Mammoth Cave Microbiology

16

Kathleen H. Lavoie

Abstract

Microorganisms are a diverse group of Bacteria, Archaea, and Eukaryotes with their very small size in common. Microbes make up the majority of organisms in numbers, biomass, and metabolic diversity and are critical component of the biosphere through geochemical cycling. Caves are models for the study of astrobiology: life on other planets. This chapter reviews intraterrestrial (inside Earth) microbes in Mammoth Cave. Despite the great size and complexity of Mammoth Cave, few microbial studies have been carried out. Great changes in methods from culture-dependent to molecular genomic studies have provided new information. Geomicrobiology is at the intersection of microbial activities and geologic processes, including sulfur-based ecosytems, formation of carbonate speleothems, saltpeter mining, and manganese oxide deposits. Microorganisms also include infectious agents like tuberculosis, and parasites of humans and cave crickets, and the devastating invasive fungus that causes white-nose syndrome in bats. Microbial nature preserves could protect communities of native cave microbes adapted to low-nutrient conditions. There are many ecological and evolutionary questions to be studied along with basic research and inventory of microorganisms in Mammoth Cave.

16.1 Introduction

Microorganisms are the only group of organisms defined by their very small size. Nearly all cells (including our own) are microscopic, but microbes live mostly as single cells; only multicellular plants, animals, and some fungi are not microorganisms (although parasites are studied in microbiology). Microbes include all prokaryotic and many eukaryotic cells. Viruses are not alive because they are not cells, but are microorganisms.

Microbes are of central importance to the biosphere and to biogeochemical cycling. They maintain the atmosphere by cycling carbon, nitrogen, and oxygen. At least half of the oxygen in the atmosphere is from phototrophic microorganisms (algae and cyanobacteria) in oceans. Microbes extend our knowledge of the strategies and limits of life. With the discovery of hundreds of new planets, it is very possible that life is abundant in the universe, microbial, uses sulfur for energy, and is located below the surface but dependent on liquid water (Domagal-Goldman and Wright 2016). Caves provide model systems for what extraterrestrial life might be. We can monitor environmental change, water pollution, the quality of an environment, and the recovery of a system to stress by studying microbes. Microbes play a major role in conservation and restoration biology, and microbial communities provide important models for understanding principles of ecology and evolution.

Because we usually cannot see microorganisms directly, they are often "out of sight, out of mind." How can such small creatures change anything? What they lack in size, they more than make up for in numbers, biomass, and metabolic diversity. There is probably more microbial biomass below the surface of the Earth than all the biomass above ground. Edwards et al. (2012) describe microbes below the surface as "intraterrestrial," life inside Earth. Caves provide access for study of shallow and deep subsurface environments.

K.H. Lavoie (✉)
Department of Biology, SUNY Plattsburgh, Plattsburgh, NY, USA

H.H. Hobbs III et al. (eds.), *Mammoth Cave*, Cave and Karst Systems of the World,
DOI 10.1007/978-3-319-53718-4_16

16.2 Intraterrestrials: Microbes in Caves

Microbial distribution is ubiquitous. They can be found in every environment, but whether they are active or not depends on factors including nutrient availability, temperature, pH, and presence of other organisms. Where are microbes in caves? The cave shows evidence of microbial activity: algae and cyanobacterial growth around entrances or artificial lights, the earthy smell, white filamentous fungi growing on scat, white microbial colonies on a wall (Fig. 16.1), a white "marshmallow" with legs—a cricket killed by a parasitic fungus, powdery soil mined for saltpeter, white filaments in a stream that smells of rotten eggs, some speleothems (formations), the limestone itself and the dissolution of passages.

What should we look for to find microbes in caves? Visual evidence of microbes and microbial activities in caves include dots, which are colonies of microbes on rock surfaces (Fig. 16.1); ferromanganese deposits, seen as discoloration of rock surfaces; precipitation of banded minerals; structural changes like a coating or crust; and biofilms, communities of microbes seen as slippery rocks or white filaments in streams with inputs of sulfur in caves (Barton 2006). Despite its large size and the growth and activity of microbes throughout Mammoth Cave, relatively few microbiological studies have been carried out, offering great potential for future research (Lavoie 2015).

16.3 It's a Small World: Methods

The first review of the microbiology of underground environments was published by Caumartin in 1963. A lot has changed in the methods used for the study of microorganisms since then leading to important insights into the ecology and distribution of microbes in caves. Their small size is of critical importance in understanding microbes. The very great surface area-to-volume ratio of microbes allows for rapid diffusion of materials in and out of cells and for rapid metabolism and cell division when food is available. It is even hard to tell when a bacterial cell is dead; microbes often exist in a dormant state with little or no metabolic activity, but those same microbes can rapidly become active if environmental conditions change.

The study of microbes has always been complicated by their small size and low morphological diversity. We cannot use conventional observations that we use for cave crickets or cavefish, yet we need to know which microbes are there, and how many, their activity and interactions. Using a microscope to look for microbes in the environment is

Fig. 16.1 A female *Hadenoecus subterraneus* cave cricket with white microbial colonies on the wall behind her in the New Discovery Entrance to Mammoth Cave

difficult. Even in a nutrient-rich agricultural soil, you would only find isolated areas with a few cells, and your chances of seeing microbes in nutrient-poor cave soils would be a thousand times lower.

16.3.1 Microscopy

Microscopes gave us our first sight of the microbial world, both traditional light microscopy with staining, like the Gram stain, and electron microscopy which allows us to examine objects at extreme close up (Fig. 16.2). Bacteria do not vary much in what they look like, so microscopy is a useful tool, though limited. We can extend the usefulness of microscopy by using fluorescent-labeled antibodies that bind to only specific bacteria, allowing for quantification.

16.3.2 Cultures

The use of traditional culture media in Petri dishes with incubation at cave ambient temperature is still critical. The use of low-nutrient media for cave microbes or media designed to grow specific types of bacteria, like sulfide oxidizers, has increased the success of cultures. The great majority of environmental microbes still cannot be grown in culture, but biochemical testing for identification can only be done on pure cultures. Presence alone does not guarantee activity, and it does not tell us whether we had one cell to start with or a million.

16.3.3 Molecular Techniques

Non-culture techniques became common in the 1990s and have revolutionized microbiology. The general idea is that a specific protein or nucleic acid is selected, its component sequence determined, and the sequences are compared among organisms. Closely related organisms have similar molecular patterns. These molecular techniques describe the molecule being compared with the suffix "-omics" (e.g., genomics, proteomics, transcriptomics). Genomic studies are showing unexpected diversity and many new and unique cave microorganisms with unknown abilities (Barton 2006). Molecular and cultural methods both have value, showing different aspects of microbial communities.

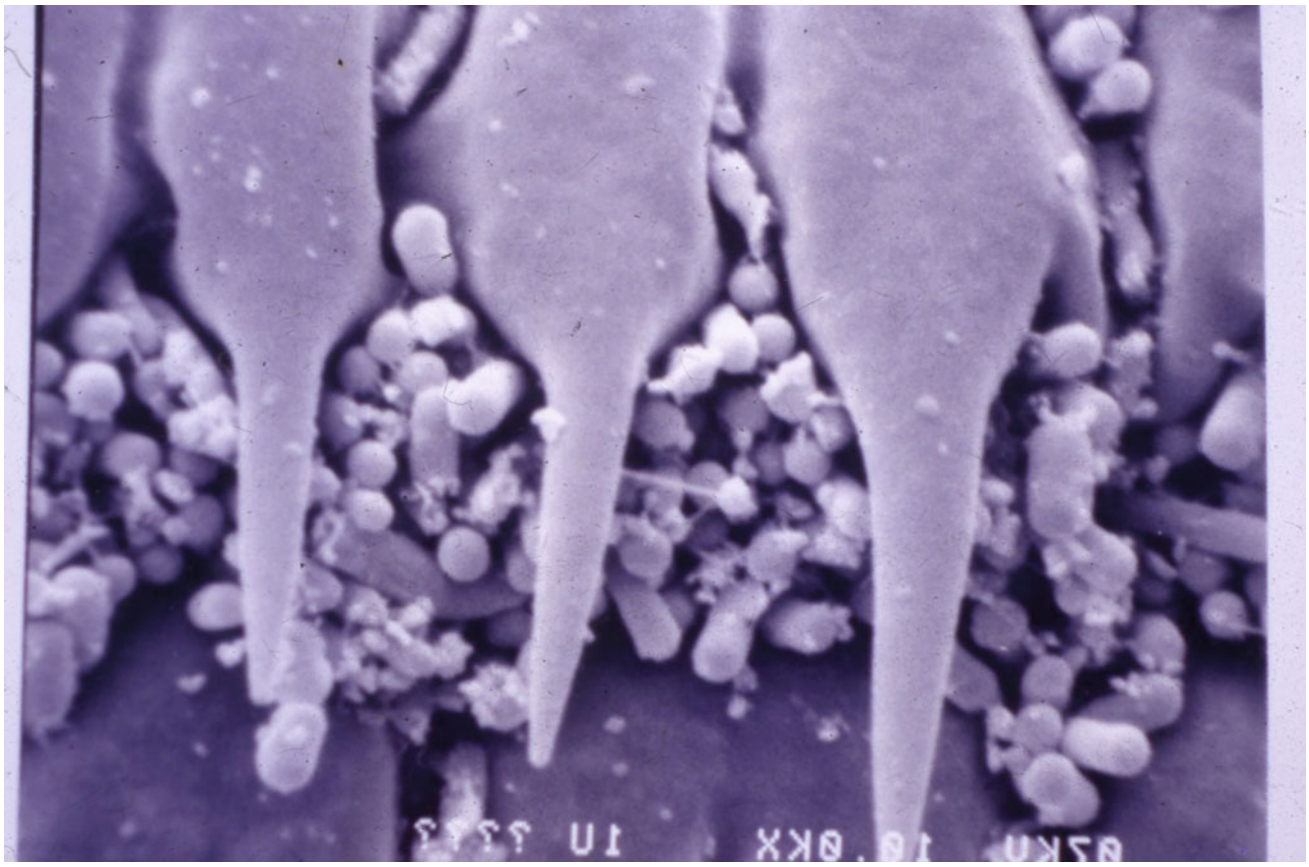

Fig. 16.2 Scanning electron micrograph of chitinous "teeth" with bacterial cells from the crop of a cave cricket

16.4 A Survey of Intraterrestrial Cave Microbes

You are probably familiar with classification of organisms at the level of kingdom, but above kingdoms are domains: Bacteria, Archaea, and Eukarya. Bacteria are prokaryotes with DNA free in the cell and are the most diverse domain. Recent discoveries of many species known only from their DNA, including from caves, have nearly doubled their known diversity (Hug et al. 2015). Archaea are also prokaryotes, but very different from the Bacteria. Eukarya include all organisms with genetic material inside a nuclear membrane, and the familiar kingdoms of animals, plants, fungi, and protists. All life comes from a common ancestor to the bacteria and a branch to a shared lineage for the Archaea and Eukarya (Hug et al. 2015) (To interpret phylogenetic trees, see A Field Guide to the Tree of Life: http://evolution.berkeley.edu/evolibrary/news/160505_treeoflife). Let us review the different types of microorganisms and some of what we know about them in Mammoth Cave.

16.4.1 Protists and Algae

The first paper on cave protists (protozoa) appeared in the mid-nineteenth century, and hundreds of species have since been identified from aquatic cave habitats and moist environments like guano, algae, soils, and parasites (Gittleson and Hoover 1969). Most species are the same as those from forest litter, but some may be truly cave-adapted, both free living and parasites of troglobionts. In Mammoth Cave, amebas were the most commonly observed protzoans, followed by ciliates, and then flagellates (Gittleson and Hoover 1969).

Protists are important in several aquatic environments in Mammoth Cave, usually located on or in bottom sediments (Barr and Kuehne 1971). Water with a direct connection to the surface, such as Echo River, shows higher densities of plankton and some seasonal changes compared to Crystal Lake, an isolated body of water perched above the current water table. Thompson and Olson (1988) found at least 13 genera of protozoa across eight orders from the stream in the upper room of Parker Cave on the sinkhole plain.

Algae are largely phototrophic and not of importance in caves except around entrances and artificial light sources as part of lamp flora (Smith and Olson 2007). Barr and Kuehne (1971) found increased algae in Mammoth Cave associated with heavy rains and spring snow melt, from algae washed into the cave and growth using organic chemicals.

Show caves are in a constant battle to remove algae and lint left by visitors without damaging underlying formations (Saiz-Jimenez 2012). Bright light results in more graffiti from visitors, and the heat dries out surfaces and decreases relative humidity, which may be lethal to cave-adapted organisms. Brightly lit areas in the Frozen Niagara entrance had no cave animals, but a switch to LED lighting made the areas habitable again.

LED lighting was tested by Olson (2006) to see whether it would reduce the growth of phototrophs in Frozen Niagara, the most heavily visited and the best-lit entrance to Mammoth Cave. He photographed a test area, cleaned it with bleach, and set up both gold-phosphor fluorescent lighting and yellow LED lamps, using existing white light as a control. After two and a half years, the traditional white lighting showed the heaviest growth of lamp flora, fluorescent lighting supported limited growth, and no growth using yellow LED lighting.

16.4.2 Fungi

Fungi growing in caves are identical to surface forms (Vanderwolf et al. 2013). They grow from spores in the cave or brought in by flooding, air, or animals. Fungi are important decomposers and recyclers. Simple filamentous forms appear first (Fig. 16.3a), and larger, more complex mushrooms appear last (Fig. 16.3b).

I did experiments in Little Beauty Cave and the Austin Entrance of Mammoth Cave to see how fungi on cave (wood) rat droppings changed with time, scat shape, and interactions with insects (Lavoie 1982). I mixed the same amount of ground up cave rat scat with water and reshaped it like the original rat fecal pellets, a single scat resembling raccoon, and spread a thin layer directly on the cave mud to simulate cricket guano. All groups of fungi were similar in the timing of appearance, but the thin layer proved difficult for the fungus to concentrate enough nutrients for mushroom formation. The numbers of beetle and fly larvae were reduced on the thin layer because the larvae had no refuge inside the scats from predators like staphylinid beetles. If early fungi get a head start, their hyphae can block colonization by invertebrates.

Food spoilage by microbial growth is a way microbes can monopolize a food resource and keep it away from much larger consumers. Microbes can produce dangerous compounds during growth, such as mycotoxins. Most animals reject moldy food if they have a choice. The abundance of cedar in cave rat (wood rat) middens, and nests may be brought in by the wood rats as a way to decrease mold growth on stored materials (Fig. 16.4).

Today the best-known fungus in caves is *Pseudogymnoascus destructans* (formerly *Geomyces destructans*) that causes white-nose syndrome (WNS) in bats, killing them by the millions across the USA and Canada. The fungus is cold-adapted and infects skin of hibernating bats. The infection is irritating and causes the bats to wake up from

Fig. 16.3 **a** Fluffy white *Mucor* fungus growing on a rat latrine in Little Beauty Cave, MCNP. Different ages of droppings have different fungi (photograph by Scott Spicer). **b** Growth of tiny white mushrooms on a highly-leached acorn in the New Discovery Entrance (photograph by Scott Spicer)

Fig. 16.4 A pack rat (*Neotoma* sp.) in her nest in White Cave showing cedar and greens (photograph by Rick Olson)

hibernation, use up their limited fat reserves faster, and leave their hibernacula early in search of food before the flying insects return. Most infected bats die of starvation.

P. desrtuctans is an introduced species from Europe where it does not cause the high mortality seen with North American bats (Puechmaille et al. 2011). Apparently the fungus coevolved with European bats over thousands of years, but it is an invasive species in North American bats that have not developed any resistance. Since its discovery in a cave in New York in 2006, WNS continues to spread across the USA and Canada, making it to MCNP in 2012–13, where it was found in a northern long-eared bat, *Myotis sepertroinalis* from Long Cave, the largest hibernacula in MCNP. Toomey et al. (2013) reviewed actions taken at MCNP starting in 2009 before white nose was detected. Continued surveillance and monitoring of hibernacula and summer bat roosts is done to document population changes. Visitor education provided an opportunity to increase understanding of bats and the value of bats in ecosystems via public announcements, pre-tour briefings by guides, and posters.

WNS has drastically changed the way we cave. Because humans may spread the fungus, great care is taken to decontaminate shoes, clothing, and equipment between caves. Caves on Federal Lands are closed or have greatly reduced access, except for a few caves open to visitors, like Mammoth Cave. WNS continues to spread and is following the major flight routes of infected bats, although it made a big jump to Washington state in 2016. For the latest on WNS, see Chap. 17.

We are not likely to find fungi unique to caves because of their high energy demands, but Vanderwolf et al. (2013) says that low nutrients, stablility, and low temperatures favor fungi adapted to cave conditions, and some may be true troglobionts. We are still looking.

16.4.3 Archaea and Bacteria

Archaea and Bacteria are both domains of prokaryotic cells, with DNA free in the cell; however, they are not closely related. Metabolically, they show huge diversity and can utilize any chemical reaction that potentially has energy. They convert energy from forms that are unusable to higher organisms and produce microbial biomass that can be eaten by animals up the food chain (For more information on food chains and pyramids, see Chaps. 13 and 14). You are familiar with bacteria as pathogens and many species that ferment foods, but the majority is beneficial to the environment and us. The focus of microbial study in caves is on the diversity of bacteria and their contributions to the ecosystem.

Archaea are often found in extreme environments, like thermal springs and salt marshes, but are widely distributed. Methanogens are Archaea that produce flammable methane gas in marshes and in the guts of mammals. Very little work

has been done on Archaea in caves, but Jarrell et al. (2011) speculate that Archaea are adapted to chronic energy stress, which might be a factor in differentiating the ecology of Bacteria and Archaea. Archaea may be important in nutrient-limited cave ecosystems by contributing to nutrient cycling, through sulfur oxidization, methane production, and nitrogen fixation and cycling. Archaea compete successfully in all mainstream environments and are dominant in soils low in nitrogen with low nitrification rates. Archaea in caves need more research.

Bacteria that commonly grow in caves are members of the *Actinobacteria*, a group of filamentous bacteria that produce exospores. *Actinobacteria* make up 10–33% of total soil microbes (Janssen 2006) and are widespread in caves. Metabolically, their main role in nature is in decomposition of organic matter.

References to cave wall slime, wall fungus, and lava wall slime all refer to the often-dense growth of *Actinobacteria* and associated microbes in many caves. Individual colonies have a branched appearance (Fig. 16.5) and are often white to yellow in color, but there are also tan, red, pink, blue, and pumpkin orange colonies. Actinobacterial colonies are often hydrophobic, with water beading up on the surface; the water reflects cavers' lights, described as "cave silver." The typical earthy smell associated with caves is a chemical called geosmin produced by *Actinobacteria*. *Actinobateria* can influence formation development by repelling water causing pitting or irregular surfaces around colonies, and by production of corrosive compounds that alter calcite deposits. Many *Actinobacteria* fix atmospheric nitrogen, particularly in extreme environments, but the role of *Actinobacteria* in nitrogen fixation in caves has not been studied.

Actinobacteria are well known for their production of secondary metabolites including antibiotics such as streptomycin and tetracycline. The majority of our antibiotics (75%) come from *Actinobacteria*. Antibiotic production by microbes in nature may give them a competitive edge over other microbes at high enough concentrations, or the chemicals may have other functions like signal molecules or for predation. Frisch et al. (2003) isolated bacteria from Mammoth Cave that produced potential drugs that blocked cancer, tuberculosis, and angiogenesis, but it takes many years before such discoveries are brought to actual treatment.

Fig. 16.5 Close-up of isolated actinobacterial colonies showing branching. Water beads up on the colonies at the bottom of the picture reflecting caver's lights ("cave silver") (photograph by Thomas Lavoie Photography)

The biomass and activity of microbes in limestone caves in MCNP were studied by Feldhake (1986). He measured microbial metabolic rates in 12 sites in four caves, with comparisons to overlying forest soils. Except for a site rich in cricket guano, Feldhake found that organic matter content, microbial activity, and biomass were much lower in the cave than in forest soil. Autotrophic activity was very low at two of twelve sites and absent at the remaining ten.

An exception to studies that show low numbers and activity of microbes in caves is one that compared the microbial activity, density, and diversity of two aquatic sediment sites in Mammoth Cave (Rusterholtz and Mallory 1994). This study was one of the first to compare high- and low-nutrient culture media. They had high numbers of bacteria and detected active metabolism in 53–58% of the population, despite very low total organic carbon. The diversity of populations was extremely high, with 42% of the isolated species similar to surface microorganisms with no dominant species and the remainder unidentified. These studies should be repeated with today's genetic techniques.

Organic chemical utilization by microbes from water samples collected at different levels in the Styx River drainage in historic Mammoth Cave was studied by Byl et al. (2013). They detected distinct community patterns with highest activity from upper level passages that were comparable to results from a surface stream. Communities from lower levels were slower and used fewer varieties of starting chemicals, but after five days the communities adapted to use almost all of the tested chemicals. The distinct community patterns they observed may vary by season or rainfall.

We are really just beginning the study of bacteria in caves, aided by advances in technology. Despite its large size and complexity, relatively few studies of Bacteria and none of Archaea have been done in Mammoth Cave. One interesting question is the origin of purple wall stains in Mammoth Cave at Mariannes Pass and major areas of purple associated with faults in Long Cave (Olson and Toomey 2016). We do not know yet if the purple deposits are microbial, mineral, or some of both; but we will know soon.

16.5 Genomic Studies

Fowler et al. (2009)[1] suspended biobeads, an inert support surface, in cave streams and pools within MCNP to grow biofilm communities. After 1 year, samples were returned to the laboratory where DNA was extracted and used to produce clone libraries to identify the types of bacteria present in each community. Phyla and class of *Proteobacteia* are compared for two samples (Fig. 16.6); Owl Cave, which has inputs of organics and possibly toxic chemicals, located in Cedar Sink near Park City, and Eyeless Fish Trail (EFT), a pristine, low-nutrient stream accessed by the Austin Entrance on Flint Ridge. The total DNA extracted from each sample is very different: 3463 ng/g from Owl Cave, with 34 clones, and only 476 ng/g from EFT, with 38 clones, supporting lower nutrient input into EFT. In both samples *Proteobacteria* dominate, with 58% from Owl and 79% of clones from EFT, but with different classes. The proportion of unclassified bacteria is 21% in Owl and 6% in EFT. The dominant bacteria from clone libraries of soils are *Proteobacteria* (39%), *Acidobacteria* (20%), and *Actinobacteria* (13%) with all other groups making up less than 10% each (Janssen 2006). The distribution in Owl Cave closely resembles soils, suggesting increased surface input compared to EFT.

The *Proteobacteria* are a large group of Gram-negative bacteria. Both sites (Fig. 16.6) are dominated by *Alphaproteobacteria*, which are a diverse group including chemohetero- and chemoautotrophs. *Betaproteobacteria* are mostly chemoheterotrophs, but include some that fix nitrogen. *Deltaproteobacteria* are found only in Owl Cave and include sulfate reducing bacteria, including anaerobes. The *Gammaproteobacteria*, particularly dominant in Owl Cave, include many familiar Gram-negative bacteria. The distribution is consistent with a polluted environment in Owl Cave, although no indicators of fecal pollution (coliforms) were identified. The different proportion of unknown bacteria shows higher diversity in Owl Cave probably due to more surface inputs. *Nitrospira* are only found in the pristine EFT and are involved in nitrogen cycling. *Planctomycetes*, nearly double in EFT, are an unusual group of bacteria that have stalks for attachment to surfaces, and some of them also oxidize nitrate.

Taking a closer look at the same data at a finer-scale classification, to the level of genus and species, results in the phylogenetic tree shown in Fig. 16.7 for EFT (Fowler 2009, see footnote 1). The higher-level groupings to the right are what we saw in the pie charts. Clones from EFT are labeled MACA-EFT# and are grouped with their closest relatives in GenBank. The *Alphaproteobacteria* include many relatives that are stalked for attachment, like the *Planctomycetes*. Many *Beta-* use one carbon compounds (e.g., methane, methanol). The few *Gamma-* are mostly novel, or related to sulfur cycling bacteria. (For more detail on bacteria of interest, consult https://microbewiki.kenyon.edu).

There are many opportunities to apply genomic and other molecular techniques to increase our knowledge of

[1]An unpublished poster titled "Concentration and Diversity of Bacteria in Clastic Sediments and Limestone Biofilms of Mammoth Cave, Kentucky" by Rick Fowler, Rick Olson, Hazel Barton, and Shivendra Sahi, a progress report to the National Cave and Karst Research Institute in Carlsbad, NM. The poster is stored in the Mammoth Cave National Park Curatorial Facility, accession number 818.

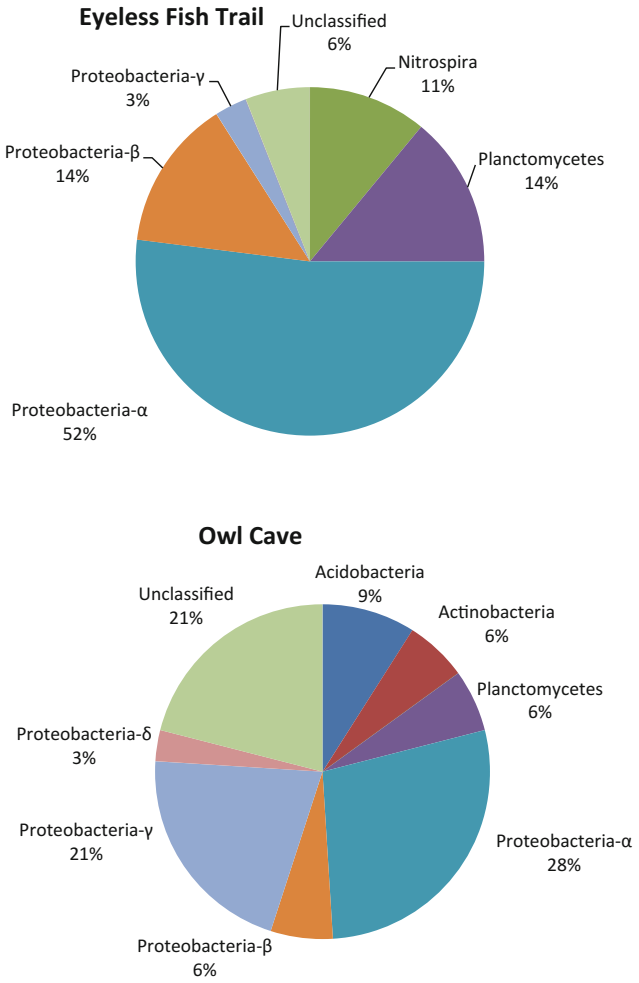

Fig. 16.6 Pie charts of bacterial phyla and Proteobacteria classes of clones from Eyeless Fish Trail and Owl Cave (Fowler et al. 2009, see footnote 1)

bacterial diversity in Mammoth Cave and for the study of Archaea.

16.6 Geomicrobiology

Geomicrobiology is a relatively new field that studies the intersection of microbial activities and geological processes. Microbes are important agents either actively or passively in chemical reactions that influence geological formations on scales from localized to landscape. Biogeochemical cycling of nutrients including carbon, phosphorous, sulfur, and nitrogen are important ecological roles of microbes. Many chemical reactions are both biotic and abiotic, but microbes are probably responsible for all or most low-energy reactions. Development of new tools and techniques in both biology and geology are contributing to a better understanding of the relative contributions of both fields.

Geomicrobiological processes are at work in caves (reviewed in Barton and Northup 2007; Engel 2010; Lavoie et al. 2010) in formation of some speleothems, mineral deposits, biokarst, and the formation of karst caves including Mammoth Cave by dissolution of carbonate rock by acidic water. Rainwater is acidic (pH 5.6), and additional acid comes from microbial activities as water moves through soil overlying limestone. Sulfuric acid speleogenesis is a chemolithotrophic microbial process for forming caves from production of sulfuric acid. The first conference in 1994 on the geomicrobiology of caves was sponsored by the Karst Waters Institute (Sasowsky and Palmer 1994).

16.6.1 Sulfur-Based Ecosystems

One of the earliest studies of microbes in caves using molecular techniques was done by Angert et al. (1998) in nearby Parker Cave, southwest of MCNP on the Sinkhole Plain that drains through Mammoth Cave (Meiman and Palmer 2009). Sulphur River is one of five parallel stream passages in Parker Cave and has a strong odor of hydrogen sulfide from the Phantom Waterfall, a soft pile of white bacterial filaments, sulfate, and elemental sulfur about 2.5 m high and 1 m wide. The likely source of sulfur is underlying oil field brines. The floor in the upper room of the cave has a highly acidic pH of 0.13 and is coated with elemental sulfur, and the ceiling has an acidic layer of microbial biofilm. The terrestrial community in the area is more diverse than other areas in the cave due to the greater microbial food base. A microscopic study of white filaments from the Phantom Waterfall by Thompson and Olson (1988) showed known sulfur-oxidizing bacteria, *Beggiatoa* and *Thiothrix*, with elemental sulfur granules. Sulfuric acid is produced as a waste product. Thompson and Olson speculate that this community is based on bacteria using energy from sulfur completely isolated from the usual indirect photosynthetic input of energy.

Comparing the sequence of a bacterial gene from the microbial filaments with known species, Angert et al. (1998) showed that the Parker Cave community had the greatest similarity to sulfur oxidizing bacteria from deep-sea hydrothermal vents and other sulfur-based environments. Others are related to species that fix CO_2 as a source of carbon. They speculate on possible impacts of growth of these microorganisms on dissolution and precipitation of minerals in caves.

Sulfur inputs are uncommon in Mammoth Cave. Hydrocarbons and hydrogen sulfide seeps near Mariannes Pass in Historic Mammoth were investigated by Olson (2013). A sulfur spring in this area was described by Bullitt in 1845. The seep is deeply weathered into the limestone and smells of hydrocarbons. White microbial biofilms in the seep

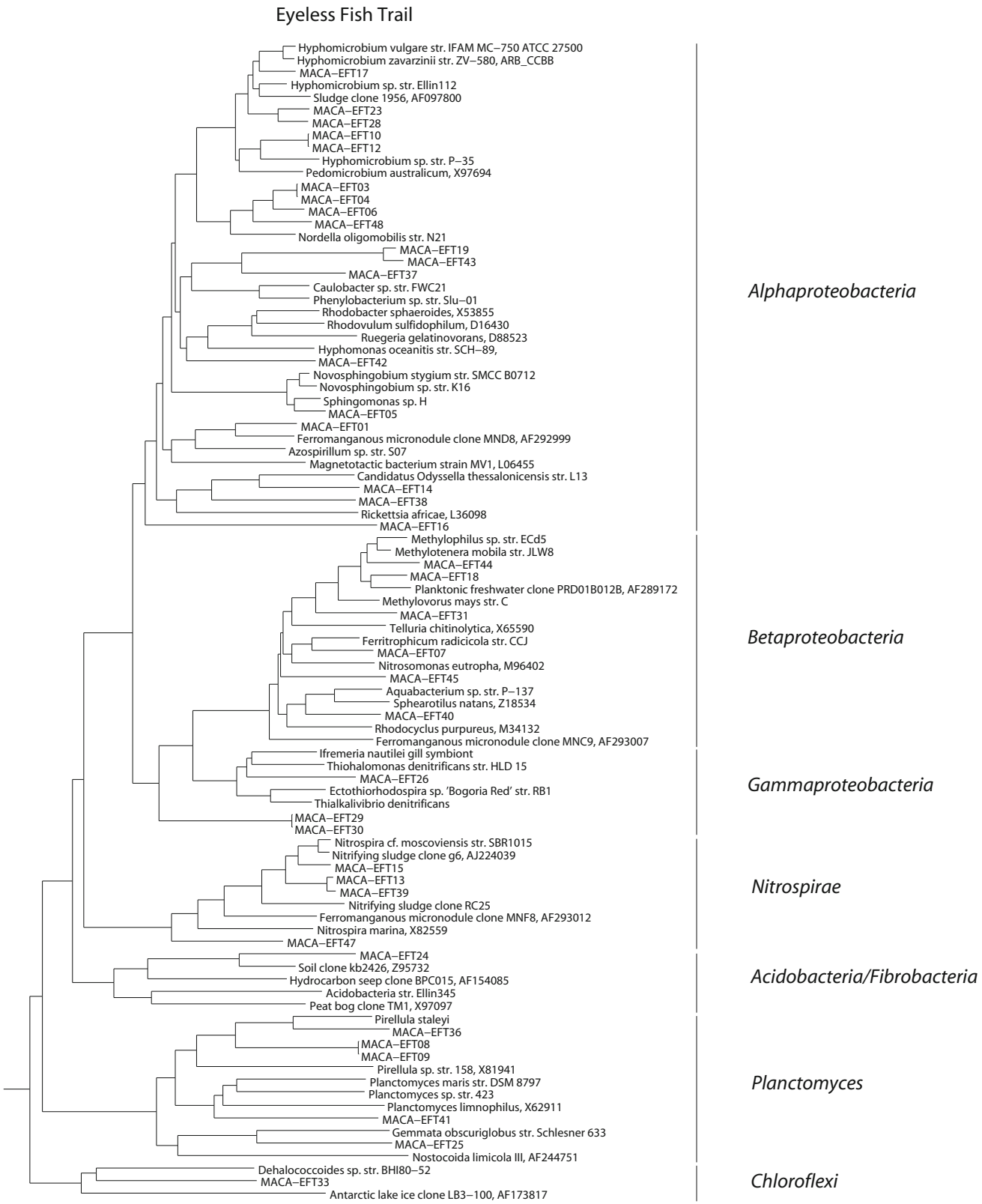

Fig. 16.7 A phylogenetic tree from Eyeless Fish Trail showing isolated clones (MACA-EFT#) and their nearest relatives (Fowler et al. 2009)

support thousands of springtails, with beetles and crickets nearby. Given the ubiquity of H_2S rich water and hydrocarbons under the entire south central Kentucky karst, sulfur inputs in Mammoth Cave need further investigation.

16.6.2 Carbonate Speleothem Formations

Most speleothems in caves are secondary calcium carbonate deposits ($CaCO_3$). A wide range of microbes and microbial processes (Barton and Northup 2007; Engel 2010; Lavoie et al. 2010) can produce extracellular polymeric substances (slime) and precipitate carbonate. Studies of microbial involvement have been carried out on stalactites, stalagmites, helectites, moonmilk, and other speleothems. Bacteria are important nucleation sites for calcite crystal growth that is influenced by the type of bacteria and abiotic factors like nutrients, temperature, and salinity. Biotic mechanisms include corrosion from release of organic acids that alter the crystal structure of the bedrock or formation, or precipitate minerals. Biotic and abiotic mechanisms can operate at the same time. I know of no studies of microbial involvement in speleothem development in Mammoth Cave.

16.6.3 Saltpeter

The best-known example of geomicrobiology in caves is saltpeter, or niter—KNO_3. Historically, caves were mined for saltpeter throughout the American Southeast to produce gunpowder for personal and strategic use during the War of 1812 when US harbors were blockaded by the British (Duncan 1997). Gunpowder is about 75% saltpeter with varying amounts of charcoal and sulfur (see Chap. 3 for more History)

The microbiology of nitre formation in cave soils is a two-step process known as nitrification that converts ammonium ion (NH_4^+) to nitrite (NO_2^-) and then bacteria add oxygen to the nitrite to produce nitrate (NO_3^-). Nitrate is also made by bacteria from insect parts in bat guano. Nitrate can be used by many organisms, or it can be converted to nitrogen gas (N_2) by denitrification. These processes are common worldwide in soils with a good source of organic compounds. Typically, there is a mix of nitrates in cave sediments, mostly with calcium and manganese. The conversion to saltpeter requires the addition of wood ash as a source of potassium and then heating of the leached solution to crystallize out the nitre (Hubbard 2005).

The biggest question in our understanding of saltpetre production at Mammoth Cave is the original source of the nitrogen. Many suggestions have been made, but the historic source was probably the large population of bats in Mammoth Cave. In shallow caves, nitrogen was probably leached from rich surface layers of organic matter and leaves that seeps down into the cave. Cave soils can regenerate niter if allowed to rest undisturbed in contact with the walls and floor of the cave, but Olson and Krapac (2001) investigated niter regeneration in Mammoth Cave for over 6 years and found regeneration rates so slow that groundwater percolation could not account for the original high niter concentrations.

One of the earliest studies of microbiology in Mammoth Cave was by Fliermans and Schmidt (1977), using species-specific fluorescent antibodies to study the presence, distribution, and population densities of *Nitrobacter*, a chemoautotrophic nitrifier. They found high population densities of *Nitrobacter* in Mammoth Cave soils and suggested that it may be responsible for the enrichment in nitrates seen in productive saltpeter soils. Leaching the soil to remove niter is the first step in production of saltpeter, and *Nitrobacter* populations did not change, while total bacteria decreased by 57%. They concluded that the original high niter content was due to bats. Repeating this study with new genetic techniques would be interesting.

16.6.4 Manganese Oxides: Ferromanganese Deposits

Black coatings exposed to flowing water in limestone caves may be poorly crystallized deposits of manganese oxide produced by microbial action (Fig. 16.8). Microbes oxidize soluble Mn^{2+} to trivalent or tetravalent manganese. White et al. (2009) did a systematic study of manganese oxide deposits from caves in central and eastern USA, including Mammoth Cave. They found that all samples contained both manganese and iron, but in different ratios; samples from Mammoth Cave had about four times more manganese than iron. They also reported enrichment of the black deposits in transition metals (copper, cobalt, nickel, vanadium, and zinc) at the fractional percent level, which are a million times greater than concentrations in the surrounding rock and water.

16.6.5 Infections and Parasites

While most microorganisms are neutral or beneficial to us, some microorganisms cause disease. Tuberculosis (TB) is a serious bacterial lung infection that even now infects (active and inactive) one third of the human population of the world. Historically known as consumption or the white plague, in the 1900s an estimated 110,000 Americans died each year from TB, second only to pneumonia and influenza (CDC 1999). Today's leading killers, heart disease and cancer were fourth and eighth, respectively. The treatments for TB in

Fig. 16.8 Black ferromanganese deposits alternating with white calcite on flowstone in White Cave (photograph by Scott Spicer)

1900 included good food, lots of fresh air, and inactivity, which led to the establishment of Sanatoriums (Sucre, n.d.). It is small wonder people were willing to live in the TB Huts deep in Mammoth Cave in hope of a cure. In 1839, Mammoth Cave was purchased by Louisville physician Dr. John Croghan (NPS History 2015). In 1841, he allowed 16 TB patients to move into wooden and stone huts in the Star Chamber beyond Giants Coffin. Cool conditions required open fires for warmth and light, resulting in soot deposits still evident today. Bushes were brought in to cheer up the patients and tours passed by the huts regularly. The deaths of some patients and the worsening conditions of others ended the experiment. Dr. Croghan died of TB in 1849 (see Chap. 3 for more History)

The diet and parasite load of ancient humans can be determined by an examination of their paleofeces. *Giardia,* a protozoan found in polluted waters that can cause diarrhea 2–4 weeks after drinking, has been reported from numerous caves and springs around the world. Human paleofeces from Salts Cave in MCNP show infestation with *Giardia*. Three paleofeces samples dated to 2420 ± BP had *Giardia* cysts (Ruppert 1994). One of eight paleofeces samples from Salts Cave in MCNP showed eggs of *Ascaris* (Fry 1974), a nematode worm that is 15–35 cm long, which is still the most common human nematode infection worldwide.

Crickets can be parasitized by horsehair worms. The infection begins when a cricket drinks from pools contaminated with worm eggs. The juvenile worm leaves the digestive tract and enters the body cavity of the cricket. The worm grows to adult size and bursts through the side of the cricket to drop into water pools under the roost where they mate and lay eggs to complete their life cycle. We Studier et al. (1991) found a horsehair worm infection rate of 9.6% among *Ceuthophilus stygius* camel crickets and 0.5% in *Hadenoecus subterraneus* cave crickets within MCNP. The difference is because *Ceuthophilus* must drink water and *Hadenoecus* gets most of their water from their food. Infected female *Ceuthophilus* had a reduction in eggs.

The cricket "marshmallow" in Fig. 16.9 is covered with a parasitic fungus (*Beauveria spp*). It is a parasite of many different insect host species that is used in insect pest management (Goettel et al. 2005). The fungal hyphae release enzymes that attack and dissolve the insect cuticle, allowing it to penetrate and grow into the insect body. Once inside the insect, it produces a toxin called beauvericin that weakens the host's immune system, and grows until it fills the entire

Fig. 16.9 A cave cricket "marshmallow" in the Frozen Niagara Entrance that has been killed by the growth of a parasitic fungus (photograph by Elizabeth Lavoie)

body cavity. When conditions are favorable (RH > 92%), the fungus will grow out through the softer parts of the insect's body, producing the characteristic "white bloom" appearance. These external hyphae produce spores that are released into the environment to infect the next insect on contact, completing the cycle.

16.6.6 Cave Cricket Microbes

Cave crickets (*Hadenoecus subterraneus*) are like little cave cows. Organisms that consume plant detritus, decaying fruit, and herbivore dung ingest a variety of microorganisms along with their food. If ingested, microbes survive and grow in the digestive tract or excrete enzymes that remain active in the gut, and then they can extend the digestive and metabolic capabilities of the organism. Cows do not actually digest their food; microorganisms in their rumen digest the food and make chemicals that feed the cow.

A similar situation is found with some orthopteran insects including crickets, grasshoppers, and cockroaches. The crop of cave crickets is a very thin-walled structure that lies between the esophagus and hindgut. The inner wall of the crop contains chitinous "teeth" that aid in mixing and movement of food through the digestive system (Fig. 16.2). Crickets can eat up to three times their body weight in food to the point of physical distortion. They waddle back to the cave and hang out, digesting their food over the next several weeks before leaving the safety of the cave to forage again (Studier et al. 1986).

Studier and Lavoie (1990) found that cave crickets rapidly lost weight in water-saturated air only 2 °C above cave ambient temperatures and die in a few hours if held above room temperature (23 °C), possibly due to loss of control over growth of crop microbes. Many bacteria and yeast make gases or toxic metabolites, like ethanol, at elevated temperatures. Some of these crickets, as well as an occasional field-collected specimen, had crops visibly distended with gas, occasionally to the point of rupture. Crop enzyme activity was optimum at 23 °C, above cave temperature (15 °C). When cave crickets were fed diets rich in either carbohydrates or protein and compared to the natural diet, enzymes responded rapidly to the different diets, as expected if microbes were producing the digestive enzymes (White 1989).

Whatever the reasons for the extreme thermal sensitivity of *H. subterraneus*, even a modest increase in cave ambient conditions could have profound negative effects on cave crickets. An increase of 2–6 °C over the next 50 years from climate changes would greatly increase metabolic demands and evaporative water loss of cave crickets, thereby forcing more frequent foraging and exposure to surface conditions and predators. Crickets could be extirpated, with loss of the major source of fixed carbon energy inputs into caves in central Kentucky. Poulson (1991) agrees and speculates that the physiological tolerance data are consistent with the narrow latitudinal distribution of *Hadenoecus* cave crickets between the Ohio River and northern Tennessee. He concludes that community change in caves may be a sensitive indicator of global climate change.

16.7 Human Impacts and Microbial Conservation

Microbes are clearly impacted by human activity. It is important to understand microbial colonization patterns, dispersal mechanisms, and potential effects on human health when studying microorganisms in caves (Saiz-Jimenez 2012). Human impacts are particularly evident in remote areas and with archeological materials.

Evidence of microbes associated with humans was done by comparing areas of Mammoth Cave and Carlsbad Cavern that had high versus low impacts from visitation (Lavoie and Northup 2006). We used swabbing and cultures to look for human-associated microbes (*E. coli* and *Staphylococcus aureus*) and bacteria that could be tracked in from the surface (high-temperature *Bacillus*). We found some trends, complicated because we do not know how long these microbes actually survive in the cave environment, but humans directly alter communities of native microbes in caves.

Shapiro and Pringle (2010) investigated human impacts on fungal diversity in caves including Dogwood Cave and Diamond Caverns that are hydrologically connected to Mammoth Cave. They sampled soils with a range of human impact, including two sites that may never have had human contact. They did not isolate any fungi from the area that had never been visited, as predicted by Caumartin (1963), who thought fungi would not naturally be found in caves without inputs from humans or animals, air, or water. In these caves, fungal diversity rises with moderate levels of disturbance and peaks in minimally disturbed sites. They concluded that impacts of human disturbance are highly localized.

Boston et al. (2006) have offered suggestions on preserving native cave microbes while removing or reducing contaminants, including wooden structures, and the difficulty of telling if materials are natural or anthropogenic (Fig. 16.10). Consideration of microbes should be a factor in choosing what cleaning methods and techniques are used to reduce collateral damage both to the microbes and speleothems. Reducing addition of organic carbon is also important in the low-nutrient cave environment to prevent overgrowth of non-native microbes. Humans continually shed hair, skin cells, and microbes, along with lint from our clothing and crumbs of food, which are great food resources

Fig. 16.10 Fungi colonizing old wood (note sneakers for scale). The white fungi are growing out from the old wood in search of new food. Is the wood brought in naturally or by humans? What invertebrates might live there? (photograph by Scott Spicer)

for non-native microbes. Microbial nature preserves could be established to protect native microbes adapted to low-nutrient conditions. We need to practice clean caving in every cave we visit or study, made all the more important by white-nose syndrome.

16.8 Conclusions

Microorganisms are critically important components of every ecosystem, including caves. Because of the extremely small size of microorganisms, we still have much to learn about their many activities. New techniques have resulted in changes in our understanding of microbial ecology and diversity, and provide many opportunities for future study.

Caves do not exist in isolation from the surface. Caves, speleothems, archeological resources, organisms, and microorganisms can all be harmed by direct visitation and any surface activity that alters quality and quantity of inputs of water, nutrients, and air exchange (Jones et al. 2003). Caves are conduits into the subterranean world, and pollution can impact water quality and cave life. In order to protect caves and what lives in them, we need to understand regional hydrology and what is happening throughout the entire drainage basin. Our knowledge of cave microbes is limited. Despite its great size and complexity, very few microbiological studies have been done in Mammoth Cave. There are many interesting ecological and evolutionary questions along with basic research and inventory to be done on intraterrestrial microbes. Microorganisms and their habitats need conservation along with larger organisms.

Acknowledgements I thank Tom Poulson, Diana Northup, Penny Boston, Louise Hose, and the late Gene Studier for many discussions that have provided insights into the contributions of microbes in caves. I thank Cody Leclair, Doug Soroka, and Lynn Kleina for comments on a draft of this chapter and the Editors for improvements.

References

Angert ER, Northup DE, Reysenbach A-L et al (1998) Molecular phylogenetic analysis of a bacterial community in Sulphur River, Parker Cave, Kentucky. Amer Mineral 83:11–12, Part 2:1583–1592

Barr TC, Kuehne RA (1971) Ecological studies in the Mammoth Cave system of Kentucky. II. The ecosystem. Annal de Spéologie 26 (1):47–96

Barton HA (2006) Introduction to cave microbiology: a review for the non-specialist. J Cave Karst Stu 68(2):43–56

Barton HA, Northup DE (2007) Geomicrobiology in cave environments: Past, current, and future perspectives. J Cave Karst Stu 69:163–178

Boston PJ, Northup DE, Lavoie KH (2006) Protecting microbial habitats: preserving the unseen. In: Hildreth-Werker V, Werker JC (eds) Cave conservation and restoration, Nat Speleol Soc, AL, pp 66–83

Bullitt AC (1845) Rambles in the Mammoth Cave during the year 1844 by a visitor. Reprint, 2nd edn. Cave Books 1985

Byl P, Trimboli SR, Toomey R et al (2013) Antibiotic resistance and substrate utilization by bacteria affiliated with cave streams at different levels of Mammoth Cave. In: Trimboli S (ed) Mammoth Cave National Park's 10th research symposium: Celebrating the diversity of research in the Mammoth Cave region, p 33–39. http://digitalcommons.wku.edu/mc_reserch_symp/10th_Research_Symposium_2013/

Caumartin V (1963) Review of the microbiology of underground environments. Nat Speleol Soc Bull 25:1–14

CDC (1999) Achievements in public health, 1900–1999: control of infectious diseases. Morbid Mort Weekly Rep 48(29):621–629 (CDC.gov)

Domagal-Goldman SD, Wright KE (eds) (2016) The astrobiology primer v2.0. Astrobio 1698:561–653 doi:10.1089/ast.2015.1460

Duncan MS (1997) Examining early nineteenth century saltpeter caves: an archaeological perspective. J Cave Karst Stud 59(2):91–94

Edwards KJ, Becker K, Colwell F (2012) The deep, hot energy biosphere: intraterrestrial life on earth. Ann Rev Earth Planet Sci 40:551–568

Engel AS (2010) Microbial diversity of cave ecosytems (Chap. 10). In: Loy A, Mandl M, Barton L (eds) Geomicrobiology: molecular and environmental perspectives. Springer, Heidelberg, pp 219–238. doi:10.1007/978-90-481-9204-5_10

Feldhake J (1986) Microbial activity and biomass of limestone caves. MS thesis, University of Cincinnati, 204 pp

Fliermans CB, Schmidt EL (1977) *Nitrobacter* in Mammoth Cave. Int J Speleol 9:1–19

Frisch RR, Schaffer P, Haak M et al (2003) Identification of three bacteria from Mammoth Cave, Mammoth Cave National Park, Kentucky, USA that are potential anti-angiogenesis, anti-tuberculosis, or anti-microbial producing organisms. Presentation 103rd general meeting of the American Society for Microbiology, Washington DC

Fry GF (1974) Ovum and parasite examination of Salts Cave human paleofeces. In: Watson PJ (ed) Archaeology of the Mammoth Cave area. Academic Press, New York

Gittleson SM, Hoover RL (1969) Cavernicolous protozoa: Review of the literature and new studies in Mammoth Cave, Kentucky. Annal de Spéléol 24(4):737–776

Goettel MS, Eilenberg J, Glare TR (2005) Entomopathogenic fungi and their role in regulation of insect populations. In: Gilbert LI, Iatrou K, Gill S (eds). Comp Mol Insect Sci 6:361–406, Elsevier, Amsterdam

Hubbard Jr DA (2005) Saltpetre mining. In: Culver DC, White WB (eds) Encyclopedia of caves. Elsevier, New York, pp 492–495

Hug LA, Baker BJ, Anantharaman K, et al (2015) A new view of the tree of life. Nat Microbiol 1:16048. doi:10.1038/nmicrobiol.2016.48

Janssen PH (2006) Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. Appl Envir Microbiol 72(1):1719–1728. doi:10.1128/AEM.72.3.1719-1728

Jarrell KF, Walters AD, Bochiwal C et al (2011) Major players on the microbial stage: why archaea are important. Microbiology 157:919–936

Jones WK, Hobbs III HH, Wicks CM et al (2003) Recommendations and guidelines for managing caves on protected lands. Special Publication 8, Karst Waters Institute

Lavoie KH (1982) The influence of biotic and abiotic factors on the successional decomposition of cave rat dung. Dissertation, University of Illinois at Chicago

Lavoie KH (2015) "A grand, gloomy, and peculiar place": microbiology in the Mammoth Cave region. In: Wagner R, Engel AS (ed) Life in extreme environments: microbial life of cave systems. DeGruyter, Berlin, pp 47–78

Lavoie KH, Northup DE (2006) Bacteria as indictors of human impact in caves. In: Proceedings of the 2005 national cave and karst management symposium. 30th anniversary issue, pp 119–124

Lavoie KH, Northup DE, Barton HA (2010) Microbial-mineral interactions: geomicrobiology in caves: a review. In: Jain SK, Kahn AA, Rai MK (eds) Geomicrobiology. Science Publisher, USA, pp 1–46

Meiman J, Palmer A (2009) The Pennyroyal Plateau, Kentucky. In: Palmer AN, Palmer MV (eds) Caves and karst of the USA. Nat Speleological Soc, Huntsville, AL pp 116–118

Olson R (2006) Control of lamp flora in developed caves. In: Hildreth-Werker V, Werker JC (eds) Cave conservation and restoration. Nat Speleological Soc, pp 343–348

Olson R (2013) Potential effects of hydrogen sulfide and hydrocarbon seeps on Mammoth Cave ecosystems. In: Trimboli S (ed) Mammoth Cave National Park's 10th research symposium: celebrating the diversity of research in the Mammoth Cave region pp 25–31

Olson R, Krapac IG (2001) Origin and regeneration of nitrates in Mammoth Cave sediment. J Cave Karst Stud 63(3):114

Olson R, Toomey RS (2016) The effect of faulting on past and present hydrogeology in Long Cave, Mammoth Cave National Park. In: Trimboli SR, Dodd LE, Young D (eds) Proceedings for celebrating the diversity of research in the Mammoth Cave region: 11th research symposium at Mammoth Cave National Park (April 18, 2016). http://digitalcommons.wku.edu/mc_reserch_symp/11th_Research_Symposium_2016/Complete_Proceedings/

Poulson TL (1991) Effects of recent and future climate change on terrestrial cave communities at Mammoth Cave. Cave Res Found Ann Rep 47–48

Puechmaille SJ, Wibbelt G, Korn V et al (2011) Pan-European distribution of white-nose syndrome fungus (*Geomyces destructans*) not associated with mass mortality. PLoS One 2011 6(4): e19167

Ruppert LK (1994) Evidence for the endoparasite *Giardia lamblia* in human paleofeces from Salts Cave, Mammoth Cave National Park, Kentucky. Thesis (MA), Western Michigan University

Rusterholtz M, Mallory LM (1994) Density, activity, and diversity of bacteria indigenous to a karstic aquifer. Microb Ecol 28:79–99

Sasowsky ID, Palmer MV (eds) (1994) Breakthroughs in Karst geomicrobiology and redox chemistry: Karst Waters Institute special publications 1. Symposium abstracts and field guide. Leesburg WV

Saiz-Jimenez C (2012) A review: microbiological and environmental issues in show caves. World J Microbio Biotech 28:2453–2464

Shapiro J, Pringle A (2010) Anthropogenic influences on the diversity of fungi isolated from caves in Kentucky and Tennessee. Am Midl Nat 163:76–86

Smith T, Olson R (2007) A taxonomic survey of Lamp Flora (Algae and Cyanobacteria) in electrically lit passages within Mammoth Cave National Park, KY. Int J Speleol 36(2):105–114

Studier EH, Lavoie KH (1990) Biology of cave crickets, *Hadenoecus subterraneus*, and camel crickets, *Ceuthophilus stygius* (Insecta: Orthoptera): metabolism and water economies related to size and temperature. Comp Biochem Physiol Part A: Physiol 95:157–161

Studier EH, Lavoie KH, Chandler CM (1991) Biology of cave crickets, *Hadenoecus subterraneus*, and camel crickets, *Ceuthophilus stygius* (Rhaphidaphoriae); Parasitism by hairworms. J Helmint Soc Wash 58(2):248–250

Studier EH, Lavoie KH, Wares WD II et al (1986) Bioenergetics of the cave cricket, *Hadenoecus subterraneus*. Comp Biochem Physiol 84A:431–436

Sucre R (nd) The Great White Plague: the culture of death and the tuberculosis sanatorium. Accessed 6 Dec 2013 at http://www.faculty.virginia.edu/blueridgesanatorium/death.htm

Thompson DB, Olson R (1988) A preliminary survey of the protozoa and bacteria from Sulphur River in Parker's Cave, KY USA. Bull Nat Speleolo Soc 50:42–46

Toomey RS, Thomas SS, Gillespie J et al (2013) White-nose syndrome at Mammoth Cave National Park: actions before and after its detection. In: Trimboli S (ed) Mammoth Cave National Park's 10th research symposium: celebrating the diversity of research in the Mammoth Cave region, pp 206–207

Vanderwolf KJ, Malloch D, McAlpine DF et al (2013) A world review of fungi, yeasts, and slime molds in caves. Int J Speleol 42:77–96

White CR (1989) Digestive enzymes of the cave cricket, *Hadenoecus subterraneus*. In: Lindsey K (ed) 1988. Cave Res Found Ann Rep 61–63

White WB, Vito C, Scheetz BE (2009) The mineralogy and trace element chemistry of black manganese oxide deposits from caves. J Cave Karst Stud 71(2):136–143