Microbial communities in carbonate precipitates from drip waters in Nerja Cave, Spain

Valme Jurado¹, Yolanda Del Rosal², Concepcion Jimenez de Cisneros³, Cristina Liñan⁴, Tamara Martin-Pozas⁵, Jose Luis Gonzalez-Pimentel⁶, Bernardo Hermosin¹ and Cesareo Saiz-Jimenez¹

¹Instituto de Recursos Naturales y Agrobiologia (IRNAS-CSIC), Sevilla, Spain
²Instituto de Investigacion Cueva de Nerja, Nerja, Spain
³Instituto Andaluz de Ciencias de la Tierra (CSIC-UGR), Armilla, Spain
⁴Departamento de Ecologia y Geologia, Facultad de Ciencias, Universidad de Malaga, Malaga, Spain
⁵Museo Nacional de Ciencias Naturales, Madrid, Spain
⁶Laboratorio Hercules, Universidade de Evora, Evora, Portugal

ABSTRACT

Research on cave microorganisms has mainly focused on the microbial communities thriving on speleothems, rocks and sediments; however, drip water bacteria and calcite precipitation has received less attention. In this study, microbial communities of carbonate precipitates from drip waters in Nerja, a show cave close to the sea in southeastern Spain, were investigated. We observed a pronounced difference in the bacterial composition of the precipitates, depending on the galleries and halls. The most abundant phylum in the precipitates of the halls close to the cave entrance was Proteobacteria, due to the low depth of this sector, the direct influence of a garden on the top soil and the infiltration of waters into the cave, as well as the abundance of members of the order Hyphomicrobiales, dispersing from plant roots, and other Betaproteobacteria and Gammaproteobacteria, common soil inhabitants. The influence of marine aerosols explained the presence of Marinobacter, Idiomarina, Thalassobaculum, Altererythrobacter and other bacteria due to the short distance from the cave to the sea. Nineteen out of forty six genera identified in the cave have been reported to precipitate carbonate and likely have a role in mineral deposition.

INTRODUCTION

In karst systems, meteoric waters percolates through rocks reaching caves where it contributes to the dissolution of carbonate rocks and the formation of speleothems as a result of water degassing and evaporation. Speleothems adopt different forms according to the factors involved in its formation: cave location, water flow, organic matter, microbial communities, etc. Although most speleothems were usually made of calcium carbonate (Fairchild et al., 2007), siliceous speleothems were reported in volcanic caves (Miller et al., 2015) and in both cases were described the contribution of microorganisms to speleothems formation (De los Ríos et al., 2011; Miller et al., 2020a). Sauro et al. (2018) reported that in
orthoquartzite caves, quartz weathering and silica mobility were affected by chemotrophic bacterial communities.

Many hydrological, geochemical and paleoclimate studies have been conducted on speleothems (e.g., Fairchild et al., 2007; Cuthbert et al., 2014; Ellis et al., 2020; Miller et al., 2020a; Miller et al., 2020b and references therein); however the microbiology of drip waters and its contribution to calcite precipitation and speleothem formation is less studied.

A few papers have described the role of bacteria and fungi in speleogenesis. Barton & Northup (2007) reviewed the past, current and future perspectives of cave geomicrobiology and stated that early in the 1960s a few authors suggested that microorganisms played an important role in cave deposits. Further evidences has accumulated across the following decades (Cañaveras et al., 1999; Sanchez-Moral et al., 2003; Cuezva et al., 2012; Maciejewska et al., 2017). It is also probable that drip water bacteria could play a role in cave carbonate deposits.

Microbially induced calcite precipitation (MICP) has been defined as the formation of carbonate minerals from a solution due to the presence of cells, microbial products or metabolic activity (Bosak, 2011). The precipitation can mainly be due to modulation of the environmental pH, nucleation sites on cell surfaces, or by the action of enzymatically driven processes involving carbonic anhydrase, urease, etc. (Achal & Pan, 2011; Hoffmann, Reeksting & Gebhard, 2021). Cañaveras et al. (2006) showed that moonmilk consisted of a network of calcite crystals and active filamentous bacteria and concluded that microbes influenced the physico-chemistry of calcite precipitation. Banks et al. (2010) proposed that the formation of speleothems in caves could involve microorganisms active in MICP. They tested the ability of cave bacteria to dissolve and precipitate carbonates and suggested that calcification required a metabolic activity because dead cells were unable to precipitate the minerals.

Cuezva et al. (2012) reported that Acidobacteria were able to capture CO₂ from the air and form calcium carbonate polymorphs. Later, Cuezva et al. (2020) linked the genus Crossiella to the ability to capture CO₂ from the cave atmosphere and precipitate calcium carbonate. Maciejewska et al. (2017) revealed that cave Streptomyces were involved in peptides/amino acids ammonification and ureolysis, which increase the pH of the bacterial environment and resulted in carbonate precipitation. Previously, Cañaveras et al. (1999) and Groth et al. (2001) reported the ability of cave Streptomyces to precipitate carbonates.

In Nerja Cave, southern Spain, karst hydrodynamic, isotopic and hydrochemical characterization and total organic carbon of drip waters were previously investigated (Liñán et al., 2002; Liñán et al., 2021; Batiot et al., 2003). Jiménez de Cisneros et al. (2020) studied drip water and carbonate precipitates in different halls and galleries of Nerja Cave. They found the presence of microorganisms forming small colonies in some precipitates, which denoted biological activity, but no identification of bacteria was carried out.

In this study we focused our attention on the microbial communities present in the carbonate precipitates along the different galleries and halls. Our aims are to determine the bacteria associated with carbonate precipitation and their source in order to determine the influence of the top soil on meteoric water reaching the cave.
**MATERIALS & METHODS**

**Geological context and research background**

Site description, geological setting, cave microclimatology and hydrology were described elsewhere (Liñán et al., 2002; Liñán et al., 2021; Batiot et al., 2003; Jurado et al., 2020a; Jurado et al., 2021).

**Sampling**

Petri dishes were placed across the galleries and halls for eight months (from October 2019 to June 2020) for collecting drip waters and carbonate precipitates. Jiménez de Cisneros et al. (2020) studied the isotopic data and mineralogy of the precipitates collected in the same sites than in this work and identified calcite in the precipitates from Touristic Galleries and calcite and aragonite in the High and New Galleries. Here, we added a new site, Bear Hall and studied the microbial communities of precipitates in five samples (Fig. 1). Bedrock thickness above the sampling points varies depending of the site: 5–8 m in the Touristic Galleries, and about 60–90 m in the High and New Galleries.

**Precipitate characterization**

The precipitates were identified by X-ray diffractometry (XRD) using a PANalytical X’Pert PRO diffractometer (PW3071) operating at 45 kV and 40 mA, and employing monochromatic Cu-Kα radiation at the Institute of Earth Sciences (IACT-CSIC). The XRD spectra were obtained from 10° to 60° 2θ using X’PertHigh Score (PANalytical) software. Samples were examined in a high-resolution scanning electron microscope (HR-SEM) AURIGA from Carl Zeiss, Germany, in the Center for Scientific Instrumentation (CIC, University of Granada).

**Drip water**

Periodically, at least once a month, the pH and the electrical conductivity (EC) of the drip points associated to the Petri dishes were measured in situ, using HORIBA portable equipment, which allowed taking measurements with a minimum sample volume (drops). pH was measured using a LAQUAtwin pH-11 pH meter (resolution: 0.1 pH, accuracy: ±0.1 pH); EC was measured with the LAQUAtwin B-771 conductivity meter (resolution: 1 µS/cm for conductivity range 9–2,000 µS/cm, accuracy ±2%). For the measure of the flow rate of the drip points a graduated cylinder was used except in Immensity Hall, in which the flow rate was measured by counting the number of drops fallen in 2 min due to its slow flow (Tables S1–S3).

**DNA extraction, sequencing and phylogenetic analysis**

The analytical protocols were described elsewhere by Gonzalez-Pimentel et al. (2021). Briefly, genomic DNA were extracted from carbonate precipitates using FastPrep matrix-E lysis tubes (Qbiogene Inc., Carlsbad, CA, USA) with glass beads and physically disrupted in a shaker (Fast Prep-24, Solon, OH, USA). The quality and concentration of the nucleic acids was measured by fluorometric quantification using the Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA, USA). Samples containing enough DNA for analyses were from Bear Hall (BEAR): 10.5 ng/µL, Bethlehem Hall (BETH): 4.4 ng/µL, and Cascade Hall (CAS1): 4.4....
(ng/µL) which appeared in the outermost part of the cave, coinciding with irrigation water locations (Fig. 1). Deeper into the cave, when the thickness of the ceiling progressively increases, DNA concentration decreased to minimal values, likely due to the fact that the water were filtered by the rock. This was not applicable to Immensity Hall (IMM): 4.1 ng/µL and Mountain Hall (MOU2): 0.6 ng/µL, located in the deeper part of the cave. Both samples corresponded to halls in which were suspected a connection with the exterior, based on geophysical and environmental data (Liñán et al., 2021).

Sequencing was performed using Next Generation Sequencing (NGS) of the V3 and V4 regions of the 16S rRNA gene (Takahashi et al., 2014). Library construction was performed using the 16S Metagenomic Sequencing Library preparation protocol (15044223 Rev.B). The generated DNA fragments (DNA libraries) were sequenced with the MiSeq V3 kit from the Illumina MiSeq platform using 300 base pair paired-end reads.

The samples generating from 138,268 (Cascade Hall) to 174,756 reads (Bethlehem Hall) were analyzed, eliminating sequences of low quality, small size and chimeras. Quality control and trimming of raw data were processed using FastQC software and QIIME2 (Bolyen et al., 2019). The elimination of primers, filtering of sequences according to their quality and the grouping of reads in amplicon sequence variants (ASV) were carried out with the DADA2 package (Callahan et al., 2016). 16S copy number corrections and primer biases may limit the data. Taxonomic assignments were based on SILVA database for prokaryotes (version 132) (Quast et al., 2013) for taxonomic identification of 16S rRNA gene sequences (threshold of 80%), and heat-maps built in R using gplots package (Warnes et al., 2016). The functionality of the microbial community in the samples was predicted using FAPROTAX (Louca, Parfrey & Doebeli, 2016). Raw data from 16S-18S rRNA metabarcoding are available at https://www.ncbi.nlm.nih.gov/sra/PRJNA798270.
RESULTS

Composition of the microbial communities in carbonate precipitates of Nerja Cave

Our results show that the microbial communities of the five samples were almost entirely composed by *Bacteria*, with percentages ranging between 99.98% and 100% (Table 1). Members of the *Archaea* domain were practically non-existent, reaching 0.01% in Cascade Hall and 0.02% in Bear Hall, both in the Touristic Galleries, but absent in Bethlehem Hall from the same galleries. Other two samples corresponded to the High Galleries (Immensity Hall) and New Galleries (Mountain Hall) where *Archaea* were not detected.

Venn diagram shows amplicon sequence variant (ASV) distribution of prokaryotes in the five samples (Fig. S1). A total of 1,161 ASVs were observed, of which 807 ASVs were unique to drip waters. Most of these unique taxa were found in Bear Hall (315), followed by Mountain Hall (168) and Cascade Hall (121). The common microbial core only comprised 6 distinct ASVs, denoting the extreme diversity of the communities from the waters collected in each hall. The highest number of ASVs was shared between the precipitates from Bethlehem and Bear Halls (35) and Bethlehem and Cascade Halls (25), while Mountain and Bethlehem Halls shared five. These data show the disparity in sample composition.

Archaeal Phyla

*Archaea’s* phyla distribution was variable, as shown in Table S4. In the precipitates collected in the Touristic Galleries appeared different phyla (*Aenigmarchaeota* in Cascade Hall and *Nanoarchaeota* in the Bear Hall), while unassigned *Archaea* were found in Bear Hall, all of them with abundances of 0.01%.

Bacterial Phyla

In the *Bacteria* domain, the microbial communities of the drip water precipitates were composed of eight phyla with percentages greater than 1% (Fig. 2). Taxa with abundances less than 1% are not shown in the table.

The most abundant phylum by far was *Proteobacteria*, which varies between 94.3% in Immensity Hall to 59.1% in Bear Hall. Other abundant phyla, between 10 and 20%, were *Actinobacteriota* (Mountain Hall, 18.9% and Bear Hall, 12.5%) and *Bacteroidota* (Bear Hall, 12.7%).

With variable abundances between 5 and 10% appeared *Bacteroidota* (6.6% in Bethlehem Hall), *Firmicutes* (9.3% in Bear Hall) and *Nitrospirae* (5.3% in Mountain Hall). The abundance of the remaining phyla: *Planctomycetes*, *Verrucomicrobia* and WPS-2 (*Candidatus Eremiobacterota*) ranged between 1 and 2%.

---

Table 1  Domains distribution in drip water precipitates from Nerja Cave.

| Domains | BETH | CAS1 | BEAR | IMM | MOU2 |
|---------|------|------|------|-----|------|
| *Bacteria* | 100.00 | 99.99 | 99.98 | 100.00 | 100.00 |
| *Archaea* | 0.00 | 0.01 | 0.02 | 0.00 | 0.00 |
The almost exclusive abundance of *Proteobacteria* in the precipitates of three Nerja halls (Bethlehem, Cascade, and Immensity) is remarkable, while in another two halls was shared with *Actinobacteriota* and *Bacteroidota* (Bear Hall) or *Actinobacteriota* (Mountain Hall).

Bacterial classes with a relative abundance >1% in at least one of the precipitates are depicted in Fig. 3. The distribution of *Bacteria* by classes showed that *Proteobacteria* dominated in Nerja precipitates with the classes *Alphaproteobacteria*, *Betaproteobacteria* and *Gammaproteobacteria*. *Alphaproteobacteria* reached 72.4% in Bethlehem Hall, 39.8% in Mountain Hall, 35.3% in Cascade Hall, 20.1% in Immensity Hall and finally 17.0% in the Bear Hall. *Betaproteobacteria* were well represented in the Immensity Hall (52.3%) and Cascade Hall (31.7%), and with less abundance in Mountain Hall (11.3%), Bear Hall (9.4%) and Bethlehem Hall (6.7%). *Gammaproteobacteria* presented a relative abundance of 32.7% in Bear Hall, followed by 23.7% in Cascade Hall, 21.9% in Immensity Hall, 18.8 in Mountain Hall and 7.3% in Bethlehem Hall.

The majority of *Actinobacteriota* were included in the *Actinobacteria* class (10.8% in Bear Hall and 17.7% in Mountain Hall). Lower percentages were obtained for *Acidimicrobiia* and *Thermoleophilia* with 1.2% in Mountain Hall and 1.5% in Bear Hall and less than 1% in other halls.

In the phylum *Bacteroidota*, two classes *Chitinophagia* and *Flavobacteria* appeared well represented in Bethlehem Hall (6.3%) and Bear Hall (8.3%), respectively, while *Firmicutes* with the class *Bacilli* reached 8.1% in Bear Hall and 1.1% in Immensity Halls. 

*Clostridia*
were only identified in Bear Hall (1.2%). The *Nitrospira* class, phylum *Nitrospirae*, attained 5.3% in Mountain Hall, while in other halls did not exceed 1%.

*Plantomycetia* was present in all the samples, but only in two halls showed relatives abundances > 1%: 1.2% in Immensity Hall and 1% in Mountain Hall.

The class *Opitutae* appeared with low relative abundances in three halls (Bethlehem, Mountain, and Cascade Halls), as well as the class WPS-2, recently proposed as *Candidatus Eremiobacterota* *(Ji et al., 2021)*, only present in Bethlehem and Immensity halls.

Ten families dominate Nerja precipitates with relative abundances over 10%: *Streptomycetaceae, Burkholderiaceae, Alcaligenaceae, Alteromonadaceae, Pseudomonadaceae, Xanthomonadaceae, Rhizobiaceae, Hyphomicrobiaceae, Caulobacteraceae* and *Sphingomonadaceae* *(Fig. 4)*. They showed the highest relative abundances in the

---

**Table 1: Taxonomic identifications of Bacteria at class level.**

| Class                    | BETH | BEAR | MOU2 | CAS1 | IMM |
|--------------------------|------|------|------|------|-----|
| Actinobacteria           | 1.3  | 10.9 | 17.7 | 2    | 0.0 |
| Acidimicrobiia           | 0    | 3.7  | 2.3  | 0    | 0.0 |
| Thermoleophilae          | 0.6  | 1.5  | 0    | 0    | 0.3 |
| Chitinophagia            | 6.3  | 3.4  | 0    | 0    | 3.1 |
| Flavobacteria            | 0.1  | 8.3  | 0    | 0    | 0.1 |
| Bacilli                  | 0.3  | 8.1  | 0    | 0    | 0.1 |
| Clostridia               | 0    | 0    | 0    | 0    | 0   |
| Nitrospira               | 0.0  | 0.8  | 5.3  | 0    | 0.0 |
| Planctomycetia           | 0.3  | 0.0  | 1    | 0.9  | 1.2 |
| Alphaproteobacteria      | 12.4 | 17   | 30.8 | 20.1 | 17  |
| Betaproteobacteria       | 6.7  | 9.4  | 11.3 | 31.7 | 52.5|
| Gammaproteobacteria      | 7.3  | 32.7 | 18.8 | 23.7 | 21.0|
| Opitutae                 | 0.8  | 0    | 1.4  | 0.1  | 0   |
| WPS-2                    | 1.5  | 0    | 0    | 0    | 0.5 |

---

**Figure 3: Heat-map of drip water precipitates from Nerja Cave.** Taxonomic identifications of *Bacteria* at class level. Classes described in the right column and their abundances included in the boxes. The colored left bar groups the classification at phylum level.

Full-size DOI: 10.7717/peerj.13399/fig-3
Figure 4  Heat-map of drip water precipitates from Nerja Cave. Taxonomic identifications of *Bacteria* at family level. Families described in the right column and their abundances included in the boxes. The colored left bar groups the classification at order level. Immensity, Cascade and Bethlehem halls, where was noticed 50.9% of *Alcaligenaceae* and 20.0% of *Pseudomonadaceae* in the Immensity Hall; 25.9% for *Burkholderiaceae*, 19.0% for *Pseudomonadaceae* and 18.1% for *Rhizobiaceae* in Cascade Hall; and 15.1% for *Hyphomicrobiaceae*, 22.9% for *Caulobacteraeaceae* and 12.3% for *Sphingomonadaceae* in Bethlehem Hall.

In Bear Hall only *Alteromonadaceae* (12.5%) and *Xanthomonadaceae* (10.4%) and in Mountain Hall *Streptomycetaceae* (12.8%) showed relative abundances > 10%.

The prediction of ecological functions using FAPROTAX tool is shown in Fig. 5. The most abundant function of the microbial community in the carbonate precipitates is heterotrophism, both under aerobic and anaerobic conditions. This is consistent with the high number of members of the *Proteobacteria* and *Bacteroidota*, two well-known copiotrophic phyla. This is followed by a group of unassigned functions, due to the lack
of information in the database. Thirdly, it highlights the bacteria involved in the nitrogen cycle, particularly abundant in the precipitates of the Immensity Hall (respiration of nitrogen and nitrate, and reduction of nitrate), although the reduction of nitrogen is higher in Cascade and Mountain Halls with respect to the two other halls.

**Calcite precipitates**

The mineralogical study revealed a calcium carbonate composition of the precipitates, appearing the two polymorphic phases of aragonite and calcite (Fig. S2). The precipitates obtained mainly responded to equidimensional crystals of calcite, with a homogeneous growth in all directions. These crystals of rhombohedral habit constituted a true "mesocrystals assembly". The precipitates of the High and New Galleries generally showed no signs of dissolution and also corresponded to well-formed calcite crystals with rhombohedral habits, with scarce presence of microbial cells (Fig. 6D). In these galleries were also detected crystals with a tabular habit that corresponded to aragonite phases. However, in the Touristic Galleries, the abundant presence of microorganisms forming biofilms was observed, as shown in Figs. 6A–6C. The biofilms can be related to some defects that appeared in the crystals, such as perforations, which were very abundant in the precipitates of some of the galleries causing the alteration of its structure and morphology.
DISCUSSION

Bacterial genera in carbonate precipitates

The sparse distribution of Archaea in precipitates is common in caves, where they do not seem to play a significant role in the composition of the community (Jurado et al., 2020a; Jurado et al., 2020b; Addesso et al., 2021; Gonzalez-Pimentel et al., 2021).

Aenigmarchaeota is a candidate phylum whose members were initially described in the waters of a deep gold mine and later found in soils and sediments of a hot spring (Rinke et al., 2013). Although there are few genomes available for study, it has been proposed that members of the phylum Aenigmarchaeota, due to their limited metabolic capacities, are symbionts of other bacteria. The phylum Nanoarchaeota is composed of obligate symbionts with other Archaea in hydrothermal environments (Clingenpeel et al., 2013) and in the precipitates is represented by the order Woesearchaeales found in Bear Hall.

Both Aenigmarchaeota and Nanoarchaeota were detected in vermiculations, mineral deposits on rock surfaces that can be found in caves all over the world (Jurado et al., 2020b; Addesso et al., 2021). Their occurrence in the precipitates is possibly related to transport by drip waters, as they were only present in the halls with least bedrock thickness.

Within the Bacteria the phyla Proteobacteria and Actinobacteriota and many of their genera were abundant in the precipitates, which is consistent with previous data from soils.
and caves (Delgado-Baquerizo et al., 2018; Jurado et al., 2020a; Jurado et al., 2020b; Farda et al., 2022). However, the low relevance of Firmicutes is in accordance with the nature of the samples. Firmicutes were abundantly represented in Spanish caves, both in air and sediments because their members form spores under adverse conditions (Domínguez-Moñino et al., 2018; Domínguez-Moñino et al., 2021). Laiz et al. (1999) found Bacillus in the drip waters of Altamira Cave. Bacillus spp. have a remarkable role in calcium carbonate precipitation (Naveed et al., 2020; Rajasekar et al., 2021).

Given the number of genera in the precipitates of Nerja Cave (Table S5), those that reached relative abundances greater than 10% in at least one of the samples will be discussed, although due to their ecological features a few more genera with lower abundances will also be considered. These genera can be distributed in three different groups according to their ecology: soil and cave, aquatic, and predatory bacteria.

With relative abundances over 10% we found seven genera (Achromobacter, Brevundimonas, Pseudomonas, Hyphomicrobium, Streptomyces, Sphingopyxis and Ensifer) which are common cave inhabitants. All except Achromobacter and Sphingopyxis were included in the global atlas of dominant soil bacteria (Delgado-Baquerizo et al., 2018). A search in genome databases confirmed that most of these genera contain carbonic anhydrase genes that could justify their involvement in the precipitates (Emameh et al., 2018).

Achromobacter represented 50.9% of relative abundance in Immensity Hall, while in other halls did not exceed 1.9% (Cascade Hall) or was less than 1%. Achromobacter was previously reported in caves (Bastian, Alabouvette & Saiz-Jimenez, 2009; Banerjee & Joshi, 2016; Gan et al., 2019) and an Achromobacter strain isolated from a coastal cave precipitate calcium carbonate (Busquets et al., 2014).

Brevundimonas was found in Bethlehem Hall (22.3%). Brevundimonas diminuta was identified in Cascade Hall and Immensity Hall, while Brevundimonas abyssalis was found in Cascade Hall. These two species of Brevundimonas were previously detected in other Spanish caves (Domínguez-Moñino et al., 2021), while unidentified species of this genus were reported in phototrophic biofilms developed on Nerja speleothems (Jurado et al., 2020a). It is noteworthy that B. diminuta and other Brevundimonas spp. can precipitate calcium carbonate (Okyay & Rodrigues, 2015; Ali et al., 2022) which could explain their occurrence in Nerja precipitates.

Pseudomonas is a genus widely distributed in soils and caves (Liu et al., 2010; Campbell et al., 2011; Bastian, Alabouvette & Saiz-Jimenez, 2009; Domínguez-Moñino et al., 2021). In Nerja Cave appeared with abundance in Cascade (19.0%) and Immensity halls (20.0%). However, while in the first hall Pseudomonas alcaligenes reached an abundance of 12.3%, in the Immensity Hall no known species was identified. Several Pseudomonas spp. genomes revealed the presence of carbonic anhydrase genes (Smith & Ferry, 2000; Sharma, Bhattacharya & Singh, 2009; Emameh et al., 2018) involved in calcium biomineralization.

Streptomyces is an abundant genus in the air and mineral surfaces of subterranean environments (Niyomvong et al., 2012; Maciejewska et al., 2017; Domínguez-Moñino et al., 2021; Farda et al., 2022) but only reached a representative abundance in Mountain Hall (12.8%) with the species Streptomyces aculeolatus (12.4%) originally isolated from soils in
Japan (Shomura et al., 1987). No report on the occurrence of Streptomyces aculeolatus in caves was found. In Bear Hall Streptomyces only attained 1.1%, while it was absent in other halls. Many Streptomyces spp. contains carbonic anhydrase genes (Emameh et al., 2018) and have been involved in calcium carbonate precipitation (Cañaveras et al., 1999; Groth et al., 2001).

Hyphomicrobium contains denitrifying methylotrophic species which are common in caves (Manolache & Onac, 2000; Northup et al., 2003; Gonzalez-Pimentel et al., 2021; Vaccarelli et al., 2021). This genus was associated with Caulobacter and Pedomicrobium (Vaccarelli et al., 2021), all of them with the ability to oxidize manganese. Northup et al. (2003) found the presence of iron and manganese oxidizing bacteria, including Hyphomicrobium, Pedomicrobium, Leptospirillum, Stenotrophomonas and Pantoea in ferromanganese deposits of Lechuguilla and Spider caves. Despite the abundance of Hyphomicrobium in Bethlehem Hall, only Hyphomicrobium vulgare was identified in the Immensity and Mountain Halls, indicating that likely other unidentified species occurred in the cave.

Sphingopyxis was identified in all the samples, with relative abundances ranging between 10.8% in Bethlehem Hall and 0.1% in Immensity Hall. Different species of Sphingopyxis were found in contaminated soils (Sharma et al., 2021) and subterranean environments. Gan et al. (2014) studied the genome of two species of Sphingopyxis, isolated from Lechuguilla Cave in New Mexico, while unidentified species were found in other caves (Marques et al., 2019). A survey on genome databases revealed that species of Hyphomicrobium and Sphingopyxis contained carbonic anhydrase, as well as Ensifer.

The genus Ensifer (= Sinorhizobium) comprises nitrogen-fixing symbiotic bacteria. Ensifer adherens has predatory activity on other bacteria (Casida, 1982) and was found in caves from India (Banerjee & Joshi, 2016), USA (Kumar et al., 2017) and Galapagos Islands (Miller et al., 2020a). In the precipitates only attained importance in Cascade Hall.

With low relative abundances was retrieved Nitrospira that reached its maximum abundance in Mountain Hall (5.3%), while in other halls the percentages were lower than 1%. This genus comprises ammonium oxidizing bacteria and is relatively frequent in caves (Jurado et al., 2020b; Martin-Pozas et al., 2020; Gonzalez-Pimentel et al., 2021). Also involved in the nitrogen cycle is Mesorhizobium, a nitrogen-fixing bacterium, found in all precipitates with relative abundances between 0.1% and 3.7% except in Immensity Hall, and previously identified in caves (Wischer et al., 2015).

The family Rhizobiaceae (order Hyphomicrobiales) is diverse with about 170 validly recognized species distributed in 17 genera, among which are four genera Allorhizobium, Neorhizobium, Pararhizobium and Rhizobium, which could not be separated in this study. Species belonging to the Rhizobiaceae have been found in subsurface environments since they can be transported from root nodules to the cave by meteoric waters or by the roots when penetrating the cave ceiling. Diaz-Herraiz et al. (2014) detected a high abundance of Hyphomicrobiales in an Etruscan tomb with many roots hanging from the ceiling.

A few genera: Limnobacter, Marinobacter, Salegentibacter, Idiomarina, Lacunisphaera, Aliihoeflea, and Altererythrobacter are of interest since were common in marine and continental aquatic environments, but only Altererythrobacter was previously found in
caves (Wischer et al., 2015). Most of these genera reflect the influence of marine aerosols on the cave environment. Limnobacter reached an abundance of 25.8% in Cascade Hall and lower in Mountain Hall (8.7%) and Bethlehem Hall (1.8%), being absent in other two halls. Marinobacter is one of the dominant genera in marine environments (Ng et al., 2014), and rarely found in terrestrial environments (Nie et al., 2021). In Nerja only appeared in Bear Hall (12.5%) and Bethlehem Hall (0.4%).

The remaining genera reached relative abundances from 6.0 to 0.1% in the halls of the Touristic Galleries, the ones closest to the sea. Most species of Salegentibacter were isolated from marine sediments (Lian et al., 2021). Members of the genus Idiomarina have a defined ecological behavior since they need sodium chloride for their growth and were found in salterns and in the oceans (Albuquerque & Da Costa, 2014). The genus Lacunisphaera was described from three new species isolated from a lake and was characterized by the fact that their cell walls have peptidoglycan, unlike other members of the Verrucomicrobia (Rast et al., 2017). This genus was one of the most abundant in wetlands with a marked role in the dissimilatory reduction of nitrate (Zhao et al., 2020). The genus Aliihoeflea, proposed by Roh et al. (2008) and not validated by the scientific community, was found in Bethlehem, Cascade and Bear halls. Another genus whose species were isolated from seawater is Altererythrobacter (Yang et al., 2014). Wischer et al. (2015) reported the methylotrophic activity of Altererythrobacter in Movile Cave, Romania.

In the group of predatory and/or bacteria associated to amoebae we included Stenotrophomonas maltophilia (Denet et al., 2018). This species is relatively common in caves (Bastian, Alabouvette & Saiz-Jimenez, 2009; Urzi et al., 2010). Other genera related to amoebas were Reyranella and Bosea (La Scola et al., 2003; Pagnier, Raoult & La Scola, 2011). The presence of Stenotrophomonas, Reyranella, and Bosea points to a likely association with amoebae in the drip waters of Nerja Cave. Previously, abundant Amoebozoa were found on the phototrophic biofilms of this cave (Jurado et al., 2020a).

Lysobacter comprises species predating on other Gram positive and negative bacteria, filamentous fungi, cyanobacteria, algae and nematodes (Reichenbach, 2006) and are frequently isolated from soils and caves (Chen et al., 2016; Kim et al., 2019). In Nerja Cave, Lysobacter appeared with significant abundance in areas from a speleothem where the biofilms had disappeared, which led to the conclusion that this genus could play a role in the control of phototrophic communities (Jurado et al., 2020a).

Prediction of ecological functions

The predicted ecological functions of the bacterial communities presented a high proportion of sequences assigned to chemoheterotrophy.

Several Achromobacter species (Doi et al., 2014) and Dechloromonas (Duffner et al., 2021) present in the precipitates of Immensity Hall, and Stenotrophomonas nitritireducens (Finkmann et al., 2000) in Bear Hall were associated with nitrogen metabolism.

In terms of nitrate reduction, Streptomyces aculeolatus found in the Mountain Hall stands out with Ensifer in Cascade Hall, Achromobacter in Immensity Hall and Dechloromonas in Bear Hall in smaller proportions. Species of Streptomyces are also through to be involved
in nitrate reduction (Feng et al., 2014; Fischer et al., 2014), as well as Ensifer (Torres et al., 2014).

Sequences assigned to hydrocarbon degradation occurs in the Bear Hall highlighted by Marinobacter and Halomonas (Gauthier et al., 1992; Gasperotti et al., 2015). In Mountain Hall the participation of Mesorhizobium in ureolysis was significant. However, all samples showed evidence of ureolysis, a process that involves calcium carbonate bioprecipitation (Omoregie, Palombo & Nissom, 2021).

**Bacteria involved in calcite precipitation**

The bacteria found in the precipitates of the different halls can have different origins: (i) from drip waters, or (ii) transported by air and deposited on the plates. Based on this, it seems appropriate to carry out a search for the genera (>1% relative abundance) found in the five samples and their possible role in calcite precipitation.

The precipitates from the five halls show a considerable abundance of bacterial genera with the ability to induce calcite precipitation. Table S6 shows 19 genera of bacteria found in the precipitates of the cave drip waters with species that have been described as inducing calcite precipitation. This represented 41.3% of the bacteria identified in Nerja Cave.

For example, in Bear Hall were identified Marinobacter (12.5%), Idiomarina (5.2%), Bacillus (3.1%), Lysobacter (3.1%) and Stenotrophomonas (2.1%), which represented 26% of the total abundance; in Bethlehem Hall Brevundimonas (22.3%) and Sphingopyxis (10.8%) amounted 33.1%; in Cascade Hall Pseudomonas (19.0%), Ensifer (10.8%) and Caulobacter (4.5%) attained 34.3%; in Immensity Hall Achromobacter (50.9%) and Pseudomonas (20.0%) reached 70.9%; and in Mountain Hall Streptomyces (12.8%) and Caulobacter (8.5%) 21.3%.

These data could support the hypothesis of a biogenic calcite precipitation, without ruling out that abiotic precipitation could also have occurred in parallel due to degassing and evaporation of the drip water. In favor of a biogenic precipitation were the SEM observations (Fig. 6) and previously published data. In fact, Jiménez de Cisneros et al. (2020) observed how the values of $\delta^{13}$C in the precipitates showed a greater variability that can be explained by the presence of vegetation on the surface and by the residence time of water in the soil. On the other hand, the presence of microorganisms in the precipitates obtained from the Touristic Galleries could explain the more negative $\delta^{13}$C values obtained in these samples.

There is a great difference in the composition of the microbial communities of the drip water precipitates in Nerja Cave. This can be attributed, without a doubt, to the diverse ecological niches existing along the different cave sectors. Above the Touristic Galleries there is an extensive garden of Casuarina, Cupressaceae (cypresses), Arecales (palm trees) and Pinaceae (pines) (Docampo et al., 2007). Due to the scarce thickness from the top soil to the cave ceiling (about 5–8 m), the waters infiltrate in favor of fissures and the hollows of roots. On the soils above the other two galleries, the predominant vegetation is natural and adapted to the ecological conditions of a poorly developed calcareous soil (Jiménez de Cisneros et al., 2020), in which the thickness of the rocks ranges between 60–70 m in the High Galleries up to 90 m in the New Galleries.
Drip water organic carbon contents were high in the garden soils near the cave entrance and the Touristic Galleries (7–9%), while in the other two galleries it was three times lower (Jiménez de Cisneros et al., 2020). This obviously has influence both on the soil microbial communities, and on the infiltration waters. In fact, Batiot et al. (2003) reported high concentrations of organic carbon in the drip water of the Touristic Galleries (1 to 5 mg/l, with a mean value of 2.2 mg/l,) and related them to the elevated content of organic matter in the top soils of the studied area.

In Bear Hall Proteobacteria reached a relative abundance of 59.1%, followed by Bacteroidota (12.7%), Actinobacteriota (12.5%) and Firmicutes (9.3%). This pattern was similar to those of soil communities, where these four phyla were usually well represented (Patel et al., 2016; Mhete et al., 2020). Mountain Hall deviates from the common pattern of all samples, with the highest abundance of Actinobacteriota (genus Streptomyces) and Nitrospirae (genus Nitrospira). Nitrospira was found in an extreme environment (Vapor Cave) at depths of up to 80 m (Martin-Pozas et al., 2020). Koch, van Kessel & Lücker (2019) suggested a metabolic versatility, and the adaptation of Nitrospira, a nitrifying bacterium, to microaerophilic and oligotrophic conditions (Daims & Wagner, 2018), which would coincide with the rock thickness in this area (90 m). Streptomyces is common in the air, rocks and sediments of show caves (Groth et al., 1999; Groth et al., 2001; Dominguez-Moñino et al., 2021). Liñán et al. (2021) suggested, based on ventilation patterns, the connection of Nerja with another cave, which could explain the abundance of Streptomyces.

The abundances of Achromobacter and Pseudomonas in the precipitates from Immensity Hall are noteworthy. This together with the presence of Hyphomicrobiales and Caulobacterales (Table S5) pointed to the influence of soil bacteria. Previous data suggested a connection of this hall with the exterior (Liñán et al., 2021).

Of the five samples studied, only the corresponding to the Touristic Galleries showed a very low relative abundance of Archaea, which can be attributed to their transport from the garden soil to the cave. Indeed, soil microbial communities were usually composed of Bacteria that dominate in metagenomes, while Archaea were rare (<3%), according to Bates et al. (2011).

What is the contribution of airborne bacteria to the precipitates, due to the plates being exposed to open air for several months? Unfortunately, we do not have data at present on airborne bacteria in Nerja Cave. Previous data showed that Bacillus and Micrococcus were the most abundant genera in the air of Touristic Galleries (Del Rosal et al., 2007; Del Rosal, Liñán & Hernández-Mariné, 2014). Bacillus was identified in the precipitates with low relative abundance, but not Micrococcus. Moreover, an aerobiological study on two caves distant 50 km (Tesoro Cave, Rincon de la Victoria) and 100 km (Ardales Cave, Ardales) was very conclusive in this respect. In fact, in both caves the most abundant bacterial genera were Micrococcus and Arthrobacter as detected along the four seasons and in all the halls. Both genera were also abundant outdoor (Dominguez-Moñino et al., 2021). Interestingly none of these two genera were identified in the precipitates which suggest a scarce or null contribution of airborne bacteria.
Other caves, Gruta de las Maravillas (at 350 km from Nerja) and Altamira (at 940 km from Nerja) also presented a similar pattern with a notable abundance of Micrococcus in all the halls and seasons (Garcia-Anton et al., 2014; Domínguez-Moñino et al., 2021).

CONCLUSIONS

The microbial communities from carbonate precipitates in Nerja Cave are quite diverse and reflect the influence of distinct drip waters, depending on the areas where the galleries and halls are located. These drip waters seem to be mainly influenced by top soil inputs. In fact, Proteobacteria, the most abundant phylum in the precipitates of drip waters of the Touristic Galleries, are related to the soil due to the low depth of this area and the direct influence of the garden and the infiltration waters. This is also supported by the important number of members of the order Hyphomicrobiales, undoubtedly originating from the roots of garden plants, and other Alphaproteobacteria and Gammaproteobacteria, common soil inhabitants.

The influence of marine aerosols could explain the presence of Marinobacter, Idiomarina, Thalassobaculum, Altererythrobacter and other bacteria due to the short distance between the cave and the sea.

The high number of genera related with carbonate precipitation and their abundance on the precipitates, as shown the SEM observations, are highly suggestive of the involvement of bacteria in the process.

ACKNOWLEDGEMENTS

The authors acknowledge and CSIC Interdisciplinary Thematic Platform Open Heritage: Research and Society (PTI-PAIS) for the professional support.

ADDITIONAL INFORMATION AND DECLARATIONS

Funding
This work was supported by the Nerja Cave Foundation, grants PID2020-114978GB-I00 and PID2019-110603RB-I00 with ERDF funds. The CSIC Open Access Publication Support Initiative through its Unit of Information Resources for Research (URICI) funded the APC. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Grant Disclosures
The following grant information was disclosed by the authors:
The Nerja Cave Foundation: PID2020-114978GB-I00, PID2019-110603RB-I00.
CSIC Unit of Information Resources for Research (URICI) funded the APC.

Competing Interests
The authors declare there are no competing interests.
Author Contributions

• Valme Jurado, Yolanda Del Rosal, Concepcion Jimenez de Cisneros and Cristina Liñan conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables, authored or reviewed drafts of the paper, and approved the final draft.

• Tamara Martin-Pozas, Jose Luis Gonzalez-Pimentel and Bernardo Hermosin analyzed the data, prepared figures and/or tables, and approved the final draft.

• Cesareo Saiz-Jimenez analyzed the data, authored or reviewed drafts of the paper, and approved the final draft.

Data Availability

The following information was supplied regarding data availability:

The raw data are available at NCBI SRA PRJNA798270.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.13399#supplemental-information.

REFERENCES

Achal V, Pan X. 2011. Characterization of urease and carbonic anhydrase producing bacteria and their role in calcite precipitation. Current Microbiology 62:894–902 DOI 10.1007/s00284-010-9801-4.

Addesso R, Gonzalez-Pimentel JL, D’Angeli IM, DeWaele J, Saiz-Jimenez C, Jurado V, Miller AZ, Cubero B, Vigliotta G, Baldantoni D. 2021. Microbial community characterizing vermiculations from karst caves and its role in their formation. Microbial Ecology 81:884–896 DOI 10.1007/s00248-020-01623-5.

Albuquerque L, Da Costa MS. 2014. The family Idiomarinaceae. In: Rosenberg E, De Long EF, Lory S, Stackebrandt E, Thompson F, eds. The Prokaryotes: Gammaproteobacteria. Berlin: Springer, 361–385 DOI 10.1007/978-3-642-30197-1.

Ali A, Li M, Su J, Li Y, Wang Z, Bai Y, Ali EF, Shaheen SM. 2022. Brevundimonas diminuta isolated from mines polluted soil immobilized cadmium (Cd^{2+}) and zinc (Zn^{2+}) through calcium carbonate precipitation: microscopic and spectroscopic investigations. Science of the Total Environment 813:152668 DOI 10.1016/j.scitotenv.2021.152668.

Banerjee S, Joshi SR. 2016. Culturable bacteria associated with the caves of Meghalaya in India contribute to speleogenesis. Journal of Cave and Karst Studies 78:144–157 DOI 10.4311/2015MB0131.

Banks ED, Taylor NM, Gulbers BR, Giarrizzo JG, Bullen HA, Hoehler TM, Barton HA. 2010. Bacterial calcium carbonate precipitation in caveenvironments: a function of calcium homeostasis. Geomicrobiology Journal 27:444–454 DOI 10.1080/01490450903485136.

Barton HA, Northup DA. 2007. Geomicrobiology in cave environments: past, current and future perspectives. Journal of Cave and Karst Studies 69:163–178.
Bastian F, Alabouvette C, Saiz-Jimenez C. 2009. Bacteria and free-living amoeba in Lascaux Cave. *Research in Microbiology* 160:38–40 DOI 10.1016/j.resmic.2008.10.001.

Bates S, Berg-Lyons D, Caporaso JG, Walters WA, Knight R, Fierer N. 2011. Examining the global distribution of dominant archaeal populations in soil. *The ISME Journal* 5:908–917 DOI 10.1038/ismej.2010.171.

Batiot C, Litián C, Andreo B, Emblach C, Carrasco F, Blavoux B. 2003. Use of Total Organic Carbon (TOC) as tracer of diffuse infiltration in a dolomitic karstic system: the Nerja Cave (Andalusia, southern Spain). *Geophysical Research Letters* 30:2179 DOI 10.1029/2003GL018546.

Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet C, Al-Ghalith GA, et al. 2019. Reproducible, interactive, scalable, and extensible microbiome data science using QIIME 2. *Nature Biotechnology* 37:852–857 DOI 10.1038/s41587-019-0209-9.

Bosak T. 2011. Calcite precipitation, microbially induced. In: Reitner J, Thiel V, eds. *Encyclopedia of geobiology*. Dordrecht: Springer, 223–227 DOI 10.1007/978-1-4020-9212-1.

Busquets A, Fornós JJ, Zafra F, Lalucat J, Merino A. 2014. Microbial communities in a coastal cave: Cova des Pas de Vallgornera (Mallorca, Western Mediterranean). *International Journal of Speleology* 43:205–216 DOI 10.5038/1827-806X.43.2.8.

Cañaveras JC, Cueva S, Sanchez-Moral S, Lario J, Laiz L, Gonzalez JM, Saiz-Jimenez C. 2006. On the origin of fiber calcite crystals in moonmilk deposits. *Naturwissenschaften* 93:27–32 DOI 10.1007/s00114-005-0052-3.

Cañaveras JC, Hoyos M, Sanchez-Moral S, Sanz-Rubio E, Bedoya J, Soler V, Groth I, Schumann P, Laiz L, Gonzalez I, Saiz-Jimenez C. 1999. Microbial communities associated to hydromagnesite and needle fiber aragonite deposits in a karstic cave (Altamira, Northern Spain). *Geomicrobiology Journal* 16:9–25 DOI 10.1080/014904599270712.

Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJ, Holmes SP. 2016. DADA2: high-resolution sample inference from amplicon data. *Nature Methods* 13:581–583 DOI 10.1038/nmeth.3869.

Campbell JW, Watson A, Watson C, Ball HL, Pirkle RS. 2011. Escherichia coli, other coliform, and environmental chemoheterotrophic bacteria in isolated water pools from six caves in Northern Alabama and Northwestern Georgia. *Journal of Cave and Karst Studies* 73:75–82 DOI 10.4311/jcks2009mb0131.

Casida LE. 1982. *Ensifer adhaerens* gen. nov., sp. nov.: a bacterial predator of bacteria in soil. *International Journal of Systematic and Evolutionary Microbiology* 32:339–345.

Chen W, Zhao Y-L, Cheng J, Zhou X-K, Salam N, Fang B-Z, Li Q-Q, Hozzein WN, Li W-J. 2016. *Lysobacter cavernae* sp. nov., a novel bacterium isolated from a cave simple. *Antonie Van Leeuwenhoek* 109:1047–1053 DOI 10.1007/s10482-016-0704-7.

Clingenpeel S, Kan J, Macur RE, Woyke T, Lovalvo D, Varley J, Inskeep WP, Nealson K, McDermott TR. 2013. Yellowstone Lake *Nanoarchaeota*. *Frontiers in Microbiology* 4:274 DOI 10.3389/fmicb.2013.00274.

Cueva S, Fernandez-Cortes A, Porca E, Pašić I, Jurado V, Hernandez-Marine M, Serrano-Ortiz P, Cañaveras JC, Sanchez-Moral S, Saiz-Jimenez C. 2012. The
biogeochemical role of Actinobacteria in Altamira Cave, Spain. *FEMS Microbiology Ecology* **81**:281–290 DOI 10.1111/j.1574-6941.2012.01391.x.

Cuezva S, Martin-Pozas T, Fernandez-Cortes A, Cañaveras JC, Janssens I, Sanchez-Moral S. 2020. On the role of cave-soil in the carbon cycle. A first approach. In: *Proceedings of the EGU General Assembly 2020, Online, 4–8 May 2020. Abstract.*

Cuthbert MO, Rau GC, Andersen MS, Roshan H, Rutlidge H, Marjo CE, Markowska M, Jex CN, Graham PW, Mariethoz G, Acworth RI, Baker A. 2014. Evaporative cooling of speleothem drip water. *Scientific Reports* **4**:5162 DOI 10.1038/srep05162.

Daims H, Wagner M. 2018. *Nitrospira*. *Trends in Microbiology* **26**:460–463 DOI 10.1016/j.tim.2018.02.001.

Del Rosal Y, Liñán C, Hernández-Mariné M. 2014. The conservation of the Nerja Cave: preserving anthropogenic impact in a tourist cave. In: Saiz-Jimenez C, ed. *The conservation of subterranean cultural heritage*. Leiden: CRC Press/Balkema, 193–206.

Del Rosal Y, Martinez-Manzanares E, Marín F, Liñán C, Simón MD. 2007. Análisis de la calidad microbiológica aérea en el interior de la Cueva de Nerja. In: Durán JJ, Robledo PA, Vázquez J, eds. *Cuevas turísticas: aportación al desarrollo sostenible*. Madrid: IGME, 181–190.

Delgado-Baquerizo M, Oliverio AM, Brewer TE, Benavent-González A, Eldridge D, Bardgett RD, Maestre FT, Singh BK, Fierer N. 2018. A global atlas of the dominant bacteria found in soil. *Science* **359**:320–325 DOI 10.1126/science.aap9516.

Denet E, Vasselon V, Burdin B, Nazaret S, Favre-Bonté S. 2018. Survival and growth of *Stenotrophomonas maltophilia* in free-living amoebae (FLA) and bacterial virulence properties. *PLOS ONE* **13**:e0192308 DOI 10.1371/journal.pone.0192308.

Díaz-Herraiz M, Jurado V, Cuezva S, Laiz L, Pallecchi P, Tiano P, Sanchez-Moral S, Saiz-Jimenez C. 2014. Deterioration of an Etruscan tomb by bacteria from the order *Rhizobiales*. *Scientific Reports* **4**:3610 DOI 10.1038/srep03610.

Docampo S, Recio M, Trigo MM, Cabezudo B. 2007. Risk of pollen allergy in Nerja (southern Spain): a pollen calendar. *Aerobiologia* **23**:189–199 DOI 10.1007/s10453-007-9063-1.

Doi Y, Shimizu M, Fujita T, Nakamura A, Takizawa N, Takaya N. 2014. *Achromobacter denitrificans* Strain YD35 pyruvate dehydrogenase controls NADH production to allow tolerance to extremely high nitrite levels. *Applied and Environmental Microbiology* **80**:1910–1918 DOI 10.1128/AEM.03316-13.

Dominguez-Moñino I, Jurado V, Gonzalez-Pimentel JL, Miller AZ, Hermosin B, Saiz-Jimenez C. 2018. *Bacillus onubensis* sp. nov. isolated from the air of two Andalusian caves. *Systematic and Applied Microbiology* **41**:167–172 DOI 10.1016/j.syapm.2018.01.001.

Dominguez-Moñino I, Jurado V, Rogerio-Candelera MA, Hermosin B, Saiz-Jimenez C. 2021. Airborne bacteria in show caves from Southern Spain. *Microbial Cell* **8**:247–255 DOI 10.15698/mic2021.10.762.

Duffner C, Holzapfel S, Wunderlich A, Einsiedl F, Schloter M, Schulz S. 2021. Dechloromonas and close relatives prevail during hydrogenotrophic denitrification.
in stimulated microcosm with oxic aquifer material. *FEMS Microbiology Ecology* 97:fiab004 DOI 10.1093/femsec/fiab004.

Ellis SA, Cobb KM, Moereman JW, Partin JW, Bennett AL, Malang J, Gerstner H, Tuen AA. 2020. Extended cave drip water time series captures the 2015–2016 El Niño in northern Borneo. *Geophysical Research Letters* 47:e2019GL086363 DOI 10.1029/2019GL086363.

Emameh RZ, Barker HR, Hytönen VP, Parkkila S. 2018. Involvement of β-carbonic anhydrase genes in bacterial genomic islands and their horizontal transfer to protists. *Applied and Environmental Microbiology* 84:e00771-18 DOI 10.1128/AEM.00771-18.

Fairchild IJ, Frisia S, Borsato A, Tooth AF. 2007. Speleothems. In: Nash DJ, McLaren SJ, eds. *Geochemical sediments and landscapes*. Oxford: Blackwell, 200–245 DOI 10.1002/9780470712917.

Finkmann W, Altendorf K, Stackebrandt E, Lipski A. 2000. Characterization of N₂O-producing *Xanthomonas*-like isolates from biofilters as *Stenotrophomonas nitritireducens* sp. nov., *Luteimonas mephitica* gen. nov. sp. nov., and *Pseudoxanthomonas broegbernensis* gen. nov., sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 50:273–282 DOI 10.1099/00207713-50-1-273.

Fischer M, Falke D, Pawlik T, Sawers RG. 2014. Oxygen-dependent control of respiratory nitrate reduction in mycelium of *Streptomyces coelicolor* A3(2). *Journal of Bacteriology* 196:4152–4162 DOI 10.1128/JB.02202-14.

Gan HY, Gan HM, Tarasco AM, Busairi NI, Barton HA, Hudson AO, Savka MA. 2014. Whole-genome sequences of five oligotrophic bacteria isolated from deep within Lechuguilla Cave, New Mexico. *Genome Announcement* 2:e01133-14 DOI 10.1128/genomeA.01133-14.

Gan HM, Wengert PC, Barton HA, Hudson AO, Savka MA. 2019. Draft genome sequences of five *Proteobacteria* isolated from Lechuguilla Cave, New Mexico, USA, and insights into taxonomy and quorum sensing. *Microbiology Resource Announcements* 8:e00913-19 DOI 10.1128/MRA.00913-19.

Garcia-Anton E, Cuezva S, Jurado V, Porca E, Miller AZ, Fernandez-Cortes A, Saiz-Jimenez C, Sanchez-Moral S. 2014. Combining stable isotope (δ¹³C) of trace gases and aerobiological data to monitor the entry and dispersion of microorganisms in caves. *Environmental Sciences and Pollution Research* 21:473–484 DOI 10.1007/s11356-013-1915-3.

Gasperotti AF, Studdert CA, Revale S, Herrera Seitz MK. 2015. Draft genome sequence of *Halomonas* sp. KHS3, a polyaromatic hydrocarbon-chemotactic strain. *Genome Announcement* 3:e00020-15 DOI 10.1128/genomeA.00020-15.
Gauthier MJ, Lafa B, Christen R, Fernandez L, Acquaviva M, Bonin P, Bertrand J-C. 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *International Journal of Systematic Bacteriology* 42:568–576 DOI 10.1099/00207713-42-4-568.

Gonzalez-Pimentel JL, Martin-Pozas T, Jurado V, Miller AZ, Caldeira AT, Fernandez-Lorenzo O, Sanchez-Moral S, Saiz-Jimenez C. 2021. Prokaryotic communities from a lava tube cave in La Palma Island (Spain) are involved in the biogeochemical cycle of major elements. *PeerJ* 9:e11386 DOI 10.7717/peerj.11386.

Groth I, Schumann P, Laiz I, Sanchez-Moral S, Cañaveras JC, Saiz-Jimenez C. 2001. Geomicrobiological study of the Grotta dei Cervi, Porto Badisco, Italy. *Geomicrobiology Journal* 18:241–258 DOI 10.1080/01490450152467778.

Groth I, Vetermann R, Schuetze B, Schumann P, Saiz-Jimenez C. 1999. Actinomycetes in kartic caves of Northern Spain (Altamira and Tito Bustillo). *Journal of Microbiological Methods* 36:115–122 DOI 10.1016/s0167-7012(99)00016-0.

Hoffmann TD, Reeksting BJ, Gebhard S. 2021. Bacteria-induced mineral precipitation: a mechanistic review. *Microbiology* 167:001049 DOI 10.1099/mic.0.001049.

Ji M, Williams TJ, Montgomery K, Wong HL, Zaugg J, Berengut JF, Bissett A, Chuvchina M, Hugenholtz P, Ferrari BC. 2021. *Candidatus* Eremiobacterota, a metabolically and phylogenetically diverse terrestrial phylum with acid-tolerant adaptations. *The ISME Journal* 15:2692–2707 DOI 10.1038/s41396-021-00944-8.

Jiménez de Cisneros C, Peña A, Caballero E, Liñán C. 2020. A multiparametric approach for evaluating the current carbonate precipitation and external soil of Nerja Cave (Málaga, Spain). *International Journal of Environmental Research* 15:1–13 DOI 10.1007/s41742-020-00278-x.

Jurado V, Del Rosal Y, Gonzalez-Pimentel JL, Hermosin B, Saiz-Jimenez C. 2020a. Biological control of phototrophic biofilms in a show cave: the case of Nerja Cave. *Applied Sciences* 10:3448 DOI 10.3390/app10103448.

Jurado V, Gonzalez-Pimentel JL, Miller AZ, Hermosin B, D’Angeli IM, Tognini P, De Waele J, Saiz-Jimenez C. 2020b. Microbial communities in vermiculation deposits from an Alpine cave. *Frontiers in Earth Sciences* 8:586248 DOI 10.3389/feart.2020.586248.

Jurado V, Del Rosal Y, Liñán C, Martin-Pozas T, Gonzalez-Pimentel JL, Saiz-Jimenez C. 2021. Diversity and seasonal dynamics of airborne fungi in Nerja Cave, Spain. *Applied Sciences* 11:6236 DOI 10.3390/app11136236.

Kim I, Choi J, Chhetri G, Seo T. 2019. *Lysobacter helvus* sp. nov. and *Lysobacter xanthus* sp. nov., isolated from soil in South Korea. *Antonie Van Leeuwenhoek* 112:1253–1262 DOI 10.1007/s10482-019-01256-w.

Koch H, Van Kessel MAHJ, Lücker S. 2019. Complete nitrification: insights into the ecophysiology of comammox *Nitrospira*. *Applied Microbiology and Biotechnology* 103:177–189 DOI 10.1007/s00253-018-9486-3.

Kumar HK, Gan HM, Tan MH, Eng WWH, Barton HA, Hudson AO, Savka MA. 2017. Genomic characterization of eight *Ensifer* strains isolated from pristine caves and a
whole genome phylogeny of *Ensifer* (*Sinorhizobium*). *Journal of Genomics* 5:12–15 DOI 10.7150/igen.17863.

La Scola B, Mallet M-N, Grimont PAD, Raoult D. 2003. *Bosea eneae* sp. nov., *Bosea massiliensis* sp. nov. and *Bosea vestrisii* sp. nov., isolated from hospital water supplies, and emendation of the genus *Bosea* (Das et al., 1996). *International Journal of Systematic and Evolutionary Microbiology* 53:15–20 DOI 10.1099/ijs.0.02127-0.

Laiz I, Groth I, Gonzalez I, Saiz-Jimenez C. 1999. Microbiological study of the dripping waters in Altamira cave (Santillana del Mar, Spain). *Journal of Microbiological Methods* 36:129–138 DOI 10.1016/S0167-7012(99)00018-4.

Liñán C, Benavente J, Del Rosal Y, Vadillo I, Ojeda L, Carrasco F. 2021. Condensation water in heritage touristic caves: isotopic and hydrochemical data and a new approach for its quantification through image analysis. *Hydrological Processes* 35:e14083 DOI 10.1002/hyp.14083.

Liñán C, Carrasco F, Andreo B, Jiménez de Cisneros C, Caballero E. 2002. Caracterización isotópica de las aguas de goteo de la Cueva de Nerja y de su entorno hidrogeológico (Málaga, Sur de España). In: Carrasco F, Durán JJ, Andreo B, eds. *Karst and environment*. Nerja: Fundación Cueva de Nerja, 243–249.

Lian F-B, Jiang S, Zhu K-L, Shang D-D, Zhang J, Du Z-J. 2021. *Salegentibacter maritimus* sp. nov. isolated from marine coastal sediment. *Systematic and Applied Microbiology* 44:126209 DOI 10.1016/j.syapm.2021.126209.

Liu Q, Wang H, Zhao R, Qiu X, Gong L. 2010. Bacteria isolated from dripping water in the oligotrophic Heshang Cave in Central China. *Journal of Earth Science* 21:325–328 DOI 10.1007/s12583-010-0250-6.

Louca S, Parfrey LW, Doebeli M. 2016. Decoupling function and taxonomy in the global ocean microbiome. *Science* 353:1272–1277 DOI 10.1126/science.aaf4507.

Maciejewska M, Adam D, Naómé A, Martinet L, Tenconi E, Calusinska M, Delfosse P, Hanikenne M, Baurain D, Compère P, Carnol M, Barton HA, Rigali S. 2017. Assessment of the potential role of *Streptomyces* in cave moonmilk formation. *Frontiers in Microbiology* 8:1181 DOI 10.3389/fmicb.2017.01181.

Manolache E, Onac BP. 2000. Geomicrobiology of black sediments in Vantului Cave (Romania): preliminary results. *Cave and Karst Science* 27:109–112.

Marques ELS, Silva GS, Dias JCT, Gross E, Costa MS, Rezende RR. 2019. Cave drip water-related samples as a natural environment for aromatic hydrocarbon degrading bacteria. *Microorganisms* 7:33 DOI 10.3390/microorganisms7020033.

Martin-Pozas T, Sanchez-Moral S, Cuezva S, Jurado V, Saiz-Jimenez C, Perez-Lopez R, Carrey R, Otero N, Giesemann A, Well R, Calaforra JM, Fernandez-Cortes A. 2020. Biologically mediated release of endogenous N$_2$O and NO$_2$ gases in a hydrothermal, hypoxic subterranean environment. *Science of the Total Environment* 747:141218 DOI 10.1016/j.scitotenv.2020.141218.

Mhete M, Eze PN, Rahube TO, Akinyemi FO. 2020. Soil properties influence bacterial abundance and diversity under different land-use regimes in semi-arid environments. *Scientific African* 7:e00246 DOI 10.1016/j.sciaf.2019.e00246.
Miller AZ, García-Sánchez AM, Coutinho ML, Pereira MFC, Gázquez F, Calaforra JM, Forti P, Martínez-Frias J, Toulkeridis T, Caldeira AT, Saiz-Jimenez C. 2020a. Colored microbial coatings in show caves from the Galapagos Islands (Ecuador): first microbiological approach. *Coatings* 10:1134 DOI 10.3390/coatings1011134.

Miller AZ, Pereira MFC, Calaforra JM, Forti P, Dionísio A, Saiz-Jimenez C. 2015. Ana Heva lava tube (Easter Island, Chile): preliminary characterization of the internal layers of coralloid-type speleothems. *Microscopy & Microanalysis* 21:68–69 DOI 10.1017/S1431927614014044.

Miller AZ, De la Rosa JM, Jiménez-Morillo NT, Pereira MFC, Gonzalez-Perez JA, Knicker H, Saiz-Jimenez C. 2020b. Impact of wildfires on subsurface volcanic environments: new insights into speleothem chemistry. *Science of the Total Environment* 698:134321 DOI 10.1016/j.scitotenv.2019.134321.

Naveed M, Duan J, Uddin S, Suleman M, Hui Y, Li H. 2020. Application of microbially induced calcium carbonate precipitation with urea hydrolysis to improve the mechanical properties of soil. *Ecological Engineering* 153:105885 DOI 10.1016/j.ecoleng.2020.105885.

Ng HJ, López-Pérez M, Webb HK, Gomez D, Sawabe T, Ryan J, Vyssotski M, Bizet C, Malherbe F, Mikhailov VV, Vrawford RJ, Ivanova EP. 2014. *Marinobacter salarius* sp. nov. and *Marinobacter similis* sp. nov. isolated from sea water. *PLOS ONE* 9:e106514 DOI 10.1371/journal.pone.0106514.

Nie Y, Su X, Wu D, Zhang R, Wang R, Zhao Z, Xamxidin M, Sun C, Wu M. 2021. *Marinobacter caseinilyticus* sp. nov. isolated from saline soil. *Current Microbiology* 78:1045–1052 DOI 10.1007/s00284-021-02351-w.

Niyomvong N, Pathom-aree W, Thamchaipenet A, Duangmal K. 2012. Actinomycetes from tropical limestone caves. *Chiang Mai Journal of Sciences* 39:373–388.

Northup DE, Barns SM, Yu LE, Spilde MN, Schelble RT, Dano KE, Crossey LJ, Connolly CA, Boston PJ, Natvig DO, Dahm CN. 2003. Diverse microbial communities inhabiting ferromanganese deposits in Lechuguilla and Spider caves. *Environmental Microbiology* 5:1071–1086 DOI 10.1046/j.1462-2920.2003.00500.x.

Okyay TO, Rodrigues DF. 2015. Biotic and abiotic effects on CO₂ sequestration during microbially-induced calcium carbonate precipitation. *FEMS Microbiology Ecology* 91:fiv017 DOI 10.1093/femsec/fiv017.

Omoregie AI, Palombo EA, Nissom PM. 2021. Bioprecipitation of calcium carbonate mediated by ureolysis: a review. *Environmental Engineering Research* 26:200379 DOI 10.4491/eur.2020.379.

Pagnier I, Raoul D, La Scola B. 2011. Isolation and characterization of *Reyranella massiliensis* gen. nov., sp. nov. from freshwater samples by using an amoeba coculture procedure. *International Journal of Systematic and Evolutionary Microbiology* 61:2151–2154 DOI 10.1099/ijs.0.025775-0.

Patel V, Sharma A, Lal R, Al-Dhabi NA, Madamwar D. 2016. Response and resilience of soil microbial communities inhabiting in edible oil stress/contamination from industrial estates. *BMC Microbiology* 16:50 DOI 10.1186/s12866-016-0669-8.
Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, Peplies J, Glöckner FO. 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research* 41:D590–D596 DOI 10.1093/nar/gks1219.

Rajasekar A, Moy CKS, Wilkinson S, Sekar R. 2021. Microbially induced calcite precipitation performance of multiple landfill indigenous bacteria compared to a commercially available bacteria in porous media. *PLOS ONE* 16:e0254676 DOI 10.1371/journal.pone.0254676.

Rast P, Glöckner I, Boedeker C, Jeske O, Wiegand S, Reinhardt R, Schumann P, Rohde M, Spring S, Glöckner FO, Jogler C, Jogler M. 2017. Three novel species with peptidoglycan cell walls form the new genus *Lacunisphaera* gen. nov. in the family *Opitutaceae* of the verrucomicrobial subdivision 4. *Frontiers in Microbiology* 8:202 DOI 10.3389/fmicb.2017.00202.

Reichenbach H. 2006. The genus *Lysobacter*. In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E, eds. *The Prokaryotes*, vol 6. New York: Springer, 939–957 DOI 10.1007/0-387-30746-X.

Rinke C, Schwientek P, Sczyrba A, Ivanova NN, Anderson IJ, Cheng JF, Darling A, Malfatti S, Swan BK, Gies EA, Dodsworth JA, Hedlund BP, Tsiamis G, Sievert SM, Liu WT, Eisen JA, Hallam SJ, Kyrpides NC, Stepanauskas R, Rubin EM, Hugenholtz P, Woyke T. 2013. Insights into the phylogeny and coding potential of microbial dark matter. *Nature* 499:431–437 DOI 10.1038/nature12352.

De los Ríos A, Bustillo MA, Ascaso C, Carvalho MR. 2011. Bioconstructions in ochreous speleothems from lava tubes on Terceira Island (Azores). *Sedimentary Geology* 236:117–128 DOI 10.1016/j.sedgeo.2010.12.012.

Roh SW, Kim KH, Nam YD, Chang HW, Kim MS, Shin KS, Yoon JH, Oh HM, Bae JW. 2008. *Aliihoeflea aestuarii* gen. nov. sp. nov., a novel bacterium isolated from tidal flat sediment. *The Journal of Microbiology* 46:594–598 DOI 10.1007/s12275-008-0196-x.

Sanchez-Moral S, Cañaveras JC, Laiz L, Saiz-Jimenez C, Bedoya J, Luque L. 2003. Biomediated precipitation of CaCO$_3$ metastable phases in hypogean environments: a short review. *Geomicrobiology Journal* 20:491–500 DOI 10.1080/713851131.

Sauro F, Cappelletti M, Ghezzi D, Columbu A, Hong P-Y, Zowawi HM, Carbone C, Piccini I, Vergara F, Zannoni D, De Waele J. 2018. Microbial diversity and biosignatures of amorphous silica deposits in orthoquartzite caves. *Scientific Reports* 8:17569 DOI 10.1038/s41598-018-35532-y.

Sharma A, Bhattacharya A, Singh S. 2009. Purification and characterization of an extracellular carbonic anhydrase from *Pseudomonas fragi*. *Process Biochemistry* 44:1293–1297 DOI 10.1016/j.procbio.2009.07.022.

Sharma M, Khurana H, Singh DN, Negi RK. 2021. The genus *Sphingopyxis*: Systematics, ecology, and bioremediation potential - a review. *Journal of Environmental Management* 280:111744 DOI 10.1016/j.jenvman.2020.111744.

Shomura T, Gomi S, Ito M, Yoshida J, Tanaka E, Amano S, Watabe H, Ohuchi S, Itoh J, Sezaki M, Takebe H, Uotani K. 1987. Studies on new antibiotics SF2415.
1. Taxonomy, fermentation, isolation, physico-chemical properties and biological activities. *The Journal of Antibiotics* **40**:732–739 DOI 10.7164/antibiotics.40.732.

**Smith KS, Ferry JG. 2000.** Prokaryotic carbonic anhydrases. *FEMS Microbiology Reviews* **24**:335–366 DOI 10.1111/j.1574-6976.2000.tb00545.x.

**Takahashi S, Tomita J, Nishioka K, Hisada T, Nishijima M. 2014.** Development of a prokaryotic universal primer for simultaneous analysis of *Bacteria* and *Archaea* using next-generation sequencing. *PLOS ONE* **9**e105592 DOI 10.1371/journal.pone.0105592.

**Torres MJ, Rubia MI, Cobadela Peña T, Pueyo JJ, Bedmar EJ. 2014.** Genetic basis for denitrification in *Ensifer meliloti*. *BMC Microbiology* **14**:142 DOI 10.1093/femsle/fny041.

**Urzì C, De Leo F, Bruno L, Albertano P. 2010.** Microbial diversity in paleolithic caves: a study case on the phototrophic biofilms of the Cave of Bats (Zuheros, Spain). *Microbial Ecology* **60**:116–129 DOI 10.1007/s00248-010-9710-x.

**Vaccarelli I, Matteucci F, Pellegrini M, Bellatreccia F, Del Gallo M. 2021.** Exploring microbial biosignatures in Mn-deposits of deep biosphere: a preliminary cross-disciplinary approach to investigate geomicrobiological interactions in a cave in Central Italy. *Frontiers in Earth Sciences* **9**:590257 DOI 10.3389/feart.2021.590257.

**Warnes GR, Bolker B, Bonebakker L, Gentleman R, Liaw WHA, Lumley T, Maechler M, Magnusson A, Moeller S, Schwartz M, Venables B, Galili T. 2016.** GGPLOTS: various R programming tools for plotting data. R Package, version 3.1.1. Available at http://cran.r-project.org/web/packages/gplots/index.html (accessed on 21 February 2021).

**Wischer D, Kumaresan D, Johnston A, El Khawand M, Stephenson J, Hillebrand-Voiculescu AM, Chen Y, Murrell JC. 2015.** Bacterial metabolism of methylated amines and identification of novel methylotrophs in Movile Cave. *The ISME Journal* **9**:195–206 DOI 10.1038/ismej.2014.102.

**Yang Y, Zhang G, Sun Z, Cheung MK, Huan C. 2014.** *Altererythrobacter oceanensis* sp. nov. isolated from the Western Pacific. *Antonie Van Leeuwenhoek* **106**:1191–1198 DOI 10.1007/s10482-014-0288-z.

**Zhao Y, Bu C, Yang H, Qiao Z, Ding S, Ni S-Q. 2020.** Survey of dissimilatory nitrate reduction to ammonium microbial community at national wetland of Shanghai, China. *Chemosphere* **250**:126195 DOI 10.1016/j.chemosphere.2020.126195.