Chapter 5
The Microbial Diversity of Caves

Olivia S. Hershey and Hazel A. Barton

5.1 Introduction

In attempting to describe microbial diversity, Baas-Becking famously stated that "Everything is everywhere, but the environment selects" (Baas-Becking 1934). His hypothesis was simple—the small size of microorganisms allows their broad environmental distribution, with the appropriate conditions selecting species growth to dominate niche space (Baas-Becking 1934). Given the many types of caves, and hence a myriad of environmental conditions, it is difficult to describe a broadly relevant microbial diversity; varying cave conditions provide opportunities to select a multitude of adaptations and hence community diversity.

Despite this drawback, caves do represent an important environment for studying microbial ecology, chiefly due to community adaptations to the resource limitation of the subsurface; the lack of photosynthetic activity in caves requires microorganisms to rely on either the heterotrophic breakdown of scant allochthonous organic carbon or autotrophic growth using in situ redox-active compounds (Peck 1986; Northup et al. 2003; Carmichael et al. 2013; Desai et al. 2013; Parker et al. 2013; Jones and Macalady 2016). While some caves, including sulfidic systems, are dominated by chemolithotrophic primary production, such cave systems are relatively rare (and their microbial diversity is reviewed in Chap. 15). In more common epigenic caves, nutrients entering from surface-derived ecosystems are limited, with allochthonous organic carbon delivered by vadose-zone groundwater generally measured below 0.5 mg/L (Barton 2015). This puts cave environments firmly in the oligotrophic (<2.0 mg/L) spectrum of energetic systems; for simplicity, we will to refer to such systems as *oligotrophic* caves (Engel et al. 2010; Harmon et al. 2013; Barton 2015).

O. S. Hershey · H. A. Barton (✉)
Department of Biology, University of Akron, Akron, OH, USA
e-mail: ose1@zips.uakron.edu; bartonh@uakron.edu

O. T. Moldovan et al. (eds.), *Cave Ecology*, Ecological Studies 235,
https://doi.org/10.1007/978-3-319-98852-8_5

Fig. 5.1 A passage within Poor Farm Cave, West Virginia, USA. Based on the age of fossil skeletons found within cave sediments, it is unlikely that the environmental conditions within this passage have significantly changed in the last 400,000 years (Grady et al. 2000). Energy for microbial growth likely comes from allochthonous organic carbon entering via cracks or fissures in the bedrock, or the presence of redox-active compounds [Mn(II), Fe(II), NO_3^-, or NH_3].

Another unique feature of caves, and one commonly overlooked by microbiologists not used to working in such systems, is the static nature of the environment; once cave forming processes have ended, the environmental conditions of a particular niche might not change for thousands (to potentially millions) of years (Fig. 5.1; Klimchouk et al. 2000; Palmer 2007). This is in stark contrast to surface ecosystems, where plant species, foraging animals, anthropogenic impacts, and even the weather can have a profound influence on microbial community structure in short (daily, seasonal) time frames (Barton 2015; Palmer 2007). The absence of these dynamic processes in caves means that the selective pressures driving diversity are dependent on the variables that are present and strongly influenced by the geologic setting and geochemical (environmental, local and regional) nature of the cave (Ortiz et al. 2013).

This review will not try to provide a synthesis of the ~400 papers published on cave microbiology (Fig. 5.2). Instead, it will focus on the microbiology of oligotrophic caves in limestone (carbonate; $CaCO_3$) settings. We will also not produce a comprehensive list of the microbial species found in caves, as this has recently been reviewed elsewhere (Vanderwolf et al. 2013; Tomczyk-Żak and Zielenkiewicz 2016). Instead, we will attempt to outline how the research history has led to our current understanding of microbial ecology in caves, the potential for a *core microbiome,* and the common ecological themes that might drive microbial diversity in caves.

5.2 Microbial Diversity in Caves Prior to 1996

Our understanding of the microbial ecology of caves is influenced by the technology of the time (Engel 2015). As microbiology was built upon the ability to culture microorganisms within the laboratory, early cave researchers used the same cultivation techniques as soil scientists and (somewhat unsurprisingly) found that caves were a weak reflection of the microbiology of surface soils (Hess 1900; Scott 1909; Høeg 1946; Caumartin 1963). The interpretation of microbial activity in caves was therefore limited, and it seemed to be of little interest to the scientific community, with less than 40 papers published prior to 1997 (Fig. 5.2; Engel 2015). Yet these papers defined our understanding of cave microbiology, suggesting that caves were essentially lifeless due to an absence of photosynthetic input, or simply home to transient microbial species introduced by the activity of animals or humans (Caumartin 1963).

When endemic cave microorganisms were putatively identified, it was through unusual metabolisms that were (incorrectly) thought to distinguish them from soil species, such as iron oxidation (Caumartin 1963).

The primary limitation of cultivation-based approaches is that the vast majority (>99%) of environmental microorganisms cannot be cultured; as in other microbial environments, the ability to accurately describe microbial diversity within caves required cultivation-independent techniques (Amann et al. 1996). Some early non-cultivation approaches did support the idea that microbiology in caves was more complex than originally thought: Fliermans and Schmidt (1977) used antibodies to identify non-culturable *Nitrobacter* in Mammoth Cave sediments; the microscopic techniques of Cunningham et al. (1995) demonstrated a rich structural diversity from samples deep within Lechuguilla Cave; and Gonzalez et al. (1999) demonstrated a rich diversity of actinobacteria in Spanish caves using fatty acid methyl ester (FAME) analyses (Fliermans and Schmidt 1977; Cunningham et al. 1995; Gonzalez et al. 1999). Nonetheless, it wasn't until the use of molecular phylogenetics in the 1990s that the potential diversity of microorganisms in cave environments emerged (Fliermans and Schmidt 1977; Cunningham et al. 1995; Pace 1997; Gonzalez et al. 1999; Barton 2006; Barton and Northup 2007; Engel 2010; Lee et al. 2012).

5.3 Microbial Diversity in Caves: The Molecular Era (1997–2012)

First introduced in the 1980s as a revolutionary way of identifying microorganisms in the environment, Pace et al. used the 16S small ribosomal subunit rRNA gene sequence as a genetic marker (phylotype) to distinguish previously uncultured species (Stahl et al. 1984; Pace et al. 1986). It was also Pace (himself an avid cave explorer) who facilitated the first molecular analysis of a microbial cave community (Fig. 5.2). These investigators used molecular phylogenetic approaches to examine the filamentous biofilms of a sulfidic stream within Sulfur River Cave,

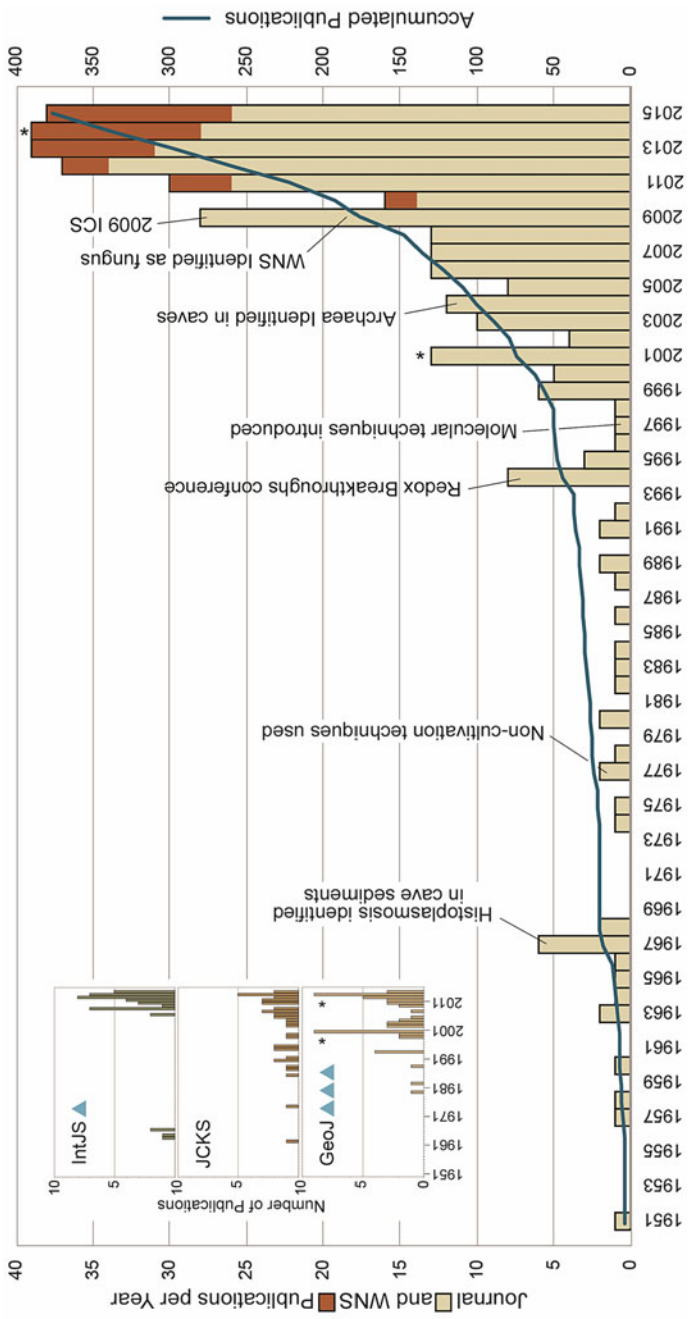

Fig. 5.2 Impact of major events in cave microbiology on research in the field, as measured by publications in peer-reviewed journals (abstracts and conference proceedings are not included in cumulative totals). The number of publications per year is indicated, cumulative total of publications, as well as the impact of the WNS epidemic on cave microbiology publications. Redox Breakthroughs Conference = Breakthroughs in Karst Geomicrobiology and Redox Geochemistry Conference (see Sasowsky and Palmer 1994). WNS = White-Nose Syndrome. 2009 ICS = International Congress of Speleology Special Section: Microbiology and Geomicrobiology of Cave and Karst Environments. The "*" symbol indicates the publication of a special issue on Cave Microbiology by the *Geomicrobiology Journal*. Inset: histogram of publication rates by the primary journals in the field, including *International Journal of Speleology* (IntJS), *Journal of Cave and Karst Studies* (JCKS), and *Geomicrobiology Journal* (GeoJ); blue triangles correspond to the number of cave microbiologists who serve as associate editors

Kentucky (Angert et al. 1998). This study revealed the surprising dominance of the *Epsilonproteobacteria,* which were previously seen only in deep, oceanic hydrothermal systems; it was also the first clue to the important influence that members of this phylum have within sulfidic cave environments (Campbell et al. 2006). Most importantly, the study also demonstrated that microbial cave communities could be remarkably distinct from their surface counterparts (Angert et al. 1998).

Through the 1990s molecular phylogenetics was the most powerful tool to study microorganisms within the environment (Pace 1997). But the tool remained limited to labs with both the molecular expertise and computing resources necessary to translate genetic difference into the robust phylogenies necessary to identify uncultivated microorganisms, with only two labs carrying out such analyses in caves (Vlasceanu et al. 1997; Angert et al. 1998). The ability to analyze cave communities was further complicated by the low biomass of these environments (routinely $<10^6$ cells/g), along with a complex geochemistry, both of which interfered with the ability to obtain sufficient DNA for analysis (Barton et al. 2006). This restricted early analyses to sites with enough biomass to overcome DNA extraction limitations, such as those found in the chemolithotrophic ecosystems of sulfidic caves, as examined by Pace (Sarbu et al. 1996; Angert et al. 1998; Engel et al. 2004a).

A lot changed over the next 15 years: new techniques improved the ability to extract DNA from the environment (Barton et al. 2006; Tan and Yiap 2009); new programs made phylogenetic analysis more accessible (Posada 2003; Wilgenbusch and Swofford 2003; Edgar 2004; Kumar et al. 2006; Pruesse et al. 2007); sequencing technologies reduced costs (Shendure et al. 2004); and high-impact journal articles demonstrated the important contributions that the study of cave microorganisms could provide (Cunningham et al. 1995; Engel et al. 2004b). A number of events further raised the profile of cave microbiology, beginning with the 1994 *Breakthroughs in Karst Geomicrobiology and Redox Geochemistry* Conference (59 conference proceedings), a special issue of the *Geomicrobiology Journal* in 2001 (10 journal articles), and a special session on the *Microbiology and Geomicrobiology of Cave and Karst Environments* at the 2009 International Congress of Speleology (32 conference papers). The cumulative impact of these changes was a fivefold increase in the number of journal articles on cave microbiology between 1997 and 2012 (from 43 to 256; Fig. 5.2).

Traditional molecular phylogenetic approaches involve PCR amplification of 16S rRNA gene sequences from environmental DNA, followed by cloning or denaturing gradient gel electrophoresis (DGGE) to generate libraries of representative 16S rRNA sequences (Pace 1997). These libraries can range in size from a few dozen to a few hundred cloned sequences. Nonetheless, given the tens of thousands to potentially millions of microbial cells in every sample, such "clone" libraries tend to identify the species/phylotypes within an environment that make the greatest contribution to total environmental DNA and/or 16S rRNA copy number (Fig. 5.4; DeSantis et al. 2007; de Araujo and Schneider 2008; Kembel et al. 2012). While this does allow a snapshot of the most successful microorganisms within an environment, it is also a limitation of the technique, potentially missing a large number of organisms that play important roles in ecosystem function (Fig. 5.4).

Box 5.1

This dramatic rise in microbial research also opened our eyes to the potential diversity of microorganisms in cave environments. Rather than supporting the idea that caves were dominated by a few specialized species adapted to nutrient limitation, caves appeared to be home to a diverse assemblage of species from multiple phyla, including the *Alpha-, Beta-, Gamma-*, and *Deltaproteobacteria, Chloroflexi, Planctomycetales, Bacteroidetes*, Acidobacteria, and Actinobacteria, with small but significant contributions from members of the *Nitrospirae, Gemmatimonadetes*, and *Verrucomicrobia* (Fig. 5.3; Northup et al. 2003; Barton et al. 2004; Chelius and Moore 2004; Barton et al. 2007; Zhou et al. 2007; Cuezva et al. 2012; Lee et al. 2012; Porca et al. 2012; Rusznyak et al. 2012; Ivanova et al. 2013; Barton 2015). These data also demonstrated the potentially significant contribution of the archaea to subsurface communities (Northup et al. 2003; Chelius and Moore 2004; Shabarova and Pernthaler 2010).

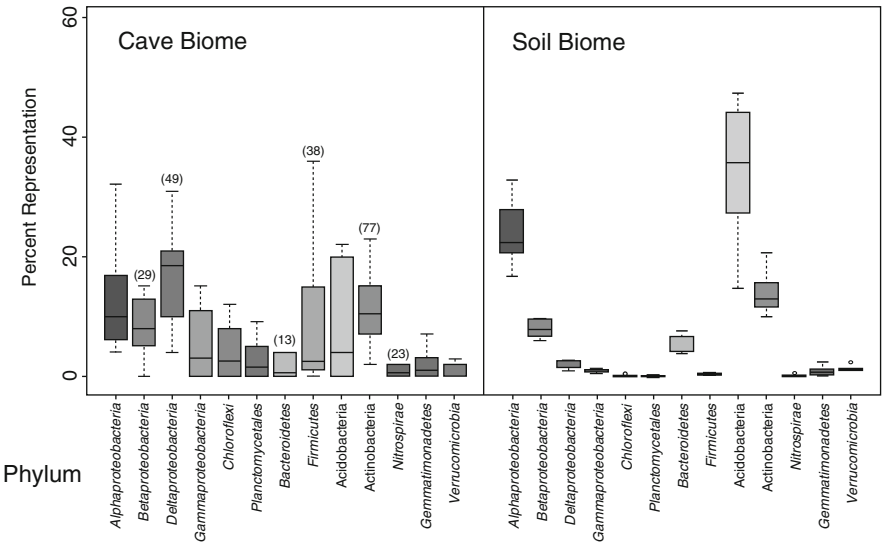

Fig. 5.3 Box plot comparison of soil microbial communities to those found in caves. The representation of major phyla of pooled soil and cave samples is shown. The soil biome was obtained from the collated data of Chu et al. (2010). The cave biome was collated from the data of Northup et al. (2003), Barton et al. (2004, 2007), Chelius and Moore (2004), Zhou et al. (2007), Cuezva et al. (2012), Porca et al. (2012), Rusznyak et al. (2012), and Ivanova et al. (2013). Only datasets including at least 100 cloned 16S rRNA phylotypes are included. The boundaries for the first and third quartile are shown, with the centerline representing the mean and whiskers representing the max/min values (outlier values for the cave biome data are shown in parentheses)

5.4 Microbial Diversity in Caves: The Genomics Era (2013–Present)

Among the many impacts of the Human Genome Project, the most powerful was the development of optically based sequencing methods—collectively referred to as "next-generation sequencing" (NGS) technologies (Ansorge 2009; Lander 2011). The dramatic increase in the number of bases that these technologies could sequence (>15 billion bases in as little as 4 h) combined with their significant cost reductions revolutionized the ability to sequence DNA (Snyder et al. 2009; Forde and O'Toole 2013). Sogin et al. (2006) were the first to use NGS to identify environmental 16S rRNA; rather than restricting the identification of phylotypes within a community to a few hundred cloned 16S rRNA genes, NGS allowed Sogin and colleagues to sequence 120,000 PCR products directly. The results were transformative and demonstrated that microbial ecosystems contained thousands of previously unidentified phylotypes (Sogin et al. 2006). Sogin et al. referred to this extensive collection of previously unidentified microorganisms as the "rare biosphere"—organisms of sufficiently low number that they cannot be identified without deep-sequencing NGS approaches (Fig. 5.4).

Due to limited access to NGS and the advanced statistical methods needed to distinguish unique DNA sequences against a background of inherent PCR error, this technology was also initially limited to a few specialized labs (Sogin et al. 2006). But as

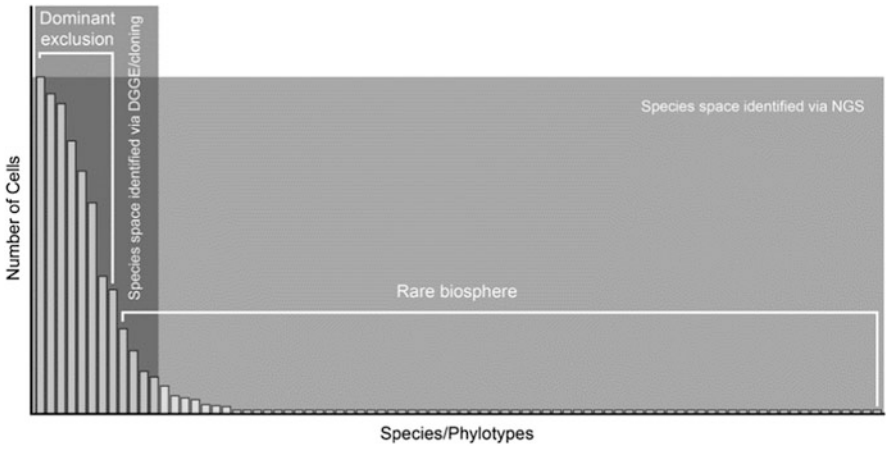

Fig. 5.4 Idealized microbial diversity in cave environments and representative portions screened via different molecular techniques. Some species are able to rapidly utilize the available nutrient and energy sources, providing a competitive advantage that allows them to become dominant in the ecosystem (similar to *r*-type selection). These dominant species are most often identified in shallow-coverage analyses, such as clone libraries and DGGE. The rare biosphere contains a combination of numerically low, slower-growing, poorly adapted, or even viable but non-growing species (similar to *K*-type selection). The rare biosphere can usually only be identified using deep-sequencing approaches, such as Illumina sequencing. NGS, next-generation sequencing

researchers developed more efficient mathematical algorithms to reduce the necessary computational power, and techniques in bioinformatics simplified the analysis of large NGS data sets, this technology rapidly became available to other researchers (Kuczynski et al. 2012; Caporaso et al. 2010; Stamatakis 2014). The first to apply these applications in cave environments were Ortiz et al. (2013) who used 454-pyrosequencing to examine ~400,000 PCR products from Kartchner Caverns, USA (Ortiz et al. 2013). Along with the 13 phyla already identified in caves by cloning approaches, Ortiz et al. demonstrated the presence of an additional 8 described and 12 candidate phyla, suggesting that caves also contained rare biosphere microorganisms. In a significant step forward, these researchers also used NGS to compare microbial communities in the cave with those in surface soils directly above. These data demonstrated that only 16% of the sequences were shared between the surface and the cave, confirming the uniqueness of microbial cave ecosystems (Ortiz et al. 2013).

In the years since this study, there have been no other published 16S rRNA NGS sequence datasets from oligotrophic caves; however, a number of studies have submitted sequence data to public databases, such as the NCBI sequence read archive (SRA) (Fig. 5.5; Leinonen et al. 2010). By processing these datasets, it is possible to expand the work of Ortiz et al. across multiple cave systems with broadly distributed geographical locations, including North America and Asia (Fig. 5.5). The results confirm the robustness of the 13 dominant phyla already identified, along with another 14 phyla consistently represented in these populations (above a 0.1% threshold); these include the *Armatimonadetes* (OP10)*, Chlorobi, Cyanobacteria, Elusimicrobia, Spirochetes*, and the candidate phyla BRC1, GN04, NC10, OP3 (Ca. *Omnitrophica*), TM6 (Ca. *Dependentiae*), WS1, and WS3 (Ca. *Latescibacteria*). Together these data support the existence of a *cave rare biosphere* (Fig. 5.5). The cave NGS datasets also contain a

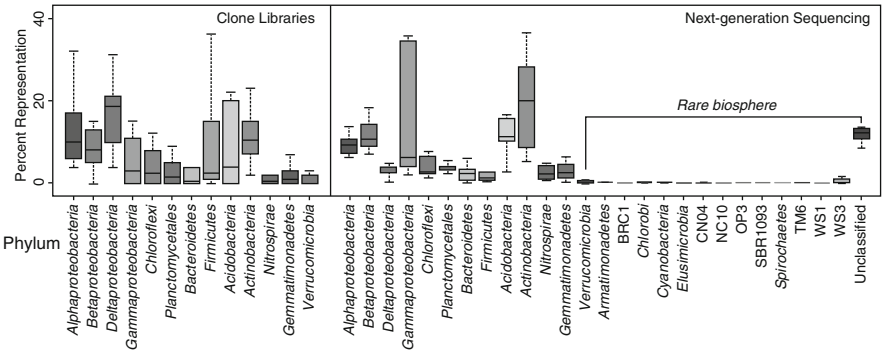

Fig. 5.5 Boxplot comparison of microbial cave community structure analyzed by 16S rRNA gene clone libraries (from Fig. 5.3) or Illumina (next-generation) sequencing. The datasets SRR1686967, SRR1686970, SRR1686976, SRR1693633, SRR1703816, SRR1703817, SRR1703818, SRR1703819, SRR1703820, and SRR1703821, obtained from the NCBI sequence read archive (SRA), were used. The datasets, which contained between 12,901 and 585,434 unfiltered sequence tags, were processed in QIIME (Caporaso et al. 2010) to assign taxonomic identity. The boundaries for the first and third quartile are shown with the centerline representing the mean and whiskers representing the max/min values

significant proportion of sequences that cannot be classified using the taxon reference databases, Greengenes and SILVA (Fig. 5.5; McDonald et al. 2012; Quast et al. 2013). These sequences, which cannot be easily placed within the current taxonomic framework, are known as *microbial dark matter* and represent the currently unexplored diversity of microbial populations (Rinke et al. 2013). Such sequences tend to have a higher representation in caves than other habitats, suggesting that the true diversity of caves requires further description (Sogin et al. 2006; Rinke et al. 2013).

While targeted PCR amplification makes it possible to rapidly screen the 16S rRNA sequences in the environment, it is also susceptible to significant technical issues, including primer and amplification biases that preferentially select certain DNA sequences for amplification (Chandler et al. 1997; Polz and Cavanaugh 1998; DeSantis et al. 2007; Kembel et al. 2012). Overcoming these limitations requires bypassing the PCR amplification step entirely and sequencing the sum of the genetic information in the environment (Miller et al. 2011). This process requires randomly fragmenting DNA into sizes appropriate for NGS sequencing (35–300 bp), either by mechanical means or using transposons (Adey et al. 2010). These fragments are then sequenced, and the overlapping ends are computationally reassembled back into a full-length DNA contig, ranging in size from a few hundred to millions of bases—a technique referred to as "shotgun sequencing" due to the randomness of the initial DNA fragmentation (Sanger et al. 1977; Adey et al. 2010). Prior to the advent of NGS, shotgun methods were not possible using environmental DNA as the complexity of the samples reduced the likelihood of obtaining sufficient coverage for assembly (Venter et al. 2004). But NGS dramatically increased sequence coverage, making it possible to examine all the genes in an environment rather than just one—a technique called *metagenomics*. Such metagenomic approaches allow the interactions that support microbial ecosystem dynamics to be identified through the functional gene composition of the community (Handelsman 2004; Tyson et al. 2004; Venter et al. 2004).

Carrying out metagenomic approaches in oligotrophic caves continues to be problematic, primarily due to the significant amounts of DNA that are needed to create shotgun libraries, from a minimum of a few hundred nanograms to multiple micrograms, depending on the method (Thomas et al. 2012). Despite these limitations, in 2014 Ortiz et al. were able to carry out metagenomic analyses of the microbial communities within Kartchner Caverns. Their data identified over 365,000 gene fragments from the microbial populations found on speleothems and walls within the cave and demonstrated that the enrichment of genes involved carbohydrate metabolism and CO_2 fixation. The enrichment of these genes suggested that both heterotrophic and autotrophic metabolic activity were important in community growth and subsistence, along with potentially novel mechanisms of nutrient cycling, especially in regard to nitrogen.

To date, the technical limitations of low biomass have prevented other researchers from publishing metagenomic studies from oligotrophic caves. Nonetheless, alternate approaches still allow researchers to use NGS to explore evolutionary adaptations, including genomic sequencing of cultured isolates (Lee 2008; Land et al. 2009; Bhullar et al. 2012; Barton et al. 2013; Saw et al. 2013; Gan et al. 2014; Jiao et al. 2015). Land et al. (2009) were the first to sequence the genome of a bacterial species

isolated from a cave: *Beutenbergia cavernae*. Their data demonstrated the significant contribution of carbohydrate catabolism and nutrient cycling genes to the genotype of this organism, mirroring the results of Ortiz et al., despite the large geographic distance between the two cave sites (China versus Arizona, USA) (Land et al. 2009). A culture study by Bhullar et al. (2012) on antimicrobial resistance phenotypes in Lechuguilla Cave (USA) sequenced the genomes of a number of isolates, revealing the presence of a novel antibiotic resistance pathway and suggesting the in situ evolution of antibiotic resistance. Finally, a comparative study between cave and soil strains of *Pseudomonas fluorescens* determined that cave isolates had adapted to living within a mineral (rather than soil) environment, demonstrating genomic traits that could be considered evidence of endemism, including horizontal gene transfer events, increased scavenging efficiency using twitching motility, and an increased ability to cycle nutrients, particularly nitrogen (Barton et al. 2013). Thus, while culturable organisms within caves do represent a small minority (<1%), they are still able to provide important clues to microbial adaptation within caves (Land et al. 2009; Bhullar et al. 2012; Barton et al. 2013).

5.5 Ecological Themes in Cave Microbial Communities

Box 5.2
Microbial communities in oligotrophic caves are dominated by the *Alphaproteobacteria, Betaproteobacteria, Deltaproteobacteria, Gammaproteobacteria, Chloroflexi, Planctomycetales, Bacteroidetes, Firmicutes,* Acidobacteria, Actinobacteria, *Nitrospirae, Gemmatimonadetes*, and *Verrucomicrobia* (Fig. 5.3). This distribution appears to be robust across a broad geographic range, indicating that the key drivers of cave community structure are consistent and that this diversity represents a core cave microbiome.

More than 80 years after Baas-Becking developed his theory of "everything is everywhere. . ." the identification of a rare biosphere appears to provide the necessary empirical support—a cosmopolitan distribution of microorganisms that can proliferate or "bloom" under the appropriate conditions (Lynch and Neufeld 2015). If all environments contain such functionally diverse populations, then the primary drivers of population dynamics and structure are simply those factors that favor one microorganism over another (Lynch and Neufeld 2015).

Yet these same 13 phyla (see Box 5.2) are also dominant in soils, which have remarkably different conditions of light, productivity, disturbance, and pH (Fig. 5.6). The simplest explanation of such similarity is that soil microorganisms seed caves. The seeding hypothesis for cave community structure would certainly provide an explanation of how cave environments are populated: the solvent action of surface (meteoric) water creates the cave, while also carrying microbial species into this newly

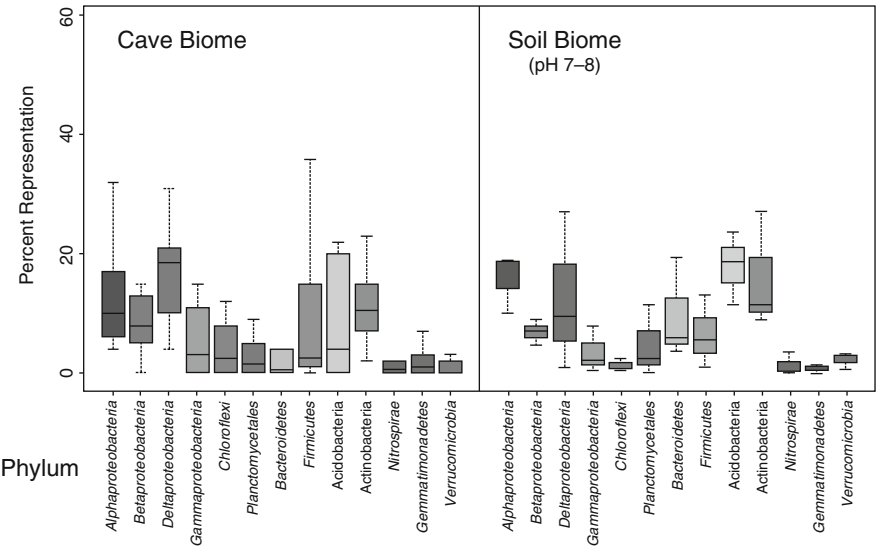

Fig. 5.6 Boxplot comparison of soil microbial communities under alkaline conditions to those found in caves. The cave biome includes the data shown in Fig. 5.3. The soil biome data was obtained from the studies of Lauber et al. (2009), Zhou et al. (2007), Ganzert et al. (2014), and Zhalnina et al. (2015). The boundaries for the first and third quartile are shown, with the centerline representing the mean and whiskers representing the max/min values (outlier values for the cave biome data are shown in parentheses)

forming habitat. Recent studies support this idea, including the identification of surface/epikarst-derived microorganisms in stalactite drip water (based on their covariance with seasonal events) and the scant (<0.1%) population of cyanobacteria observed in cave samples via deep sequencing (Fig. 5.5) (Gerič et al. 2004; Harmon et al. 2013; Yun et al. 2015). Nonetheless, several pieces of evidence suggest that the long-term impact of surface species on cave community structure may be diminished over time, including (1) the finding by Ortiz et al. that less than 16% of the microorganisms found in caves share taxonomic identity with soil species, (2) the significant evolutionary adaptations observed in the genomes of indigenous microorganisms, and (3) a study by Johnston et al., which demonstrated that human commensal species introduced into a cave habitat are quickly lost (Land et al. 2009; Barton and Barton 2012; Johnston et al. 2012; Ortiz et al. 2013). Thus, while soil may seed these environments, unique selective pressures within caves "sort" microbial species into the observed cave populations (Fig. 5.3).

5.6 An Incomplete View

This review has focused on the contributions of bacteria to cave microbial community structure as most studies likewise focus on the members of this domain (Peck 1986; Vlasceanu et al. 1997; Schabereiter-Gurtner et al. 2002; Laiz et al. 2003; Barton et al. 2004; Engel et al. 2004b; Ikner et al. 2007; Zhou et al. 2007; Banks et al. 2010; Iker et al. 2010; Bhullar et al. 2012; Cuezva et al. 2012; Ortiz et al. 2013). Nonetheless, focusing on the bacteria provides an incomplete view of microbial populations, which commonly include contributions from the archaea and microscopic eukarya—in caves, primarily in the form of filamentous fungi (Pace 1997; Barton and Northup 2007; Vanderwolf et al. 2013). While some studies have incorporated the archaea into their analyses and others have focused exclusively on mycology, none have attempted to determine community structure and metabolic relationships across all three domains of life (Woese and Fox 1977, Northup et al. 2003; Tetu et al. 2013; Vanderwolf et al. 2013; Barton et al. 2014; Ortiz et al. 2014). Given the significant contributions that members of these domains can make in other microbial ecosystems, it is reasonable to assume that the archaea and fungi help shape community metabolic interactions and diversity in caves.

5.6.1 Archaea

The first demonstration of archaea in caves was by Northup et al. (2003), who identified members of the *Thaumarchaeota* (at the time still phylogenetically grouped within the *Crenarchaeota*) in the ferromanganese deposits of Lechuguilla Cave, USA. Other studies supported the presence of archaea in caves, including the significance of the *Thaumarchaeota* across multiple cave habitats (Fig. 5.7; Chelius and Moore 2004; Barton et al. 2007; Barton et al. 2014; Ortiz et al. 2014). In all cases, the numerical abundance of the archaea remains small (<2%), although these studies are based on sequence-only approaches, rather than direct cell counts (Fig. 5.7). In our work, which incorporated direct cell counts using archaeal-specific fluorescent in situ hybridization, we have observed a strong correlation between the availability of nitrogen and presence of archaea; when nitrogen levels are at their lowest (ng/L), the contribution of the *Thaumarchaeota* to total population size can exceed 15% (Johnston and Barton unpublished data), indicating that the contribution of archaea to population structure covaries with resource limitation. The small size of *Thaumarchaeota* (up to 100-fold smaller than their bacterial counterparts), their slow growth rate, and innate resistance to severe energetic stress could certainly make the archaea more competitive under extreme nutrient limitation and explain why they make a larger contribution to microbial community structure in some cave environments (Könneke et al. 2005; Valentine 2007; Brochier-Armanet et al. 2008; Brochier-Armanet et al. 2012).

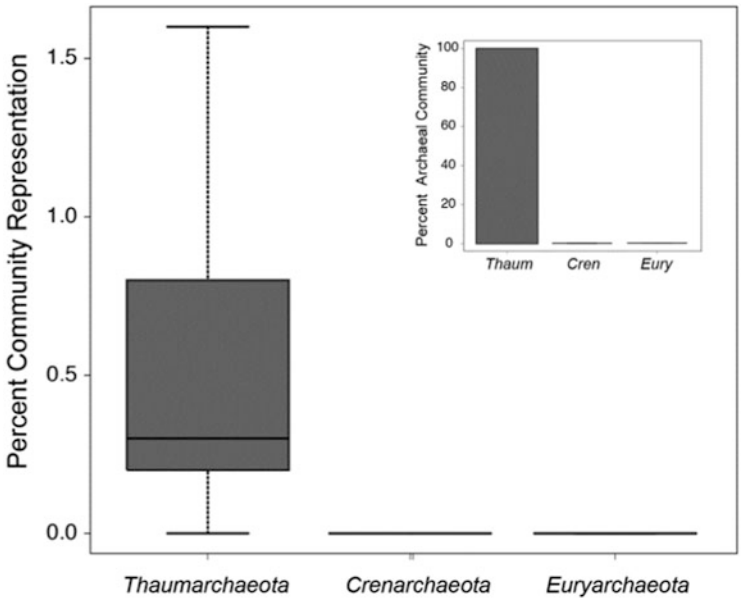

Fig. 5.7 Boxplot comparison of the relative distribution of three major Archaea phyla, *Thaumarchaeota, Crenarchaeota*, and *Euryarchaeota* in cave environments as determined by Illumina sequencing. The datasets used were SRR1686967, SRR1686970, SRR1686976, SRR1693633, SRR1703816, SRR1703817, SRR1703818, SRR1703819, SRR1703820, and SRR1703821, obtained from the NCBI sequence read archive (SRA) and processed in QIIME (Caporaso et al. 2010) as described in Fig. 5.5. The boundaries for the first and third quartile are shown with the centerline representing the mean and whiskers representing the max/min values. Inset: A histogram of the relative distribution of all archaeal 16S rRNA sequences identified from caves

Despite this, the dominance of *Thaumarchaeota* in archaeal populations should be difficult to reconcile with the low levels of nitrogen found in caves (Barton 2015); the *Thaumarchaeota* play an important role in nitrification, where they utilize the oxidation of mineralized nitrogen (NH_3/NH_4^+) to generate energy for autotrophic growth (Brochier-Armanet et al. 2008). In order to compete with bacterial species for available NH_3, the *Thaumarchaeota* express high affinity (nM) transport mechanisms, making them better adapted to the low-nitrogen level found in caves, and providing them an alternate growth strategy in an environment where competition for other resources is likely to be high (Martens-Habbena et al. 2009)**.** Given the increased dominance of nitrogen cycling and recycling metabolic pathways observed in bacterial populations in caves, the almost exclusive identification of *Thaumarchaeota* in archaeal populations may suggest that nitrogen is one of the most overlooked drivers of microbial community structure in caves (Fig. 5.7; Ortiz et al. 2013; Tetu et al. 2013).

5.6.2 *Fungi*

One aspect of diversity that has generally lagged behind our understanding of cave microbiology is the role of fungi. While the human pathogen *Histoplasma capsulatum* was found in guano from cave hibernacula in 1957, very few papers had examined the mycology of caves beyond this organism (Ajello et al. 1960; Hasenclever et al. 1967; McMurray and Russel 1982; Sterflinger 2000; Burford et al. 2003); however, in 2009 this rapidly changed, when a fungal agent was found to be responsible for the devastating White-Nose Syndrome (WNS) epidemic in bats (Fig. 5.2; Reynolds and Barton 2014b). This disease was first identified in the winter of 2006–2007, when a cave in New York State, USA, contained a number of dead and dying bats. Every one of the sick bats appeared to have an unusual, white-powdery substance on their muzzles and wing membranes (Frick et al. 2010). This powdery substance was subsequently identified as the conidia (asexual spores) of a previously undescribed fungal pathogen, *Pseudogymnoascus* (known as *Geomyces*) *destructans* (*Pd*) (Gargas et al. 2009; Frick et al. 2010; Minnis and Lindner 2013). Since this initial outbreak, WNS has spread to infect bats across 38 US states and 5 Canadian provinces with mortality rates approaching 71%, making it one of the most devastating wildlife diseases of North America in modern history (Boyles et al. 2011; Reynolds and Barton 2014b). The WNS fungus (*Pd*) originated in Europe, where it likely emerged from a *Pseudogymnoascus* sp. endemic to cave environments (Peuchmaille et al. 2011; Warnecke et al. 2012; Reynolds and Barton 2014a; Reynolds et al. 2015, 2016). The identification of such an important mycosis demonstrated a significant lack of our understanding of cave mycology, and there has been a surge in studies attempting to determine how the *Geomyces/Pseudogymnoascus* fit into the ecology of cave systems (Fig. 5.2). By attempting to produce a broader ecosystem prospective, such studies have dramatically increased our understanding of the diversity of fungi in caves (Fig. 5.1; Vanderwolf et al. 2013).

The most commonly identified fungal species in caves are members of the phyla *Ascomycota* (~70%), *Basidiomycota* (20%), and *Zygomycota* (~7%) (Vanderwolf et al. 2013); however, the relevance of these findings should be viewed with caution, as these studies utilize cultivation-dependent techniques, which are susceptible to the same sample bias that once plagued cave bacteriology (Anderson and Cairney 2004; Tedersoo et al. 2014). The dominance of the phylum *Ascomycota,* which contains filamentous (mold) species such as *Aspergillus* and *Penicillium,* would suggest an environmental advantage for members of these genera; however, these fast-growing species readily utilize the nutrients found in media, often outcompeting other species during cultivation. One cave study based on molecular techniques suggested that the *Ascomycota* and *Basidiomycota* were almost equal in abundance (52 and 48%, respectively), although the investigators could not rule out contamination for dominance of the observed *Basidiomycota* (Connell and Staudigel 2013). Other investigators have suggested that fungal spores found in cave locations have been introduced by human or animal activity, reiterating the hypothesis that microbial populations in caves do not represent endemic species, but environmental

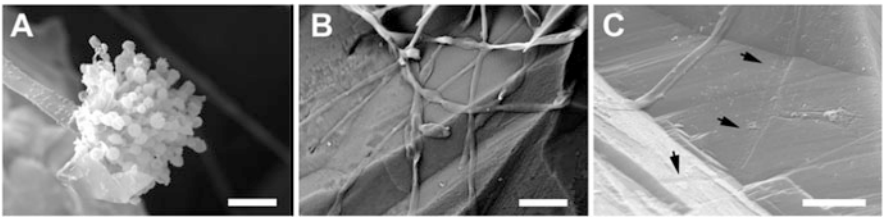

Fig. 5.8 Scanning electron microscopy images of pristine surfaces from Lechuguilla Cave, USA. Clearly visible on calcite mineral surface are fungal conidia (**a**), fungal hyphae (**b**), and etch marks left behind by fungal growth (**c**; arrows), demonstrating how the fungi modify mineral surfaces within the cave. Such etching of calcite by fungal species has been demonstrated before (e.g., Burford et al. 2003). Scalebars = 10 μm

contaminants (Shapiro and Pringle 2009, Vanderwolf et al. 2013). The use of molecular techniques to identify the true fungal ecology of caves therefore remains a high priority (Tedersoo et al. 2014).

Whatever the true diversity of fungi in caves, it is likely that they play a significant role in ecosystem processes. Fungi have been described from pristine cave environments, ruling out the idea that they are introduced contaminants, while there is evidence that they play an important role in altering mineral chemistry (Fig. 5.8; Cunningham et al. 1995). In other geologic settings, fungi are known to be important weathering agents, whether mechanically sugaring or chemically dissolving the surface in an attempt to access nutrients, or concentrating important nutrients and trace metal ions, which can be readily utilized by other microorganisms for growth (Sterflinger 2000; Burford et al. 2003). The growth of these filamentous fungi across mineral surfaces (Fig. 5.8) also generates microfabrics that support the growth of other microbial species (Burford et al. 2003). Together these diagenetic processes change the mineral matrix to form other deposits, such as calcite, goethite, halloysite, and montmorillonite, all of which have been detected in caves (Polyak and Güven 2000, 1996). Within surface soils, fungi play a most dominant role in breaking down macromolecular structures, and it is likely that their dominance in caves is greatest where particulate detritus (such as sticks and leaves) is brought in through flooding or direct anthropogenic impact (Jurado et al. 2010; Schneider et al. 2012). The effective ability of fungi to breakdown these recalcitrant carbon sources could subsequently provide a pool of nutrients for the growth of other microorganisms (Barton 2015).

5.7 Toward a Better Understanding of Microbial Cave Diversity

There has been a rapid increase in the pace of research in cave microbiology (Fig. 5.2). While just a handful of labs in North America, Europe, and Australia published on the topic in the 1990s, a tally of current publications reveals over 47 separate research

groups, including numerous labs in Asia and the emergence of the discipline in South America. Such increased contributions have started to impact the broader microbiological sciences, where interest is growing in the ability of cave environments to provide important clues into the emergence of infectious mycoses, novel antibiotics, and the evolution of antibiotic resistance (Bhullar et al. 2012; Fisher et al. 2012; Derewacz et al. 2013, 2014).

Despite this increased interest, there remain several barriers that need to be overcome by new researchers wanting to enter the field. These barriers include the difficulty in accessing and carrying out research in the challenging environment of caves and the technical limitations of working with low biomass samples. Cave access limitations can be overcome by reaching out to local cavers and speleologists, who often have the best information on appropriate caves for access and can even help identify microbial habitats for research; however, working with low biomass samples remains challenging, particularly in regard to preventing contamination, DNA extraction, and low DNA template levels (Barton et al. 2006). Such limitations can be overcome by using targeted cultivation approaches that take into account potential bias or circumvented by access to technologies that make it possible to work with low biomass samples (Summons et al. 2014). Currently, advances in DNA extraction and analysis make it possible to extract and work with nanogram to picogram- levels of DNA from geochemically complex environmental samples, while a new Nextera protocol only requires 1 ng/μL of template DNA to prepare Illumina libraries for metagenomic sequencing (Pel et al. 2009; Grunenwald et al. 2010; Rinke et al. 2013). Low biomass limitations may be overcome in the future by emergent technologies, such as new NGS approaches; PacBio (sequencing individual DNA fragments >40,000 bp in length) or Nanopore (sequencing single DNA molecules up to millions of bases in length) sequencing could be combined with single-cell whole-genome sequencing (SCWGS) (Branton et al. 2008; Rinke et al. 2013; Rhoads and Au 2015). While these methods have not yet been used in caves, such technologies could revolutionize the way low biomass environments are examined.

Over the next decade, it is likely that cave research will adopt many of the technology trends currently advancing the field of environmental microbiology, including the use of metabolomics (studying whole community metabolic products), metatranscriptomics (studying changes in whole community transcription), and metaproteomics (studying whole community protein expression patterns). Such data could help us understand the relative contributions of heterotrophy, autotrophy, mutualism, and competition to community energetics, or the unique role geochemistry plays on microbial community structure, with a goal of integrating the domain-level contributions of bacteria, archaea, and fungi that make it possible for microbial communities to subsist in such nutrient-limited habitats (Barton et al. 2007; Banks et al. 2010; Engel 2010; Lee et al. 2012; Barton 2015). Such studies may identify the fundamental ecological principles and adaptations that drive community dynamics and diversity and provide a more satisfying answer as to what constitutes a cave microbiome.

Acknowledgements The authors would like to thank Dr. Raina Maier for access to sequencing data, Drs. Soumya Ghosh and Naowarat Cheeptham in compiling the list of international research groups, and Dr. Max Wisshak for the SEM images used in Fig. 5.8.

References

Adey A, Morrison HG, Xun X et al (2010) Rapid, low-input, low-bias construction of shotgun fragment libraries by high-density in vitro transposition. Genome Biol 11:1–17

Ajello L, Manson-Bahr PEC, Moore JC (1960) Amboni Caves, Tanganyika, a new endemic area for Histoplasma capsulatum. Am J Trop Med Hyg 9:633–638

Amann RI, Snaidr J, Wagner M et al (1996) In situ visualization of high genetic diversity in a natural community. J Bacteriol 178:3496–3500

Anderson IC, Cairney JWG (2004) Diversity and ecology of soil fungal communities: increased understanding through the application of molecular techniques. Environ Microbiol 6:769–779

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

Ansorge WJ (2009) Next-generation DNA sequencing techniques. New Biotechnol 25:195–203

Baas-Becking LGM (1934) Geobiologie; of inleiding tot de milieukunde. WP Van Stockum & Zoon NV, Den Haag

Banks ED, Taylor NM, Gulley J et al (2010) Bacterial calcium carbonate precipitation in cave environments: a function of calcium homeostasis. Geomicrobiol J 27:444–454

Barton HA (2006) Introduction to cave microbiology: a review for the non-specialist. J Cave Karst Stud 68:43–54

Barton HA (2015) Starving artists: bacterial oligotrophic heterotrophy in caves. In: Summers Engel A (ed) Life in extreme environments: microbial life of cave systems, vol 1. DeGruyter, Berlin, Germany

Barton MD, Barton HA (2012) Scaffolder—software for manual genome scaffolding. Source Code Biol Med 7:4

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

Barton HA, Taylor MR, Pace NR (2004) Molecular phylogenetic analysis of a bacterial community in an oligotrophic cave environment. Geomicrobiol J 21:11–20

Barton HA, Taylor NM, Lubbers BR et al (2006) DNA extraction from low-biomass carbonate rock: an improved method with reduced contamination and the low-biomass contaminant database. J Microbiol Methods 66:21–31

Barton HA, Taylor NM, Kreate M et al (2007) The impact of host rock geochemistry on bacterial community structure in oligotrophic cave environments. Int J Speleol 36:93–104

Barton MD, Petronio M, Giarrizzo JG et al (2013) The genome of *Pseudomonas fluorescens* strain R124 demonstrates phenotypic adaptation to the mineral environment. J Bacteriol 195:4793–4803

Barton HA, Giarrizzo JG, Suarez P et al (2014) Microbial diversity in a Venezuelan orthoquartzite cave is dominated by the *Chloroflexi* (Class *Ktedonobacterales*) and *Thaumarchaeota* Group I.1c. Front Microbiol 5:1–14

Bhullar K, Waglechner N, Pawlowski A et al (2012) Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS One 7:e34953

Boyles JG, Cryan PM, McCracken GF et al (2011) Conservation. Economic importance of bats in agriculture. Science 332:41–42

Branton D, Deamer DW, Marziali A et al (2008) The potential and challenges of nanopore sequencing. Nat Biotechnol 26:1146–1153

Brochier-Armanet C, Boussau B, Gribaldo S et al (2008) Mesophilic *Crenarchaeota:* proposal for a third archaeal phylum, the *Thaumarchaeota*. Nat Rev Microbiol 6:245–252

Brochier-Armanet C, Gribaldo S, Forterre P (2012) Spotlight on the Thaumarchaeota. ISME J 6:227–230
Burford EP, Kierans M, Gadd GM (2003) Geomycology: fungi in mineral substrata. Mycologist 17:98–107
Campbell BJ, Engel AS, Porter ML et al (2006) The versatile epsilon-proteobacteria: key players in sulphidic habitats. Nat Rev Microbiol 4:458–468
Caporaso JG, Kuczynski J, Stombaugh J et al (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods 7:335–336
Carmichael MJ, Carmichael SK, Santelli CM et al (2013) Mn (II)-oxidizing bacteria are abundant and environmentally relevant members of ferromanganese deposits in caves of the upper Tennessee River Basin. Geomicrobiol J 30:779–800
Caumartin V (1963) Review of the microbiology of underground environments. NSS Bull 25:1–14
Chandler DP, Fredrickson JK, Brockman FJ (1997) Effect of PCR template concentration on the composition and distribution of total community 16S rDNA clone libraries. Mol Ecol 6:475–482
Chelius MK, Moore JC (2004) Molecular phylogenetic analysis of Archaea and bacteria in Wind Cave, South Dakota. Geomicrobiol J 21:123–134
Chu H, Fierer N, Lauber CL et al (2010) Soil bacterial diversity in the Arctic is not fundamentally different from that found in other biomes. Environ Microbiol 12:2998–3006
Connell L, Staudigel H (2013) Fungal diversity in a dark oligotrophic volcanic ecosystem (DOVE) on Mount Erebus, Antarctica. Biology 2:798–809
Cuezva S, Fernandez-Cortes A, Porca E et al (2012) The biogeochemical role of *Actinobacteria* in Altamira Cave, Spain. FEMS Microbiol Ecol 81:281–290
Cunningham KI, Northup DE, Pollastro RM et al (1995) Bacteria, fungi and biokarst in Lechuguilla Cave, Carlsbad Caverns National Park, New Mexico. Environ Geol 25:2–8
de Araujo JC, Schneider RP (2008) DGGE with genomic DNA: suitable for detection of numerically important organisms but not for identification of the most abundant organisms. Water Res 42:5002–5010
Derewacz DK, Goodwin CR, McNees CR et al (2013) Antimicrobial drug resistance affects broad changes in metabolomic phenotype in addition to secondary metabolism. Proc Natl Acad Sci USA 110:2336–2341
Derewacz DK, McNees CR, Scalmani G et al (2014) Structure and stereochemical determination of hypogeamicins from a cave-derived Actinomycete. J Nat Prod 77:1759–1763
Desai MS, Assig K, Dattagupta S (2013) Nitrogen fixation in distinct microbial niches within a chemoautotrophy-driven cave ecosystem. ISME J 7:2411–2423
DeSantis TZ, Brodie EL, Moberg JP et al (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. Microbiol Ecol 53:371–383
Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32:1792–1797
Engel AS (2010) Microbial diversity in cave ecosystems. In: Loy A, Mandl M, Barton LL (eds) Geomicrobiology: molecular and environmental perspective. Springer, New York, pp 219–238
Engel AS (2015) Bringing microbes into focus for speleology: an introduction. In: Engel AS (ed) Microbial life of cave systems. DeGruyter, Germany, pp 1–18
Engel AS, Porter ML, Stern LA et al (2004a) Bacterial diversity and ecosystem function of filamentous microbial mats from aphotic (cave) sulfidic springs dominated by chemolithoautotrophic "*Epsilonproteobacteria*". FEMS Microbiol Ecol 51:31–53
Engel AS, Stern LA, Bennett PC (2004b) Microbial contributions to cave formation: new insights into sulfuric acid speleogenesis. Geology 32:369–372
Engel AS, Meisinger DB, Porter ML et al (2010) Linking phylogenetic and functional diversity to nutrient spiraling in microbial mats from Lower Kane Cave (USA). ISME J 4:98–110
Fisher MC, Henk DA, Briggs CJ et al (2012) Emerging fungal threats to animal, plant and ecosystem health. Nature 484:186–194
Fliermans C, Schmidt E (1977) Nitrobacter in Mammoth Cave. Int J Speleol 9:1–19

Forde BM, O'Toole PW (2013) Next-generation sequencing technologies and their impact on microbial genomics. Brief Funct Genomics 12:440–453
Frick WF, Pollock JF, Hicks AC et al (2010) An emerging disease causes regional population collapse of a common North American bat species. Science 329:670–682
Gan HY, Gan HM, Tarasco AM et al (2014) Whole-genome sequences of five oligotrophic bacteria isolated from deep within Lechguilla Cave, New Mexico. Genome Announc 2:6
Ganzert L, Bajerski F, Wagner D (2014) Bacterial community composition and diversity of five different permafrost-affected soils of Northeast Greenland. FEMS Microbiol Ecol 89:426–441
Gargas A, Trest MT, Christensen M et al (2009) *Geomyces destructans sp.* nov. associated with bat White-nose Syndrome. Mycotaxon 108:147–154
Gerič B, Pipan T, Mulec J (2004) Diversity of culturable bacteria and meiofauna in the epikarst of Škocjanske Jame Caves (Slovenia). Acta Carsol 33:301–309
Gonzalez I, Laiz L, Hermosin B et al (1999) Bacteria isolated from rock art paintings: the case of Atlanterra shelter (south Spain). J Microbiol Methods 36:123–127
Grady F, Garton R, Homes MG (2000) The Pleistocene peccary *Platygonus vetus* from Poorfarm Cave, Pocahantas County, WV. J Cave Karst Stud 62:41
Grunenwald H, Baas B, Caruccio NC et al (2010) Rapid, high-throughput library preparation for next-generation sequencing. Nat Methods 7:8
Handelsman J (2004) Metagenomics: application of genomics to uncultured microorganisms. Microbiol Mol Biol Rev 68:669–685
Harmon DR, Rannen KM, Keenan SW et al (2013) Drip water chemistry from the Cascade Cave system, Kentucky, and implications for epikarst-derived microbial communities. In: GSA Annual Meeting, Denver, CO, 27–30 October, p 778
Hasenclever HF, Shacklette MH, Young RV et al (1967) The natural occurrence of *Histoplasma capsulatum* in a cave. 1. Epidemiologic aspects. Am J Epidemiol 86:238–245
Hess WH (1900) The origin of nitrates in cavern earths. J Geol 8:129–134
Høeg OA (1946) Cyanophyceae and bacteria in calcareous sediments in the interior of limestone caves in Nord-Rana, Norway. Nytt Mag Naturvidensk 85:99–104
Iker BC, Kambesis P, Oehrle SA et al (2010) Microbial atrazine breakdown in a karst groundwater system and its effect on ecosystem energetics. J Environ Qual 39:509–518
Ikner LA, Toomey RS, Nolan G et al (2007) Culturable microbial diversity and the impact of tourism in Kartchner Caverns, Arizona. Microb Ecol 53:30–42
Ivanova V, Tomova I, Kamburov A et al (2013) High phylogenetic diversity of bacteria in the area of prehistoric paintings in Magura Cave, Bulgaria. J Cave Karst Stud 75:218–228
Jiao JY, Liu L, Park DJ et al (2015) Draft genome sequence of *Jiangella alkaliphila* KCTC 19222T, isolated from Cave Soil in Jeju, Republic of Korea. Genome Announc 3:4
Johnston MD, Muench BA, Banks ED et al (2012) Human urine in Lechuguilla Cave: the microbiological impact and potential for bioremediation. J Cave Karst Stud 74:278–291
Jones DS, Macalady JL (2016) The snotty and the stringy: energy for subsurface life in caves. In: Hurst CJ (ed) Their World: a diversity of microbial environments. Springer, New York, pp 203–224
Jurado V, Porca E, Cuezva S et al (2010) Fungal outbreak in a show cave. Sci Total Environ 408:3632–3638
Kembel SW, Wu M, Eisen JA et al (2012) Incorporating 16S gene copy number information improves estimates of microbial diversity and abundance. PLoS Comput Biol 8:e1002743
Klimchouk AB, Ford DC, Palmer AN et al (2000) Speleogenesis: evolution of Karstic Aquifers. National Speleological Society, Huntsville, AL
Könneke M, Bernhard AE, de la Torre JR et al (2005) Isolation of an autotrophic ammonia-oxidizing marine archaeon. Nature 437:543–546
Kuczynski J, Stombaugh J, Walters WA et al (2012) Using QIIME to analyze 16S rRNA gene sequences from microbial communities. Curr Protocol Microbiol 27:E1–E5

Kumar Y, Westram R, Kipfer P et al (2006) Evaluation of sequence alignments and oligonucleotide probes with respect to three-dimensional structure of ribosomal RNA using ARB software package. BMC Bioinformatics 7:240

Laiz L, Gonzalez JM, Saiz-Jimenez C (2003) Microbial communities in caves: ecology, physiology, and effects on Paleolithic paintings. In: Koestler RJ, Koestler VH, Charola AE, Nieto-Fernandez FE (eds) Art, biology and conservation: biodeterioration of works of art. Metropolitan Museum of Art, New York, pp 211–225

Land M, Pukall R, Abt B et al (2009) Complete genome sequence of *Beutenbergia cavernae* type strain (HKI 0122). Stand Genomic Sci 1:21–28

Lander ES (2011) Initial impact of the sequencing of the human genome. Nature 470:187–197

Lauber CL, Hamady M, Knight R et al (2009) Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the Continental Scale. Appl Environ Microbiol 75:5111–5120

Lee SD (2008) *Jiangella alkaliphila* sp. nov., an actinobacterium isolated from a cave. Int J Syst Evol Microbiol 58:1176–1179

Lee NM, Meisinger DB, Aubrecht R et al (2012) Caves and karst environments. In: Bell EM (ed) Life at extremes: environments, organisms and strategies for survival. CAB International, Egham, UK, pp 320–344

Leinonen R, Sugawara H, Shumway M (2010) The sequence read archive. Nucl Acids Res 39: D19–D21

Lynch MDJ, Neufeld JD (2015) Ecology and exploration of the rare biosphere. Nat Rev Microbiol 13:217–229

Martens-Habbena W, Berube PM, Urakawa H et al (2009) Ammonia oxidation kinetics determine niche separation of nitrifying Archaea and Bacteria. Nature 461:976–979

McDonald D, Price MN, Goodrich J et al (2012) An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. ISME J 6:610–618

McMurray DN, Russel LH (1982) Contribution of bats to the maintenance of *Histoplasma capsulatum* in a cave microfocus. Am J Trop Med Hyg 31:527–531

Miller CS, Baker BJ, Thomas BC et al (2011) EMIRGE: reconstruction of full-length ribosomal genes from microbial community short read sequencing data. Genome Biol 12:1–14

Minnis AM, Lindner DL (2013) Phylogenetic evaluation of *Geomyces* and allies reveals no close relatives of *Pseudogymnoascus destructans*, comb. nov., in bat hibernacula of eastern North America. Fungal Biol 117:638–649

Northup DE, Barnes SM, Yu LE et al (2003) Diverse microbial communitiens inhabiting ferromanganese deposits in Lechuguilla and Spider Caves. Environ Microbiol 5:1071–1086

Ortiz M, Neilson JW, Nelson WM et al (2013) Profiling bacterial diversity and taxonomic composition on speleothem surfaces in Kartchner Caverns, AZ. Microb Ecol 65:371–383

Ortiz M, Legatzki A, Neilson JW et al (2014) Making a living while starving in the dark: metagenomic insights into the energy dynamics of a carbonate cave. ISME J 8:478–491

Pace NR (1997) A molecular view of microbial diversity and the biosphere. Science 276:734–740

Pace NR, Stahl DA, Lane DJ et al (1986) The analysis of natural microbial populations by ribosomal RNA sequences. Adv Microb Ecol 9:1–55

Palmer AN (2007) Cave geology. Cave Books, Dayton, OH

Parker CW, Wolf JA, Bresser WJ et al (2013) Microbial reducibility of Fe(III) phases associated with the genesis of iron ore caves in the Iron Quadrangle, Minas Gerais, Brazil. Fortschr Mineral 3:395–411

Peck SB (1986) Bacterial deposition of iron and manganese oxides in North American caves. NSS Bull 48:26–30

Pel J, Broemeling D, Mai L et al (2009) Nonlinear electrophoretic response yields a unique parameter for separation of biomolecules. Proc Natl Acad Sci USA 106:14796–14801

Peuchmaille SJ, Wibbelt G, Korn V et al (2011) Pan-European distribution of White-nose Syndrome (*Geomyces destructans*) not associated with mass mortality. PLoS One 6:e19167

Polyak VJ, Güven N (1996) Alunite, natroalunite and hydrated halloysite in Carlsbad Cavern and Lechuguilla Cave, New Mexico. Clays Clay Miner 44:843–850
Polyak VJ, Güven N (2000) Clays in caves of the Guadalupe Mountains, New Mexico. J Cave Karst Stud 62:120–126
Polz MF, Cavanaugh CM (1998) Bias in template-to-product rations in multitemplate PCR. Appl Environ Microbiol 64:3724–3730
Porca E, Jurado V, Žgur-Bertok D et al (2012) Comparative analysis of yellow microbial communities growing on the walls of geographically distinct caves indicates a common core of microorganisms involved in their formation. FEMS Microbiol Ecol 81:255–266
Posada D (2003) Using MODELTEST and PAUP* to select a model of nucleotide substitution. Current Protocols in Bioinformatics Chapter 6:Unit 6.5
Pruesse E, Quast C, Knittel K et al (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196
Quast C, Pruesse E, Yilmaz P et al (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res 41:D590–D596
Reynolds HT, Barton HA (2014a) Comparison of the White-nose Syndrome agent *Pseudogymnoascus destructans* to cave-dwelling relatives suggests reduced saprophytic enzyme activity. PLoS One 9:e86347
Reynolds HT, Barton HA (2014b) White-nose Syndrome: human activity in the emergence of an extirpating mycosis. In: One health: people, animals, and the environment. ASM Press, Washington, DC, p 167
Reynolds HT, Ingersoll T, Barton HA (2015) The environmental growth of *Pseudogymnoascus destructans* and its impact on the White-nose Syndrome epidemic in Little Brown Bats (*Myotis lucifugus*). J Wildl Dis 51:318–331
Reynolds HT, Barton HA, Slot JC (2016) Phylogenomic analysis supports a recent change in nitrate assimilation in the White-nose Syndrome pathogen, *Pseudogymnoascus destructans*. Fungal Ecol 23:20–29
Rhoads A, Au KF (2015) PacBio sequencing and its applications. Genomics Proteom Bioinformatics 13:278–289
Rinke C, Schwientek P, Sczyrba A et al (2013) Insights into the phylogeny and coding potential of microbial dark matter. Nature 499:431–437
Rusznyak A, Akob DM, Nietzsche S et al (2012) Calcite biomineralization by bacterial isolates from the recently discovered pristine karstic Herrenberg cave. Appl Environ Microbiol 78:1157–1167
Sanger F, Air GM, Barrell BG et al (1977) Nucleotide sequence of bacteriophage φX174 DNA. Nature 265:687–695
Sarbu SM, Kane TC, Kinkle BK (1996) A chemoautotrophically based cave ecosystem. Science 272:1953–1955
Sasowsky ID, Palmer MV (1994) Breakthroughs in karst geomicrobiology and redox geochemistry, vol 1. Karst Waters Institute, Charles Town, WV
Saw JHW, Schatz M, Brown MV et al (2013) Cultivation and complete genome sequencing of *Gloeobacter kilaueensis* sp. nov., from a lava cave in Kīlauea Caldera, Hawai'i. PLoS One 8: e76376
Schabereiter-Gurtner C, Saiz-Jimenez C, Pinar G et al (2002) Altamira cave Paleolithic paintings harbor partly unknown bacterial communities. FEMS Microbiol Lett 211:7–11
Schneider T, Keiblinger KM, Schmid E et al (2012) Who is who in litter decomposition? Metaproteomics reveals major microbial players and their biogeochemical functions. ISME J 6:1749–1762
Scott W (1909) An ecological study of the plankton of Shawnee Cave, with notes on the cave environment. Biol Bull 17:386–407
Shabarova T, Pernthaler J (2010) Karst pools in subsurface environments: collectors of microbial diversity or temporary residence between habitat types. Environ Microbiol 12:1061–1074

Shapiro J, Pringle A (2009) Anthropogenic influences on the diversity of fungi isolated from caves in Kentucky and Tennessee. Am Midl Nat 163:76–86
Shendure J, Mitra RD, Varma C et al (2004) Advanced sequencing technologies: methods and goals. Nat Rev Genet 5:335–344
Snyder LA, Loman N, Pallen MJ et al (2009) Next-generation sequencing—the promise and perils of charting the great microbial unknown. Microb Ecol 57:1–3
Sogin ML, Morrison HG, Huber JA et al (2006) Microbial diversity in the deep sea and the underexplored "rare biosphere". Proc Natl Acad Sci USA 103:12115–12120
Stahl DA, Lane DJ, Olsen GJ, Pace NR (1984) Analysis of hydrothermal vent-associated symbionts by ribosomal RNA sequences. Science 224:409–411
Stamatakis A (2014) RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30:1312–1313
Sterflinger K (2000) Fungi as geologic agents. Geomicrobiol J 17:97–124
Summons RE, Sessions AL, Allwood AC et al (2014) Planning considerations related to the organic contamination of Martian samples and implications for the Mars 2020 Rover. Astrobiology 14:969–1027
Tan SC, Yiap BC (2009) DNA, RNA, and protein extraction: the past and the present. J Biomed Biotechnol 2009:574398
Tedersoo L, Bahram M, Põlme S et al (2014) Global diversity and geography of soil fungi. Science 346:1256688
Tetu SG, Breakwell K, Elbourne LD et al (2013) Life in the dark: metagenomic evidence that a microbial slime community is driven by inorganic nitrogen metabolism. ISME J 7:1227–1236
Thomas T, Gilbert JA, Meyer F (2012) Metagenomics—a guide from sampling to data analysis. Microb Inform Exp 2:1–12
Tomczyk-Żak K, Zielenkiewicz U (2016) Microbial diversity in caves. Geomicrobiol J 33:20–38
Tyson GW, Chapman J, Hugenholtz P et al (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. Nature 428:37–43
Valentine DL (2007) Adaptations to energy stress dictate the ecology and evolution of the Archaea. Nat Rev Microbiol 5:316–323
Vanderwolf KJ, Malloch D, McAlpine DF et al (2013) A world review of fungi, yeasts, and slime molds in caves. Int J Speleol 42:9
Venter JC, Remington K, Heidelberg JF et al (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66–74
Vlăsceanu L, Popa R, Kinkle BK (1997) Characterization of *Thiobacillus thioparus* LV43 and its distribution in a chemoautotrophically based groundwater ecosystem. Appl Environ Microbiol 63:3123–3127
Warnecke L, Turner JM, Bollinger TK et al (2012) Inoculation of bats with European *Geomyces destructans* supports the novel pathogen hypothesis for the origin of White-nose Syndrome. Proc Natl Acad Sci USA 109:6999–7003
Wilgenbusch JC, Swofford D (2003) Inferring evolutionary trees with PAUP*. Current Protocols in Bioinformatics Chapter 6:Unit 6.4
Woese CR, Fox GE (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. Proc Natl Acad Sci USA 74:5088–5090
Yun Y, Xiang X, Wang H et al (2015) Five-year monitoring of bacterial communities in dripping water from the Heshang Cave in central China: implication for paleoclimate reconstruction and ecological functions. Geomicrobiol J 33:1–11
Zhalnina K, Dias R, de Quadros PD et al (2015) Soil pH determines microbial diversity and composition in the Park Grass Experiment. Microb Ecol 69:395–406
Zhou JP, Gu Y, Zou C et al (2007) Phylogenetic diversity of bacteria in an earth-cave in Guizhou Province, southwest of China. J Microbiol 45:105–112