

Review

Contributions of DNA Sequencing Technologies to the Integrative Monitoring of Karstic Caves

Zélia Bontemps ^{1,2} , Yvan Moënné-Loccoz ^{1,3}  and Mylène Hugoni ^{3,4,*} 

¹ Université Claude Bernard Lyon 1, CNRS, INRAE, VetAgro Sup, UMR5557 Ecologie Microbienne, F-69622 Villeurbanne, France; zelia.bontemps@imbim.uu.se (Z.B.); yvan.moenne-loccoz@univ-lyon1.fr (Y.M.-L.)

² Department of Medical Biochemistry and Microbiology, Science for Life Laboratories, Uppsala University, 75310 Uppsala, Sweden

³ Institut Universitaire de France (IUF), F-75005 Paris, France

⁴ Université Claude Bernard Lyon 1, INSA Lyon, CNRS, UMR5240 Microbiologie Adaptation et Pathogénie, F-69621 Villeurbanne, France

* Correspondence: mylene.hugoni@univ-lyon1.fr

Abstract: Cave microbiota knowledge has greatly expanded in the past decades, driven by the development of molecular techniques, which allow an in-depth characterization of diversity and its metabolic potential. This review focuses on the contribution of DNA sequencing technologies to depict the cave microbiome for the three domains of life (*Bacteria*, *Archaea*, and *Microeukaryotes*), assessing their advantages and limits. Cultural methods do not provide a representative view of cave microbial diversity but do offer, subsequently, the possibility to genomically characterize the strains isolated from caves. Next-generation DNA sequencing permits an exhaustive description of microbial biodiversity in caves, using metabarcoding (for taxonomic assessment) or metagenomics (for taxonomic and functional assessments). It proved useful to compare caves, different rooms, or substrata (water, soil, bedrock, etc.) within a cave, or the effect of cave disturbance in Lascaux and elsewhere. The integration of next-generation DNA sequencing with cultivation techniques, physico-chemical characterization, and other complementary approaches is important to understand the global functioning of caves and to provide key information to guide cave conservation strategies.

Keywords: microbial ecology; next-generation DNA sequencing; metabarcoding; metagenomics



Citation: Bontemps, Z.; Moënné-Loccoz, Y.; Hugoni, M. Contributions of DNA Sequencing Technologies to the Integrative Monitoring of Karstic Caves. *Appl. Sci.* **2024**, *14*, 9438. <https://doi.org/10.3390/app14209438>

Academic Editors: Ramona Iseppi, Cesareo Saiz-Jimenez and Valme Jurado Lobo

Received: 1 July 2024

Revised: 26 September 2024

Accepted: 1 October 2024

Published: 16 October 2024



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1. Introduction

Karstic caves are important and complex ecosystems representing from 15% to 20% of the global Earth surface [1]. These peculiar ecosystems are the result of the karstification process, which consists of the dissolution of carbonates or sulfated rocks caused by infiltration water charged with carbonic acid (H_2CO_3) [2] or, in a few cases, by sulfuric acid [3]. Caves represent the most karstic environments compared with underground rivers or abysses, and are associated with atypic features leading to highly original ecosystems: (i) absence of light, (ii) high relative humidity (close to saturation), (iii) high concentration of CO_2 , (iv) stable temperature, and (v) oligotrophic conditions (less than 2 mg TOC L^{-1}) [4]. While these combined environmental variables could represent an unfavorable set for microbial life establishment and activity, it has been shown that diverse microorganisms thrive in caves, reaching in the order of $10^6 \text{ cells g}^{-1} \text{ rock}$ [4–6]. However, holistic studies of microbial diversity, structure, and dynamics in underground karstic ecosystems are still rare [7].

It has been well documented that microorganisms play an important role in cave development as key players of (i) many processes from karstification to biogeochemical transformations (including chemoautotrophy) and (ii) biotic interactions and cave trophic networks [2,5,6,8–10]. Moreover, microorganisms may be responsible for alteration

processes that can occur on cave walls. For example, in many caves presenting famous paintings or engravings such as Lascaux Cave (France) or Altamira Cave (Spain), *Fungi* and *Bacteria* are reported as causing black or yellow stains [8,11]. In Lascaux Cave, these colored stains were the result of fungal pigmentation, i.e., *Ochroconis lascauxensis* (syn. *Scolecobasidium lascauxense*) and *Ochroconis anomala* synthesize black melanin pigments [12], while in Altamira Cave, the yellow pigmentation was attributed to *Betaproteobacteria* and *Gammaproteobacteria* activity [11]. While each cave is unique due to the particularity of its biological, chemical, and physical features [1,5,13], knowledge about microbial diversity, its interactions, and its impact on ecosystem functioning represents a challenge to address fundamental microbial ecology questions, but also conservation issues associated with caves.

The number of cave microbiota studies has greatly expanded in the last decade, driven by large-scale conservation programs [14] and enabled by the development of high-resolution molecular techniques coupled with DNA Next-Generation Sequencing (NGS). Indeed, beyond classical microbial ecology methods relying on microbial cultivation, NGS techniques have promoted the emergence of new high-throughput sequencing-based approaches, such as metabarcoding, metagenomics, or metatranscriptomics [15], with the potential for (i) an in-depth characterization of microbial diversity in caves, (ii) the assessment of their genetic potential and metabolic capacities, and (iii) the possibility of focusing even on individual strains by using genomics or transcriptomics. It is now essential to consider complementary and multidisciplinary techniques to investigate microbial diversity thriving in caves, including culture-dependent and -independent methods, allowing scientists to gain insights into a global microbial functioning of caves and offering the possibility to guide conservation management politics.

This review summarizes the contribution of the various technologies, from cultural methods to NGS sequencing, to our current knowledge on microbial diversity associated with cave ecosystems. A particular emphasis is put on molecular techniques integrating genomic reconstruction approaches as a perspective for non-cultivated microorganisms. Their relevance in interdisciplinary science to understand cave ecology is also discussed.

2. Cultivation Methods, the First Assessments of Cave Microbial Diversity

Whatever the ecosystem, a microbial diversity assessment was historically based on classical microbial ecology techniques such as laboratory cultivation, often coupled with microscopy (Table 1).

The cultivation of microorganisms appears a simple and cheap method to isolate microbial members from diverse ecosystems. However, the success of microbial cultivation is highly dependent on many factors, such as the ability to reproduce ideal microbial life conditions [16,17]. The difficulty also lies in avoiding nutritional shock or inadequate substrate concentrations that might accelerate cellular death and/or inhibit the growth of microorganisms [17]. Thus, as cultivation conditions do not always match in situ conditions, many microorganisms will not be cultured, and the microbial diversity documented might not reflect the in situ diversity, implying biases in many studies. Indeed, microbiologists estimate that at the most 1 to 10% of microorganisms can be cultured, and comparisons between cultivation and molecular methods showed that only a minority of bacterial phyla identified through direct PCR amplification of 16S rRNA genes had yielded culturable representatives [18].

Table 1. Comparison of methods to document the taxonomic diversity of microorganisms.

Cultural Methods	Cloning Sequencing	Metabarcoding	Shotgun Metagenomics
Principle			
Microbial isolation followed by characterization	DNA extraction, PCR cloning and sequencing of a taxonomic marker	DNA extraction, PCR amplification and sequencing of a taxonomic marker	DNA extraction and PCR-free sequencing of all DNA fragments
Scale			
Typically hundreds to thousands of colonies per study	Typically hundreds to thousands of clones per study	Typically 2000 to 70,000 sequences per marker per sample	Typically 2000 to 70,000 sequences per marker per sample
Advantages and added value			
Isolates enable also to assess strain physiology experimentally and to perform genomics, inexpensive	No particular advantage now that NGS is available	Enables to document the rare biosphere, inexpensive, well-established bioinformatics tools, requires less DNA than metagenomics, reference databases usually well developed	High-throughput, taxonomy not restricted to single markers, documents functional properties also, several NGS technologies available
Drawbacks and limits			
Requires culturability, tedious and low-throughput	DNA extraction not effective for certain taxa, PCR primers not fully universal, tedious and low-throughput, expensive Sanger sequencing	DNA extraction not effective for certain taxa, PCR primers not fully universal, amplicon size limits taxonomic resolution	DNA extraction not effective for certain taxa, minor taxa hard to document, expensive, bioinformatics-intensive, reference databases far from exhaustive, requires more DNA than metabarcoding

Most of the time, the isolation of cave microorganisms has been performed in a two-step screening process [19]. The first step consists of an incubation period often using a nutrient-rich medium (i.e., CN agar or CN gellan gum [20]) that allows the isolation of strains presenting different morphological characteristics. Because of the oligotrophic status of caves, this procedure can be adapted by using culture media with low nutrient amounts (i.e., Tryptic Soy Agar and Reasoner's 2A Agar (R2A) [20]) or diluted media. Using cultivation on seven different media, i.e., Glycerol-Asparagine Agar (GAA), Peptone Yeast Extract–Brain Heart Infusion (PY-BHI) agar, 1000-fold-diluted Tryptic Soy Agar (TSA), Starch-Casein Agar (SCA), Malt Yeast extract Agar (MYA), Soil Extract Agar (SEA), and Tap Water Agar (TWA), all supplemented with the fungal inhibitor nystatin, Herzog Velikonja et al. (2014) identified microorganisms from the genera *Lysobacter*, *Pseudomonas*, *Sphingomonas*, *Bosea*, etc., in the Pajsarjeva jama Cave in Slovenia [21]. While all seven growth media allowed microbial isolation, microorganisms were most abundant on low-nutrient TWA. By using low-nutrient medium $0.1 \times$ R2A instead of high-nutrient media CN agar or CN gellan gum, Brar and Bergmann (2019) showed that a gold mine located in South Dakota hosted irregular thin and iridescent biofilms, similar to silver biofilms described in limestone caves [22,23]. They evidenced the dominance of *Alphaproteobacteria* (67% and 42% of isolates on high- and low-nutrient media, respectively), *Actinobacteria* (24% and 47%), and, to a lesser extent, *Bacilli* (4% and 2%). *Betaproteobacteria* were only present on high-nutrient media (5% of isolates) and *Gammaproteobacteria* on low-nutrient media (9% of isolates). Other studies used Potato Dextrose Agar (PDA) to promote sporulation and pigment production of fungi often retrieved from caves (e.g., *Alternaria alternata*, *Cladosporium cladosporioides*, *Aspergillus niger*, etc.) [8,24,25]. Previous work identified yeast-like fungi in Harmaneká Cave (Slovakia) through cultivation on Sabouraud agar or Czapek-Dox medium, and the density of airborne fungi was 810 ± 124 CFU per m^3 air outside vs. 129 ± 17 CFU per m^3 of air inside the cave [25]. These examples show the importance of the media chosen to isolate microorganisms. In addition, overgrowth of fast-growing microorganisms (r-strategists) and short incubation times limit the recovery of ecologically valuable slow-growing microorganisms (K-strategists) [17]. Previous work showed that cave *Cyanobacteria*, usually slow growers in these ecosystems, require from

150 to 200 cultivation days to produce enough biomass for further analysis of phylogeny, antimicrobial activity screening, or scanning electron microscopy (SEM) [13]. In the absence of inhibitory chemicals such as antibiotics or toxins in the culture medium, the rapid growth of certain microorganisms often inhibits or delays the growth of the others, yielding a narrow range of microorganisms. This can be amplified by several other factors including niche and nutrient competition, production of secondary metabolites, and production of toxins by these fast growers [13,17]. Some media only support the growth of fast-growing microorganisms while inhibiting slow-growing oligotrophic microorganisms due to nutritional shock [13,17,21]. It is important to note that most members of the archaeal domain in caves are still not cultivated [26].

The majority of cultivation studies include a second screening step performed either by microscopy or molecular methods [1,19]. To go further with two *Streptomyces* isolates obtained from Iron Curtain Cave (Canada) using two broth media (R2A and Hickey-Tresener), whole genome sequencing and metabolomic analyses showed a high number of biosynthetic gene clusters for secondary metabolites (polyketides and non-ribosomal peptides), thought to contribute to their fitness [27]. Work conducted on Lechuguilla Cave (New Mexico) allowed the identification of antibiotic resistance among the culturable microbiome and especially in *Streptomyces* spp. and *Brachybacterium paraconglomeratum* [28]. This was consistent with another study of the walls of a pristine karstic cavity named the Yumugi river cave, where cultivated members of the *Pseudomonas* genus were identified for their antibiotic resistance properties [29]. Using microscopy coupled with Fluorescent In Situ Hybridization (FISH), previous work identified the microbial structures and taxa composing yellow biofilms, especially *Actinomyces*, in Pindal Cave (Spain) [30]. Others identified more diverse microbial members, such as *Bacteria* and *Fungi*, as well as *Cyanobacteria* and *Algae* (in relation to light exposition) in biofilm samples from Fornelle Cave (Italy) [31].

Since effective cultivation requires the reproduction of both abiotic (i.e., pH, temperature, etc.) and biotic (i.e., syntrophy, symbiosis, etc.) conditions that microorganisms experience in situ [17], new methods of cultivation have emerged, such as the in situ cultivation of natural microorganisms using diffusion chambers or cultivation of soil microorganisms in soil extract agar medium [32,33]. Microbial culturomics is also used. It is based on the multiplication of culture conditions aimed at facilitating the growth of fastidious species and the use of growth inhibitors against the majority species, allowing the selection of minority species, subsequently identified with MALDI-TOF mass spectrometry [34] or other methods. Such culturomic approaches were applied in Maxwellton Sink Cave (USA) to recover a wider microbial diversity for better screening of bioactive compound production and/or to confirm in situ metabolic strategies [35].

To conclude this section, all microorganisms might be cultivable, at least in theory, but successful isolation of the majority of microorganisms is hard to achieve, both in caves and elsewhere. The most abundant taxa (as indicated by cultivation-independent methods) could be isolated, such as *Proteobacteria* or *Actinobacteria* (see Sections 3 and 4), but members of the rare biosphere were only isolated once in a while. Consequently, cultivation methods offered a partial view of microbial diversity occurring in caves. However, cultivation is a powerful tool for delivering reference strains and identifying their metabolic capabilities, providing new genome sequences that assist, for example, in designing better primers and probes for refinement of molecular and microscopy detection methods [17], or providing microbial strains used for their metabolites or enzymes in a biotechnological context. It is also useful to complement findings obtained by sequencing methods (see below).

3. Molecular Cloning, the First Culture-Independent Approach to Investigate the Microbial Ecology of Caves

Cloning has been widely applied to environmental rRNA genes over previous decades [36]. Briefly, classical cloning is a technique by which environmental genes are inserted into a vector and subsequently introduced into a host such as *Escherichia coli* cells (via transformation). *E. coli* are then grown on rich media and cells containing the environmental

fragment are screened and selected for Sanger sequencing (Table 1). In detail, clone library construction proceeds as follows: (i) DNA extraction, (ii) amplification of targeted rRNA genes via PCR, (iii) ligation into a plasmid vector, (iv) transformation into competent cells, (v) selection on antibiotic-supplemented agar plate of *E. coli* cells that contain a plasmid insert, (vi) selection of individual *E. coli*, and (vii) growth of *E. coli* for replication of environmental gene inserts to provide sufficient copies for sequencing by capillary Sanger technology [37,38].

Prior to sequencing, environmental DNA must be extracted from cave samples, considering that environmental samples contain DNA in a variety of forms (i.e., free DNA, DNA in cells). Thus, extraction methods must be driven according to the environmental matrix and the targeted microbial species to optimize extraction efficiency [39]. The use of DNA extraction kits is very common, but the presence of ubiquitous contaminants is widely recognized in these kits and varies considerably in composition between different kits and batches of kits [40]. These microbial DNA contaminants can impact estimates of microbial richness and diversity by contributing to incorrect interpretations and identifications, especially in low biomass samples [40–43]. To avoid this bias, simultaneous sequencing of extraction blanks and negative controls is required for accurate interpretation of microbial diversity results. Contamination is a general challenge for environmental sequencing studies and the identification and removal of sequence contaminants is particularly problematic [44]. In addition, unsuitable lysis techniques (i.e., chemical, mechanical, enzymatic) can provide a biased view of the microbial community studied by (i) not efficiently lysing all microbial membranes and walls, and/or (ii) leading to DNA degradation for some microorganisms [39,45–47]. Indeed, some lysis methods led to a reduced retrieval of Gram-positive bacteria compared with Gram-negative bacteria (due to the cellular composition of their membrane) [39,47]. Finally, the presence of humic acids and/or salts co-extracted with environmental DNA that can inhibit enzymes used for library construction and/or sequencing is a shortcoming. These issues are even more critical in the case of meta-omic approaches (see Sections 4 and 5 below).

In the 2000's, 16S rRNA gene cloning was largely applied to cave studies. One of earliest applications of molecular cloning in a cave was achieved in a sulfidic stream located in Parker Cave (Kentucky, USA), showing that bacterial clones were affiliated to phylotypes known to obtain energy for CO₂ fixation from the oxidation of inorganic compounds [48]. Cloning Sanger sequencing method was also used for the first characterization of the microbial environment of Lascaux Cave, where as many as 696 clones of bacterial 16S rRNA genes were retrieved from 11 samples in different galleries of the cave [49,50]. Rarefaction analysis on these clones showed 90% clone coverage, indicating that a large part of the bacterial diversity was detected [49]. By focusing on Lascaux's fungal community on altered surfaces (black stains) colonized by arthropods and unaltered surfaces, 607 clones of 18S rRNA genes were also obtained [50]. Among these 607 clones, 8 of the 10 most abundant phylotypes (representing approximately 50% of the clones) corresponded to *Fungi* known to associate with arthropods, such as *Cladosporium cladosporioides*, *Geomyces pannorum*, *Lecanicillium psalliotae*, etc. Other studies applied cloning sequencing to explore the microbiology of underground ferromanganese corrosion residues [51] or highly acidic caves [52]. The 16S rRNA analyses evidenced that clones associated with water drips in a highly acidic cave were affiliated to *Thiobacilli*, which may obtain energy from the oxidation of sulfur compounds, or *Acidimicrobium ferrooxidans*, a sulfide-oxidizing bacterium [52]. In Lechuguilla Cave, sequence analysis of clones showed the presence of *Hyphomicrobium*, *Pedomicrobium*, *Leptospirillum*, *Stenotrophomonas*, and *Pantoea*, which have known iron- and manganese-oxidizing/reducing bacteria among close relatives [51].

While providing the advantage to give long fragments (about 1500 bp for 16S rRNA genes), cloning sequencing is expensive and time consuming. In consequence, new sequencing methods have been developed to replace cloning sequencing techniques, allowing simpler library construction (without cloning), faster sequencing, and generating more data for more in-depth diversity analyses. These methods provide much more information,

allowing scientists to document rarer taxa, and also benefit from improvements made in microbial taxonomy and in database quality.

4. NGS Taxonomic Assessment of Cave Microorganisms by Metabarcoding

Applied to caves since the 2010's, high-throughput DNA sequencing allows detailed description of microbial diversity and of the genetic potential of microbial communities by acquiring hundreds of thousands of sequences simultaneously [53]. Using NGS allows elimination of cloning steps and/or the creation of genomic banks by sequencing (i) targeted genes after a PCR step, known as metabarcoding, or (ii) directly environmental DNA, known as metagenomics (see Section 5 below) (Table 1).

4.1. Metabarcoding-Based Knowledge of Caves

At a global cave scale, data obtained across anthropized and non-anthropized caves indicate that the microbial community is largely dominated by *Proteobacteria* and *Actinobacteria* among *Bacteria*, *Thaumarchaeota* and *Euryarchaeota* among *Archaea*, and *Ascomycota* when considering *Fungi* among microeukaryotic communities. However, many differences exist in microbial diversity and structure considering the contrasted compartments present in caves (i.e., water, air, speleothems, sediments, etc.), which are detailed below.

Metabarcoding has been useful in documenting microorganisms in cave water. Water infiltration through carbonate rocks is an important process in cave functioning, as it can cause CO₂-mediated chemical dissolution of limestone, resulting in the formation of karstic caves, and plays a role in the microbial colonization of caves after their formation (for example, via rhizospheric channels going through the epikarst). Water has been studied in both non-anthropized and anthropized caves where a high prevalence of *Proteobacteria* was reported (Figure 1) [54,55], among which the *Gammaproteobacteria* class was overwhelmingly dominant [56,57]. Generally, water samples from karstic caves present highly diverse microorganisms [57,58], which could be positively correlated with the water content of the epikarst, as it promotes microbial transportation, as found below farmland [59] and forest [60]. Bacterial co-occurrence networks suggested richer and more complex interactions, in terms of number of nodes, edges, clustering coefficient, and average degree, in cave water compared with mineral substrates (i.e., sediments and rocks) [61]. However, cave water studies are still scarce and lack the consideration of *Archaea* and microeukaryotes.

Metabarcoding has identified airborne microorganisms in caves. Microorganisms may enter in caves and karst ecosystems following air currents. Air flow transporting organic particles and gaseous organics as condensation [5,62] forms clouds of water microparticles (hydroaerosols) that support adhesion of microorganisms on walls [63,64]. *Bacteroidetes*, *Firmicutes*, and *Ascomycota* were significantly enriched in air samples compared with other compartments in anthropized and non-anthropized caves [61,65]. Cave fungal dynamics were investigated by Zhu et al. (2019), and co-occurrence networks metrics showed that the bacterial community in air samples was less connected than those in cave sediments and water. Being mainly transported as spores, *Fungi* in air samples were extensively described in comparison with *Bacteria* and *Archaea*, which are more likely to enter caves as cells adhering to dust particles [64,66]. Investigations into airborne *Fungi* in caves showed that the quantity of fungal spores decreased with the distance from the entrance of the cave [25,67] and the numbers of human visitors [68]. Yet, anthropized caves present a more diversified fungal microbiota, with a greater proportion of *Basidiomycota* (+11%) and *Mucoromycota* (+19%) than in non-anthropized caves (Figure 1) [69].

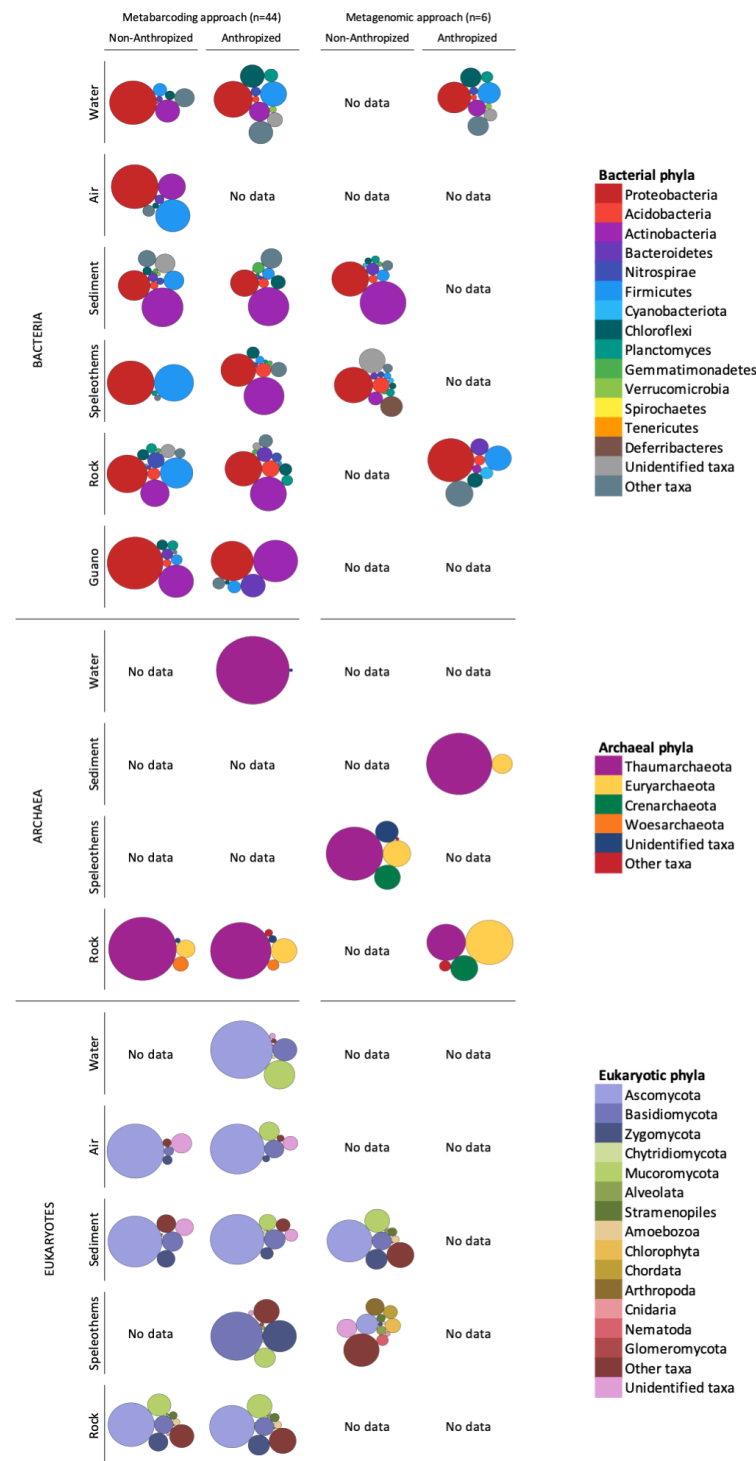


Figure 1. Composition of bacterial, archaeal, and eukaryotic communities based on metabarcoding and metagenomic data. The size of the circles represents the mean relative abundance of each lineage across the caves studied. Only lineages with relative proportion > 0.5% are shown. Sequences were retrieved in the results sections or supplementary data of research articles or in sequence databases (e.g., RDP classifier, Greengenes, 119 SILVA, 123 SILVA, 123.1 SILVA, UNITE 7.0, etc.). These sequences were affiliated according to the best hit obtained by BLASTn against Nucleotide collection (nr/nt) and the relative abundance of phyla was calculated. Bubble plots were generated using R (v.4.0.2) software. 'No data' is indicated when data are not available. See Supplementary Table S1 for further information on the experimental conditions that are included in the comparison.

Metabarcoding has been applied to characterize microorganisms associated to speleothems (e.g., stalagmites, stalactites, and flowstones). Biomineralization is a naturally occurring process in which microbial activity influences the formation of geological structures like speleothems [70–72]. Bacteria have been reported as important actors for carbonate mineral nucleation and growth [73]. *Proteobacteria* and *Actinobacteria* were found to be the predominant phyla associated with speleothems (Figure 1). *Actinobacteria* are known to be involved in biomineralization processes in karstic ecosystems [74–78] and are dominant in rock and sediment samples, while proteobacteria were more abundant in air and water samples, explaining their retrieval in speleothems [61]. The metabarcoding study of bacterial communities associated with the different cave speleothems highlighted specific variations in assemblages that were related to mineralogy and geochemistry [79]. In Lake Cave (Australia), *Cyanobacteria*, *Firmicutes*, and *Planctomycetes* were less abundant in the stalactites, where a greater proportion of aragonite and less dolomite was reported compared with stalagmites [79]. Recent work focused on the microbial entrapment in stalactites and their possible environmental origin in the Agios Athanasios cave (Greece) showing bacterial transport from the upper ground cave environment through water movement, especially from plant roots, when considering *Rhizobiales*, *Pseudarthrobacter* (*Micrococcales*) or *Gaiella* (*Thermoleophilia*), *Pseudomonas* (*Gammaproteobacteria*), *Ralstonia* (*Betaproteobacteria*), and *Pantoea* (*Gammaproteobacteria*) [80]. This study also reported that some bacterial genera retrieved in cave speleothems, such as *Polaromonas*, *Thiopfundum*, or the *Verrucomicrobia* phylum, support the concept that stalactites could be used as proxies of paleoecology to infer paleoclimate variations [80]. In the same cave, another work investigated the diversity of the rarest *Actinobacteria* families and genera, suggesting that stalactites represent microbial arks, entrapping and preserving DNA signal of such microorganisms at a paleomicrobiological scale [81].

Metabarcoding has documented microorganisms present in soil at the cave floor. Many studies focusing on cave microbial ecology targeted the soil in these caves. *Actinobacteria* and *Proteobacteria* dominate metabarcoding dataset (an average of 44% and 24%, respectively) in anthropized and non-anthropized cave soil. For the microeukaryotic community, reads affiliated to *Ascomycota* were largely preponderant (an average of 67%), followed by *Basidiomycota* phyla (an average of 14%). However, tourist activity can have an impact on the composition of soil microbial communities by bringing organic matter and dust from the outside on shoes, fallen hair, and flakes of skin [76]. A tourist that became sick during his visit to Castañar de Ibor Cave (Spain) raised the organic carbon content of the cave soil from 0.10% to 0.28% where he vomited [82]. Surprisingly, no major difference in structure and composition of soil microbial communities between anthropized and non-anthropized caves was observed at the phyla level (Figure 1). Only the *Firmicutes* phylum displayed a difference, with 9.7% of sequences in soil of non-anthropized caves compared with 4.1% of sequences in anthropized caves. This shows the necessity to go to lower taxonomic levels to observe possible differences. Currently, there is a crucial lack of knowledge on archaeal diversity in cave floors, whether anthropized or not.

Metabarcoding also identified microorganisms associated to arthropods and bats living in caves. These animals can act as biotic vectors facilitating the colonization of microorganisms in karstic environments [5,26,66,83]. They can promote the dispersion of *Bacteria*, *Archaea*, and *Fungi* by carrying them on their bodies or hosting them in their gut [84–86]. However, studying both the body- and gut-associated microbiota present is rather difficult due to sampling and contamination risks. Many studies focused on bats and guano in caves, particularly for microorganisms involved in zoonoses (viruses such as the European bat lyssavirus [87]), epizootics (*Pseudogymnoascus destructans* responsible of white-nose syndrome [88]), or human pathologies (*Bartonella* [89]). Bat gut samples hosted diverse bacterial phyla, dominated by *Proteobacteria* followed by *Firmicutes* [90]. Anthropization favors cave colonization by arthropods corresponding to *Collembola* [91]; *Folsomia candida* present on black stains in Lascaux Cave exhibited gut darkening (as did *F. candida* raised in vitro on plates where black fungi were grown), suggesting fungal

consumption by these arthropods [8]. Accordingly, fungal conidia can be present in their feces [8], which points to the ability of collembolans to disseminate bacteria and fungi [50]. Metabarcoding confirmed these observations, with a total of 470 bacterial OTUs (including the endosymbiont *Wolbachia*) and 148 fungal OTUs (including *Ochroconis* black fungi) associated to collembolans sampled in Lascaux Cave, which included 369 of the 382 bacterial OTUs and 21 of the 24 fungal OTUs found in the black stains underneath [86]. Metabarcoding approaches therefore make it possible to investigate the microbiota of arthropods and chiroptera in caves, but do not necessarily allow the identification of disseminated pathogens at the species level (Figure 1) [50,90,92].

Metabarcoding has been carried out to characterize the microorganisms of rock walls in karstic caves. The study of cave walls is often completed to address conservation issues. Indeed, many Paleolithic caves show wall alterations mainly due to ecosystem anthropization, which can cause, for example, the abnormal proliferation of microorganisms and the formation of visual stains on the walls (e.g., Lascaux Cave, Altamira Cave, etc.) [8,11,93,94]. Many microorganisms potentially involved in these alterations have been identified, but the microbial diversity studies often missed comparisons with neighboring control areas, making such comparisons uncertain. The black stains and dark zones of Lascaux, the yellow and gray spots of Altamira, and the red spots of the Cave of Bats are partly due to the growth of black fungi (e.g., *Ochroconis*, *Exophiala*, *Acremonium*, etc.), *Gammaproteobacteria* from the order *Xanthomonadales*, and *Betaproteobacteria* from the genus *Thauera*, respectively [8,11,95–97]. Pfendler et al. (2018) showed contrasted fungal communities in different caves [94]. Most of the time, cave walls, whether anthropized or not, are mainly colonized by *Proteobacteria*, *Actinobacteria*, *Thaumarchaeota*, and *Ascomycota* (Figure 1). Moreover, previous studies indicated that the anthropization of caves lead to a higher relative abundance of *Bacteroidetes* and a lower abundance of *Nitrospirae* (Figure 1), suggesting their use as bioindicators of disturbance in karstic environments [26,98–100]. A study comparing eight Chinese caves showed that temperature and sampling distance from the entrance have a significant influence on fungal communities on cave walls [65]. Indeed, fungal communities were more similar between the outside environment and the proximal entrance zone, while, when entering deeper into the cave, fungal communities were more dissimilar compared with the outside [65].

4.2. Workflow, Pitfalls, and Care for the Metabarcoding Approach

Metabarcoding allows the specific analyses of targeted genes (or gene regions) on many samples in parallel (i.e., multiplexing), generating thousands of gene sequences [43]. While metabarcoding can be applied either on taxonomic markers and/or on functional genes, most studies targeted taxonomic markers to investigate archaeal, bacterial, and/or microeukaryotic communities. For example, the 16S rRNA gene can be directly amplified from environmental DNA for the exploration of prokaryotic diversity. This technique presents many advantages: (i) it is cost effective, (ii) data analysis can be carried out using established pipelines, and (iii) large amounts of data are archived in reference databases [101].

However, the PCR step induces several issues: (i) variable amplification efficiency, (ii) formation of chimeras (hybrid products between multiple sequences, which can be falsely interpreted as new microorganisms, artificially increasing the richness of a sample), (iii) punctual mutations, and (iv) potential primer aspecificity [43,47,101–103]. To limit these biases, the selection of appropriate primers (different primers may uncover different microbial diversity ranges [104,105]), optimization of the number of PCR cycles, and establishment of a physical separation of the pre- and post-PCR steps are required [43,47], as well as the use of synthetic communities.

Moreover, the number of gene copies per genome can considerably vary from one taxa to another, distorting quantitative estimates of diversity. For example, an average of 160 18S rRNA gene copies is present in the genome of the dinoflagellate *Symbiodinium kawagutii*, as large and repetitive genomes are typical of dinoflagellates [106]. Single-copy

targeted genes (e.g., *rpoB*, *recA*, *gyrB*, etc.) have been proposed as alternatives, but the lack of a well-represented database for these genes is an important limiting factor [45].

Previous studies showed that different ribosomal hypervariable regions of the 16S rRNA and/or 18S rRNA genes were not equivalent for taxonomic affiliation of taxa and community diversity estimation, leading to a debate in the choice of the optimal regions to use for precise phylogenetic and taxonomic analyses [107,108]. However, the recent literature suggests that there is no ideal hypervariable region to target [55,107].

In the same way, amplicon size remains a crucial issue for a better assignment of taxonomy, as longer amplicons will provide a more reliable taxonomic affiliation. However, this parameter not only depends on the primer set used, but also on the sequencing technical limitation of the available platforms. For example, the V4 region (250 bp) of the 16S rRNA gene allows complete overlap of DNA sequences using the most commonly used platform to date (Illumina MiSeq 2 × 250 bp or 2 × 300 bp), unlike the hypervariable V3-V4 and V4-V5 regions, which cannot completely overlap [47]. Large overlapping regions allow merging of R1 and R2 pairs fixing a maximal mismatch rate, thus discarding sequencing errors and avoiding an overestimation of the real microbial diversity [43]. A key issue is the trimming of raw sequences, including overlapping but also the curation of sequences presenting ambiguous bases and/or an unexpected size.

The current debate is rather on the treatment of cured sequences, which can be (i) grouped into operational taxonomic units (OTUs) using global or local thresholds or (ii) processed with the Amplicon Single Variant (ASV) method [47,109]. While powerful rapidly evolving bioinformatic pipelines are available, they also display limits and biases in data processing, such as large differences in sensitivity and specificity. On the one hand, QIIME-ucrust attributes more than 12% of the sequences to false OTUs against 0.17% on average in the other tested pipelines, i.e., Mothur and Usearch-Uparse [109]. On the other hand, the pipelines dedicated to the treatment of ASVs differ in their sensitivity and the generation of parasitic ASVs; DADA2 has the best sensitivity but the highest proportion of parasitic ASVs compared with USEARCH-UNOISE3 and QIIME2-Deblur [109]. The choice and settings of bioinformatic pipelines must be adapted as well as possible to the biological question to limit biases in data interpretation. This must be completed with the establishment of a minimal abundance threshold filter that will eliminate OTUs/ASVs with extremely low abundance, as they have a high probability of being false.

To conclude, the analysis of metabarcoding sequence data must be considered with care. In this context, it is of interest to confirm or at least complete metabarcoding findings with complementary approaches such as metagenomics, which does not include a PCR step.

5. Taxonomic and Functional Assessment of Cave Microorganisms by Metagenomics

Sequencing of total environmental DNA allows the simultaneous characterization of both taxonomic markers and genetic potential in complex communities. Thus, this technique permits to answer the following questions: who is there and what are they capable of doing? Because of the complexity in analyzing metagenomic data and because of the high cost of this approach, only a few studies have used metagenomics in caves so far. Moreover, most of the studies using metagenomics exploited their data partially by only considering (i) taxonomic information, (ii) functional information, (iii) very large functional pathways (i.e., nucleic acids synthesis, energy metabolism, lipids metabolism, carbohydrates metabolism, etc.) that are not very informative, or (iv) very specific metabolic pathways (i.e., methanogenesis, carbon fixation [103]).

5.1. Cave Metagenomics Tells Us Who Is There

The analysis of taxonomic diversity by shotgun metagenomic sequencing is typically carried out by (i) considering taxonomically informative marker genes simultaneously for all microorganisms present in a community (providing an estimate of their contribution to the microbial assemblage), (ii) assigning every metagenomic sequence to a taxonomic group,

or (iii) reconstructing Metagenome-Assembled Genomes (MAGs), which is a powerful tool to build synthetic representatives for uncultured microorganisms.

Marker gene analysis is an effective way to quantify taxonomic diversity within metagenomes. This method involves comparing reads to a database of taxonomically informative gene markers (i.e., SILVA database for 16S/18S rRNA genes, UNITE database for ITS regions, etc.). It needs to be noted that a high proportion of reads could not be classified using this method. Two studies conducted on Kartchner Cave speleothems (Arizona, USA) [101] and Manao-Pee Cave sediments (Thailand) [103] assessed rRNA gene diversity using BLAST analysis against the NCBI Non-Redundant (NR) nucleotide database and SILVA database, respectively (Supplementary Table S1). On the speleothem surfaces of Kartchner Cave, 85% of the assigned rRNA genes (among those classified) corresponded to *Bacteria*, 10% to *Archaea*, and 5% to *Eukaryota*. In the sediments of Manao-Pee Cave, 96.6% of the assigned rRNA genes pointed to *Bacteria*, 2.6% to *Archaea*, and 0.8% to *Eukaryota*. In both ecosystems, the dominant bacterial phylum was *Proteobacteria* (52.0% and 32.9% in Kartchner and Manao-Pee caves, respectively), followed by *Actinobacteria* (13.0% and 51.2%, respectively), whereas *Thaumarchaeota* represented the major archaeal phylum with 76.0% and 90.7% of the archaeal sequences in Kartchner and Manao-Pee caves, respectively. The dominance of these three phyla in both caves suggests their ecological importance and potential role in C and N biogeochemical cycles [9,103,110]. Those results contrast with recent NovaSeq sequencing work conducted in Congo Cave (Africa), which reported an overwhelming dominance of *Bacteria* (99.66% of sequences) with only 0.05% and 1.20% of archaeal and eukaryotic sequences, respectively [111]. Among *Bacteria*, the *Proteobacteria*, *Bacteroidetes*, and *Actinobacteria* were the most abundant phyla, representing 81.74, 10.57, and 4.16% of sequences, respectively [111].

An alternative strategy, known as binning, attempts to group together the metagenomic sequences originating from the same taxonomic population [112]. To this end, each sequence is either (i) classified into a taxonomic group (e.g., genus, family, etc.) through comparison to some referential data, or (ii) clustered into groups of sequences that correspond to taxonomic groups based on shared characteristics (e.g., GC content). This method may provide insight into the genomic composition of uncultured organisms found in communities, and finally gives a way to reduce the complexity of the data, such that post-binning analysis (e.g., assembly) can be performed independently on each set of binned reads rather than on the entire set of data [112–114]. Using this method, Kumaresan et al. (2018) evidenced a high proportion of *Proteobacteria* in microbial mats and sediments from Movile Cave (Romania). *Gallionellaceae* was the dominant family within sediment microbial communities of caves, suggesting a major role for these microaerophilic iron-oxidizing bacteria in nutrient cycling [115,116]. However, there is usually a trade-off between the number of reads that are binned and the taxonomic specificity of the annotations assigned to each bin. Additionally, there may be a large variance in the accuracy and specificity of the annotations across reads. Also, genes that have undergone horizontal transfer may not be represented adequately in the training dataset, and experimental data are needed to validate the algorithms predictions made for novel organisms.

A third sequence analysis method consists in reconstructing MAGs (Metagenome-Assembled Genomes). It relies on an assembly that merges collinear metagenomic reads from the same genome into a single contiguous and longer sequence (i.e., contig) [102]. In some instances, complete or nearly complete genomes can be assembled, which provides insight into the genomic composition of uncultured organisms found in a community. The major challenge associated with assembly is the possible generation of chimeras, wherein sequences from two distinct genomes are spuriously assembled into a contig due to shared sequence similarity. This can lead to MAGs that aggregate sequence information from different strains of the same species, or from different species or even genera. In Weebubbe Cave (Australia), a high proportion of reads were taxonomically assigned to the phylum *Thaumarchaeota*. From the assembly of the metagenomic data, a set of 20,233 contigs with a minimum of $\times 4$ coverage, ranging in size from 100 bp to 67 kb,

was generated. The subset of contigs that binned to *Thaumarchaeota* was used to generate alignments, and the global alignment showed that the greatest degree of synteny of the Weebubbie *Thaumarchaeota* contigs was with *Nitrosopumilus maritimus* SCM1 [117]. While several studies have investigated microbial cave communities using assembly methods, the reconstruction of genomes is limited to the most abundant taxa in the community. Without extensive sequencing, it may be difficult to assemble genomes of rare microbiota. Also, the variation in repeat copy number between assemblies should be carefully evaluated. Finally, the assembly can be computationally intensive, especially in its requirements for RAM, but binning sequences prior to assembly can be a good way to cut down on computational complexity.

5.2. Cave Metagenomics Tells Us What They Are Capable of Doing

As for other environments, shotgun metagenomic DNA sequencing is an asset for the study of karstic ecosystems. Metagenomes can provide insight into the functional and ecological roles of microorganisms by investigating their genes with annotation tools (e.g., COG, KEGG, Pfam) [118]. Thus, cave metagenomes can reveal how microbes develop under specific cave conditions which are energetically unfavorable and limited in nutrient content [26,103]. Functional annotation involves two major steps, i.e., gene prediction and gene annotation [47,102].

Gene prediction determines which metagenomic reads contain coding sequences. Once identified, coding sequences can be functionally annotated. For metagenomes assembled with complete coding sequences, gene prediction can be classic (with default parameters specific to each tool), while some prediction algorithms require specific parameters. In the case where the coding sequences are partial, the prediction is more difficult. There are three ways to predict genes in metagenomes: (i) recruiting gene fragments, (ii) classifying protein families, and (iii) predicting genes de novo. The biggest problem with this prediction is the level of database information, since not all predicted genes will exhibit homology with already identified sequences [119], highlighting the presence of new genes.

Once coding sequences in a metagenome are predicted, they can be subjected to functional annotation. Metagenomic proteins are classified, and families of proteins are predicted (protein sequences related to evolution). As proteins in the same family are believed to encode similar biological functions [102], if a metagenomic sequence is determined to be a homologue of this family, then it is deduced that the sequence corresponds to the function of the family. The classification of a metagenomic protein sequence into a family of proteins is usually performed by comparing it to a protein database. Then, the protein is classified into either (i) a single family, (ii) a series of families (all families with a significant classification score), or (iii) no family, suggesting that the protein may be new, very divergent, or false. There are several pitfalls in functional annotation. First, most databases contain families that have no known functional annotation. Second, the database of protein families used to annotate the sequences may be subject to phylogenetic bias, or some communities may be disproportionately annotated. New strategies are needed for functional metagenome annotation and improvements in the way predicted metagenomic proteins are integrated into protein family databases.

Few metagenomic studies have investigated functions associated with cave microorganisms. Analysis of the metabolic potential of these microorganisms is performed by mapping the reads into a general database, the Kyoto Encyclopedia of Genes and Genomes (KEGG). For caves, six functional modules are commonly used (metabolism, genetic information processing, environmental information processing, cellular processes, organ systems, and human diseases) [120]. Usually in caves, predictions of the metabolic potential of microorganisms have shown the presence of genes involved in methane metabolism, nitrogen metabolism, sulfur metabolism, carbon fixation, biodegradation, xenobiotic metabolism, and production of secondary metabolites [103,110,117,121]. More recently, shotgun metagenomics has suggested the presence of microbial communities able to change Fe and Mn redox states, especially members of the Comamonadaceae and

Hyphomicrobiaceae families, in Monte Cristo Cave (Brazil) [122]. In Borra Caves (India), metagenomics suggested the vital role of sulfur reducers and sulfur-disproportionating microorganisms in energy generation in these caves and also the role of various nitrogen modifications for nitrogen pools conservation [123]. This knowledge on the metabolic capabilities of microorganisms is very useful to understand their survival under energetically unfavorable conditions.

5.3. Workflow, Pitfalls, and Care for the Metagenomic Approach

Metagenomic data represent complex and large datasets, which complicates computational analyses. The type of sequencing platform used is a crucial issue, as it generates highly contrasted numbers of reads as well as contrasted read sizes. Illumina HiSeq or MiSeq technologies are used most of the time. Ranjan et al. (2016) performed a direct comparison of Illumina HiSeq 2000 (that generated 67.2×10^6 reads; 6.7 Gb of sequences), MiSeq v-300 (37.5×10^6 reads; 5.8 Gb), and MiSeq v3-600 (59.0×10^6 reads; 15.6 Gb) [101]. MiSeq technology provided longer reads (150 bp for v-2 chemistry, 300 cycles and even 300 bp for v-3, 600 cycles, versus 100 bp for HiSeq), which may improve the efficiency of de novo assembly of contigs, and thus improve the detection of the uncultivated fraction in the MiSeq dataset. A previous study conducted in Tjuv'Ante's Cave (Sweden) generated 39 million sequences with HiSeq technologies, allowing the detection of 29 different bacterial phyla (Supplementary Table S1) [121]. More recently, NovaSeq Illumina platforms greatly expanded sequencing capabilities and sequencing depth, reaching 6 Tb and 20 billion sequences in less than 48 h, and making it thus possible to fully investigate the rarest taxa present in ecosystems. The application of NovaSeq sequencing in caves gave the first insights into Movile Cave sediment functioning [124].

Sequencing depth is also an element to be considered for the study of the diversity of microbial communities in caves. Shallow Shotgun Metagenome Sequencing (SSMS) involves sequencing samples at a shallower depth than in full shotgun metagenome sequencing, and most of the time is preferred because of lower sequencing cost. By combining many more samples into a single sequencing run and using a modified protocol that uses a lower volume of reagents for sequencing library preparation, SSMS is an economical way to provide compositional and functional sequencing data of the most abundant microorganisms. It gives more accurate functional profiles and more precise taxonomic resolution than 16S rRNA gene sequencing [56]. SSMS was used in Movile Cave (Romania) and elucidated the distribution of aerobic methylotrophic microorganisms within microbial mats and sediments [116]. However, shallow shotgun sequencing is not intended to replace deep whole-genome shotgun sequencing for strain-level analysis or novel gene and genome assembly.

5.4. Comparison between Metabarcoding and Metagenomic Approaches in Cave Ecosystems

Few studies have compared metabarcoding and metagenomic approaches to document microbial diversity, and even fewer in caves. However, the compilation of various studies presented in Figure 1 allows us to establish a comparison between these two methods for the three domains of life in different cave compartments. Four literature comparisons can be made for the bacterial community (water and rock walls for anthropized caves and sediments and speleothems for non-anthropized caves), one for the archaeal community (rock walls for anthropized caves), and one for the microeukaryotic community (sediment for non-anthropized caves). Whatever the comparison, differences in community structure and composition were found. For example with the sediments of non-anthropized caves, the relative abundance of the *Basidiomycota* microeukaryotic phylum was higher with the metagenomic approach, where it represented >5% of sequences (Figure 1 and references therein). In speleothems, 4 bacterial phyla were detected by metabarcoding versus 16 using metagenomics, with a 35% decrease in the relative abundance of the *Firmicutes* phylum when considering metagenomic data compared with metabarcoding. Another comparison in the sediments of Manao-Pee Cave identified 123 bacterial families by metagenomics

against 55 families with the 16S rRNA dataset [103,125]. For the archaeal community, the proportion of sequences affiliated to the *Thaumarchaeota* and *Euryarchaeota* phyla was also very different between the two approaches. In Weebubbie Cave (Australia), the relative abundance of *Archaea* was ~40% in the 16S rRNA amplicon dataset versus only ~20% in the binned metagenomic dataset [117]. The different copy numbers of the 16S and 18S rRNA genes in microbial genomes can lead to contrasted results. Since the number of 16S rRNA gene copies per genome for each single species or strain present in microbial assemblages is not predictable, consequently no fully effective corrections in metabarcoding and/or metagenomic datasets can be implemented to better reflect environmental reality.

Metabarcoding and metagenomic approaches display different advantages and drawbacks to characterize taxonomic diversity. On the one hand, metabarcoding is a powerful tool for exhaustively describing the diversity of *Archaea*, *Bacteria*, and *Eukaryota* in caves and other ecosystems. In addition, metabarcoding sequencing is less expensive than metagenomics. On the other hand, metabarcoding does not allow quantitative comparisons when different markers are used. In addition, metabarcoding is PCR based, whereas DNA is directly fragmented and sequenced in metagenomics, without any PCR bias. Finally, metagenomics has the advantage of assembling large fragments, sometimes entire reconstructed genomes of high quality (in terms of contamination and coverage), allowing scientists to discriminate microorganisms at the species or even strain level. This is not possible with the short amplicons used for metabarcoding, whose resolution is generally limited to the genus level [56,103]. Therefore, metagenomics is more appealing to uncover the contribution of each microbial domain to the microbial assemblage in caves.

In addition to taxonomic information, shotgun metagenomics also provides information on the genetic potential of microorganisms, allowing a better understanding of microbial adaptation and functional capacities in the environment investigated. The functional diversity encoded in the metagenome can only approximate the functionality of the community, as the presence of a gene does not mean that it is expressed at the time of collection. Analysis of metatranscriptomic and metaproteomic data can provide an additional insight into pathways that are actively expressed in the community, but this remains to be implemented in caves.

6. Contribution of NGS Assessments of Microorganisms to Cave Integrative Studies

A majority of environmental studies only consider one disciplinary side of the question at stake (i.e., biology, geochemistry, or physics, etc.), with, consequently, (i) gaps in our understanding at the interface between disciplines, (ii) a disciplinary focus on specific scales or levels of organization, and (iii) the development of specific disciplinary lexicons and methods that are difficult for non-experts to understand [126,127]. For a better understanding of the underground world, the use of an integrative approach through the interdisciplinarity of projects seems necessary. Interdisciplinary science is an integrated approach that synthesizes the perspectives of multiple individual disciplines during all phases of the research [128]. Thus, cave studies require a holistic, interdisciplinary approach that simultaneously considers the physical, chemical, and biological components of the underground environment (Figure 2) [126,129]. One of the ultimate aims of integrative investigations is the development of a database that regroups information about water, air, rock surfaces, speleothems, microorganisms, fauna, etc. (Figure 2) [129]. Some caves suffer from an imbalance in their environment due to construction works, excessive presence of tourists, and/or chemical/biological treatments (Figure 2) [26,50,91]. This may induce rock wall alterations and complicates cave conservation, especially in Paleolithic caves. The lack of interface between disciplines constitutes a major issue for understanding ecosystem functioning and conservation of rock art in caves. Another aim of interdisciplinarity is to compile information on cave wall alterations, their characterization, methods of diagnosis, the mechanism of their formation and the parameters ruling their evolution, in order to better identify, prevent, or control them in painted caves [129].

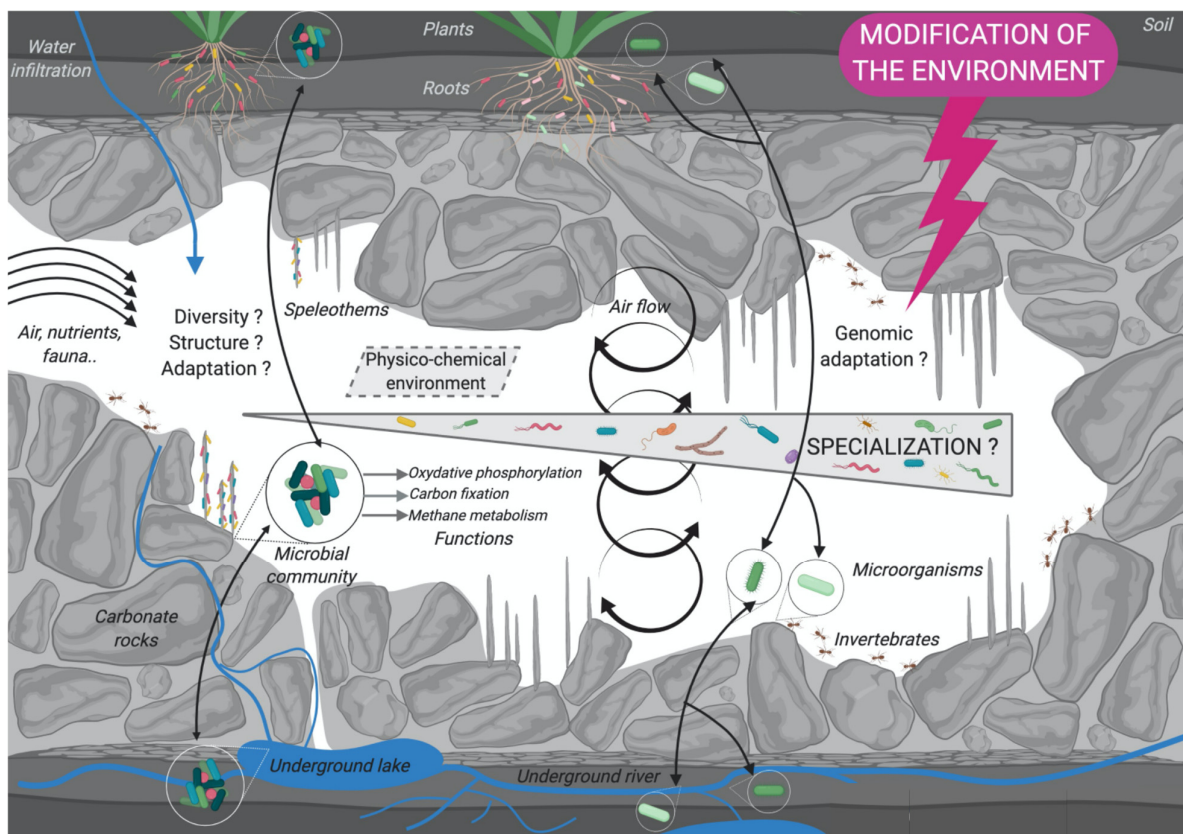


Figure 2. Schematic representation of a karstic environment integrating the challenges of interdisciplinarity.

One of the first interdisciplinary cave studies was carried out in 1983, and included specialists in karstology, the micromorphology of walls, and underground climatology to understand how tourism activities were responsible for artwork wall alteration [130,131]. This collaborative work determined the cause of artwork damage in the Black Salon of Niaux Cave, France. To make further progress, scientists established a laboratory cave in Vézère Valley (Dordogne, France) [129], which was set to conduct experiments for in situ analyses and obtaining samples to develop interdisciplinary approaches in caves. Using this pilot cave, Lacanette et al. (2013) focused on microclimate analysis, showing that there are special conditions for large exchanges between the inside and outside of the cavity, and after a year of monitoring the cavity, a link between microbiology and microclimate could be made to understand the dynamics of microbial communities (Figure 2) [129]. Such interdisciplinary studies of caves have been performed in many countries, especially in Spain [132,133].

While interdisciplinarity is powerful, challenges stem from disciplinary barriers, which including the challenge of competing and conflicting uses of scientific terms, but also institutional difficulties of organization and logistics [126,128]. Integrating disparate disciplines with different paradigms, priorities, inherent questions, research methods, approaches, and metrics of success is a fundamental challenge in environmental science [126]. Consequently, the results of interdisciplinary efforts may be emergent as well. Ultimately, true collaboration is essential for successful interdisciplinary science and a better comprehension of the underground ecosystem.

7. Conclusions and Perspectives

Each method for investigating the microbial ecology of caves has advantages, biases, and limitations. The choice of these methods will therefore be oriented in relation to the scientific question and/or hypothesis. The cultivation approach associated with high-throughput sequencing offers opportunities for understanding the precise physiology of cer-

tain cave microorganisms and remains a useful complement to sequence-based approaches. Metabarcoding sequencing has allowed major advances to better document the microbial ecology of caves, with comparisons of anthropized and non-anthropized caves as well as different compartments within a cave (e.g., water, soils, wall, etc.), but the archaeal community remains largely understudied. The development of non-targeted high-throughput sequencing techniques (i.e., metagenomics) is more expensive than metabarcoding, but it provides higher resolution power, enabling a more reliable taxonomic classification and also a functional profiling of the cave community. Metagenomics can reveal crucial information about the lifestyle of cave microorganisms. However, it should be noted that these techniques are based on a genetic and metabolic potential which may not be expressed in each environmental condition. To gain a more holistic understanding of cave microorganisms, transcript-based (metatranscriptomics) as well as metabolome-based (metabolomics) studies need to be conducted as well to provide more complete information on the cave microbiota. Despite the constant improvement of sequencing techniques, the total understanding of the microbial ecology of caves can be achieved on the condition of following a multidisciplinary approach that also includes geology, physics, climatography, hydrology, and chemistry. Such an integrative view to explore and understand microbial ecological processes in caves has been little implemented so far.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app14209438/s1>. Table S1. Detailed information on the selected literature related to the bacterial, archaeal, and microeukaryotic communities in cave microbiota all around the world. References [9,12,50,55,57,58,61,65,79,94,99,103,110,116,117,121,125,134–148] were cited in the Supplementary Files.

Author Contributions: Conceptualization, Z.B., M.H. and Y.M.-L.; Formal analysis, Z.B.; Investigation, Z.B.; Writing—original draft preparation, Z.B.; Writing—review and editing, Y.M.-L. and M.H.; Visualization, M.H.; Supervision, M.H. and Y.M.-L.; Funding acquisition, Y.M.-L. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by DRAC Nouvelle Aquitaine (Bordeaux, France).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: This review was completed using data from published sources, all of which are cited in the ‘References’ section of this article.

Acknowledgments: We thank S. Géraud, T. Baritaud, and M. Mauriac (DRAC Nouvelle Aquitaine), and Lascaux Scientific Board for helpful discussions.

Conflicts of Interest: The authors declare no conflicts of interest.

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