in areas with the greatest visitation levels in both wild caves and commercial caves, although recovery rates were very low at Mammoth. A reduction in numbers was seen in Lechuguilla with *S. aureus* and *E. coli* if the areas are given a rest from human visitation. Survival of Human Indicator Bacteria under idealized lab conditions in a variety of cave soil types showed that *S. aureus* died off within two to four weeks. *E. coli* K survival was similar, but varied, and increased in numbers in some soils. Bacteria can be used as indicators of human impact in caves and as such, provide cave managers with a useful tool to measure human impact.

Introduction

Human Indicator Bacteria are those that would not normally be present in a cave unless there has been substantial impact by humans in terms of presence, activities, or pollution. We know from studies of lint deposition that visitors can leave behind a mix of biodegradable and non-biodegradable materials (Jablonsky, Kraemer, and Yett 1995). These organics provide new microhabitats and nutrients that can support the growth of contaminating microbes, and even alter the underlying surface.

The microbes used in our studies are *Staphylococcus aureus*, a member of the normal flora of skin; *Escherichia coli*, a normal inhabitant of the digestive tract of warm-blooded animals; and

high-temperature *Bacillus*, found in soils heated by sunlight. Limited additional work was done with fungi. Fungi are limited to areas contaminated from the surface, and require large inputs of organic matter (Dickson and Kirk 1976). We expect that human impacts will vary in different environments, and report on studies from two very different locations, Lechuguilla Cave in Carlsbad Caverns National Park, New Mexico, and three locations in Mammoth Cave National Park, Kentucky: Great Onyx Cave, Historic Mammoth Cave, and the Frozen Niagara–New Entrance area. Information will be used to improve the scientific information base to allow for informed management decisions.

Studies of microbes in caves are limited due to the technical difficulties of studying organisms that can't be seen. Comparisons of cave soils with surface soils were done by Gounot in 1967 at the CNRS in France. She compared the numbers of bacteria culturable from cave silts compared to rich agricultural land. Cave soil bacterial counts tended to be lower, yielding several million per gram, while the agricultural soil bacterial counts yielded several hundreds of millions from a gram. Most other studies have supported her general conclusions. Many studies of microbes in caves have relied on limited plate count methods, which greatly underestimate the total population and tell us nothing about the activity of the microbes in that environment. Culture techniques work best for non-resident microbes, including the human indicator microbes reported on in this study.

Studies of the biomass and activity of microbes in limestone caves in Kentucky were conducted by Feldhake (1986). He concentrated his studies on actual measurements of microbial metabolic rates in 12 sites in four caves, along with comparisons to overlying forest soils. Except for a site rich in cricket guano, Feldhake found that organic matter content, microbial activity, and biomass was much lower in the cave than in forest soil. He also found significant variations among sample sites within the cave, and methodological problems when samples were removed from the cave, transported to the lab, and assays were attempted more than 24 to 48 hours after collection.

One notable exception to studies that show low numbers and activity of microbes in caves has been work done by Mallory and his students. A major study compared the microbial activity, density, and diversity in two aquatic sediment sites in Mammoth Cave (Rusterholtz and Mallory 1994). The study included counts of cells in the sediment, staining to determine metabolic activity of soil microbes, plate counts using both high and low nutrient media, followed by extensive physiological testing of isolates from the plate counts. They recovered between 11 to 58% of the total cell count on culture medium. The recovery rate for most surface soils is typically 0.4 to 1.7%. They also detected active metabolism in 53 to 58% of the population, despite very low nutrient levels of total organic carbon per liter of water. The diversity of populations was extremely high, with 42% of the isolated species similar to surface organisms, and the remainder unidentified. There were no dominant species, and the type of growth medium used strongly influenced the types isolated.

Studies of bacteria and fungi in Lechuguilla Cave by Northup and others (Northup *et al*. 1992, 1995, 2003; Mallory, Spokas, and Northup 1995, Cunningham *et al.* 1995; Spilde *et al*. 2005) from a variety of habitats, including sediments, pools, speleothems, and speleosols (corrosion residues), show microbial communities that are indigenous to the cave. These microbes are adapted to extremely low nutrient conditions. Chemolithotrophs were suggested to play important roles in formation of speleosol deposits in Lechuguilla. The report expressed concern about the potential of increasing inputs of organic matter into the pools to protect the native oligotrophic bacteria and provides suggestions for limiting human organic input. Northup's suggestion for establishing "microbial cave preserves" would allow for study of indigenous populations of microbes without human impacts. Application of new molecular biological techniques pioneered by Norm Pace is revealing potentially novel bacteria that may serve as a marker for native cave microbial communities (for example, Barton *et al*. 2003).

Northup (1997) *et al*. (1997) studied the same three groups of organisms on which we focus in this proposal in high and low impact areas in Lechuguilla Cave. They found significantly more Human Indicator Bacteria in the high impact areas. A major focus of her work is seeking the balance between cave exploration and scientific discovery, particularly in terms of human introduction of non-indigenous microorganisms

and organic carbon.

The situation in Mammoth Cave is very different. Mammoth has multiple entrances, high visitation by tourists, and significant impact by water from the surface; pristine areas are few. Studies of unique communities are limited to studies of microbes in saltpeter soils (Hill *et al.*1983, Olson and Krapac 1997).

The objectives of this study are:

A. Can numbers of Human Indicator Bacteria (*S. aureus*, *E. coli*, and high-temperature *Bacillus* spp.) be used to compare the impact of humans in caves (Lavoie and Northup, 2002)?

B. How long will Human Indicator Bacteria survive when added to a range of cave soil types?

Due to the complexity of microbial communities and the specialized techniques for their study, development of an Index of Biological Integrity (IBI) to assess the impacts of humans on cave microbes has not been developed. This study will provide important baseline information on the feasibility of detecting a select group of bacteria that may be able to serve as indicators of human presence and activity, using simple and relatively inexpensive techniques. These Human Indicator Bacteria were detected and quantified in areas known to have high human impact compared to areas with low human impact in both Lechuguilla Cave and Mammoth Cave. The survival of indicator bacteria will be modeled in the lab. We will extend this study to Carlsbad Caverns and Spider Cave at a later date.

Material and Methods

Description of study areas:

Lechuguilla Cave is a largely pristine cave environment, with one small entrance and has limited and highly regulated visitation. The cave is in a desert region, protected by a siltstone caprock, with very low surface impacts from water (Davis 2000). In an attempt to preserve the cave and native microbial communities, expeditions utilize flagged trails and established camp areas with defined drinking pools and urine dump areas. The study compared low-impact areas off-trail, in alcoves, and drinking water pools with high-impact sites in camps, trade routes, trails, urine dumps, and drinking water pools in the East, West, and Southwest Branches. A particular branch was placed off-limits to exploration for a month following each sampling, allowing comparison of Human Indicator Bacteria during typical visitation impacts and after a recovery period.

Mammoth Cave is in a temperate region with high levels of rainfall. There are multiple entrances, with large communities of invertebrates that can enter and leave the cave on a daily basis. The study sites in Mammoth Cave are all associated with tourist trails, and have variable levels of visitation. All of these locations are also frequently impacted by water from flooding or direct surface inputs. Great Onyx Cave is visited by an average of 36 people per day during the summer months. The cave branches, and tourists are limited to one branch, allowing for comparison of high and low impact areas in an environment that largely has low impact. The Frozen Niagara–New Entrance is visited by an average of 847 people per day in the summer. Directly adjacent to the Frozen Niagara formation is a drop down into a very seldom visited area named the Radio Room, again allowing for a high and low impact comparison. The Historic Mammoth Cave receives an average of 1,002 people per day in the summer that all pass through the Rotunda on their way to different destinations within the cave. We sampled from the Historic Tour route, and used areas off the trail in branch passages for low impact comparison.

Sampling, Plating, and Incubation:

Objective A. Human Indicator Microbes. Swabs of defined areas (1 cm^2 areas and later 10 cm^2) were collected from a range of locations with high exposure to humans and low exposure in both Lechuguilla Cave and the Mammoth Cave National Park sites (see Results). Samples were plated on EMB agar, Mannitol Salt Agar, and Sabaroud Dextrose Agar (Mammoth Cave sites only) for detection of *E. coli*, *S. aureus*, and fungi, respectively, with incubation at 37° C. Lechuguilla samples were inoculated in Brilliant Green Bile Broth with Durham tubes (Difco) onsite and transported to the lab where they were monitored for gas production. Those that were positive for gas production were plated on EMB

agar and monitored for production of the characteristic metallic-green sheen of *E. coli*. High salt media was used to enrich for *S. aureus,* which is highly resistant to high-salt content media, in Lechuguilla samples; those that showed growth were subjected to a coagulase test to confirm the presence of *S. aureus*. To test for high-temperature *Bacillus* spp., Lechuguilla swab samples were inoculated into sterile distilled water and heated in 70° C for 10 minutes on-site and transported to the lab where they were plated onto Antiobiotic Medium 1 (Bacto) and incubated at 45° C. Mammoth swabs were heated at 65° C for ten minutes to kill most bacteria, and plated on Nutrient Agar or Nutrient Agar with antibiotics added, and incubated at 45° C for detection of high-temperature *Bacillus*. Samples from Lechuguilla Cave were incubated in the cave for a minimum of 24 hours prior to removal to the surface for incubation. Samples from Mammoth Cave were brought to the surface, plated, and incubated.

Objective B. Survival. This part of the study has been done so far only for the Mammoth Cave sites. Soil samples were collected from appropriate locations to represent a range of typical cave soil types. We aseptically collected approximately 150 g of clay-silt mud from the right hand passage in Great Onyx Cave, sand near Bubbly Pit in Great Onyx, gypsum from the Great Kentucky Desert of Great Onyx Cave, and saltpeter soil from the Rotunda in Historic Mammoth. Except for the mud, soils were screened to remove large materials, which were left *in situ* to minimize disturbance in the area. Soils were refrigerated and returned to the lab at Plattsburgh State University.

We set up three replicates of each soil using 60 g of soil and 120 ml of water mixed in a 250 cc Erlenmeyer flask. We marked the level of the water line in each flask for future adjustments. Each soil type was plated to test for the background level of *S. aureus* and *E. coli*. One set of each soil type was inoculated with *Escherichia coli K* and one set with *Staphylococcus aureus*, and incubated at room temperature. At regular intervals (24 hours and then weekly) each microcosm was cultured using appropriate dilution and spread-plating in triplicate onto EMB Agar for *E. coli*, and Mannitol Salt Agar or Nutrient Agar with 20% added NaCl for *S. aureus*. Plates were incubated at 37° C for 24 hours before counting individual CFU. We continued this until the bacteria are below the limits of detection.

Results

In Lechuguilla, *E. coli* were recovered only from urine dumps and urine control sites (Table 1). No pools were contaminated. *S. aureus* was found at a much higher frequency in high impact sites (Table 2). With both organisms the number of positive samples declined after humans were excluded from the area (Table 1 and 2). Results from Mammoth Cave are presented in Table 3. The difference in number of colonies of *Bacillus* spp. between low and high human impact areas in Lechuguilla Cave was marked. On average there were 56.4 colonies from samples from high human impact areas (trails and camps) and 4.60 colonies from low human impact areas (off trail, alcoves), a difference that is statistically significant.

Table 1. Lechuguilla sites contaminated with *E. coli* by impact level, comparing the initial sample and the results post-exclusion. Numbers in parentheses indicate total number of sample sites.

Impact Level	Initial Sample	Post-exclusion
Urine dumps	4 (18)	1 (12)
Urine controls (low impact)	1 (15)	1 (12)
Old urine dump	0 (15)	0 (6)
Drinking pools	0 (24)	0 (9)
Non-drinking pools (low impact)	0 (30)	0 (15)

Table 2. Lechuguilla sites contaminated with *Staphylococcus aureus* by impact level, comparing the initial sample and the results post-exclusion. Numbers in parentheses indicate total number of sample sites.

Impact Level	Initial Sample	Post-exclusion
High impact	13 (42)	0 (21)
Low impact	1 (36)	0 (21)

Table 3. Summary of the number of positive sites (total number of samples in parentheses) from Frozen Niagara, Great Onyx Cave, and Historic Mammoth Cave. High and Low impact sites. Samples are either 1 cm^2 or 10 cm^2. ND = not determined.

Frozen Niagara	E. coli	S. aureus	Fungi	HT *Bacillus*	
High 1 cm^2	2 (16)	1 (16)	5 (16)	ND	
High 10 cm^2	2 (10)	0 (10)	4 (6)	ND	
Low 10 cm^2	0 (10)	1 (10)	8 (10)	ND	
Great Onyx	*E. coli*	*S. aureus*	Fungi	HT *Bacillus*	
High 10 cm^2	1 (15)	1 (15)	8 (15)	1 (12)	
Low 10 cm^2	0 (14)	0 (14)	1 (014)	0 (12)	
Mammoth	*E. coli*	*S. aureus*	Fungi	HT *Bacillus*	HTB Count
High 10 cm^2	2 (15)	2 (15)	1 (15)	8 (9)	>500 CFU
Low 10 cm^2	1 (7)	0 (7)	0 (7)	5 (7)	5 CFU

Survival with time of *S. aureus* (Figure 1) and *E. coli* (Figure 2) in the different soil types from the Mammoth Cave sites show an initial rapid die-off. *S. aureus* were all gone or below the limits of detection in two to four weeks. *E. coli* showed a similar pattern, except for an apparent increase in numbers in three of the four soil types after two weeks.

Discussion

In Lechuguilla, urine dumps and urine control sites were the only areas where *E. coli* was recovered (Table 1). An old urine dump site and drinking and non-drinking pools of water were all negative. *S. aureus* was found (Table 2) at much higher frequency in high-impact sites (13 of 42 samples) compared to low-impact sites (1 of 36 samples). The numbers of contaminated samples decreased after a period of closure of the cave to exploration, indicating that these non-resident human indicator bacterial species die off over time.

The number of sites in Lechuguillla contaminated with high-temperature *Bacillus* was statistically significant, with the average number of colonies from high-impact sites 54.6 compared to only 4.6 at low-impact sites. These contaminant bacteria are expected to persist for years because of their production of highly environmentally resistant endospores.

Recovery rates for *E. coli* and *S. aureus* were low at all of the Mammoth Cave sites (Table 3), despite sampling immediately following tour groups in high-impact sites. Distinctions between low and high impact areas were weak. High-temperature *Bacillus* were almost undetectable in Great Onyx Cave. The cave receives very few visitors and visitors are dropped off by bus just a few feet from the entrance, and probably simply have no opportunity to pick up these bacteria from the soil and track them into the cave. The results at Mammoth Cave are simply confounding, with no real distinction in recovery between high and low impact sites, although the number of colonies was much higher in high-impact sites. We believe the situation merits further study. Our low-impact sites may have been too close to high-impact sites, allowing for contamination by air currents. There may also be fewer high-temperature *Bacillus* in the soils at Mammoth compared to the desert areas around Lechuguilla.

Preliminary work with fungi provided interesting results, with higher positive sites reported from Great Onyx and Frozen Niagara–New Entrance sites, which both have higher impacts from water. There was no distinction between high and low impact areas with Frozen Niagara–New Entrance sites, possibly reflecting the high surface impacts at all of the sites and proximity to entrances. The number of positives was much greater in high impact sites in Great Onyx Cave. Sites closer to the entrance are included in the high impact sites.

The low impact site is a side passage at the limits of the tourist trail. Results at Mammoth were very low, with no distinction between high and low impact sites.

We intend to follow-up at Mammoth with a survey of frequency of high-temperature *Bacillus* and fungi by distance into the cave. We will also conduct a similar study at Carlsbad Caverns.

Survival of *S. aureus* (Figure 1) and *E. coli* (Figure 2) bacteria with time was conducted in the lab using four soil types common in Mammoth Cave; a clay-silt mud, sand, gypsum, and saltpeter. *S. aureus* bacteria died off quickly in the first 24 hours to one week, then showed a lower rate of decline, and were below the limits of detection in two to four weeks. Survival was higher in saltpeter soil only. *E. coli* showed the same initial pattern of a rapid die-off in the first 24 hours to one week. Numbers were below the limits of detection in four weeks in the clay-silt mud soil. The three remaining soils had an unexpected increase in the number of cells after two weeks. These findings lend support to the results reported by Hunter *et al*. (2004) with further discussion by Barton and Pace (2005) and response by Hunter *et al*. (2005). We simply may not know how these bacteria behave in nature in contaminated ecosystems. This portion of the study will be repeated with soils from Carlsbad Caverns.

In summary, higher numbers of Human Indicator Bacteria (*E. coli*, *S. aureus*, and high-temperature *Bacillus*) are found in areas with greater human impact in Lechuguilla, with less distinctive results from Mammoth Cave sites. Recovery rates are higher in Lechuguilla possibly because of the intense, sustained impacts of people in camps, while human impact at the Mammoth Cave sites may be much more limited due to the rapid movement of visitors through the caves. Differences may also be due to temperature, moisture, and soil types. Given time with no humans present, the numbers of *E. coli* and *S. aureus* did die off at Lechuguilla. Laboratory microcosm studies show that *S. aureus* and *E. coli* are below the limits of detection in two to four weeks, with some influence of soil type. *E. coli* may be able to grow under field conditions with high levels of contamination to provide nutrients, contrary to expectations. Northup (1997) stresses the importance of maintaining low nutrient conditions to preserve native cave microbial communities.

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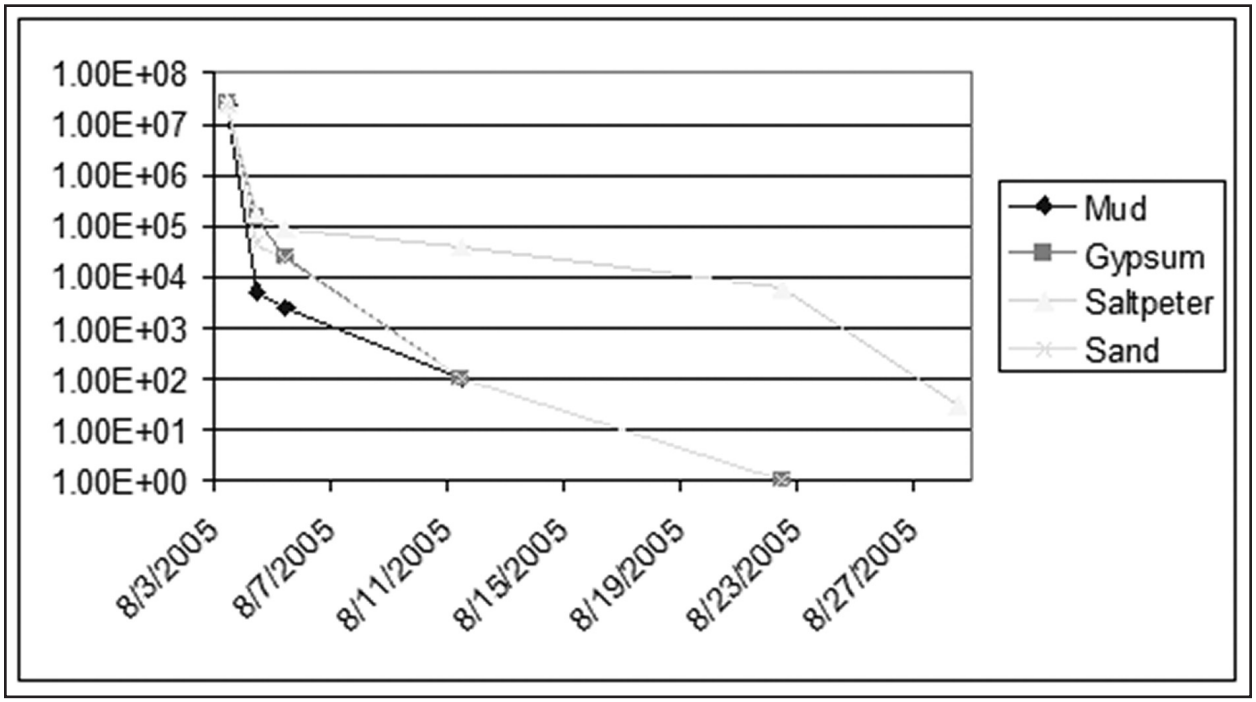

Figure 1. Numbers of S. aureus with time in different soil types from Mammoth Cave.

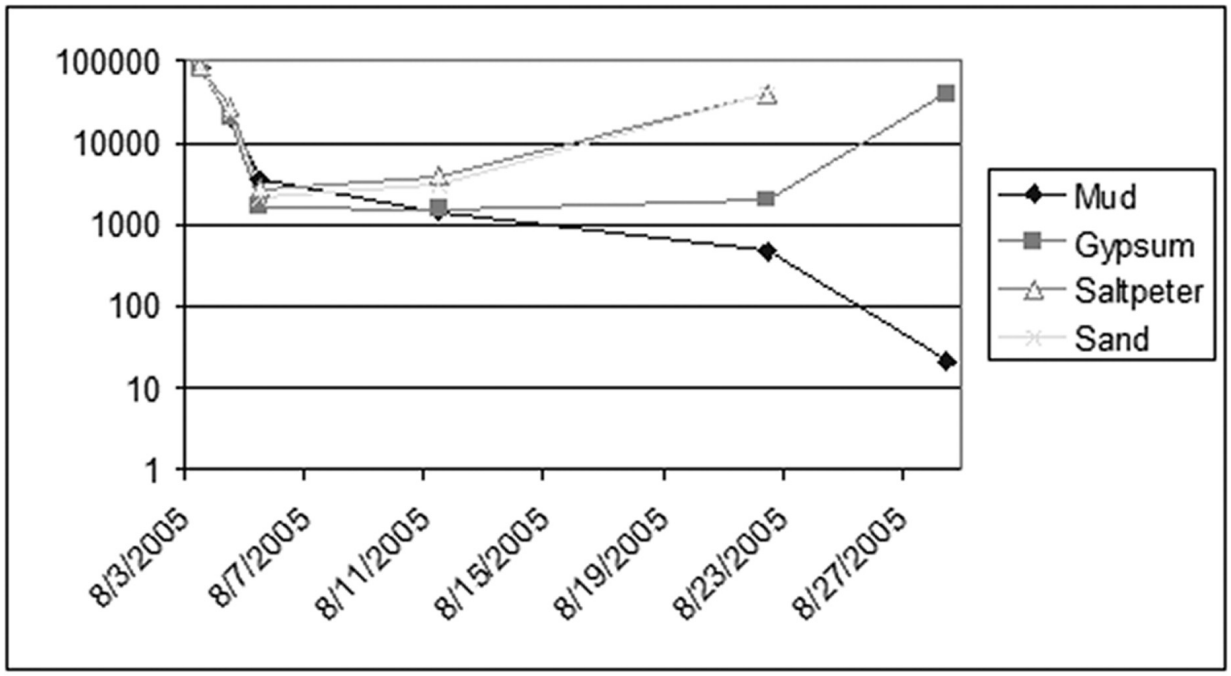

Figure 2. Numbers of E. coli with time in different soil types from Mammoth Cave.

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References

Barton, H.A. and Pace, N.R. 2005, Discussion: Persistent coliform contamination in Lechuguilla Cave pools. Journal of Cave and Karst Studies v. 67, p. 55-57.

Cunningham, K.I.; Northup, D.E.; Pollastro, R.M.; Wright, W.G.; and LaRock, E.J. 1995,

Bacteria, fungi and biokarst in Lechuguilla Cave, Carlsbad Caverns National Park, New

Mexico. Environmental Geology v. 25, p. 2-8.

Davis, D.G. 2000, Extraordinary features of Lechuguilla Cave, Guadalupe Mountains, New Mexico. Journal of Cave and Karst Studies v. 62, p. 147-157.

Dickson, G.W. and P.W. Kirk. 1976, Distribution of heterotrophic microorganisms in relation to detritivores in Virginia caves. (with supplemental bibliography on cave microbiology and mycology). In B.C. Parker and M.K. Roane (eds.) The distributional history of the biota of the Southern Appalachians. Part IV. Algae and Fungi. p 205-226. University Press of Virginia, Charlotteville, Va.

Feldhake, D.J. 1986, Microbial activity and biomass of limestone caves. Master of Science Thesis, University of Cincinnati.

Gounot, A.M. 1967, La microflore des limons argileaux souterains: son activité productrice dans la biocénose cavernicole. Annales de Spéléologie v. 22, p. 23-143.

Hill, C.A.; Eller, P.G.; Fliermans, C.B.; and Hauer, P.M. 1983. Saltpeter conversion and the origin of cave nitrates. National Geographic Society Research Report 15:295-309.

Hunter, A.J.; Northup, D.E.; Dahm, C.N.; and Boston, P.J. 2004, Persistent coliform contamination in Lechuguilla Cave pools. Journal of Cave and Karst Studies v. 66, p 102-110.

Hunter, A.J.; Northup, D.E.; Dahm, C.N.; and Boston, P.J. 2005, Persistent coliform contami-

nation in Lechuguilla Cave pools. Response: Barton and Pace Discussion. Journal of Cave and Karst Studies v. 67, p 133-135.

.Jablonsky, P; Kraemer, S; and Yett, B. 1995, Lint in caves. Proceedings of the 1993 Cave Management Symposium. Ed. D.L. Pate, Carlsbad Caverns, New Mexico. p. 73-86.

Lavoie, K.H. and D.E. Northup. 2002, Microbial diversity in caves: Balancing cave exploration and scientific discovery. Invited presentation, Hamilton Valley Symposium, Mammoth Cave National Park, KY. November.

Northup, D.E. 1997, Balancing conservation of unusual cave microbial communities with exploration and research in Lechuguilla Cave, Carlsbad Caverns National Park, New Mexico. Final Report to the Lindburgh Foundation and the National Park Service.

Northup, D.E.; Carr, D.L.; Crocker, M.T.; Hawkins, L.K.; Leonard, P.; Welbourn, W.C. 1992, Lechuguilla Cave Biological Inventory. Report to the National Park Service. 177 pp. University of New Mexico.

Northup, D.E.; Beck, K.M.; and Mallory, L.M. 1997, Human impact on the microbial communities of Lechuguilla Cave: Is protection possible during active exploration? Selected Abstracts from the 1997 National Speleological Society Convention in Sullivan, MO. Journal of Cave and Karst Studies p 166.

Olson, R. and Krapac, I. 1997, Origin and regeneration of nitrates in Mammoth Cave sediment. In:Karst-O-Rama 1997, The Electric Caver 34:51-60.

Rusterholtz, K.J. and L.M. Mallory. 1994, Density, activity and diversity of bacteria indigenous to a karstic aquifer. Microbial Ecology, v. 28, p. 79-99.

Spilde, M.N.; Northup, D.E.; Boston, P.J.; Schelble, R.T.; Dano, K.E.; Crossey, L.J.; and Dahm, C.N. 2005, Geomicrobiology of cave ferromanganese deposits: A field and laboratory investigation. Geomicrobiology Journal, v. 22, p. 99-116.