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16S rRNA DIVERSITY OF MIRROR LAKE IN GILINDIRE CAVE (TURKEY) SHOWS ABUNDANT *NITROSPIRA*

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Abstract

We present the prokaryotic microbial diversity of Mirror Lake, located at the end of Gilindire Cave (Turkey), whose geomorphology shows development in multiple geologic periods and by multiple mechanisms. The lake comprises brackish water with both fresh and seawater inputs. In total, 5 liters of water was sampled from Mirror Lake and was filtered through a 0.22 µm membrane, and after the DNA isolation, 16S amplicon sequencing was conducted to get whole prokaryotic diversity. The bacterial community of this system is predominately composed of nitrite-oxidizing *Nitrospira* with a relative abundance of 28 %. We hypothesize that *Nitrospira* recovered in our samples mediates nitrification by reciprocal feeding with ammonia-oxidizing archaea (*Nitrososphaeria*). We found *Nitrospira* had a close association with Planctomycetes CL500-3 clade and Marinimicrobia (SAR406) in the cave habitat, with a relative abundance of 8.3 % and 5.7 %, respectively. To our knowledge, this is the first time that the presence of marine clade SAR324 has been reported from brackish cave waters.

INTRODUCTION

Gilindire Cave, also known as Aynalıgöl Cave (Mirror Lake Cave, is located in the Aydıncık district, part of the Mersin province of Turkey (Fig. 1A). The name Aynalıgöl comes from the lake located at sea level in the deepest part of the cave that reflects images like a mirror (Fig. 1B). A shepherd discovered the cave by chance in 1999. He noticed a hedgehog in the steep rocky slopes of Aydıncık when he was trying to protect himself from the scorching Mediterranean sun. He followed the hedgehog disappearing among the rocks and found the cave entrance. After baseline characterization of the environment, the cave opened for visitors. It receives approximately 50,000 visitors annually (personal communication with Aydıncık Municipality). Visitation is limited to between 8:00 a.m. and 5:00 p.m. from November to April, and 8:00 a.m. and 7:00 p.m. in the other months. Visitors go down 560 steps to reach the lake. Safety measures include cage-like railings around the stairs (Fig. 1C). Visitors are directed to a balcony by the lake by the steel path with

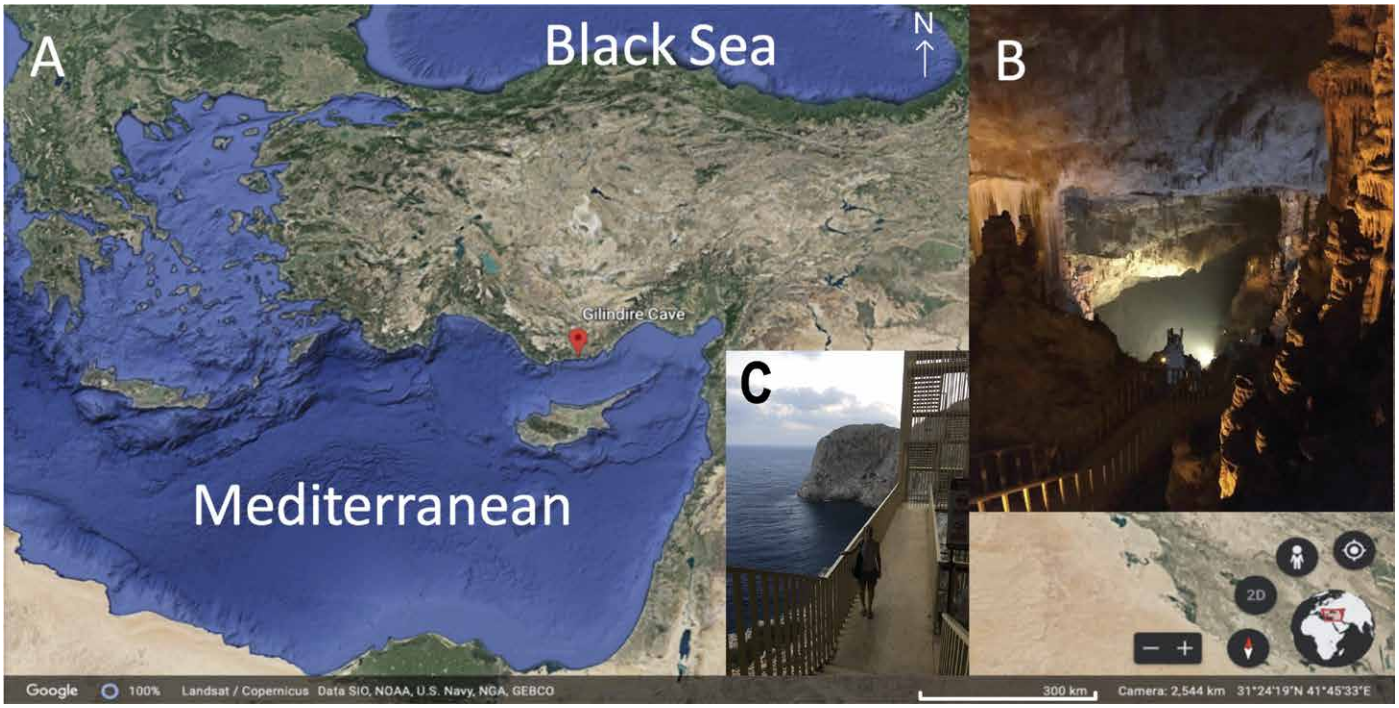

Figure 1. (A) Location of the Gilindire Cave (source: GoogleEarth https://earth.google.com/web). (B) A picture from inside the cave with lake view, in 2017. (C) A picture from the entrance section of the cave, 2017.

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handrails from the cave entrance, but they cannot come in contact with the lake water. There are artificial light sources along with the stairs around the lake, and the lights are on only if there is a visitor.

The first comprehensive study of the geological and hydrological features of Gilindire Cave was conducted by Nazik et al. (2001). The cave contains stalagmites, stalactites, and pillars that divide the interior into many small chambers. There are three main chambers in the cave: 1) the entrance, 2) the main gallery, and 3) the gallery with Mirror Lake. The lake is located at the northeast end of the cave (Fig. 2), 46 m below the cave entrance. The cave formed during the Würm glacial stage that began about 70,000 years ago when the Mediterranean Sea was at its lowest level.

Prokaryotes show high resilience coping with changing environmental conditions; they evolved different strategies from heterotrophy to autotrophy and survived under various conditions, both anoxic to oxic. Recent advances in molecular techniques, such as next generation sequencing, enable scientists to discover microbial diversity in various habitats without the need to culture them.

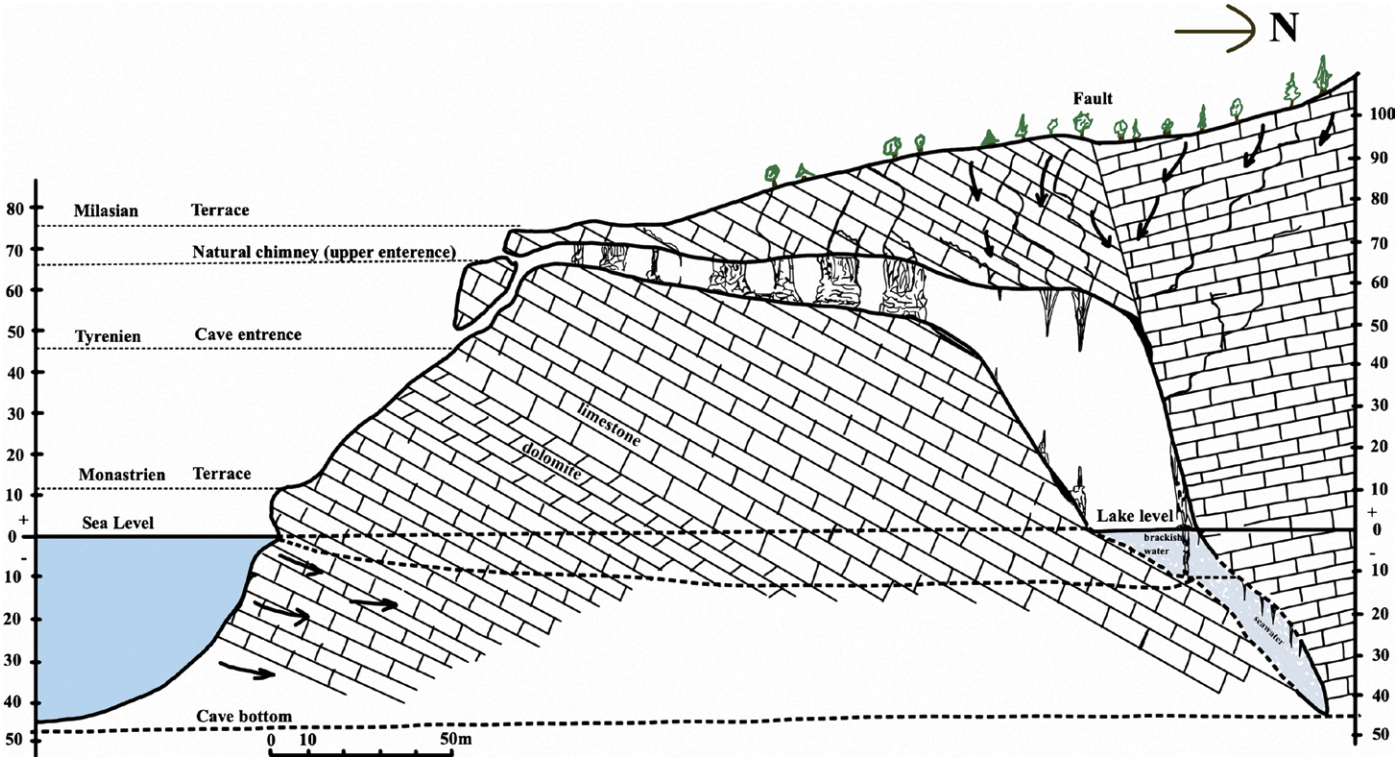

Figure 2. Geomorphological section of the Gilindire Cave (redrawn from Nazik et al. 2001). Arrows indicate infiltrating water.

Here, we present the first culture-independent amplicon sequencing study from the surface water of Mirror Lake in Gilindire Cave to identify the microbiota of the brackish lake. We recognize the nitrification potential of the cave environment, because the most dominant bacteria are the nitrite-oxidizing *Nitrospira*.

MATERIALS AND METHODS

Sampling Site

Gilindire Cave is located at 36°07'58.08"' north latitude and 33°24'11.04" east longitude. The area exhibits a typical Mediterranean climate, and the temperature reaches around 40 ºC during the summer with an average of 80 % humidity. The surface vegetation of the study area is dominated by maquis. The cave was developed within Cambrian limestone or dolomitic limestone as a result of two faults intersecting each other in a northeast-southwest direction. The cave developed in multiple geologic periods and by multiple mechanisms. Erosion surfaces belonging to the Monastrien-I, Thyrrenian, and Milazzian periods are observed in the cave. The main chambers in the cave were formed by different processes. The entrance of the cave is located on the steep rocky slopes 46 m above present-day sea level. The first part of the cave might have been connected to the surface during the Thyrrenian period. The second part (the main gallery) of the cave is dominated by the speleothems developed during the Pliocene. The lake chamber is the youngest part of the cave and developed along a prominent fault during the Würm glaciation due to the 90 m lowering of the sea. This part initially developed as a result of karstification and then filled with seawater due to the rising sea level again. Mirror Lake is located at the end of the cave, 46 m below the cave entrance and 250 meters inland from the coast.

The total depth of the lake is 46 m. While the first 10 m are brackish, salty seawater can be found below this depth from intrusion along fracture zones (Nazik et al., 2001). According to physicochemical measurements, the salinity increases with depth (2.4 ppt at the surface and 31.7 ppt at a depth of 27 m), whereas temperature is observed to be relatively constant (about 21 °C). Saturated oxygen concentrations also decreased throughout the depth (86.6 % at the surface and 43.5 % at a depth of 27 m) (Nazik et al., 2001). Physicochemical measurements from the deepest part of the lake (46m) were not reported elsewhere.

Sampling

The water sample was collected from Mirror Lake on July 31, 2017. In total, 5 L of water were collected using a sterile bottle from the lake's surface (approximately the first 15 cm of depth). The bottle's lid was closed immediately to avoid contamination, and the bottle was carried out in a sterile plastic bag. The water sample was then transported to the Institute of Marine Sciences, Middle East Technical University (IMS-METU) laboratory, which is an hour away, and then filtered through a 0.22 µm-pore MoBio polyethersulfone membrane.

DNA Extraction, Sequencing, and Analysis

Total DNA extraction from the filter was processed in the IMS-METU genetic laboratory. DNA extractions were carried out using the protocol of Paz et al. (2003). The filter was placed into 2 mL vial and 1000 µL of lysis solution (0.25 M Tris Borate, pH 8.2, 0.1 M EDTA, 2 % sodium dodecyl sulfate, 0.1 M NaCl) and the same volume of phenol/chloroform/isoamyl alcohol solution (25:24:1 v:v:v) was added. The sample was incubated for a week at room temperature and then mixed thoroughly (1 min.) before centrifugation (14,000 rpm, 10,000 *g*, 5 min.). The aqueous phase was collected, added to the same volume of chloroform-isoamyl alcohol solution (24:1 v:v), thoroughly mixed (1 min.), and centrifuged (14,000 rpm, 10,000 *g*, 5 min.). Genomic DNA was then precipitated by further centrifugation with cold 100 % ethanol (14,000 rpm, 10,000 *g*, 15 min.). The alcohol was removed and the DNA was twice washed with 2 mL of 70 % ethanol, then centrifuged again (14,000 rpm, 10,000 *g*, 15 min.). The DNA pellet was then dried in a ventilated hood, dissolved in 50 µL sterile, molecular-grade water, and quantified using a nano drop spectrophotometer. DNA samples were kept at −20 °C until sending to the sequencing company. PCR amplification and amplicon sequencing were performed by Macrogen Inc. (Macrogen-Europe) for both directions (forward and reverse) by using the Illumina MiSeq platform (300 bp paired-end sequencing). Herlemann et al. (2011) primer pairs (341F- CCTACGGGNGGCWGCAG and 805R-GACTACHVGGGTATCTAATCC) were used to amplify two variable sites (V3-V4) of the 16S rRNA gene. All sequence reads were processed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (Quast et al., 2013). Each read was aligned using the SILVA Incremental Aligner version 1.2.10 for ARB SVN (revision 21008) (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and quality controlled (Quast et al., 2013). Reads shorter than 50 aligned nucleotides and reads with more than 2 % of ambiguities or 2 % of homopolymers were excluded from further processing.

Reads were dereplicated and the unique reads were clustered into operational taxonomic units (OTUs), and the reference read of each OTU was classified. Dereplication and clustering were done using cd-hit-est (version 3.1.2; http://www. bioinformatics.org/cd-hit) (Li and Godzik, 2006) running in accurate mode, ignoring overhangs, and applying identity criteria of 1.00 and 0.95, respectively. The classification was performed by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 132) using BLASTN (version 2.2.30+) with standard settings (Camacho et al., 2009). Reads without any BLAST hits or reads with weak BLAST hits remain unclassified. These reads were assigned to the meta group "no relative" in the SILVAngs fingerprint and Krona charts (Ondov et al., 2011). The associated sequencing data was deposited in the NCBI BioProject under the accession ID PRJNA789137.

RESULTS AND DISCUSSION

In total, 162,559 sequences were recovered from the brackish water of Mirror Lake's surface. The number of OTUs was 12,839, and 94.9 % of all sequence reads were classified. At the phylum level, the three dominant taxa in our sample were Proteobacteria (36 %), *Nitrospirae* (28 %), and Planctomycetes (12 %). Phylum Marinimicrobia (SAR406 clade), Bacteroidetes, Patescibacteria, and Verrucomicrobia were represented by relative abundances between 2.7 % and 5.7 % (Fig. 3). In total, 35 phyla were detected, of which 3 were archaeal. The relative abundances of the rest of the taxa recovered from our sample were each less than 1 %. The water sample was taken from the surface of the brackish zone, which extends to a depth of 10 m. The groundwater, which reaches the lake through rainfall, is mostly mixed with seawater at the surface of the lake (Nazik et al., 2001). We recorded the presence of many marine isolates in our samples. The presence of marine isolates might be as a result of seawater inflow from the fracture zones.

At the phylum level, Proteobacteria were the most abundant (36 %) taxa in our study and were represented by Alphaproteobacteria (20 %), Gammaproteobacteria (10 %) and Deltaproteobacteria (6 %). The genus *Gemmobacter* is represented by 4.2 % relative abundance in the recovered OTUs. The utilization of methanol and formate by various

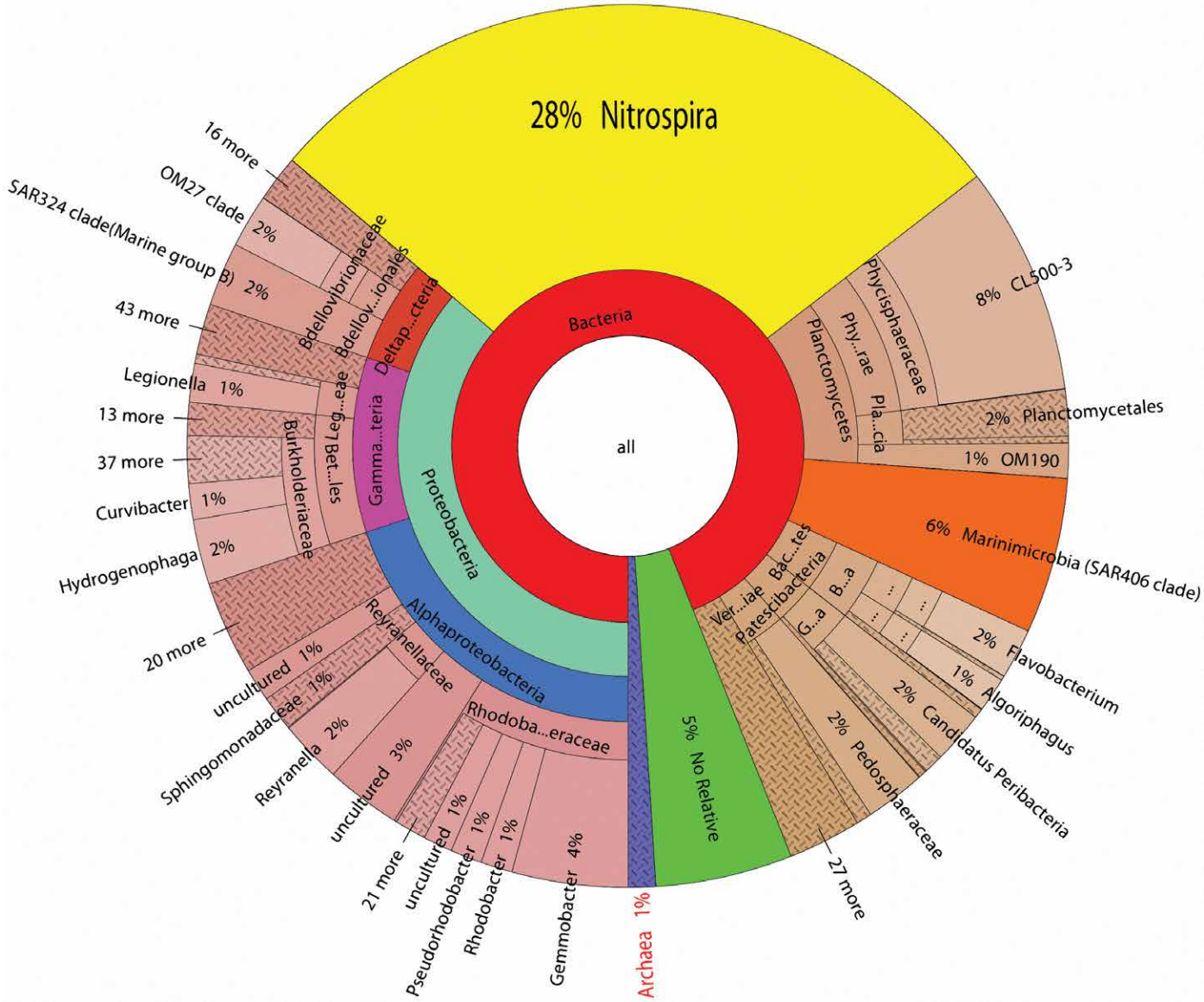

Figure 3. Taxonomic abundances of phyla observed in the Gilindire Cave. Dark blue colored pie slice at the bottom represents the Archaea (relative abundance of 1 %). OTUs belonging to the archaeal sequences were affiliated with Thaumarchaeota and Nanoarchaeaeota.

species of *Gemmobacter* genus has been reported previously (Chen et al., 2013; Hameed et al., 2020; Kröber et al., 2021). While they comprise a relatively small portion of total abundance in Gilindire Cave's Mirror Lake, the degree to which cave ecosystems contribute to the global carbon sink should be further studied. The genus *Hydrogenophaga*, which is capable of using hydrogen as an energy source (Willems et al., 1989), was represented by 2.3 % of relative abundance. We also recovered OTUs which belong to deltaproteobacterial SAR324 clade (marine group B) with a relative abundance of 2.3 %. The clade SAR324 is ubiquitous in the marine environment and has been reported from various depths of marine waters (Wright et al., 2012). Their versatile metabolism has been attributed to sulfur oxidation, carbon fixation, hydrocarbon utilization, and heterotrophy (Wright et al., 2012; Haroon et al., 2016). This study is the first time clade SAR324 has been reported from any brackish water system, to the best of the authors' knowledge

We recorded the dominance of globally-distributed, nitrite-oxidizing bacteria (Bock and Wagner, 2013); *Nitrospira* was the dominant genus in our sample (Table 1). The success of *Nitrospira* has been associated with nitrite oxidation (Lücker et al., 2010; Bock and Wagner, 2013), metabolic diversity (Watson et al., 1986; Daims et al., 2001; Lücker et al., 2010; Koch et al., 2015), nitrification by reciprocal feeding with ammonia-oxidizing microbes (Koch et al., 2015; Palatinszky et al., 2015), chemolithoautotrophic aerobic hydrogen oxidation (Koch et al., 2014), and complete nitrification (complete ammonia oxidation, or "comammox") (Daims et al., 2015). We did not measure any of the nutrient (ammonia or nitrate) concentrations in our study, so we are not able to conclude if the *Nitrospira* representative in our sample performs nitrite oxidation or comammox.

Table 1. The relative abundances of recorded taxa.

Relative Abundance	Taxonomy
28.3	*Nitrospirae; Nitrospira; Nitrospirales; Nitrospiraceae; Nitrospira*
8.4	*Planctomycetes; Phycisphaerae; Phycisphaerales; Phycisphaeraceae; CL500-3*
5.7	*Marinimicrobia (SAR406 clade)*
5.0	*No Relative*[a]
4.2	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Gemmobacter*
2.8	*Proteobacteria; Alphaproteobacteria; uncultured*
2.4	*Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; Hydrogenophaga*
2.3	*Proteobacteria; Deltaproteobacteria; SAR324 clade (Marine group B)*
2.3	*Proteobacteria; Alphaproteobacteria; Reyranellales; Reyranellaceae; Reyranella*
2.2	*Verrucomicrobia; Verrucomicrobiae; Pedosphaerales; Pedosphaeraceae*
2.0	*Proteobacteria; Deltaproteobacteria; Bdellovibrionales; Bdellovibrionaceae; OM27 clade;*
1.9	*Patescibacteria; Gracilibacteria; Candidatus Peribacteria*
1.8	*Bacteroidetes; Bacteroidia; Flavobacteriales; Flavobacteriaceae; Flavobacterium*
1.4	*Proteobacteria; Gammaproteobacteria; Legionellales; Legionellaceae; Legionella*
1.3	*Proteobacteria; Gammaproteobacteria; Betaproteobacteriales; Burkholderiaceae; Curvibacter*
1.3	*Planctomycetes; OM190*
1.3	*Bacteroidetes; Bacteroidia; Cytophagales; Cyclobacteriaceae; Algoriphagus*
1.2	*Proteobacteria; Alphaproteobacteria; Acetobacterales; Acetobacterales Incertae Sedis; uncultured*
1.2	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Rhodobacter*
1.2	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Pseudorhodobacter*
1.1	*Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; uncultured*
20.7	Other (≤1 %)[b]

[a] Reads without any BLAST hits or reads with weak BLAST hits remain unclassified and assigned to the meta group "No Relative."
[b] Taxa having relative abundance less than 1% grouped together and annotated as "Other (<1 %)."

The other dominant taxon recovered in our sample was CL500-3, belonging to the Planctomycetes and represented by 8.3 % relative abundance. According to 16S amplicon sequencing studies, Planctomycetes CL500-3 clade was reported from the deeper water columns of ultra-oligotrophic Crater Lake in Oregon, United States (Urbach et al., 2001) and of the oxygenated hypolimnion deep lakes in Japan (Okazaki et al., 2017). In contrast with the earlier studies, we recovered CL500-3 from the surface of the Gilindire Cave's Mirror Lake. However, environmental conditions at the surface of our sampling site (such as low light, low saline, and oxygenated water) might be similar to the conditions in earlier studies' deeper water-column lake conditions. According to Urbach et al. (2001), who first described the CL500-3 in the Crater Lake, Oregon, the distribution of relative abundance throughout the water column suggests that this clade functions in the remineralization of detrital particles or processes associated with sediments or hydrothermal waters. Here, we suggest that the products of the remineralization process mediated by the CL500-3 might be used by the dominant *Nitrospira* and linking carbon and nitrogen cycles in the brackish waters of Mirror Lake.

The deep-branching bacterial phylum Marinimicrobia (SAR406) comprised 5.7 % of all 16S rRNA affiliated sequence reads at Mirror Lake. A recent study showed that most of the *Marinimicrobia* clades participate in the biogeochemical cycling of sulfur and nitrogen. Additionally, two of these clades use nitrous oxide and act as a global sink for the greenhouse gas nitrous oxide (Hawley et al., 2017). We speculate that Marinimicrobia representatives recorded at Mirror Lake might also use nitrous oxide, which is a by-product of nitrification.

The uncultured OM190 Planctomycetes (Silva taxonomy) was also recovered from the brackish waters of Mirror Lake at 1 % of relative abundance. The OM190 sequence was reported previously from a variety of environments like kelp surfaces (Bengtsson and Øvreås, 2010), seawater (Rappé et al., 2003), soil (Elshahed et al., 2008), and sponges (Mohamed et al., 2010). The taxon OM190 was reported to have a negative correlation with salinity, and some representative sequences were distantly related to anammox-like environmental sequences (Ye et al., 2016). Environmental conditions in our sampling area, such as low salinity and oxygenated water, support those findings.

Archaea sequences were only represented by Thermoplasmata, Woesearchaeia, and *Nitrososphaeria* in our sample, and they constituted only 1% of relative abundance of all OTUs. It is critical to stress here that the primer pairs used in our study have limited coverage over the Archaea domain (Fischer et al., 2016). This is the possible reason for

such low coverage. Among the archaeal sequences, *Nitrososphaeria* constituted the highest relative abundance (0.96 %). *Nitrososphaeria* is one of the two genera of Thaumarchaeota mediating ammonium oxidation and is known as an ammonia-oxidizing Archaea (AOA). The presence of *Nitrososphaeria* (Candidatus *nitrosoarchaeum*, Candidatus *nitrosopumilus*, Candidatus *nitrosotenuis*) in the brackish waters of Gilindire Cave's Mirror Lake is consistent with other studies conducted in freshwater sediments (Xie et al., 2014). Methanomassiliicoccales belonging to the Thermoplasmata is one of the archaeal lineages that retain the methanogenesis pathway (Evans et al., 2019). However, Zinke et al. (2021) analyzed twelve metagenome-assembled genomes (MAGs) belonging to the Methanomassiliicoccales. They further recovered 16S rRNA from three of the MAGs and showed that if these sequences were recovered by 16S rRNA-based approaches, they were most likely classified as Methanomassiliicoccales*,* which leads to wrong assumptions that these sequences represent methanogens (Zinke et al., 2021). We recovered archaeal sequences from the lake waters of Gilindire Cave that are classified as Methanomassiliicoccales*.* However, when Zinke et al. (2021) is considered, their assignment to a methanogenesis mediating group is uncertain. Marine group II Archaea (Thermoplasmata) were also recovered from the brackish waters of Gilindire Cave. They are known to be ubiquitous in marine surface waters (Rinke et al., 2019) and have different salinity preferences (Xie et al., 2018). Marine Benthic Group D, which belongs to Thermoplasmata and plays a role in protein remineralization in anoxic marine sediments (Lloyd et al., 2013), was also recovered in our study. Our findings regarding the archaeal lineage suggest further investigation of metabolic diversities in Gilindire Cave using Archaea-specific primers or metagenomic approaches. Lastly, *Nitrososphaeria* (which are AOA) and *Nitrospira* recorded in the Gilindire Cave's Mirror Lake might mediate nitrification by reciprocal feeding.

Gilindire Cave's Mirror Lake shows some major features of anchialine caves, which have both freshwater and seawater influences due to their sea and groundwater connections, may form in limestone (Bishop et al., 2015; Sawicki, 2003), and have similar prokaryotic communities (Kajan et al., 2022). Anchialine caves have sinkholes where they connect directly with the surface (Sawicki, 2003, figure 1). However, the non-sinkhole entrance to Gilindire Cave is its only connection to the surface. For that reason, even if Gilindire Cave shares some features with anchialine caves, we hesitate to define it as an anchialine cave.

Kajan et al. (2022) studied the diversity of four anchialine caves in the Mediterranean and identified the phyla Proteobacteria as the most abundant taxa. In the present study, we found that Nitrososphaeria dominated. This AOA was also recovered with high abundance above and in the halocline of two of the caves studied by Kajan et al. (2022). A 5-year study of the cave waters of the Emilia Romagna region, Italy, has demonstrated that the microbial community variation depends on location. Additionally, that study showed seasonality is responsible for the community variation (D'Angeli et al., 2017). Based on the findings of Kajan et al. (2021) and D'Angeli et al. (2017), we can say that the composition of Gilindire Cave's microbial community should be further investigated by including water column diversity and seasonality.

CONCLUSION

In this study, we evaluated the prokaryotic microbial diversity in the brackish surface waters of Mirror Lake in Gilindire Cave, which formed when the Mediterranean Sea was at its lowest level. One methodological drawback to our study is the lack of physicochemical measurements, which limits our conclusion. Keeping in mind the methodological limitation, our results point to the nitrification by reciprocal feeding between *Nitrospira* and *Nitrososphaeria,* which are ammonia-oxidizing *Archaea.* Investigating microbial diversity in such a unique environment showed the close relationship between *Nitrospira*, Planctomycetes CL500-3 clade, and Marinimicrobia (SAR406), which dominate the cave's surface water.

This is the first study conducted in Mirror Lake. However, more comprehensive and integrated studies should be carried out in the lake to uncover the complex relationship between recorded taxa and their small-scale contribution compared to global-scale biogeochemical cycles.

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