Life in the Hidden Atmosphere: Culturing and Genomic Perspectives on Aerobiology in Gypsum Caves

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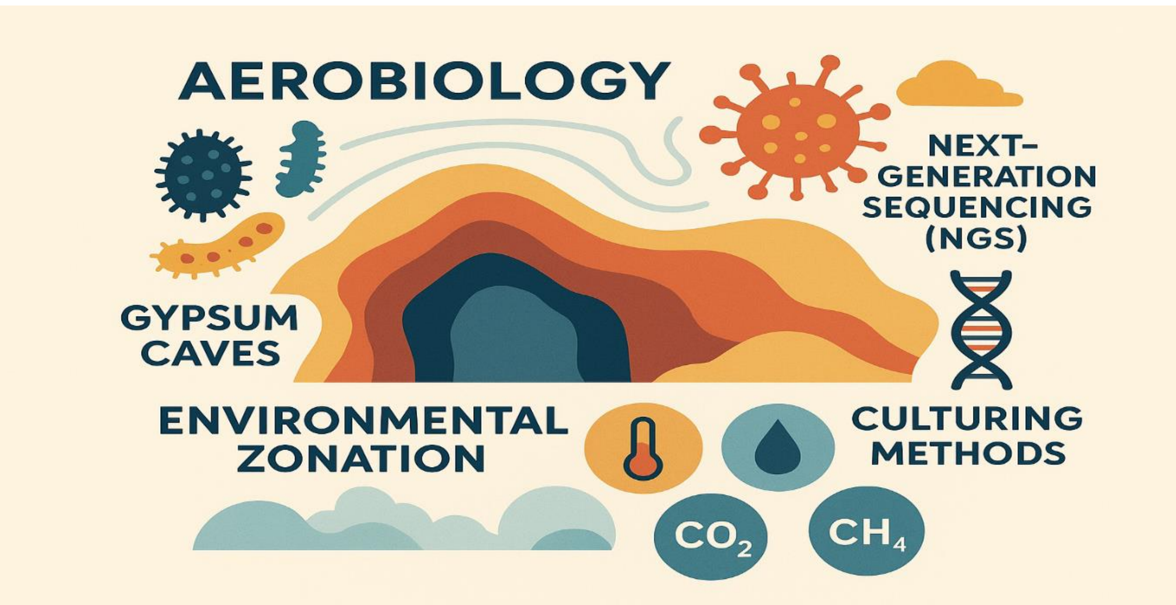
Abstract

Aerobiology, the study of airborne microorganisms and particles, provides a crucial lens through which subterranean ecosystems can be understood. In gypsum caves unique low-energy environments with potent microclimatic gradients—the airborne microbiome reflects a dynamic interaction between geology, ventilation, and biological processes. Traditional culture-dependent studies have characterized primarily spore-forming and resilient taxa, whereas next-generation sequencing (NGS) technologies now uncover the enormous hidden diversity of both culturable and non-culturable microorganisms suspended in cave air. This review synthesizes ongoing knowledge on aerobiology and environmental zonation in gypsum caves, with emphasis on the comparative performance of culturing and NGS approaches. It examines methodological advances, ecological implications, and the role of bioclimatic parameters temperature, relative humidity, CO_2, and CH_4 concentrations in shaping microbial dispersion and persistence. Integrating molecular biology with microclimate monitoring not only broadens the understanding of cave aerobiomes but also informs conservation strategies for fragile subterranean habitats increasingly affected by tourism and climate change.

Keywords: aerobiology, gypsum caves, next-generation sequencing (NGS), culturing methods, cave microbiome, environmental zonation, microclimate

Graphical Abstract



Introduction

Caves are among the most stable yet intricate ecosystems on Earth. Devoid of sunlight and area to restricted nutrient input, these subterranean voids maintain finely balanced physical and biological regimes. Within them, microorganisms bacteria, fungi, and archaea perform fundamental ecological functions, from mineral dissolution to the recycling of organic matter. In gypsum caves, formed through the dissolution of calcium sulfate minerals, environmental conditions such as high solubility of rock, limited air exchange, and low moisture buffering generate distinct physicochemical niches. The aerobiome the population of microorganisms suspended in air acts as both a reservoir and a vector of microbial dispersal within such systems. The scientific area of aerobiology firstly developed to study airborne pollen and spores implicated in allergies(Barton & Jurado, 2007). Only later did researchers recognize the ecological and biogeochemical significance of airborne microbes. Studies in show caves across Europe and Asia have since demonstrated that air serves as a critical conduit connecting surface and subsurface microbiomes. Air currents, visitors, and even animal movement can introduce or redistribute microorganisms across cave sectors. Yet for decades, technical limitations confined cave aerobiology to classical culture-based analyses. Culturing methods, while essential for isolating viable strains and assessing physiological traits, detect only a tiny fraction often estimated at less than 1 % of the total microbial diversity found in any natural environment(Ijoma et al., 2025). Many airborne microbes are either dormant, non-culturable under normal laboratory conditions, or form transient stages during aerosolization. The arrival of next-generation sequencing (NGS) platforms has revolutionized this field by enabling high-throughput detection of microbial communities from environmental DNA without the need for cultivation(de Groot et al., 2021). NGS approaches, particularly 16S rRNA gene amplicon sequencing for bacteria and ITS sequencing for fungi, allow scientists to examine entire microbial assemblages and to correlation diversity patterns with environmental gradients. In caves, these tools have elucidated remarkable complication and revealed connections between air, sediments, and biofilms that were formerly invisible. For example, sequences recovered from cave air often match taxa dominating nearby biofilms or soils, indicating that aerosolization within the cave environment repeatedly redistributes microbial life(Bontemps et al., 2024). Gypsum caves, such as those found in the Sorbas Karst of southeastern Spain, represent ideal natural laboratories for investigating the interaction between bioclimatology and airborne microbiology. Their stratified galleries, varying depths, and distinct ventilation regimes generate microhabitats ranging from ventilated ecotones near entrances to stagnant, isolated chambers deeper within(Calaforra & Pulido-Bosch, 1996). Temperature, humidity, and trace gases like CO_2 and CH_4 fluctuate predictably along these gradients, influencing microbial survival, growth, and dispersal. The comparative study of culturing and NGS approaches in such systems therefore provides dual insight: first, into methodological efficacy, and second, into the environmental structuring of microbial life. Culturing isolates viable organisms with potential biotechnological value and allows precise species identification via full-length 16S rRNA sequencing, while NGS captures the broader ecological context and uncovers unculturable taxa. Integrating these approaches within a bioclimatic framework facilitates elucidate how physical parameters and microbial communities co-evolve within the confined airspace of caves(Jones, 2015). This review synthesizes the state of knowledge on aerobiology and environmental zonation in gypsum caves.

Bioclimatology of Gypsum Caves

Geological and Physical Background

Gypsum caves are distinctive among subterranean environments due to the high solubility of calcium sulfate ($CaSO_4.2H_2O$) and the delicate equilibrium between temperature, humidity, and mineral precipitation. Their formation occurs primarily through sulfate dissolution and evaporitic procedures in semi-arid or arid regions(Wali et al., 2024). As a result, they typically display thin walls, extensive microcrystalline surfaces, and low mechanical stability compared with carbonate caves. These geological characteristics directly influence the bioclimatic framework in which microbial communities develop. Gypsum bedrock is less capable of buffering temperature and moisture fluctuations than limestone(Jehlička et al., 2024). Consequently, gypsum caves exhibit greater microclimatic sensitivity temperature and humidity vary more rapidly with external weather conditions, although deep chambers may remain quite stable over annual cycles. The porosity and friability of gypsum also permit air and moisture exchange through fissures and capillaries, leading to heterogeneity in relative humidity and gas composition. This permeability provides numerous microhabitats for microbial colonization and contributes to the diversity of airborne microorganisms(C. Gaylarde, 2020).

Temperature and Humidity Dynamics

The thermal regime in gypsum caves relies primarily on cave geometry and ventilation. Temperature generally reduces with distance from the entrance, following a gradient from external atmospheric conditions to stable interior zones. In Covadura Cave (Sorbas, Spain), for instance, air temperatures range from ~19 °C near the entrance to ~12 °C in the deepest sections (Gázquez et al., 2015).Relative humidity, often above 90 % throughout the system, displays less variation than temperature but may fluctuate seasonally with surface aridity or rainfall infiltration. Humidity and temperature act synergistically to regulate microbial viability. High humidity favors bacterial survival and spore germination, while temperature stability corroborates long-term persistence of airborne taxa. Conversely, condensation zones particularly those associated with gypsum recrystallization tend to concentrate aerosols and organic material, generating localized microbial "hotspots." These areas are often characterized by dense biofilms and measurable increases in CO_2 from microbial respiration(Jehlička et al., 2024).

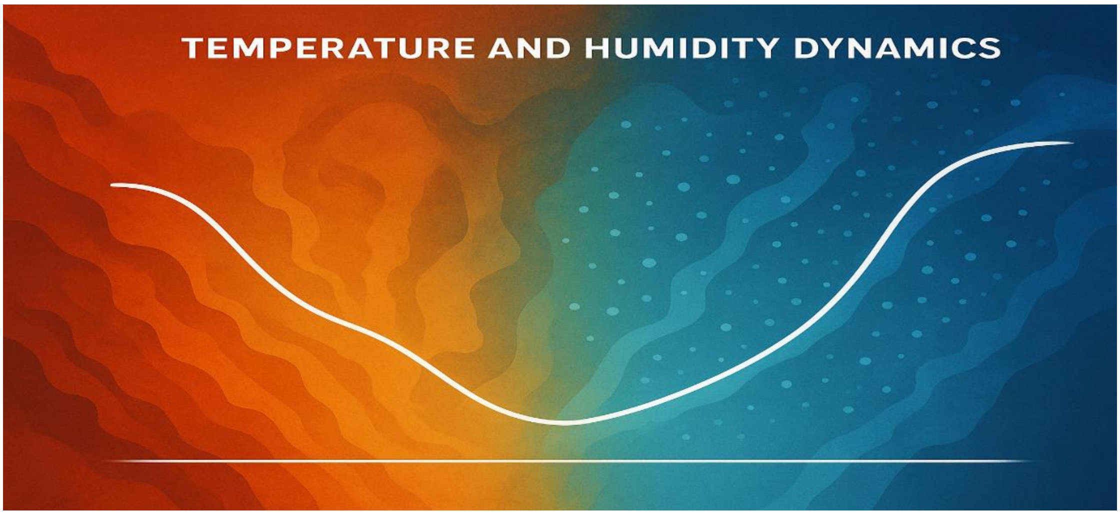


Figure1: The dynamics of temperature and humidity in the cave, where incoming heat reaches the cold of the depths and stable humidity shapes microbial viability.

Gas Composition and Ventilation Patterns

Concentrations of CO_2 and CH_4 serve as key indicators for comprehending ventilation patterns and microbial activity in caves. CO_2 mainly originates from soil respiration and microbial decomposition of organic matter, while CH_4 levels are shaped by atmospheric input and oxidation by methanotrophs. The ratio and isotopic composition of these gases ($\delta^{13}C$-CO_2 and $\delta^{13}C$-CH_4) reveal the scope of air exchange between the cave, soil, and atmosphere(Buursink). In gypsum caves, ventilation follows a seasonal cycle: during winter, dense cold surface air sinks into the cave, driving convective circulation, whereas in summer, temperature inversion reduces airflow and promotes stratification(Mahyuddin & Awbi, 2012). This cycle generates three environmental zones an entrance region with potent air exchange and high variability, a middle zone with greater stability and restricted ventilation, and a deep zone with isolated, stagnant air. These zones exhibit distinct gas concentration patterns, with ventilated areas characterized by higher CH_4 and lower CO_2, and isolated regions showing the reverse. Such gradients closely align with shifts in the composition of cave microbial communities(Volpe et al., 2008).

Implications for Airborne Microbial Dispersal

Airflow in caves functions as the principal vector for microbial transport. Even small pressure differences can move air masses through narrow conduits, carrying microorganisms from soil, guano deposits, or biofilms. Physical parameters such as air velocity, particle size, and turbulence ascertain the residence time of airborne cells. In gypsum caves, where airflows are slower than in limestone systems, microbial particles can remain suspended longer, enhancing possibilities for deposition onto cave surfaces or resuspension from sediments. Thus, bioclimatology and aerobiology are inseparably linked: ventilation controls not simply gas chemistry but also the spatial distribution and temporal dynamics of airborne microbial assemblages(Garcia-Anton et al., 2014).

Airborne Microbial Ecology in Gypsum Caves

Sources and Pathways of Airborne Microorganisms

The microbial load of cave air originates from both external and internal sources. External inputs encompass windborne soil dust, pollen, and anthropogenic particles introduced by visitors. Internal sources consist of resuspended cells from cave sediments, water droplets, biofilms, and faunal activity such as bats or insects. Each source contributes differently depending on cave ventilation and season. Surface soils in arid regions like those surrounding the Sorbas Karst harbor diverse actinobacteria, many of which form resistant spores that survive aerosolization. During dry periods, strong winds transport these spores into cave entrances. Conversely, during humid or stagnant periods, cave-internal sources dominate, with microorganisms detaching from biofilms and entering the air through mechanical disturbance or air currents(Martin-Pozas et al., 2025).

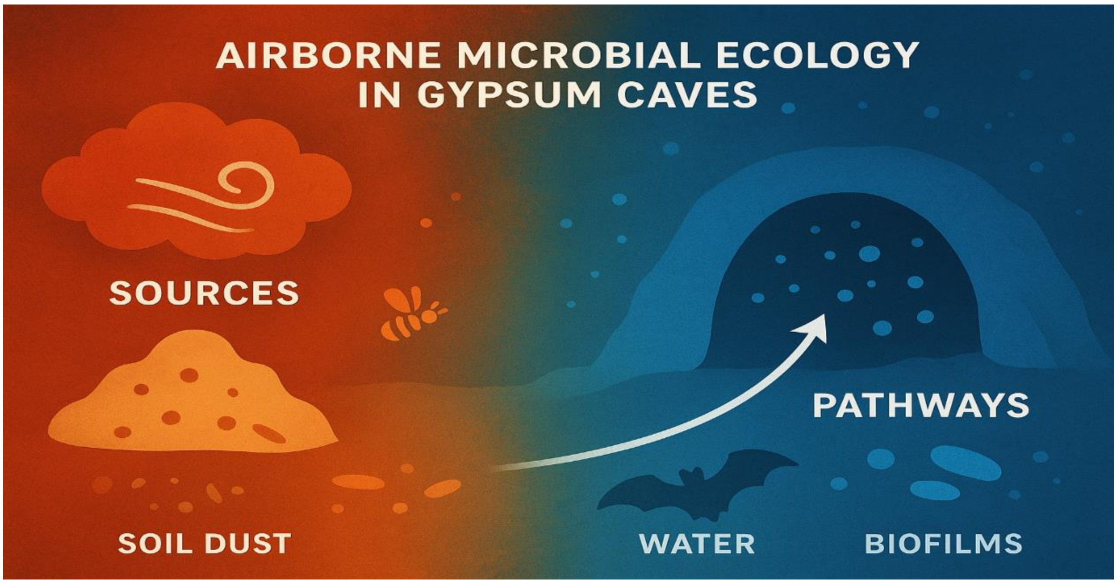


Figure2: Origin and pathways of aerobic microorganisms in gypsum caves: from surface dust to separation from biofilms; a hidden journey into air currents and internal cave dynamics

Composition and Diversity of Cave Aerobiomes

Studies employing culture-based methods have invariably reported dominance of Gram-positive, spore-forming taxa, particularly from the phyla *Bacillota* (formerly Firmicutes) and *Actinomycetota*. Genera such as *Bacillus*, *Streptomyces*, *Micrococcus*, and *Paenibacillus* are commonly isolated (Dominguez-Moñino et al., 2021).These bacteria are adapted to desiccation and ultraviolet resistance, enabling survival during airborne transit. However, culture-based data represent only a small proportion of the total diversity. Next-generation sequencing has revealed a far richer microbial community, including Gram-negative and non-spore-forming taxa such as *Acinetobacter*, *Massilia*, *Pseudomonas*, *Rubrobacter*, and *Sphingomonas*. In gypsum caves, NGS surveys have identified more than 700 bacterial genera in air samples orders of magnitude higher than culture counts(Martin-Pozas et al., 2025). The dominance of

Pseudomonadota (formerly Proteobacteria) and *Actinomycetota* in these datasets indicates that cave air acts as an interface between soil-derived and cave-endemic microbial communities(Mandic-Mulec et al., 2016).

Environmental Controls on Microbial Diversity

Environmental zonation, as produced by microclimate parameters, shapes the structure of airborne microbial communities.

Ventilated zones contain higher percentages of soil- and surface-associated taxa, reflecting direct interaction with the external atmosphere.

Intermediate zones illustrate mixed communities, where transient airflows exchange cells between surface and deep areas.

Isolated zones often harbor taxa typical of biofilms or sediments, such as *Crossiella*, *Blastococcus*, and methanotrophic groups (*Methylobacterium–Methylorubrum*).

Statistical analyses, such as non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarities, consistently indicate clear discrepancy between ventilated and stagnant sectors. Shannon diversity indices also correlate positively with ventilation and adversely with CO_2 concentration(Martin-Pozas et al., 2025).

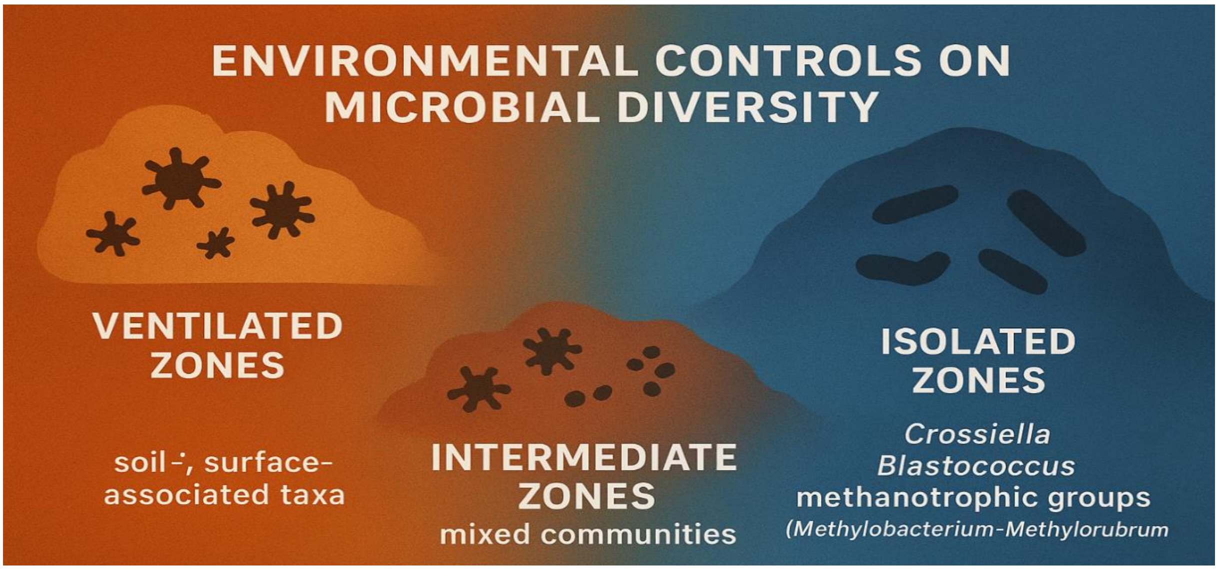


Figure 3: Microbial diversity in Gypsum caves under microclimate control; from wind-exposed areas with surface communities to isolated sections hosting specialized, less mobile groups a layered ecology shaped by ventilation, CO_2, and the hidden rhythm of airflow

Functional Roles and Ecological Interactions

Although direct metabolic studies are limited, inferred functional profiles propose that airborne microbes in caves participate in carbon cycling, methane oxidation, and mineral transformation. Methanotrophic bacteria contribute to CH_4 depletion in isolated chambers, whereas chemoorganotrophic actinobacteria degrade organic residues from animal activity(Zhu et al., 2022). Cyanobacteria and other phototrophs diagnosed even in dark zones may represent dormant cells or remnants from external influx. Microbial dispersal through air correlations different cave habitats. Cells settling from the air colonize speleothems, sediments, or biofilms, establishing feedback loops between surface and subsurface microbiomes. This connectivity implies that the cave atmosphere acts not merely as a passive medium but as an ecological corridor maintaining microbial continuity across space and time(Louca et al., 2018).

Anthropogenic and Climatic Influences

Human visitation alters cave aerobiomes by increasing particulate matter, introducing exogenous microbes, and changing airflow through door operations. Studies in illustrate caves demonstrate rapid shifts in airborne microbial composition following visitor activity, Similarly, global climate change, by altering temperature and humidity gradients, can influence cave ventilation regimes, potentially reshaping microbial communities. Gypsum caves, located in semi-arid climates, are particularly sensitive to such changes due to their limited buffering capacity. Continuous aerobiological monitoring is consequently critical for conservation and management of subterranean ecosystems(Martin-Pozas et al., 2024).

Literature Review Approach

To provide a scientific foundation for this review, a comprehensive literature search was conducted across peer-reviewed articles and reports published in the last two decades, with a special concentrate on aerobiological assessments in cave ecosystems(Piano et al., 2022). The approach reinforces critical comparisons between traditional culturing methods and next-generation sequencing (NGS) methods, utilizing representative studies from gypsum and limestone caves worldwide.Aerobiological sampling in caves is challenged by very low microbial abundance and unpredictable microclimatic conditions. The literature reveals two primary sampling strategies: active methods (using devices like Surface Air System, Andersen impactor, and liquid impingers) and passive sedimentation(Dominguez-Moñino et al., 2021). Culturing studies typically employ nutrient-rich media such as TSA or ISP2, incubating samples at moderate temperatures to recover a extensive range of bacteria, mainly Gram-positive spore-formers. Molecular approaches, on the other hand, rely on high-volume membrane filtration, DNA extraction, amplification of taxonomic marker genes (16S rRNA), and bioinformatic analysis to capture both culturable and non-culturable taxa(Xavier, 2014). Recent studies highlight NGS as transformative, uncovering hundreds of genera per air sample, including “microbial dark matter” not observed through culturing. For example, the analysis by Martin-Pozas et al. (2025) identified upwards of 749 genera, while traditional culturing revealed fewer than 40. Comparative tables show culturing excels at species-level identification and physiological assays, whereas NGS provides broad taxonomic coverage and functional inference. The review also discusses advances in sampling technology, data analysis, and the appearance of multi-omics schemes for integrated ecological insight. Key limitations such as contamination risks and methodological artifacts are described, specifically relevant in low-biomass cave environments. Emphasis is placed on the demand for interdisciplinary collaboration and standardized protocols to ensure reproducibility and rigorous interpretation across investigation efforts. By synthesizing methodological advancements and comparative evaluations in the literature, this review offers an updated framework for studying cave aerobiomes and identifies promising directions for future studies in subterranean microbial ecology(Martin-Pozas et al., 2025).

Comparative Evaluation: Strengths and Limitations

Table: A critical comparison of culturing and NGS reveals complementary advantages and limitations:

Feature	Culturing	NGS
Detects	Only viable, cultivable microbes	Both culturable and non-culturable taxa
Resolution	Species-level via full 16S rRNA	Genus-level (amplicon-based)
Quantification	CFU counts (absolute abundance)	Relative abundance (proportional)
Functional insight	Physiological and biochemical tests possible	Functional inference (metagenomic prediction)
Bias	Medium and incubation selection bias	Primer and database bias
Applications	Strain isolation, antimicrobial discovery	Diversity analysis, ecological modeling

8. Conclusions

Gypsum caves represent exceptional natural laboratories for exploring the interaction between geological, climatic, and biological procedures in subterranean ecosystems. Their mineral composition, solubility, and ventilation patterns generate sharp environmental gradients that, in turn, structure the distribution and diversity of airborne microbial communities. Research conducted over the past two decades, particularly with the inclusion of molecular technologies, has transformed our understanding of cave aerobiology from a descriptive to a mechanistic science. Traditional culturing techniques continue to play an essential feature by enabling the isolation and characterization of viable microorganisms, including rare actinobacteria and other taxa with potential biotechnological applications. However, such approaches alone provide only a narrow view of the cave atmosphere, typically limited to a few spore-forming genera. The introduction of next-generation sequencing (NGS) has unveiled a previously hidden diversity, demonstrating that cave air harbors a complex assemblage of microorganisms originating from both external and endogenous sources. These findings reveal the existence of a “core aerobiome” that reflects regional soil microbiota as well as cave-specific adaptations. Environmental zonation, defined by temperature, humidity, and trace gas gradients (CO_2 and CH_4), exerts a fundamental influence on microbial community composition. Ventilated entrance zones host diverse, surface-derived taxa, while deep, isolated chambers harbor more specialized and metabolically constrained groups, including methanotrophs and oligotrophic actinobacteria. Temporal variations in ventilation further produce cyclic shifts in microbial assemblages, linking cave aerobiology to regional climate dynamics. Methodologically, the convergence of culturing, molecular sequencing, and environmental monitoring has produced an integrated framework for understanding cave aerobiomes. Future research should continue to develop interdisciplinary approaches that unite microbiology, geology, and climatology. High-resolution sequencing, coupled with in situ microclimatic sensors and isotopic tracers, will elucidate microbial contributions to biogeochemical cycles such as carbon and sulfur turnover. At the same time, anthropogenic impacts particularly tourism, artificial lighting, and microclimatic alteration pose growing challenges for subterranean ecosystems. Incorporating aerobiological monitoring into cave management policies can help preserve the delicate ecological equilibrium of these environments. As global temperatures rise and precipitation patterns shift, gypsum caves may serve as sentinels for studying microbial responses to environmental change. In summary, the comparative study of culturing and NGS approaches in gypsum caves demonstrates that cave air is not a sterile void but a dynamic ecological system. The synergy of physical, chemical, and biological processes within these spaces underscores the need for holistic, multidisciplinary exploration. Aerobiology thus emerges as a bridge discipline linking the atmospheric and geological sciences, providing critical insights into microbial resilience, dispersal, and adaptation in one of Earth's most extreme and least accessible habitats.

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