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Subterranean marvels: microbial communities in caves and underground mines and their promise for natural product discovery

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Covering: 2014 to 2024

Since the dawn of human history, caves have played an intimate role in our existence. From our earliest ancestors seeking shelter from the elements to more recent generations harnessing cave substances for medicinal purposes, caves have served as essential resources and havens. The last 40 years of geomicrobiology research has replaced the outdated perception of subterranean environments as lifeless and unchanging with the realization that vibrant microbial communities have adapted to thrive in extreme conditions over millions of years. The ability of subterranean microbial communities to withstand nutrient deprivation and darkness creates a unique reservoir of untapped biosynthetic potential. These communities offer exciting prospects for medicine (e.g., antimicrobial and antitumor therapies) and biotechnology (e.g., redox chemical properties and biomineralization). This article highlights the significance of caves and mines as reservoirs of microbial diversity, the potential impact of their bioactive compounds on the fields of healthcare and biotechnology, and the significant challenges that must be overcome to access and harness the biotechnological potential of subterranean microbial communities. Additionally, it emphasizes the conservation efforts needed to protect these delicate ecosystems, ensuring the preservation of both ancient traditions and tomorrow's medicines.

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- 1. Introduction
- 2. Subterranean ecosystems
- 2.1 The anatomy of caves
- 2.2 Human history in caves: folk medicines and cultural significance
- 3. Microbial diversity in subterranean environments
- 3.1 Bacterial diversity
- 3.2 Fungal diversity
- 3.3 Factors influencing microbial diversity in subterranean environments
- 3.3.1 Extreme environments
- 3.3.2 Human impacts
- 3.3.3 White-nose syndrome (WNS)
- 4. Natural products discovered from subterranean environments
- 4.1 Examples of natural products
- 4.2 Approaches to natural products discovery
- Biotechnological and medicinal potential of subterranean microorganisms

- 5.1 Antibacterial properties
- 5.2 Antifungal properties
- 5.3 Cytotoxic and antiproliferative properties
- 5.4 Other bioactivities and potential applications
- 6. Accessing the untapped potential of subterranean microbial chemistry
- 6.1 Culture-dependent approaches
- 6.1.1 Isolation and cultivation
- 6.1.2 Identification and elicitation of biosynthetic pathways
- 6.2 Culture-independent approaches
- 6.2.1 Targeted approaches
- 6.2.2 Non-targeted approaches
- 7. Challenges and future outlook
- 8. Data availability
- Conflicts of interest
- 10. Acknowledgements
- 11. References

1. Introduction

Subterranean environments have long held a significant place in human history. Caves have both served as shelters for our

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earliest ancestors and repositories of traditional knowledge. As early as 40 000 years ago, humans began excavating their own subterranean environments, creating man-made mines that have provided essential resources that have shaped societies throughout history.1 This is unsurprising given that around 15% of the ice-free surface of the Earth is karstified and close to 17% of the world's population lives in karst environments.² Despite the extensive utilization of caves and mines by humans, many subterranean environments remain undisturbed by anthropogenic activities and represent pristine environments with remarkable biodiversity. Beneath their seemingly unchanging façade, subterranean environments host intricate microbial communities that have evolved to thrive in extreme oligotrophic conditions over millions of years. The adaptations of these cave-dwelling microorganisms to survive under extreme darkness and nutrient scarcity are made possible, at least in part, through the development of specialized metabolic pathways encoding natural product molecules with diverse biological activities.

In recent years, a number of notable review papers have been published on the topic of microbial communities in caves and

their biotechnological applications.3-7 These reviews provide important background information on the medicinal and biotechnological properties of prokaryotic communities, primarily actinomycetes, in caves, and we encourage the interested reader to explore these manuscripts for additional commentary on the subject. Our review concentrates on the underground communities in terrestrial settings (i.e., caves and mines). While previous reviews have primarily focused on bacteria, this review also covers studies of fungal communities, including bioactive cave-dwelling fungi and the devastating pathogen Pseudogymnoascus destructans. We highlight the challenges associated with accessing the untapped bio- and chemodiversity of underground systems, with particular emphasis on cultivation techniques for maximizing microbial diversity as well as strategies to "turn on" cryptic biosynthetic gene clusters in laboratory settings. Finally, we emphasize the importance of cave conservation and environmental stewardship, highlighting the unfortunate negative impacts of mining and tourism and how to sustainably access fragile communities for the discovery of bioactive metabolites.



Paris S. Salazar-Hamm

Paris S. Salazar-Hamm graduated with her PhD from the University of New Mexico in Biology in 2021 under the guidance of Dr Donald Natvig where she focused on the ecology of several human and animal fungal pathogens. Her research on bat and cave microbiomes and their responses to whitenose syndrome began in 2015 as a master's student at Western University with Dr Illinois Andrea Porras-Alfaro.

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Evelyn G. Haugh, Shyleigh A. Good, and Frances E. Homan (left to right)

Evelyn G. Haugh, Shyleigh A. Good, and Frances E. Homan (left to right) are senior undergraduate students (class of 2025) majoring in chemistry at James Madison University. All are working on projects investigating the induction of natural products from bat-associated, subterranean, and/ or epigean microorganisms through coculture utilizing mass spectrometry-based metabolomics.



Jennifer J. M. Hathaway

caves.

Jennifer J. M. Hathaway has been working in caves for over 15 years. She earned a Master's in Biology from the University of New Mexico in 2010, studying the lava caves of the Azores. Since then, she has been working as a Research Scientist at UNM DrDiana Northup, exploring the geomicrobiology of caves. Her interests include the drivers of diversity of microbial life in caves, and the role of microbes in nitrogen cycling in



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white-nose syndrome. They are currently pursuing a PhD in Chemistry at Vanderbilt University.

Subterranean ecosystems

Subterranean ecosystems are comprised of distinct ecological zones ranging from the illuminated entrance to the pitch-black deep interior that have profound effects on both natural ecological communities and human culture. This section provides a detailed examination of the structure of subterranean ecosystems, specifically terrestrial caves, as well as the ways in which ancient peoples engaged with these unique environments. By investigating the roles of caves in shaping human practices and beliefs, we uncover the deep cultural connections that have persisted throughout human history.

2.1 The anatomy of caves

Cave systems consist of a network of subterranean, interconnected, oligotrophic environments that are characterized by high humidity, low consistent temperatures, and limited organic nutrients.8 These environments can form under a variety of geological settings including volcanic, limestone, granite, gypsum, mud, marble, glacial, and boulderous, with most formations occurring by water erosion.8 The three most well-studied cave types are epigenic limestone caves (formed by water-driven dissolution of limestone), hypogenic sulfuric acid caves (formed from the bottom up by sulfuric acid), and lava tubes (formed from the cooling, evacuation, and crusting of the lava drainage channel).9 Because caves are largely isolated from surface energy input, they are considered nutrient-limited environments. The level of nutrients and organic matter is largely influenced by mineralogical composition, tourism, animal activity, runoff, and drip water.10

Caves habitats can be divided into four distinct zones: the entrance zone, the twilight zone, the transition zone, and the deep interior (Fig. 1). The entrance zone is where the surface and subterranean environments intersect. This is followed by the twilight zone which is characterized by variable temperatures and low levels of light. While plants and other autotrophic



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metabolite products. She is currently an Assistant Professor at James Madison University where she leads her undergraduate research group in chemical ecology, metabolomics, and natural products discovery, with an emphasis on discovery from cave microorganisms.

organisms struggle to grow in the twilight zone, photosynthetic organisms such as lichens and algae have the capacity to grow here as well as other heterotrophic and mixotrophic organisms. The transition zone of the cave experiences complete darkness, but still has some temperature variability. The deep interior of caves is characterized by complete darkness, relatively constant temperature, high humidity, and fixed CO2 pressure regardless of surface conditions. This section of the cave is of particular interest because of the highly specialized microbes capable of surviving in the extreme nutrient-limited environments using energy from the surrounding rocks, infiltrating water, and air.5,11,12 While the microbial communities of the entrance and twilight zones are highly mediated by outside forces, such as the movement of water, wind, soils, and animals, a high barrier of dispersion has largely preserved the transition and deep interior zones, resulting in unique microbial and metabolic diversity.

2.2 Human history in caves: folk medicines and cultural significance

The association between humans and caves is so profound that our earliest human ancestors are commonly referred to as 'cavemen'. Indeed, there is considerable anthropologic evidence that prehistoric people, including now-extinct relatives of modern humans (e.g., Neanderthals and Cro-Magnon), found physical protection in caves. 13-16 The first evidence of early Neanderthals was found in a cave in Germany,14 and additional evidence of Neanderthal-like skulls found inside Spy Cave in Belgium have provided key insights into when Neanderthal populations disappeared from Eurasia over 40 000 years ago. 16 Although caves were utilized extensively as shelters, they were appreciated for more than mere physical protection. Some viewed caves as spiritual portals, uncontaminated from the outside world. Human remains suggest caves served as burial sites for some Indigenous communities. 17,18 Numerous artifacts have also been recovered from caves, for example, ceramic vessels coated in copal incense residue from the caves of Naj Tunic,19 indicating that Indigenous peoples including the Mayans conducted ritual events in them. 17,18 One of the most widely performed ritual events in caves was the rite of passage, which was required for Indigenous males to enter adulthood.9,10 Now, show caves host more than 150 million visitors worldwide;20 thus they are considered places with great geoheritage significance.21

Cave walls have long held stories of ancient civilizations including depictions of animals, handprints, and geometric patterns,⁹ and many revere prehistoric caves as the world's first museums.¹⁵ Gypsum, a soft sulfate mineral within caves, was used by some Indigenous groups for paint, as evidenced by Mammoth and Salt caves in central Kentucky (USA).¹⁸ One of the most famous caves for paleolithic art is Lascaux Cave in Montignac, France, containing over 600 paintings dating between 16 000–18 000 years ago.¹⁵ The majority of paintings in Lascaux depict bison, aurochs, and horses. Interestingly, anthropomorphic images in Western European paleolithic cave art are exceedingly rare and simplistic, usually located in the deepest

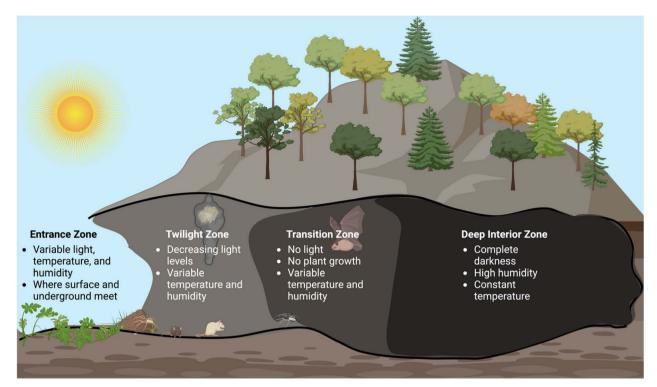


Fig. 1 Schematic representation of cave zones. Created in BioRender. Caesar, L. (2025) https://BioRender.com/q65d669

galleries among dense concentrations of drawings. In fact, the only known anthropomorphic image in Lascaux, a simple stick figure with a bird-like head, is hidden at the bottom of a hardto-reach well.22

In addition to their spiritual and artistic uses, caves have a long-intertwined history with medicine. For example, the presence of indigenous artifacts and preserved human fecal matter near deposits of selenite and mirabilite suggest these minerals may have been consumed for their laxative effects. 17,18 Salt caves have been used to treat respiratory illnesses due to their high humidity and presence of anti-inflammatory ions (e.g., Ca²⁺, Mg²⁺, and I⁻), a practice known as speleotherapy.²³ This efficacy of speleotherapy is in part supported by a small study of 22 participants who stayed at "Wieliczka" Salt Mine Resort where the health of all participants significantly improved after three weeks.23 However, the most famous and widely utilized medicinal cave substance is moonmilk. Moonmilk is a viscous white substance primarily composed of CaCO₃ that forms small pools in caves when hydrated. Etruscans and Romans used moonmilk as an emollient to induce lactation, while followers of Christianity revered moonmilk as blessings from angels (first century to present day).24 European peasants applied moonmilk on their livestock between the 11th and 15th centuries, realizing that moonmilk healed wounds at an exponentially faster rate than letting the wound resolve on its own. Because of these healing powers, they believed that moonmilk was created by supernatural entities such as gnomes.25 When Conrad Gesner published a document about the healing properties of moonmilk in 1555, moonmilk became more prominent in the pharmaceutical industry.11 Pharmacies prescribed and sold moonmilk to treat heartburn until the early 19th century.24

While the diverse microbial communities inhabiting moonmilk are influenced by microclimatic conditions including temperature and CO2 availability,26 the healing properties of moonmilk likely stem from the bioactive compounds produced by symbiotic Streptomyces species which are involved in the biomineralization of calcite in these unique formations.5 Moonmilk-associated Streptomyces strains have exhibited antibacterial and antifungal properties, although only a few bioactive compounds have been identified (discussed in Sections 4.1 and 5.1).5,27

Microbial diversity in subterranean environments

It is with the utmost difficulty that we attempt to describe microbial diversity in subterranean systems. The definition of subterranean environments is quite broad with differences in geology, climate, and organic inputs that can have immense influences on microbial diversity. Although the diminished light and nutrients are constraints, they have also created unique conditions for convergent evolution to subterranean environments. Microbial species richness and diversity generally decreases with increasing distance from the aboveground entrance, likely tied to available biomass;28 however, there have been instances reported of high biodiversity and richness even compared to regional surface environments.29 Microbes are essential members of these communities playing important roles in primary production, decomposition, and biogeochemical processing. Here, we discuss the biotic and abiotic factors that shape subterranean microbial communities. We focus on

bacterial and fungal diversity because of their importance in natural product production; nonetheless, we also acknowledge that archaea, cyanobacteria, and microalgae are present in subterranean systems with notable roles.^{30–32} For example, archaea contribute greatly to, and possibly modulate, nitrogen cycling in caves.³³ However, the role of these groups in natural product production is scarce at best.

3.1 Bacterial diversity

Microbial research of subterranean environments has largely focused on bacteria. This is both because of their wide-ranging metabolic capabilities allowing them to thrive in nutrientlimited environments but also their roles in sustaining these ecosystems. For example, a study of karst caves in southwest China leveraged over 200 bacterial genomes and eight metagenomic datasets, identifying genes involved in carbon, nitrogen, and sulfur metabolism essential for biogeochemical cycling.34 Chemolithotrophs contribute to mineral weathering and formation of secondary structures in caves by breaking down inorganic compounds in the rocks and walls for energy. The chemical composition of the cave bedrock modulates the diversity and metabolic responses of these cave bacteria.35 For example, ferromanganese deposits deep in Lechuguilla Cave (New Mexico, USA) were in part produced by manganese and iron-oxidizing bacteria.36,37 Microbial biofilms are recognized to aid in cave pearl formation,38 and bacteria-induced carbonate precipitation is important for formation of soda straw and popcorn speleothems.39

Early cultivation from the 1900s revealed that caves contained undescribed bacteria different from aboveground soil communities. 40,41 As with other ecosystems, the development of DNA-based sequencing has allowed for culture-independent studies revealing that the cave microbiome is more complex than a few species of highly specialized bacteria. 42,43 In fact, recent studies demonstrate that cave communities are still largely undescribed. Research from Hawaiian lava caves (USA) and fumaroles found that ~70% of the taxa found could not be classified at lower taxonomic levels. 44 Similar results have been found in karst caves where up to 19% of the sequences recovered belonged to unclassified phyla. 45 The percentage of sequences belonging to unknown taxa was >90% in the Sukinda chromite mine in India. 46

Caves across the world are inhabited by members of the phyla Pseudomonadota, Actinomycetota, and Acidobacteriota. ^{42,47,48} However, the abundance of these groups shifts based on geological history of caves, as illustrated in a recent review of bacterial metabarcoding (16S rRNA) studies of limestone caves, sulfuric acid speleogenetic caves, and volcanic caves. ⁹ While limited in its scope (105 samples from 22 caves), it reveals the distinct differences in bacterial composition and structure of higher taxonomic groups in caves based on rock type. ⁹ Other variables, such as sample type (*i.e.*, air, water, rock, and sediment), native minerals (*e.g.*, carbon, nitrogen, and copper), seasonality, and bat activity, also influence bacterial communities. ^{35,48–51} While the weight of these factors as key determinants of microbial composition and function is not

simple, salinity and pH have been highlighted across other ecosystems to particularly affect bacterial constituents.^{52,53}

Bacterial residents of mines similarly are inhabited by diverse members of Pseudomonadota, Actinomycetota, Bacillota, Bacteroidota, which are largely driven by contamination of heavy metals and pH.⁵⁴ Effluents draining from abandoned mines, known as acid mine drainage, are environmental pollutants due to their high acidity and presence of dissolved metals (*e.g.*, iron, manganese, copper, nickel, and zinc). Chen *et al.*⁵⁵ documented a shift in bacterial community composition correlating to pH of an acid mine drainage, specifically by members within Pseudomonadota and Nitrospirota. Bioprospecting efforts have targeted heavy metal tolerant bacteria in mines across Europe,⁵⁴ and this may be an avenue worth continued investigation for bioremediation potential.

Taxa within Actinomycetota are estimated to produce over 10 000 bioactive compounds of which 70–80% are produced by members of the genus *Streptomyces*. ⁵⁶ Actinomycetota are renowned for their cellular and metabolic versatility, allowing for specialization and emerging speciation in subterranean environments. ^{57–63} Volcanic caves in particular have been praised for their high diversity of Actinomycetota and their bioactive metabolites (discussed in Sections 5.1 and 5.2). ^{64–66} While many species are harmless to humans, a few actinomycetes from caves can opportunistically cause infections including *Nocardiopsis dassonvillei*, which can cause a subcutaneous skin infection (actinomycetoma), and *Inquilinus limosus*, which has been associated with cystic fibrosis. ⁶⁷

Pseudomonadota, among the most dominant taxa in caves, vary in abundance and distribution within and between cave systems. For example, Gammaproteobacteria, mainly represented by the genus *Pseudomonas*, have been shown to be particularly abundant in tourist caves. ⁶⁸ Conversely, the genera *Sphingomonas*, *Lysobacter* and *Polaromonas* were more prevalent in pristine caves. ⁶⁸ Species of *Pseudomonas* isolated from caves have been documented for their antibacterial and antifungal activities ^{65,69-74} (discussed in Sections 5.1 and 5.2). Rare members within this phylum are human pathogens, including *Aurantimonas altamirensis*, which was first described from Altamira Cave in Spain⁷⁵ and was subsequently connected to nosocomial infections of cystic fibrosis patients. ⁷⁶

Acidobacteriota is a highly diverse phylum found across multiple ecosystems;⁷⁷ however, they are poorly understood due to the difficulty of isolating them in culture.⁷⁸ Acidobacteriota appear to be prominent in hydrogen sulfide rich environments, including the springs of Lower Kane Cave (Wyoming, USA)⁷⁹ and cave wall biofilms of Frasassi cave system (Italy).⁸⁰ This group requires further investigation for their metabolic and functional capabilities, but members may be of biotechnological interest. For example, members of the genus *Blastocatella*, found in moonmilk of both carbonate⁸¹ and lava caves,⁸² have been shown to contribute to ammonium removal in wastewater.⁸³

3.2 Fungal diversity

Fungi that can persist in subterranean environments generally function as parasites or decomposers and play important roles in biogeochemical cycling of carbon, nitrogen, phosphorus, iron, manganese, and sulfur. Fungi can also participate in speleogenesis including the formation of needle fiber calcite.84-86 Endolithic species penetrate the rock substratum, which can cause biological weathering of rock surfaces, but also can contribute to stabilization and preservation over time.87 Due to limited organic matter in most subterranean environments, fungal richness tends to be lower than bacterial richness. 49,88 Despite this, there have been over 1000 species of fungi reported from karst caves alone,89 and this is likely an underestimate of cave-associated fungi given the exclusion of glacier, lava, and sea caves. Fungal surveys of caves consistently report dominance of members within the phylum of Ascomycota followed by members of Basidiomycota and Mucoromycota.89,90 Fungi (especially within the phylum Ascomycota) thrive in anthropogenically impacted caves with additional human-mediated inputs of organic matter (discussed in Section 3.3.2).91 Like humans, bats also serve as vectors transporting environmental fungi into caves;92 however, transient spores may also be introduced by drip water or air currents. Natural sources of subterranean organic materials preferred by fungi include bat guano93-95 and cave insects or arachnids.96-100 Bat and arthropod populations likely have some influence on cave fungal community composition, but it fluctuates between caves and cave structures. 49,101 Significant variability between fungal communities has been observed between caves of one system, locations within a cave, and sample type. 28,102,103

Subterranean mines offer similar conditions to caves, sometimes with the additional factor of heavy metals. Coal mines have been frequently used for timber storage in the USA and Europe, resulting in the dominance of wood decaying fungi, mainly within the phylum Basidiomycota.104 The Soudan Mine in Tower, Minnesota (USA) has both high iron-ore concentrations as well as an abundance of wood that remained from mining activities, supporting several phylogenetically distinct fungal species, 105 some of which have been recently explored for bioactive potential (discussed in Section 5.2).106,107 Fungi tolerant of heavy metals and metalloid compounds isolated from subterranean mines may be useful for bioremediation. Armillaria rhizomorphs observed in Soudan Mine105 as well as Champion Mine, a copper rich mine in Michigan (USA), 108 are suspected to play a role in absorption of metal ions and protection.109 Trichoderma harzianum isolated from Libiola Mine (Italy) demonstrated the highest efficiency of the native fungal population for silver bioaccumulation. 110 Trichoderma virens and several members of Penicillium (P. griseopurpureum, P. janthinellum, P. canescens, and P. soppii) cultured from the soils of Pestarena gold mine (Italy) were tolerant to arsenic levels of 10 000 mg L⁻¹.¹¹¹

Despite a growing body of research, subterranean environments remain one of the largely underexplored areas on this planet. Notorious pathogens such as Histoplasma capsulatum, causing human histoplasmosis, and Pseudogymnoascus destructans, causing white-nose syndrome (WNS) (discussed in Section 3.3.3), have created a somewhat cynical view of caveassociated fungi. However, caves and mines offer copious opportunities for discovery of novel fungal diversity112-117 and

fungal-derived natural products with both medicinal and biotechnological applications (discussed in Section 5).

3.3 Factors influencing microbial diversity in subterranean environments

Subterranean ecosystems present a diverse range of conditions that significantly influence the microbial life inhabiting them. Here, we discuss the impacts of harsh conditions on the development of specialized niches that support unique microbial communities adapted to thrive in extreme environments, as well as the impact of mining and tourism industries on microbial diversity through the introduction of non-native species and alteration of otherwise stable environmental conditions. We address the impact of WNS on bat populations and its broader implications for cave ecosystems. Understanding these factors is crucial to understand the complexity and fragility of subterranean microbial ecosystems and to develop strategies to protect and study these habitats.

3.3.1 Extreme environments. Subterranean environments have immensely variable conditions with extremes in temperature, salinity, pH, heavy metals, minerals, and/or oxygen availability that require a high degree of specialization and adaptation to survive. For example, high temperature hot springs, such as those in the Naica Underground System (Chihuahua, Mexico), are inhabited by thermophilic microbes that can withstand temperatures >50 °C.118 Other specialized microbes have adapted to extremely cold temperatures (0-4 °C), such as those documented in European limestone ice caves^{119–121} and icy volcanic caves in Mt. Erebus, Antarctica. ^{122,123} Heavy metals and metalloids can be highly toxic to humans even in minor quantities. Bacteria, archaea, and fungi that inhabit subterranean mines can often tolerate these pollutants at high concentrations, and play an important role in biogeochemical cycling and detoxification. 124 Morassina caves (Schmiedefeld, Germany) contain elevated metal loads (aluminum, manganese, vanadium, and uranium) attributing to extremely acidic conditions (pH 2.6-3.7) that are tolerated by extremophiles, 125 while Poole's Cavern (Buxton, England) offers a different challenge for microorganisms to adapt to a hyperalkaline environment (pH 9.3-12.2).126 Halophilic microbes have adapted to highly saline environments such as within halite crystals and deep shale deposits in the Appalachian basin (USA).127,128

While our review primarily focuses on terrestrial environments, it is worth mentioning that anchialine ecosystems, consisting of both microeukaryotic and prokaryotic communities, represent reservoirs of new biodiversity anticipated to host unique biometabolic activity. 129,130 Such ecosystems are unique in their anoxic conditions, which vary based on ocean depth, and their distinct salinity, temperature, and pH zones that influence species distribution.131

Finally, the unique conditions of caves may offer insight into microbial life beyond planet Earth. Subsurface environments on Mars hold particular promise for astrobiology given their protection from extreme winds and ultraviolet, cosmic, and solar ionizing radiation.132 Identifying Martian caves through remote sensing is challenging due to poor visibility and unknown structural integrity. Analogous formations on Earth, including lava tubes and basaltic caves, may provide insights into volcanic terrain on Mars, allowing for development of exploration strategies to investigate Martian subsurface environments. 82,133,134

3.3.2 Human impacts. A growing interest in subterranean environments has led to the marketing of show caves, allowing tourists to safely explore cave systems. Physical augmentation to caves for human visitation such as adding artificial lights and creating pathways affect relative temperature, organic matter, humidity, and illumination which shape cave biodiversity. 135 Increased light can lead to the appearance of lampenflora, complex phototrophic biofilms composed of microbes and algae that can deteriorate speleothems and other natural cave structures. 136-138 Human visitation also alters cave microbial environments by introducing non-indigenous organisms that may shift the relative abundance of natural microbial community members.139-142 In Lechuguilla Cave (New Mexico, USA) levels of bacterial human commensals such as Staphylococcus aureus and Escherichia coli were increased at times of high human traffic versus when visitors were absent. 43 Similarly, fungal commensals such as Candida and Malassezia were significant indicators of human visitation in a comparison of Italian pristine versus show caves. 68 Investigations of caves in Kentucky and Tennessee (USA) as well as France discovered a trend of lower microbial diversity and altered community structure in anthropized caves. 144,145 Additional surveys of show caves in Slovenia and Spain found higher bacterial counts (CFUs m⁻³) were indicative of recent human visitation. ^{146,147} A culture-independent study of Appalachian caves (Tennessee, USA) similarly documented fungal and bacterial enrichment in show caves compared to pristine caves.91 Taken together, human presence can introduce non-native microorganisms, decreasing the natural diversity and sometimes causing shifts towards domination by a few foreign organisms that may even surpass previous microbial loads.

In some delicate cases, subterranean artifacts have been disrupted or destroyed by even brief human contact. For example, 17 000 years-old cave paintings in Lascaux Cave (Montignac, France) were contaminated with algae, bacteria, and fungi contributed by human visitation that introduced humidity, warmth, and light.84 Aerosolized bacterial and fungal spores, in part attributed to human activities, have also contributed to biodeterioration of paleolithic paintings in the Cave of Altamira (Cantabria, Spain). 148 Castañar Cave (Cáceres, Spain), notable for its spectacular mineral morphologies including aragonite and calcite speleothems, has suffered from two human-mediated fungal outbreaks. 149,150 The different nature of organic carbon introduced into Castañar Cave in 2008 (human vomit) and 2021 (environmental debris transported by construction workers) provoked unique disturbances and ecological changes to delicate cave fungal communities. 151 Within caves, deep interior zones may be the most fragile because total organic carbon concentrations are less than 2 mg L⁻¹, 152 making even cursory human interactions risky. While education and exploration can be worthwhile ventures,

efforts should be taken to minimize human impacts and preserve the natural conditions of cave systems.¹⁵³

3.3.3 White-nose syndrome (WNS). WNS, caused by the fungal pathogen Pseudogymnoascus destructans, has resulted in devastating North American bat die-offs since its introduction during the winter 2006-2007. Over 6 million bats have perished across 44 U.S. states and ten Canadian provinces (https:// www.whitenosesyndrome.org/). Cave roosting bats are vehicles for local microorganisms, including pathogens, moving them in and out of caves. Because bat microbial communities are influenced by their habitat, 92,155-157 fungal and bacterial communities of bats and caves are exchanged; however, the directionality and stability is unclear.49 Cave managing agencies have intervened to prevent human-mediated WNS spread and protect vulnerable cave-roosting bat populations. 158 Naturally occurring bacteria on bat fur surfaces produce antifungal metabolites. 69,159-162 WNS-resistant bats in China have a high abundance of bacteria with antifungal activity163 which could suggest natural microbial barriers. Related beneficial taxa were enriched on the skin microbiota of several North American bats at WNS-positive sites164 affording these bats similar protections from WNS. In efforts to replicate these natural defenses, initial probiotic testing has been performed in vitro165 and in field trials. 166 Continuing research is critical to determine how natural host microbiota can be implemented as a biocontrol treatment to improve WNS disease outcomes and, further, if augmentation of bat communities will have effects on caves or other cave dwelling organisms. Non-pathogenic species of Pseudogymnoascus are frequently isolated from bats' fur as well as bat hibernacula.167-169 In a challenge study testing resource capture and competitive ability of non-pathogenic Pseudogymnoascus from a WNS-positive underground mine versus P. destructans, all non-pathogenic Pseudogymnoascus outcompeted P. destructans, suggesting P. destructans may have difficulty establishing on non-host surfaces in similar environmental niches.¹⁷⁰ Still, it is undeniable that the presence of this lethal pathogen has grossly affected subterranean systems and their microbial communities.

4. Natural products discovered from subterranean environments

In the last decade, exploration of natural products derived from subterranean environments has significantly advanced our understanding of subterranean microbiota. In this section, we review the diverse natural products discovered from cave- and mine-dwelling bacteria and fungi between 2014–2024 and examine the methodologies utilized in their discovery. While most compounds have been discovered using traditional bioactivity- or taxonomy-directed approaches, the potential of omics-guided strategies is high and remains underutilized.

4.1 Examples of natural products

Prior to 2014, only eight compounds were discovered and characterized from caves, and these are covered in previous reviews.⁴⁻⁶ Searches in PubMed, Google Scholar, Scopus, and

Table 1 List of bacterial compounds identified from subterranean environments between 2014–2024 and their producing organisms

No.	Compound name	Strain name	Cave of origin	Analytical technique	Ref.
Nov	vel compounds				
1	Xiakemycin A	Streptomyces sp. CC8-201	Chongqing City, China	NMR	171
2	Hypogeamicin A	Nonomuraea specus	Hardin's cave system, Ashland City,	111111	172 and
3	Hypogeamicin B	Tronomaraca specias	Tennessee, USA		173
4	Hypogeamicin C		Termessee, 6521		170
5	Hypogeamicin D				
6	Funisamine	Strentosnorangium sp. KDCAGF35	Various cave systems, Tennessee, USA		172
7	(2 <i>S</i> , 3 <i>S</i> , 4 <i>S</i>)-4-methyl-1-phenylhexane-2,3-diol	Streptomyces sp. CB09001	Karstic cave in Xiangxi, China		174
8	(2 <i>S</i> , 3 <i>S</i>)-4-methyl-1-phenylpentane-2,3-diol				
9	Huanglongmycin A				175
10	Huanglongmycin B				
	Huanglongmycin C				
12	Huanglongmycin D				176
	Huanglongmycin F				
	Lunaemycin A	Streptomyces lunaelactis MM109	Grotte des Collemboles, Comblain-au-		177
	Lunaemycin B1		Pont, Belgium		
17	Lunaemycin D				
	own compounds				
18	Diazepinomicin	Streptomyces sp.	Iron Curtain Cave, Canada	LC-MS/MS	178
19	14-Deoxychaxalactin B	Streptomyces sp. IB 2014/I/78-8	Bolshaya Oreshnaya Cave, Siberia	LC-MS ^a	179
	Cyclodysidin D				
	Stylissazole B				
22	Gyrophoric acid				
	Okicenone	Micromonospora sp. BBHARD22	Various cave systems, Tennessee, USA	NMR	172
24	Aloesaponarin II				
	Actinomycin C2	Streptomyces sp. BCCAGE06			
	Propeptin 1	Microbispora sp. BCCAGE54			
	Propeptin 2				
28	•				
29	Xenocyloin B	Streptomyces sp. CB09001	Karstic cave in Xiangxi, China		174
	2				
	Xenocyloin D				
32	Lumichrome				
33	,				
	Hexadecanamide	Paenibacillus sp. 23TSA30-6	Krubera-Voronja Cave, Georgia	GC-MS	180
	Octadecanamide	Paenibacillus spp. 23TSA30-6 and			
	(Z)-Octadec-9-enamide	28ISP30-2			
	Cyclic dipeptide cyclo(Pro-Phe)				
	(1-Methyl-2,2-diphenylcyclopropyl) sulfanylbenzene	Paenibacillus sp. 28ISP30-2			
	Diisooctyl phthalate	Streptomyces sp. GLD25	Gueldaman Cave, Akbou-Algeria		181
	6-Hydroxy-heptanoic acid				
	Hexadecanoic acid				
	Benzeneacetic acid				
43	3-(3,5-di- <i>tert</i> -Butyl-4-hydroxyphenyl) propionic acid				
44	Cycloheximide	Streptomyces sp. MM99	Grotte des Collemboles, Comblain-au-	LC-MS/MS	177
45	Dehydrocycloheximide	-	Pont, Belgium		
46	Ferroverdin A	Streptomyces lunaelactis MM109	-		
47	Phenazine-1-carboxylic acid	Pseudomonas yamanorum	Bats swabbed in Ge-zi Cave and Temple	GC-MS	182
48	Octanoic acid	GZD14026	Cave, China		
40	Isoprenol				
49					

 $^{{\}it a} \ {\it Compounds were identified by matching accurate masses to natural products databases. Without MS/MS fragmentation patterns or NMR spectra,}$ these compounds should be considered putative.

Table 2 List of fungal compounds identified from subterranean environments between 2014–2024 and their producing organisms. All compounds were identified using NMR

No	Compound name	Strain name	Cave of origin	Ref.
No	vel compounds			
51	•	Aspergillus fumigatus	Fangjing mountain, Guizhou province,	184
52	Sulfurasperine B	GZWMJZ-152	China	
53	Sulfurasperine C	•		
54	Sulfurasperine D			
55	4-Methoxy-7-methylbenzo[d]thiazole-5,6-diol			
56	2-Hydroxymethyl-4-methoxy-7-methylbenzo[<i>d</i>]thiazole-5,6-diol			
57	Pseudoanguillosporoin C	Cadophora sp. 10-5-2 M	Soudan underground iron mine,	106
58	Soudanone A		Minnesota, USA	
59	Soudanone B			
60	Soudanone C			
61	Soudanone D			
62	Soudanone E			
63	Soudanone F			
64	Soudanone G			
65	Oidiolactone G	Oidiodendron truncatum		107
66	Epi-oidiolactone G			
67	Oidiolactone H			
68	Oidiolactone I			
69	Oidiolactone J			
70	Oidiolactone K			
71	Oidiolactone L			
72	5-Chloroparietin			
Kn	own compounds			
73	Sulochrin	Aspergillus fumigatus	Fangjing mountain, Guizhou province,	184
74	Monomethylsulochrin	GZWMJZ-152	China	
75	3-Hydroxy-5-methoxy-2-methylbenzoquinone			
76	Pseudoangillosporin A	Cadophora sp. 10-5-2 M	Soudan underground iron mine,	106
77	Nectriapyrone		Minnesota, USA	
78	Isosclerone			
79	3,8-Dihydroxy-3-hydroxymethyl-6-methoxy-4,5-			
	dimethylisochroman-1-one			
80	7-Hydroxy-3-(1-hydroxyethyl)-5-methoxy-3,4-			
	dimethylisobenzofuran-1(3H)-one			
81	3-Acetyl-7-hydroxy-5-methoxy-3,4-dimethylisobenzofuran-1(3 <i>H</i>)-			
	one			
82	PR 1388	Oidiodendron truncatum		107
83	Oidiolactone C			
84	Oidiolactone D			
85	Oidiolactone E			
86	Oidiodendronic acid			
	LL-Z1271α			
88	LL-Z1271β			
89	Physcion			
90	Emodin			

ScienceDirect resulted in 17 articles that describe the isolation and/or characterization of 90 polyketides, peptides, terpenoids, and hybrid molecules (Tables 1 and 2). Of the compounds, 50 (56%) are derived from bacteria (Fig. 2 and 3) and 40 (44%) were of fungal origin (Fig. 4 and 5). Thirty-seven (41%) demonstrated one or more bioactivities (discussed in Section 5). Thirty-four (38%) compounds were novel, having only been discovered from subterranean environments (Fig. 2 and 4).

Streptomyces are the most prominent source of natural products discovered from caves in the last decade. Thirty-one (34%) of the compounds were from Streptomyces, which also represented 62% of the bacterial-derived compounds. Of the 19 strains whose natural products have been investigated since

2014, 16 are bacterial and only 3 are fungal, illustrating that fungi are underrepresented in natural products studies of subterranean environments. Further, of the 553 natural products derived from fungi published in 2023, none were reported from caves.¹⁸³ The importance of exploring this underresearched niche is emphasized by the fact that the three subterranean fungal strains in this review yielded 40 total natural products (44% of total compounds), of which more than half were novel. Interestingly, a strain of *Aspergillus funigatus* isolated from cave soil collected near Fanjing Mountain, China, produced six new compounds,¹⁸⁴ suggesting that even well-studied species,¹⁸⁵ when adapted to cave environments, may possess novel biosynthetic potential.

Table 3 Studies of antibacterial activity in caves and mines from 2014–2024

Bioactive strain(s)	Pathogens tested	Bioactive agent(s)	Cave of origin	Ref
Aspergillus fumigatus, Trichoderma yunnanense	S. aureus, P. aeruginosa	Not determined	Sthreepura Cave – Kuruwita, Sri Lanka	193
Streptomyces sp. CC8-201	S. aureus	Compound 1	Karst cave in Chongqing City, China	171
Six strains of <i>Bacillus</i> spp., <i>Rhodococcus</i> sp. P209	S. aureus	Not determined	Rogers Belmont Cave, Warren County, Virginia, USA	194
Brevibacterium frigoritolerans, Bacillus thuringiensis, B. weihenstephanensis, B. cereus, Bacillus sp., Pseudomonas sp., Saccharopolyspora erythraea	S. epidermidis, B. subtilis, S. aureus, E. coli	Not determined	Kadıini Cave, Antalya, Turkey	70
Four <i>Streptomyces</i> spp. and <i>Erwinia</i> sp.	M. luteus, M. smegmatis, ESBL-producing E. coli, S.aureus, A. baumanni	Not determined	Helmcken Falls Cave, Wells Gray Provincial Park, British Columbia	195
Fictibacillus nanhaiensis, Bacillus humi, B. eiseniae, Pseudomonas mosselii	S. typhi, S. aureus	Not determined	Hindu Kush, India	71
Nine Streptomyces spp.	B. subtilis, S. carnosus, E. coli, P. putida	Not determined	Badzheyskaya and Okhotnichya caves in Siberia	196
Toxopsis calypsus, Phormium melanochroun	S. aureus, E. faecalis, E. coli, P. aeruginosa	Not determined	Francthi Cave in Peloponnese, Greece	197
Streptomyces spp. M4_24 and M5_8	S. aureus, S. enterica, Enterococcus sp., E. coli, B. subtilis, B. megaterium, B. cereus, P. aeruginosa	Not determined ^a	Szczelina Chochołowska cave, Tatra mountains, Poland	188
11 strains belonging to nine genera (Microbacterium, Arthrobacter, Candidimonas, Dietzia, Pseudarthrobacter, Caulobacter, Delfia, Pseudomonas, Bacillus)	S. aureus, E. coli, E. cloacae, Pseudomonas sp., E. falcium	Not determined	Scarisoara Ice Cave, Romania	72
Streptomyces sp. GLD22	E. coli, P. aeruginosa, B. subtilis, B. cereus, S. aureus	2-tert-Butyl-4,6-bis(3,5-di- tert-butyl-4-hydroxybenzyl) phenol, dibutyl phthalate, Cyclo(leucyloprolyl) ^b	Gueldaman cave, Algeria	181
Streptomyces sp. CB09001	S. aureus, E. coli, K. pneumoniae, P. aeruginosa	Compounds 9–11	Xiangxi, China	175
Paenibacillus spp. 23TSA30-6 and 28ISP30-2 w	E. coli, M. luteus, B. thuringiensis, Pseudomonas sp.	Fusaricidins, polymyxins, and tridecaptins c	Krubera-Voronja Cave, Western Caucasus	180
Actinomycetota strains GSF102, and GSF201	B. subtilis, K. pneumoniae	Not determined	Parque Nacional dos Campos Ferruginosos National Park, southeastern Amazon	198
Five strains belonging to three genera (Pseudomonas, Flavobacterium, Rhodococcus)	E. coli, S. aureus	Not determined	Raspberry rising Cave located in the Columbia mountain range, British Columbia, Canada	73
Streptomyces sp. GLD25	P. aeruginosa, E. coli, K. pneumoniae, B. subtilis, B. cereus, S. aureus	Compounds 39–43	Algeria	181
Bacillus spp. 1350R2-TSA30-6 and 1410WF1-TSA30-2	B. cereus, E. faecalis, L. monocytogenes, S. aureus, Rhodococcus sp	Diisobutyl phthalate and pyrrolopyrazines b	Krubera-Voronja Cave	199
Paenibacillus polymyxa AC30 and Paenibacillus peoriae AC32	S. aureus, Salmonella sp., Klebsiella sp., E. coli, P. aeruginosa., Acinetobacter sp.	Not determined	Mossy cave in Summan region, Saudi Arabia	200
21 strains belonging to 11 genera (Streptomyces,	S. aureus, E. faecalis, B. cereus, K. pneumoniae	Not determined	Oceania, Fiji	201

Bioactive strain(s)	Pathogens tested	Bioactive agent(s)	Cave of origin	Re
Psychrobacillus, Lysinbacillus, Cupriavidus, Micromonospora, Fontibacillus, Nonomuraea, Kocuria, Pseudonocardia,				
Mesorhizobium, Bacillus) Five Streptomyces spp.	S. aureus, M. luteus, B. subtilis, E. coli, L.	Not determined	Chaabe Cave, Algeria	202
38 strains belonging to ten genera (Agrobacterium, Aerococcus, Bacillus, Kocuria, Lysobacter, Micrococcus, Pseudomonas, Rhodococcus, Sphingomonas, Streptomyces)	monocytogenes E. coli, S. enterica, B. cereus, K. pneumoniae, B. subtilis, S. aureus, L. monocytogenes, S. pseudointermedius	Not determined	Slovenian karst caves	74
Bacillus spp., five sp., Nocardia sp., and Erwinia sp.	M. luteus, S. aureus, M. smegmatis, E. coli, A. baumannii, P. aeruginosa, K. pneumoniae	Not determined	Helmcken Falls Cave, Wells Gray Provincial Park, British Columbia	65
38 Strains belonging to six families (Streptomycetaceae, Nocardiaceae, Micrococcaceae, Microbacteriaceae, Micromonosporaceae, Pseudonocardiaceae)	S. aureus, B. subtilis, M. luteeaus, K. pneumoniae, E. coli, C. freundii, P. aeruginosa	Not determined	Grotte des Collemboles, Belgium	192
Streptomyces lunaelactis	K, rhizophila, B. subtilis, S. aureus	Compounds 15–17		177
28 Streptomyces spp. and three unidentified strains	K. pneumoniae, E. coli, C. freudii, P. aeruginosa, S. aureus, B. subtilis, M. luteus	Not determined		182
Micrococcus sp.	S. aureus and S. epidermidis	Azaserine, adefovir, dipivoxil, valclavam and leucomycin A7/A4 ^b	Parsık Cave, Turkey	203
Two Crossiella spp.	B. cereus, A. baumannii, S. aureus, E. coli, P aeruginosa	Not determined	Six caves in Spain, one of which is Altamira Cave	47
Two Streptomyces spp.	E. coli, P. aeruginosa, and B. subtilis	Diketopiperazines ^d	Iron Curtain Cave, Chilliwack, Canada	178
12 Streptomyces spp. and two Arthrobacter spp.	S. typhimurium, E. coli, P. aeruginosa, Proteus sp., L. monocytogenes, L. innocua, S. aureus	Not determined	Two Canadian caves and 12 Portuguese volcanic cave	204
16 Actinobacteria from six genera (Streptomyces, Nocardioides, Agromyces, Oerskovia, Micromonospora, and Actinoplanes)	S. aureus, E. coli, B. cinerea	Not determined	Shuanghe Cave, China	205
23 Actinobacteria from five genera (Streptomyces, Kocuria, Micromonospora, Saccharomonospora, and Streptosporangium)	M. luteus, E. coli, B. subtilis, S. aureus	Not determined	Hampoeil Cave, Iran	206
136 Bacterial isolates, including members of Streptomyces, Micrococcus, Actinobacteria, Actinomycetales, Virgibacillus, and Kocuria genera	S. aureus, P. aeruginosa, E. coli, M. luteus, B. subtilis	Not determined	Pukzing Cave, India	207

Authors identified active constituents as dichloranthrabenzoxocinones using accurate masses and database matching. However, the isotope patterns of the detected ions did not contain the 3:1 isotope pattern characteristic of chlorine-containing molecules, and as such, were likely misidentified. ^b Putative bioactive compounds identified by GC-MS analysis of bioactive extracts. ^c Putative bioactive compounds identified by presence of biosynthetic gene clusters in the microbial genomes. ^d Putative bioactive compounds identified by LC-MS based molecular networking.

Structures of novel bacterial natural products isolated from caves between 2014-2024.

4.2 Approaches to natural products discovery

Natural products discovery strategies typically fall into one of two categories: traditional and omics-guided approaches. Traditional approaches have been used for nearly 100 years and include prioritization of natural products by bioactivity-guided fractionation and/or taxonomic novelty of their producing organism.¹⁸⁶ Omics-guided approaches, while still incorporating some of the steps utilized in traditional approaches, take advantage of genomics, metabolomics, proteomics, and/or transcriptomics datasets to guide natural products discovery efforts.183

Although researchers have increasingly turned to omicsguided discovery in the last decade, 187 the majority of studies involving natural products discovery from subterranean ecosystems utilized traditional approaches. Indeed, of the 17 total studies conducted between 2014 and 2024, 12 (71%) use approaches. $^{69,106,107,171,173-176,179,181,184,188,189}$ studies account for the identification of 72 total compounds (80%), including 30 novel compounds (81% of total novel compounds). Only three studies utilize omics-guided approaches (18%), 172,177,180 while two additional studies utilize a combination of traditional and non-traditional approaches (12%).178,182 Hybrid approaches have led to the identification of only three compounds in the last decade (3%, none of them novel), and omics-guided strategies have led to the identification of 15 compounds (17%, four of them novel, accounting for 11% of all novel compounds discovered in this timeframe).

While these numbers may cause one to question the advantages of using non-traditional approaches to natural products discovery, it is worth noting that over 30% of novel bacterial compounds were discovered using omics-guided strategies (from just three studies total), particularly those involving genome mining to evaluate biosynthetic potential177,180 and/or mass spectrometry-based comparative

metabolomics to identify target metabolites. 172,177,180 Thus far, no studies involving subterranean fungi have utilized hybrid or omics-guided strategies, and incorporating these new approaches could accelerate discovery of novel fungal natural products.

Regardless of approach, researchers must utilize analytical tools including gas chromatography-mass spectrometry (GC-MS), liquid chromatography-mass spectrometry (LC-MS), and/ or nuclear magnetic resonance (NMR) to identify natural product molecules. While the majority of studies covered in this review utilized robust identification and dereplication methods, including full structure elucidation using NMR and matching fragmentation spectra of experimental data to those of authentic standards using GC-MS or LC-MS/MS, some studies only utilized accurate masses obtained by LC-MS to those found in natural products databases. 179,188 In one such case, authors identified dichloranthrabenzoxocinones as putative bioactive constituents from subterranean Streptomyces spp. by matching experimentally determined accurate masses to those in the Dictionary of Natural Products. 188 However, when inspecting the mass spectrometry data, it is clear that the associated ions do not contain the isotopic distribution patterns characteristic of chlorine-containing molecules and that the molecules were misidentified. This case study emphasizes the limitations of simple database matching for dereplication of natural products to avoid incorrect annotation of identified natural products.

Biotechnological and medicinal potential of subterranean microorganisms

Extracts from cave microorganisms, and in some cases, purified compounds from them, have been evaluated for numerous biological activities. In this section, we describe antibacterial,

Fig. 3 Structures of known bacterial natural products isolated from caves between 2014–2024.

(48)

(47)

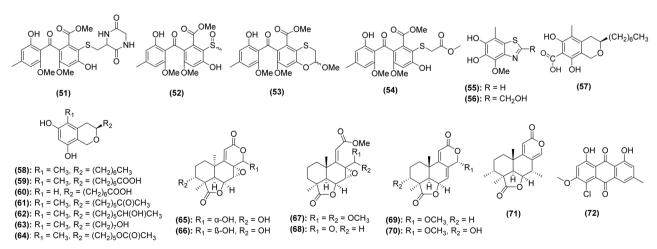


Fig. 4 Structures of novel fungal natural products isolated from caves between 2014-2024.

(50)

(49)

Fig. 5 Structures of known fungal natural products isolated from caves between 2014-2024

antifungal, cytotoxic, antioxidant, anti-inflammatory, and other biological activities of cave microorganisms with potential use in human society. Although most studies evaluating biotechnological potential of cave microbiota do not investigate the chemical constituents responsible for their activity, when possible, known active constituents and structure-activity relationships are described. A summary of in vitro and in vivo bioactivity studies on cave microorganisms can be found in Tables 3-6.

Antibacterial properties

Antibacterial resistance is a global health and economic issue. In 2019, antibacterial resistance was directly attributed to 1.27 million deaths worldwide. 190 Drug-resistant strains of Escherichia coli and Staphylococcus aureus are among the World Health Organization's major concerns, contributing to more than 900 000 infections and 230 000 deaths each year.191 Microbial communities in caves have demonstrated in vitro antibacterial activity against such bacterial pathogens. Since 2014, bacterial isolates from caves have been investigated for bioactivity against methicillin-resistant S. aureus (MRSA) (25 papers) and E. coli (20 papers), among others (Table 3). A compilation of studies investigating the antibacterial potential of subterranean microorganisms since 2014 are provided in Table 3. It is worth noting that most of these studies evaluate antimicrobial activities of strains or strain extracts using cross-streak or disk diffusion assays, and the identity and strength of individual active constituents remains unknown.

Four studies investigated bacterial isolates collected from moonmilk for antibacterial activity. 177,182,188,192 Streptomyces spp. (M4_24 and M5_8) were collected from the Szczelina Chochołowska Cave in the Tatra Mountains, Poland and evaluated using the cross-streak method.188 Both strains exhibit strong antibacterial activities against Salmonella enterica (inhibition zone: M4_24 = 11.5 mm; M5_8 = 8.0 mm), and M5_8 additionally inhibited E. coli (inhibition zone = 8.5 mm). Bacterial isolates from La Grotte des Collemboles, Belgium were also evaluated using the cross-streak method against a variety of

Gram-positive and Gram-negative bacterial pathogens. 182,192 The majority of these strains (67.5%) were identified as Streptomyces spp. with varied bioactive potential. While many strains showed greater than 10 mm zones of inhibition against Bacillus subtilis (58%) and Micrococcus luteus (61%) under at least one growth condition, only 13% of tested strains inhibited growth of S. aureus with more than a 10 mm zone of inhibition, with a maximum inhibition zone of 30 mm compared to the 45 mm maximum for both other Gram-positive organisms. Although a good portion of the tested strains showed activity against Klebsiella pneumoniae (45%, maximum inhibition zone of 44 mm), activity against the other Gram-negative organisms was limited, with only 15%, 16%, and 9% of bacterial isolates showing activity (zone of inhibition ≥ 10 mm) against *E. coli*, Citrobacter freundii, and Pseudomonas aeruginosa, respectively.182,192

The majority of studies investigating the antibacterial properties of cave microbiota do not explore the chemistry behind these bioactivities; however, a small subset of studies have identified the bioactive molecules responsible for the observed activities. For example, researchers studying Streptomyces lunaelactis isolated from moonmilk in La Grotte des Collemboles (Belgium) utilized genomic data from multiple S. lunaelactis strains to identify biosynthetic gene cluster (BGC) sequences that were not conserved across the species. They overlaid this data with LC-MS/MS fragmentation data to identify suite of antibacterial molecules called lunaemycins (compounds 15-17) which were associated with this gene cluster. In silico analysis of the lunaemycin BGC along with LC-MS/MS, 1H, and 13C NMR data enabled structural elucidation of this novel group of molecules.177 Agar diffusion assays showed that lunaemycins A and B1 exhibited stronger antibacterial activity against Gram-positive bacteria than lunaemycin D. Lunaemycin A was further studied by in vitro experiments to determine MIC values; this compound exhibited the greatest activity against B. subtilis, E. faecalis, and Staphyloccoccus. spp. (MIC = $0.12 \mu g \text{ mL}^{-1}$ for all strains). Additional bioactive compounds produced by Streptomyces include xiakemycin A

(compound 1) and huanglongmycins A–C (compounds 9–11). Compound 1 was found to exhibit antibacterial activity against Gram-positive bacteria such as *Enterococcus faecalis* (MIC = 16 μg mL⁻¹),¹⁷¹ while compounds 9–11 exhibited only weak antibacterial activity, with MICs against *Staphylococcus* spp., *E. coli, K. pneumonia*, and *P. aeruginosa* \geq 64 μg mL⁻¹.¹⁷⁵

5.2 Antifungal properties

It is estimated that 6.5 million fungal infections occur annually, directly leading to around 2.5 million deaths.²⁰⁸ Despite this, there are only three main classes of antifungal drugs available: azoles, echinocandins, and polyenes.209,210 A multitude of bacterial and fungal strains isolated from caves, underground mines, or bats have been found to possess in vitro antifungal activity against fungal pathogens. Since 2014, 22 papers have investigated the antifungal capacity of subterranean microorganisms, 11 of which focus on members of genus Streptomyces (Table 4). Thirty other genera have been investigated, only of four of which were fungal. Over half of the studies investigated in vitro antifungal activity against Candida albicans, a yeast that naturally occurs in the human microbiome but whose overgrowth can cause candidiasis. One additional study reported antifungal activity against C. glabrata, 188 which causes 28% of Candida bloodstream infections, second only to C. albicans (39%).211 These studies report moderate to weak antifungal activities from the tested organisms/purified compounds, with zones of inhibition ranging from 5-22 mm and MICs ranging from 12-40 µg mL⁻¹. Given the lack of antifungal medications effective against Candida spp., the discovery of new antifungals should be a critical priority.212 In addition to anti-Candida activities, crude extracts from cave microbes have been tested against nine genera of opportunistic fungal pathogens. 47,106,107,182,196 In vitro zones of inhibition ranged from 3 mm to >20 mm and MICs from 12.5–50 µg mL⁻¹ (Table 4). It is worth noting that studies in which bioactive constituents were not identified utilized co-culture assays (e.g., cross-streak or agar plug) and not chemical extracts, so the potency of individual chemical constituents cannot be estimated.

To date, only three studies have identified bioactive compounds responsible for the observed antifungal activities. Two additional studies identified putative bioactive constituents from antifungal bacterial strains using GC-MS181 or LC-MS/ MS, 182 but individual constituents were not purified or tested individually. This includes cycloheximide, a known inhibitor of eukaryotic protein synthesis, and its precursor (compounds 44-45), which were identified as major constituents from Streptomyces spp. isolated from a cave moonmilk deposit in Grotte des Collemboles in Belgium¹⁸² as well as compounds 39-43, identified in extracts of cave-derived Streptomyces from Gueldaman Cave in Akbou-Algeria. 181 The three studies that have definitively identified antifungal constituents discovered weak to moderate antifungal activity, at best. For example, the fungus Cadophora sp. 10-5-2 M collected from the Soudan Mine (Minnesota, USA) vielded 14 secondary metabolites (compounds 57-64 and 76-81), four of which exhibited weak antifungal activity. Only isosclerone (compound 78) inhibited the growth of both C.

albicans (MIC = 40 $\mu g \text{ mL}^{-1}$) and Cryptococcus neoformans (MIC = 30 μ g mL⁻¹), while pseudoanguillosporin C (compound 57), soudanone A (compound 58), and nectriapyrone (compound 77) only inhibited the growth of C. neoformans with MICs from 20-40 μg mL⁻¹.¹⁰⁶ A concerted effort has been undertaken to investigate the bat microbiome for antifungal activity against P. destructans, the cause of WNS (discussed in Section 3.3.3). P. destructans has the ability to cause skin lesions on bats, 213 weakening regulatory processes including thermoregulation, gas exchange, and water balance,214,215 and decreasing their likelihood of surviving hibernation. Several authors have identified candidate bacteria69,159-162 and fungi216 with antagonism against P. destructans in vitro. Follow-up studies, though few, have shown particular promise of bat-derived strains of the bacterium Pseudomonas fluorescens, which has successfully been used as a treatment in situ. 165,166 Although the bioactive compounds from bat-derived strains of P. fluorescens have not yet been identified, other authors have identified promising secondary metabolites in other strains of the bacterium.217 Another species of *Pseudomonas*, *P. yamanorum*, isolated from bats in China, was found to produce four compounds that inhibited P. destructans (compounds 47-50).69 The main inhibitory compound, phenazine-1-carboxylic acid (compound 47), was determined to have a MIC of 50.12 μg mL⁻¹ and an IC₅₀ of 32.08 $\mu g \text{ mL}^{-1}$. Compounds 48-50, all volatile organic compounds, demonstrated inhibition of P. destructans at concentrations of 10 ppm (compound 48) and 100 ppm (compounds 49-50). Though they demonstrate only moderate antifungal abilities, the production of these compounds supports the role of the bat microbiome in protection from WNS. Several researchers have leveraged standard genome mining approaches to explore the secondary biosynthetic potential of bat-associated Streptomyces; 218,219 however, they have yet to confirm which natural products were directly correlated to the inhibition of P. destructans. A few fungi have also shown bioactivity against P. destructans. For example, a preliminary screening of bat-associated yeasts yielded two strains of Cutaneotrichosporon moniliiforme that inhibited P. destructans under certain conditions.216 Non-pathogenic Pseudogymnoascus spp., also isolated from bat hibernacula, have been shown to inhibit the growth of P. destructans. 170 Notably, pH, salinity, temperature, and nitrogen source appear to have an effect on antifungal activity, and additional chemical analyses are required to identify the associated products.²²⁰

Beyond the bat microbiome, the fungus *Oidiodendron truncatum*, isolated from wood in the Soudan Mine (Minnesota, USA), demonstrated antifungal activity against multiple zoonotic fungal pathogens including *P. destructans*. Fourteen secondary metabolites produced by *O. truncatum* were identified (compounds **65–72** and **82–90**), the strongest being PR 1388 (compound **82**) with antifungal activity against *P. destructans* (MIC = 7.5 μ g mL⁻¹), *C. albicans* (MIC = 20 μ g mL⁻¹), and *C. neoformans* (MIC = 17.5 μ g mL⁻¹). Compound **82** was determined to be non-cytotoxic toward primary fibroblast cell cultures from bat species *Myotis septentrionalis* (IC₅₀ = 75.6 μ M) and *Myotis grisescens* (IC₅₀ = 102.7 μ M) as well as humans (IC₅₀ > 100 μ M).¹⁰⁷ Although this *in vitro* screening for cytotoxicity

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Table 4 Studies of antifungal activity in caves and mines from 2014-2024

Bioactive strain(s)	Pathogens tested	Bioactive agent(s)	Cave of origin	Ref.
Streptomyces spp.	Rasamsonia argillacea, Penicillium chrysogenum, Aspergillus fumigatus, Trichophyton mentagrophytes, Candida albicans and C. albicans 'R'	Compound 44	Grotte des Collemboles, Comblain-au- Pont, Belgium	182
Cadophora sp. 10-5-2 M	Cryptococcus neoformans and	Compounds 57, 58, 77, and 78	Soudan underground iron mine, Tower, Minnesota. USA	106
Oidiodendron truncatum	C. neoformans, C. albicans, and Pseudoevmnoascus destructans	Compounds 68 , 69 , 82 , and 83		107
Pseudogymnoascus spp.	P. destructans	Not determined		170
Pseudomonas yamanorum, P. brenneri, and P. fragi	P. destructans	Compounds 47–50	Bats swabbed in Ge-zi Cave in Jilin, China	69
36 Bacterial strains from 5 genera (Luteipulveratus, Streptomyces Nocardiopsis,	P. destructans	Not determined	and rempie Cave in Liaoning, Crima Bats swabbed in New Mexico, USA (Carlsbad Caverns National Park, El	160
Rhodococcus, and Streptosporangium)			Malpais Conservation Area, Fort Stanton- Snowy River Cave National Conservation Area, and Bureau of Land Management caves 45 and 55) and Arizona, USA (Grand Canyon-Parashant National Monument)	
Streptomyces sp. MM56	Candida spp.	Not determined	Szczelina Chochołowska Cave, Tatra Mountains, Poland	188
Streptomyces spp.	Candida spp.	Not determined	Chaabe Cave, Algeria	202
Actinobacteria isolate TB64, actinomycetes isolate TA62, and Bacilli isolates TB48, SB1, and SC3	C. albicans	Not determined	Parsık Cave, Turkey	221
Streptomyces spp.	Saccharomyces cerevisiae, C. albicans	Not determined	Badzheyskaya Cave, Krasnoyarsk Krai, Siberia, Russia	196
Pseudomonas fluorescens	P. destructans	Not determined	Virginia, USA	165 and 166
Crosiella spp. ON669108 and ON669109	Aspergillus versicolor, Penicillium chrysogenum, Cladosporium cladosporioides, Ochroconis lascauxensis	Not determined	Altamira Cave, Spain	47
Brevibacillus borstelensis and Pseudomonas mosselii	C. albicans	Not determined	Koat Maqbari and Smasse-Rawo Caves, Hindu Kush Mountain Range, Pakistan	71
42 Bacterial strains from 11 genera (Lactococcus, Bacillus, Paenibacillus, Curtobacterium, Rhodococcus, Streptomyces, Psychrobacter, Achromobacter, Erwinia, Serratia, and Pseudomonas)	P. destructans	Not determined	Bats swabbed in various locations across Western Canada	162
18 bacterial strains from 16 genera (Arthrobacter, Lysobacter, Bacillus, Agromyces, Rhodococcus, Rhizobium, Achromobacter, Aminobacter, Sphingomonas, Pseudomonas, Luteibacter, Streptomyces, Microbacterium, Nocardia, Corynebacterium, Enterococcus)	P. destructans	Not determined	Bats swabbed in Eastern and central Tennessee, USA	161
Pseudomonas spp.	P. destructans	Not determined	Bats swabbed in New York and Virginia, USA	159
Two isolates of Cutaneotrichosporon moniliiforme	P. destructans	Not determined		216

Bioactive strain(s)	Pathogens tested	Bioactive agent(s)	Cave of origin	Ref.
			Bats swabbed in Arkansas, West Virginia, Iowa, Pennsylvania, Wisconsin, Alabama, Kentucky, New York, Missouri, and	
Streptomyces sp. GLD25	C. albicans and F. oxysporum	Compounds 39–43	Oklanoma, OSA Gueldaman Cave, Akbou-Algeria	181
Actinomycete strain PM100	C. albicans	Not determined	Helmcken Falls Cave, Wells Gray	195
65 Strains of Streptomyces spp. and five strains of	C. albicans	Not determined	Provincial Park, British Columbia,	65
Bacillus spp., Pseudomonas spp., Nocardia spp., and Erwinia spp.			Canada	
3 Streptomyces strains	P. anomala	Not determined	Hampoeil cave, Iran	206
106 Bacterial strains including members of Streptomyces, Micrococcus, Actinobacteria, Actinomycetales, Virgibacillus,	C. albicans	Not determined	Pukzing Cave, India	207

may not accurately reflect potential irritation or toxicity toward bat skin, the strong anti-*P. destructans* and non-cytotoxic activity of *O. truncatum* indicates its promise as a treatment for WNS.

5.3 Cytotoxic and antiproliferative properties

Cancer is a leading cause of death worldwide, accounting for nearly 10 million deaths in 2020.222 Both bacterial extracts from caves and purified natural products from them have been shown to possess chemopreventive properties in vitro against a variety of cancer cell types, including colon, 173 breast, 171,188 lung, 171,175,223 and melanoma cells (Table 5).224 For example, the cytotoxic effects of two cave-derived Bacillus subtilis strains were evaluated against murine melanoma cells (B16F10). Organic extracts produced during the stationary phase of growth showed highest cytotoxicity, with an IC₅₀ value of 83.99 μg mL⁻¹ against B16F10 cells and no impact on the normal cell lines evaluated, indicating a high degree of selectivity.224 In another study, a Nonomuraea strain was isolated from cave soil in Pha Tup Cave Forest Park in Thailand and tested against human small cell lung cancer (NCI-H187), human oral cavity cancer (KB), and human breast cancer (MCF7) cell lines and found to have IC50 values of 3.48 μg mL⁻¹, 16.11 μg mL⁻¹, and >50 μg mL⁻¹ respectively.²²³ Although the extracts in these studies were only weakly or moderately active, it is possible that further efforts to purify cytotoxic agents would result in concentration of activity.

Several researchers have studied the inhibitory effects of purified compounds from cave microorganisms as well, with IC₅₀ values of individual constituents in the micromolar or high nanomolar ranges. Hypogeamicins A-D (compounds 2-5) were purified from the cave-derived actinomycete Nonomuraea specus and subjected to a suite of biological assays. Interestingly, compound 2, the only dimeric hypogeamicin, was the only compound to possess cytotoxic activity against colon cancer cells (IC₅₀ = 6.4– $12.8 \mu M$), indicating that dimerization is essential for chemopreventive activity. 173 Huanglongmycins (compounds 9-13) from Streptomyces sp. CB09001 were evaluated against non-small cell lung cancer (A549), epithelial cancer (SKOVV3), and epithelial colorectal adenocarcinoma (Caco-20) cells and demonstrated moderate cytotoxicity against A549 $(IC_{50} = 13.8 \mu M)$ and weak activities against all other cell lines tested (IC₅₀ = 40-45 μ M).¹⁷⁵ The most potent cytotoxic natural product yet discovered from caves is the pyranapthoquinone xiakemycin A (compound 1), which was found to have in vitro activity against A549, MCF7, hepatoma (HepG-2), cervical cancer (HeLa), colon carcinoma (HCT-116), neuroblastoma (SH-SY57), and human prostate cancer (PC-3) cells with IC₅₀ values ranging from 0.43-2.77 μM.¹⁷¹ Several in vitro activities have been conducted on the cytotoxic effects of cave-derived natural products; however, the efficacy of these compounds in in vivo systems has yet to be determined. As such, no conclusive evidence yet exists to confirm the use of cave-derived natural products as anticancer agents, and more robust animal studies followed by clinical trials are essential to support the utilization of these constituents for cancer treatment.

Review

Bioactive strain(s)	Cell lines evaluated	Bioactive agent(s)	Cave of origin	Ref.
Nonomuraea specus	TCT-1 cells	Compound 2	Hardin's Cave, Tennessee	173
Two isolates of <i>Streptomyces</i> sp. MM56	T47D cells	Not determined ^a	Szczelina Chocholowka Cave, Poland	188
Bacillus subtilis	B16F10 cells	Not determined	Pedra da Chaoeria Cave, Brazil	224
Streptomyces sp. CB09001	A549, SKOVV3, Hela, Caco- 20 cells	Compound 9	Karstic cave in Xiangxi, China	175
Nonomuraea sp. PT708	NCI-H187 cells	Not determined	Pha Tup Cave Forest Park, Thailand	223
Streptomyces sp. CC8-201	A549, MCF-7, HepG-2, HeLa, HCT-116, SH-SY57, PC-3	Compound 1	Karst cave in Chongquing City, China	171

^a Authors identified active constituents as dichloranthrabenzoxocinones using accurate masses and database matching. However, the isotope patterns of the detected ions did not contain the 3:1 isotope pattern characteristic of chlorine-containing molecules, and as such, were likely misidentified.

5.4 Other bioactivities and potential applications

Oxidative damage to cellular components may cause downstream complications leading to cardiovascular diseases, carcinogenesis, and neurodegeneration.181 In addition to their antimicrobial and chemopreventive activities, compounds isolated from caves have been shown to possess antioxidant and anti-inflammatory properties (Table 6). Modes of action include radical scavenging activity, 181,184 attenuation of oxidative stress, 184 and inhibition of inducible nitric oxide synthase (iNOS) proteins.¹⁷⁴ Multiple assays exist to evaluate radical scavenging through evaluation of hydrogen transfer and/or electron transfer ability, including oxygen radical absorbance capacity (ORAC), 2,2diphenyl-1-picrylhydrazyl (DHHP), and ferric reducing antioxidant power (FRAP) assays. Chemical investigation of fermented A. fumigatus GZWMJZ-152 revealed a suite of anti-inflammatory compounds (compounds 52-56 and 73-75). Compounds 55, 56, 73, and 75 showed radical-scavenging activity in the DPPH assay with IC₅₀ values ranging from 3-17 μ M, and compounds 52, 54, and 73-74 showed oxygen radical absorbance capacity values in the low µM range. Compounds 52-53 also protected PC12 cells against H2O2 oxidative damage.184 In another study, a crude extract from Streptomyces sp. GLD25 illustrated weak antioxidant activities in both DHHP and FRAP assays. While individual constituents were not isolated, authors were able to identify several putatively antioxidant metabolites (compounds 40-43) via GC-MS.181 Finally, Jiang et al.174 investigated compounds 29-33 for anti-inflammatory effects via iNOS inhibition and found that compound 29 showed potent iNOS inhibition, while compounds 31 and 33 showed only moderate iNOS inhibitory effects.

Cave microorganisms are known to produce various enzymes with potential uses in environmental bioremediation as well as in the detergent, cosmetic, and textile industries. ^{203,225} For example, ten microbial strains isolated from different zones (entrance/twilight, transition, and deep interior) of the GEM-1462 cave in the southeastern Amazon exhibited proteolytic activity, along with varying degrees of cellulolytic, amylolytic, phosphate solubilization, and starch/casein degradation activities. Strains isolated from the deep interior zone produced the highest enzymatic indices (particularly proteolytic activities), followed by those from

the transition zone and twilight/entrance zones. ¹⁹⁸ The enzymatic activities of 49 isolates from Gumki Cave, India belonging to *Paenibacillus, Staphylococcus, Streptococcus, Salimicrobia, Lysinibacillus, Aeromonas, Proteus*, and *Clostridium* genera also showed high promise for enzymatic production. Of the 90% of isolates with some enzyme production, 75% were lipase producers, 47% were amylase producers, 24% produced protease, and 12% produced cellulase. ²²⁵ In Parsik Cave (Turkey), 28 Actinomycetota strains showed amylase, gelatinase, casein hydrolase, cellulase, DNase, and/or urease activities, with *Streptomyces exfoliatus* showing the greatest enzymatic potential. ²⁰³

Mining activities produce vast quantities of toxic metal wastes, including copper, nickel, and arsenic, which significantly contaminate our soils and waterways and pose serious risks to the environment. The effective detoxification and removal of metal contaminants from polluted environments has increasingly moved towards bioremediation by specialized microorganisms as a sustainable solution to mitigate the negative environmental impacts of mining.226 Given the presence of toxic pollutants, mines house organisms that have adapted unique enzymatic activities to function in harsh conditions and break down toxic pollutants, priming them for utilization in bioremediation. For instance, Rhodococcus erythropolis, isolated from the Sossego Mine in Brazil, demonstrates impressive copper biosorption capabilities, reaching up to 101.90 mg of copper absorption per gram of biomass. Physical adsorption and ion exchange mechanisms by this bacterium are responsible for its notable ability to capture Cu2+ ions, and highlight its potential for use in environmental treatment of metal residues from waterways.227 Fungi isolated from mines have also showed promise for use in bioremediation. 110,111,228 Trichoderma harzanium, isolated from sulfide-rich waste rock dumps from the Libiola Mine in Italy, showed remarkable Ni²⁺ tolerance, capable of hyperaccumulating up to 11 000 mg of nickel per kg of biomass.228 In a later study, this same strain possessed significant silver accumulation capabilities, with an uptake capacity of 46.36% taken at an initial concentration of 330 mg L⁻¹. Additional fungal isolates from the decommissioned Pastarena gold mine complex located in the Anzasca

Table 6 Additional bioactivities identified from cave microorganisms from 2014-2024

Bioactive strain(s)	Bioactivity tested	Bioactive agent(s)	Cave of origin	Ref
Aspergillus fumigatus	Antioxidant capacity (DPPH	Compounds 52–56 and 73 –	Cave near Fanjing Mountain	184
GZWMJZ-152	assay, ORAC assay, and cell viability assay in PC12 cells)	75	of Guizhou province, China	
Streptomyces sp. GLD25	Antioxidant capacity (DPPH assay, FRAP assay)	Compounds 40–43	Gueldaman Cave GLD1, Akbou-Algeria	181
Streptomyces sp. CB09001	Anti-inflammatory activity (iNOS inhibition, COX-2 protein expression)	Compounds 29, 31, and 33	Karstic cave in Xiangxi, China	174
10 Microbial strains (eight gram-positive bacteria, one gram-negative bacterium, and one yeast fungus)	Enzymatic activity (proteolytic, cellulolytic, amylolytic, nitrogen fixation, and phosphate solubilization activities)	Not determined	Cave GEM-1462 in Parque Nacional dos Campos Ferruginosos National Park, Brazil	198
49 Bacterial strains from 9 genera (Paenibacillus, Staphylococcus, Streptococcus, Salimicrobium, Lysinibacillus, Aeromonas, Proteus, and Clostridium)	Enzymatic activity (proteolytic, cellulolytic, amylolytic, and lipolytic activities)	Not determined	Gumki Cave, Garhwhal Himalaya, India	225
28 Strains of Actinomycetota belonging to 13 genera (Streptomyces, Agromyces, Nocardioides, Propionicimonas, Microbacterium, Arthrobacter, Nocardia,	Enzymatic activity (amylase, gelatinase, cellulase, DNase, urease, and casein hydrolysing activities)	Not determined	Parsık Cave, Turkey	203
Pseudoarthrobacter, Micrococcus, Rhodococcus, Kocuria, Oerskovia, Microterricola) 61 Strains of Actinobactera belonging to 11 genera (Micromonospora, Kocuria, Streptomyces, Micrococcus, Promicromonospora, Rhodococcus, Actinomadura, Nonomuraea, Nocardia, Cornebacterium,	Enzymatic activity (amylase, protease, esterase, lipase, DNase), and resistance to heavy metals (Zn, Cu, Cd, Ni, Pb)	Not determined	Hampoeil cave, Iran	206
Streptosporangium) 15 Bacterial isolates belonging to three genera (Serratia, Dickeya, Nissabacter)	Biocontrol activity against phytopathogens, plant growth promoting activity	Not determined	Seven caves from the iron Quadrangle, Minas Gerais, Brazil	229
Four bacterial isolates	Biocontrol activity against phytopathogens, plant growth promoting activity	Not determined	Lime Cave of Andaman and Nicobar Islands, India	230
Rhodococcus erythropolis	Copper biosorption capacity	Not determined ^a	Sossego mine, Brazil	227
rate are constant of the openio	Nickel accumulation	Not determined	Libiola mine, Italy	228
Trichoderma harzanium	capacity Silver accumulation capacity	Not determined		110

^a Although individual compounds were not identified, FT-IR analysis revealed the presence of carbonyl and carboxyl functional groups, indicating that organic compounds including carboxylic acids, amides, and ketones, were available for copper ion capture.

Valley, Italy, belonging to Penicillium, Trichoderma, and Chaetomium genera, showed promise to effectively manage arsenic contamination, primarily through volatilization.111 While these results are promising, the chemical mechanisms behind these

activities remain poorly understood, and further investigations are warranted.

One particularly promising yet understudied area of investigation is the agricultural uses of cave microorganisms. Farda et al.3 published an excellent review outlining the unique adaptations of cave microorganisms that make them amenable to use in soil environments. The use of plant growth promoting (PGPR) bacteria is a promising method to enhance crop productivity and manage plant diseases. PGPR bacteria promote plant growth through mechanisms including phosphorus solubilization, hormone production, and phytopathogen antagonism.3,230 Given that caves are rich in carbonates, phosphates, sulfates, and potassium-rich sediments, they are a promising source of mineral-solubilizing microbes with PGPR activities.3 Despite this potential, only two studies have investigated cave isolates as bioinoculants and plant growth promoters.^{229,230} In one study, 563 cave isolates from ferruginous caves in Brazil were tested against Xanthomonas citri subsp. citri (citrus canker) and later evaluated for bioactivity against fusariosis (Fusarium oxysporum) and bean anthracnose (Colletotrichum lindemuthianum). Twenty strains inhibited F. oxysporum, 15 of which also inhibited C. lindemuthianum. These strains were also evaluated for their ability to solubilize inorganic phosphates, fix nitrogen, and produce siderophores and hydrolytic enzymes. All strains fixed nitrogen, produced proteases and siderophores, and showed motility and biofilm forming abilities, and all but one solubilized phosphates. These bacteria, primarily from the genera Serratia, Nissabacter, and Dickeya could be important candidates for future investigations into sustainable agriculture.229 A similar study of four strains isolated from Lime Cave on the Island of Baratang (Andaman and Nicobar Islands, India) showed that one strain had antagonistic effects against Sclerotium rolfsii, Pythium aphanidermatum, and Rhizoctonia solani, three strains produced indole acetic acid and solubilized phosphate, two strains had protease activity, and one strain produced siderophores.230

Accessing the untapped potential of subterranean microbial chemistry

Molecular approaches employing metabarcoding or metagenomics are unparalleled in their ability to provide a bird's-eye perspective of microbial communities in an environment. While these culture-independent approaches can provide insight into the biosynthetic potential of microbial communities, cultivation is required to discern the functional metabolic activity under the growth conditions studied. 192,231 In this section, we review the culture-dependent and culture-independent approaches to access potential of cave microbial chemistry and some of the lingering challenges of these techniques.

6.1 Culture-dependent approaches

Culture-dependent approaches play a crucial role in studying microbial diversity and functionality. Cultures enable diverse downstream analyses including bioactivity assays, metabolomics analysis, and whole genome sequencing (WGS). Unfortunately, uncultivability is a major challenge in microbiology, as many species exist in the environment in a viable but non-cultivable state. 192 This so-called "great plate count anomaly" highlights the discrepancy between the total number of microbial cells in an environmental specimen and the

isolable population of that sample. It has been estimated that only 0.1-1% of species can be cultured under common laboratory conditions, and for cave microorganisms, this percentage is at an even lower 0.02%. 192,232 To overcome this challenge, innovations in cultivation techniques are being developed to increase the recovery yield of microorganisms in a laboratory setting.

6.1.1 Isolation and cultivation. Successful cultivation of cave microorganisms depends largely on factors involving sample collection and processing methods, media composition, and culture conditions. In recent years, a number of studies have evaluated the impacts of sample buffer and time between sample collection and processing,232 storage temperature and time,233 inoculum dilution,192,231 and sample pre-treatment methods. 66,231,234 For example, Bender et al. 232 investigated several factors that influenced both colony counts and bacterial diversity from a single sample location, finding that sample processing time had a significant impact on cultivability. Samples crushed and plated immediately showed no significant differences compared to those plated six hours later, but samples crushed six hours after collection produced no bacterial colonies, highlighting the importance of immediate on-site processing for maximizing microbial cultivability.232 The impact of sample storage (both time and temperature) on the isolation of geophilic cave fungi has also been documented, with storage time being the most critical factor in influencing fungal diversity.233 Storage conditions affected not only abundance but also types of isolated taxa, with certain groups, such as psychrophilic fungi, only being isolated from samples stored at temperatures below 0 °C. Additionally, some genera were solely isolated from samples stored for less than one month or more than six months, emphasizing the importance of varying storage conditions to isolate diverse fungal communities.

The pre-treatment of microbial samples requires careful thought by researchers to maximize cell counts, diversity, and/ or novelty depending on the project goals. Physical pretreatments of samples include air drying, moist heat, dry heat, and microwave irradiation. 66,231 Moist heating (50 °C for 5-6 minutes) can stimulate or inhibit different Actinomycetota, typically favoring slow-growing bacteria at the expense of dominant fast-growers.231 For researchers aiming to isolate rare Actinomycetota, air drying is a useful method given that dry spores have low respiration rates and can survive for longer periods of time. Fang et al.234 found that drying samples at 40 °C for two days yielded the highest cultivability compared to those dried at higher temperatures or at room temperature. Their team also noted a positive correlation between the cultivability of spore-forming Actinomycetota and pre-treatment temperatures.234 Interestingly, numerous researchers have documented a paradoxical effect of inoculum dilution on final plate counts, in which the median viable cell counts obtained in 1000-fold dilutions were an order of magnitude higher than those obtained with only a 10- or 100-fold dilution, potentially due to a negative impact of overcrowding or antibiosis on cell viability.192,231

Cultivation media used to isolate cave microbiota range from routine media including soil extract agar (SEA), malt-yeast extract agar (MYA; ISP2), glycerol-asparagine agar (GAA; ISP5), or tryptic soy agar (TSA) to selective media like Actinomycete isolation agar (AI), Hickey-Tresner medium (HT), pyruvate agar, and Reasoner's 2 agar (R2A).66,73,231,232,234,235 While many novel organisms have been isolated from these sources, media preparations that reduce organic carbon levels to those more accurately mimicking the low concentrations in caves have found great success in targeting oligotrophic organisms.²³² Inconveniently but perhaps unsurprisingly given the immense diversity of cave environments themselves, there is no consistent "winner-takes-all" medium for maximizing microbial cultivability. While some studies have found low-nutrient TWA as the best medium for isolating oligotrophs from caves, 231,232 others have found that full-strength R2A yielded higher numbers of bacteria than diluted versions of the same medium or other minimal media.71 Selective media like HV agar or even pyruvate agar showed maximum cultivability and bacterial diversity in some cases.232,234 Bender et al.232 found that the most nutrientrich media, including soil agar and ISP2, were particularly poor in culturing isolates from cave environments. While this could be explained by osmotic stress of cave-associated bacteria in the presence of high levels of nutrients, it could also be explained by the fact that standard preparation procedures of nutrient rich media, particularly those containing added phosphates, can result in the formation of toxic reactive oxygen species during autoclaving that can impact cell growth. Adam et al. 192 found that nutrient-rich ISP5 media performed quite well for isolating hard-to-culture and rare Actinomycetota from moonmilk when components of the media were autoclaved separately but not when they were prepared using standard procedures.

Supplementation of isolation media with chemical modifiers has also been shown to influence microbial cultivability. In a study evaluating the impact of pH and calcium salts on isolation of cave Actinomycetota, Fang et al.234 found that the highest number of colony-forming units were obtained at a neutral pH as opposed to alkaline or nearly neutral pH, suggesting that neutral conditions facilitate easier maintenance of cytoplasmic pH within cells. In the same study, both the type and concentration of calcium salts significantly affected isolation efforts, with CaCO₃ yielding more colony forming units than CaCl₂ or Ca(CH₃COO)₂. Higher CFUs were observed at 0.1% or 0.01% than at 1% w/v or in the absence of salts. Calcium ions are crucial for spore-forming microorganisms, with CaCO₃ stimulating the most growth of rare heterotrophic bacteria.²³⁴ Supplementation of culture media with low concentrations of antibiotics has shown promise for the selection of slow-growing microbial species. Bender et al.232 found that although antibiotic treatment with chloramphenicol and nalidixic acid reduced overall colony counts and species diversity, it increased the proportion of slow-growing oligotrophs that may represent rare species, emphasizing the differential selection pressures exerted by nutrient composition and antibiotic presence.

Finally, incubation temperature and time significantly impact the cultivability of cave microorganisms, with optimal conditions varying both by species and location. While

temperatures between 28–30 °C generally yield more isolates, temperatures around 5 °C can improve isolation of rare psychrophiles. 66,236 Optimal temperatures are also media specific. For example, recovery of bacteria from caves in the Hindu Kush mountain range in Pakistan was highest at 37 °C when samples were plated on full-strength R2A, but when plated on half-strength R2A, 17 °C incubation temperatures resulted in higher colony counts. Notably, extending incubation time from two to four (or even ten) weeks consistently increases colony counts and diversity across cave systems, allowing for the isolation of rare and slow-growing species, 192,232 so a combination of varying temperatures and prolonged incubation time is recommended for researchers aiming to maximize diversity of cave isolates.

6.1.2 Identification and elicitation of biosynthetic pathways. After an organism has been successfully cultivated in the laboratory, chemical analysis can begin. However, phenotypic investigation is often stymied due to the complex challenges associated with natural products discovery-namely, rediscovery of known compounds, inconsistent production of target metabolites under laboratory settings, lack of knowledge about biological activities, and material limitations of purified compounds.237 WGS and mining of cultivable microorganisms from extreme environments such as caves have become important strategies to identify novel biosynthetic pathways and target organisms with maximum biosynthetic potential. 180,237 Although studies evaluating the biosynthetic potential of cave microorganisms are few, the takeaway message is clear: cave bacteria are a rich source of untapped chemodiversity. For example, analysis of 16 Actinomycetota genomes from bat skin microbiota indicated that 69-93% of their BGCs were novel and encode a variety of uncharacterized natural products.²³⁸ In Krubera-Voronja Cave, two antimicrobial Paenibacillus spp. contained 19 and 21 BGCs (much higher than the average number of 8.5 BGCs for Paenibacillus genomes),239 two-thirds of which had no similarity to known pathways. 180 In another study, 91 bacterial strains from the same cave demonstrated widespread presence of BGCs with the potential to synthesize yetuncharacterized natural products.237 Finally, draft genome assemblies of rare moonmilk Actinomycetota revealed a significant predisposition of these organisms to produce bioactive secondary metabolites. All strains encoded multiple nonribosomal peptide synthetase (NRPS) BGCs, while 97% encoded Type I polyketide synthase (PKS-I) genes, 94% had PKS-II genes, and 48% PKS-III genes.182

Cultivation, WGS, and LC-MS can be effective strategies for prioritizing bacterial strains for natural product discovery, but for fungal genomes that are larger with more repetitive elements and often poor annotation, this strategy requires considerable time and financial resources. Initial investigations of alternative low-cost methodologies suggest ketoacyl synthase alpha subunit (KS α) gene homology may be used as a proxy for a strain's total biosynthetic capacity. PKS II systems, the simplest type of PKS pathways, contain only a single representative of each domain: ketosynthase alpha (KS α), ketosynthase beta (KS β), and the acyl carrier protein, and the presence of one of these domains can be representative of an entire PKS II gene

cluster. Researchers isolated 467 bacterial isolates from the bat skin microbiome and found that between 34-60% contained KSα sequences, depending on bat species. Among these, 21% of KSα sequences had less than 85% homology to known sequences, suggesting that the associated BGCs may encode novel polyketide products. WGS of a 16-strain subset of these bacterial isolates revealed that lower KSa homology correlated with higher overall BGC novelty. These findings (although notably discovered using a small sample size) suggest that KSa gene homology may predict a strain's biosynthetic capacity, allowing for quicker strain prioritization through a simple and cost-effective PCR screening.238 However, this approach has its biases because KSα is not distributed evenly across all bacterial diversity, thus selecting for known natural product producers.

A bottleneck to accessing this untapped biosynthetic potential is the well-documented observation that most microbial BGCs are transcriptionally inactive under laboratory conditions, likely because biosynthesis is energetically expensive and organisms grown in controlled monocultures lack the environmental cues required to induce metabolite formation. 172,237 Numerous strategies have been taken to activate silent gene clusters under laboratory conditions, including "brothological" methods involving varying cultivation parameters such as medium composition, pH, and temperature to more effectively mimic the organisms' natural environment (or challenge the organism in unique ways), assessing changes to secondary metabolism under different phases of microbial growth, or by adding defined chemical or biological stressors to cultivation media to model environmental stimuli. 172 Such additives include histone deacetylase inhibitors, 240 subinhibitory concentrations of microbially-derived antibiotics,241 heavy metals,242 and co-cultures with other microorganisms.243

Although studies evaluating the impact of culture conditions on biosynthetic gene expression in cave microorganisms are limited, they emphasize that growth conditions have significant impacts on both BGC transcription180,237 and bioactivity.182,195 For instance, Lebedeva et al.180 conducted transcription analysis of two cave-derived Paenibacillus strains and found that certain genes were transcribed at significantly higher rates during the transition phase, while others peaked during the stationary phase. Higher overall transcription was also noted in halfstrength medium compared to full-strength medium, although results varied significantly from one BGC to another. In a related study, transcriptional analysis of 91 additional strains from the same location similarly demonstrated that for a subset of BGCs, growth phase and nutrient levels impacted transcription, with some genes showing higher expression in the stationary phase and others in the exponential phase.237 Biological activity, presumably resulting from the change in expression of secondary metabolites, can also be impacted by cultivation conditions. For example, Streptomyces spp. isolated from moonmilk deposits possessed strong antimicrobial activities against a suite of microorganisms.182 Researchers compared BGC profiles to observed bioactivities and found that there was no correlation between the global antimicrobial activity of a strain and the number of NRPS and PKS genes in the genome. In some strains, antimicrobial activity was elicited by culturing them in minimal

medium supplemented with GlcNAc, a known elicitor of antibiotics under nutrient-poor conditions. Other isolates did not have antimicrobial phenotype under any tested condition but still possessed numerous BGCs, indicating that the lack of bioactivity was due to inappropriate culture conditions rather than a lack of biosynthetic potential.182 Perhaps unsurprisingly given their location in lightless conditions, exposure to UV light can also change the behavior of cave microorganisms. Rule and Cheeptham¹⁹⁵ tested 176 actinomycetes against microbial pathogens with and without UV light exposure and found that 70% of the strains had antimicrobial activity under at least one growth condition. Approximately 20% were active under both conditions, 17% were active only with UV light exposure, and 33% were active only under no light. Notably, Streptomyces spp. exhibited the most significant change in antibacterial activity in UV light versus darkness. These isolates lost activity against Acinetobacter baumannii, Mycobacterium smegmatis, multi-drug resistant (MDR) S. aureus, extended-spectrum beta-lactamases-containing (ESBL) E. coli, M. luteus, and C. albicans when placed in UV light.

Somewhat surprisingly, only a single study has evaluated changes in expressed secondary metabolites using untargeted metabolomics.¹⁷² In this study, 20 phylogenetically diverse Actinomycetota from caves in Tennessee (USA) were exposed to subinhibitory concentrations of antibiotics (rifampin and streptomycin), rare earth metals (lanthanum or scandium), or co-cultured with mycolic acid-containing bacteria Tsukamurella pulmonis or Rhodococcus sp. BBSNAI13 and evaluated for changes to their secondary metabolite profiles. Comparative metabolomics using LC-MS analysis revealed significant changes in secondary metabolism, with over 30% of detected features increasing at least tenfold under at least one treatment (Fig. 6A). Among these upregulated features, several known natural products were identified, compounds 2-5 and 23-28, along with a novel aminopolyol polyketide, funisamine (compound 6) (Fig. 6B). Notably, the specific stimuli that triggered upregulation were both strain- and metabolite-specific, highlighting the somewhat unpredictable microbial responses to environmental conditions. 172

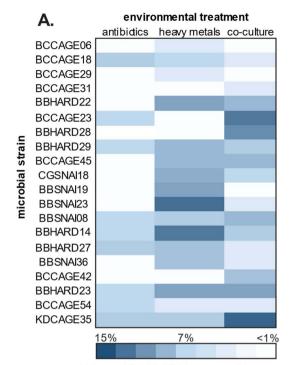
6.2 Culture-independent approaches

Modern sequencing approaches circumvent the necessity of culturing by providing a comprehensive view of the genetic and/ or metabolic diversity of the community members contained within a complex sample. Depending on the specific aims, investigators may leverage targeted or untargeted approaches involving short- or long-read sequencing for in silico prioritization and characterization of natural products reviewed below.

6.2.1 Targeted approaches. The dominant targeted sequencing approach in subterranean environments has been amplicon-based sequencing in which PCR primers amplify highly conserved gene regions. These metabarcoding studies have divulged great diversity of bacteria (16S rRNA), fungi (18S or ITS rRNA), cyanobacteria, mosses, and algae (23S rRNA) (discussed in Sections 3.1 and 3.2). This method has uncovered unknown specialized communities, such as those in the Allchar mineral mine (Republic of North Macedonia), which contains arsenic, thallium, gold, and antimony,²⁴⁴ or the Sukinda chromite mine in India.⁴⁶ Additionally, metabarcoding investigations allow for multi-location comparisons, such as compounding similarities of tourism-impacted Paleolithic caves¹⁴¹ or contrasting aboveground and belowground microbial communities.²⁴⁵

In natural product discovery, amplicon sequencing can be leveraged for large-scale screening of a targeted gene region. Signature genes and conserved protein domains of enzymes responsible for the biosynthesis of diverse families of bioactive compounds have been identified as putative targets.246 Researchers have employed degenerate PCR primers that target the ketosynthase (KS) and adenylation (AD) domains of the PKS and NRPS pathways, respectively.247-249 PKS and NRPS pathways are known for prolific production of bioactive compounds.²⁵⁰ For example, they are among the most common BGC classes of Streptomyces isolated from bats in caves from New Mexico and Arizona (USA).251 Rego et al.252 provides a proof-of-concept that these targets (KS and AD domains) are anticipated to divulge metabolites biosynthesized by cryptic genes or by uncultured microorganisms that have adapted to unique niches. One comparative study screened the AD domain to explore diversity and richness of NRPS biosynthesis in cave-derived sediments from a lava tube and a limestone cave in Canada. They discovered that the sequence clusters could be distinguished based on if they were limestone or volcanic cave origin.²⁰⁴ This approach can therefore both identify ecological variables that influence biosynthetic capacity and identify metabolites that are biosynthesized by uncultured microorganisms within the constraints of known BGC classes.

6.2.2 Non-targeted approaches. Non-targeted sequencing approaches capture nucleic acids of a community without a priori knowledge. Shotgun metagenomics, for example, aims at deep non-targeted sequencing to gain insights into microbial community structure, metabolic potential, and BGC diversity. A study by Wiseschart et al. 253 illustrates the wealth of knowledge gained by using high-throughput shotgun metagenomic sequencing of soil in Manao-Pee Cave (Thailand). In addition to taxonomic profiling, they were able to quantify genes involved in energy metabolism (i.e., oxidative phosphorylation, methane metabolism, carbon fixation, nitrogen metabolism, and sulfur metabolism), and identify 27 biosynthetic pathways of secondary metabolites.²⁵³ In another study, researchers retrieved 451 putative BGCs from metagenomic sequencing of speleothems from the aphotic zone of Borra Caves (Andhra Pradesh, India), and subsequent phylogenetic analysis of NRPS sequences (24%) found representation of all six function categories with several novel subclades.254 Novelty is generally predicted when sequences share low homology with references; however, a rising issue is the unresolved function of known sequences. For example, PKS and NRPS genes amplified from bacteria in Krubera-Voronja Cave (Western Caucasus, Russia) had high sequence similarity to those in databases, but because many of these genes were missing functional annotations, they were not able to predict encoded products.²³⁷ Despite this, Bukeskis et al.237 maintained genome mining for PKS and NRPS genes in parallel with transcriptional analyses was a more



% ions with ≥10-fold increase with treatment

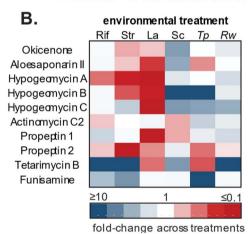


Fig. 6 Impacts of environmental stimuli (antibiotics, rare earth metals, and co-culture) on secondary metabolism of subterranean microorganisms. (A) Percent of total detected features with 10-fold or higher increase in abundance in stimuli vs. control conditions. (B) Foldchanges of identified natural products across stimuli conditions of subinhibitory concentrations of rifampicin (Rif) and streptomycin (Str), rare earth metal exposure of lanthanum (La) and scandium (Sc), and co-culture with *T. pulmonis* (Tp) or *R. wratis* (Rw). Adapted with permission from Covington *et al.* 2018.¹⁷²

effective strategy to analyze bioactivity than culture-dependent assays which underestimated the potential of strains collected in Krubera-Voronja Cave. 199

7. Challenges and future outlook

Subterranean microbial communities represent a largely untapped reservoir of natural products with diverse activities useful in biotechnology, including antimicrobial, anticancer, Review

and bioremediative properties. In the last ten years, there have been 59 studies exploring the biotechnological potential of thousands of strains of subterranean bacteria and fungi, more than 750 of which have shown at least one bioactivity. Despite these significant findings, only 30% of these studies have identified the natural products produced by these microorganisms (and these studies investigate only 19 microorganisms, <3% of the bioactive strains investigated since 2014), highlighting a considerable gap in our knowledge. Fungi are particularly underrepresented in the subterranean research landscape, as only three studies focusing on natural products discovery from subterranean microorganisms have involved fungi. Strikingly, the three strains investigated in these studies were responsible for the production of more than half of the novel compounds discovered from these environments in the last decade, highlighting the tremendous unrealized potential of subterranean fungi.

It is clear based on existing studies that microbial diversity and biosynthetic potential of cave microorganisms is high. However, accessing this untapped biosynthetic potential presents significant challenges. Bacterial uncultivability remains one of the key problems in modern-day microbiology, and a large majority of "known" microorganisms have been identified only through genome-based approaches with no culturable representatives. 192,255 This challenge is compounded by the fact that each microorganism has their own optimal nutrient and physical growth requirements, and that for most novel species, these requirements are unknown. 11 Factors such as sample collection and processing methods, media composition, and storage temperature and time all have significant impacts on the cultivation of microorganisms, and tailored approaches to maximize microbial recovery are required. Even when microorganisms are successfully cultured, the production of secondary metabolites is inconsistent as the majority of BGCs remain transcriptionally inactive under laboratory conditions. Strategies to activate these silent BGCs are many but require significant experimentation and optimization. It is worth noting that while many natural products chemists have turned to heterologous expression as a valuable tool for accessing metabolite products of cryptic BGCs, 187 such technologies have not yet been exploited in subterranean microorganisms.

In recent years, cultivation-independent techniques have enhanced our understanding of microbial diversity and evolution, allowing for genome-based identification of novel bacterial groups and assessment of their biotechnological potential without the limitations of culturing and single organism isolation.256,257 However, despite ongoing improvement of standard methods, all platforms inevitably miss mutations and contain sequencing artifacts.²⁵⁸ Many computational tools are limited to algorithms that search for conserved enzyme motifs; however, improved strategies of data training or incorporating phylogenomics could offer discovery of novel natural products.²⁵⁹ Additionally, metagenomic sequence data can provide insights into traits involving primary metabolism, substrate utilization, and oxygen requirements, allowing researchers to design and optimize specialized media tailored to specific metabolic needs.260 Such strategies in studies involving

subterranean microorganisms remains underutilized, but could help to unlock the potential of previously unculturable microorganisms from these unique environments. The larger issue now, beyond identification, is prioritization of laborious experimental procedures to characterize the compounds with the greatest biomedical or biotechnological potential. Studies that combine culture-dependent and culture-independent methodologies, such as those utilized by Suárez-Moo et al.261 who leveraged culturing, metagenomic sequencing, and genome mining of microbial communities in a karst coastal sinkhole in Yucatán, Mexico, may provide the greatest opportunities to elucidate unexplored microbial genomes and metabolic functions.

Of course, the study of natural products in subterranean ecosystems is only possible if the delicate communities contained within these environments are protected, and it is essential that researchers adopt a conservation-oriented mindset when exploring caves and mines. Alien species pose significant threats to subterranean habitats, threatening biodiversity and access to the untapped biotechnological potential contained within these environments.262 Mining, in particular, introduces substantial environmental hazards, including acid mine drainage and toxic element contamination, which can impact neighboring ecosystems and persist long after mining activities cease.263 Interestingly, the same mines that produce such environmental hazards contain microbes with specialized metabolic pathways privileged for bioremediative activities 124 including the detoxification of pollutants such as arsenic, vanadium, and cyanide.264,265 Specific conservation methods, such as limiting visitor numbers in show caves, enforcing hygiene protocols to prevent the introduction of non-native species, and establishing guidelines for rehabilitation of mining sites, are essential to safeguard indigenous subterranean communities. 153,266,267

Data availability 8.

No primary research results, software, or code have been included and no new data were generated in this review.

Conflicts of interest

There are no conflicts to declare.

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