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Depth Related Structure and Microbial Composition of Microbialites in a Karst Sinkhole, Cenote Azul, Mexico

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ABSTRACT

Microbialites are sedimentary structures that represent modern models of the oldest life forms, stromatolites (<3.5 Ga), and are relevant for evolutionary and ecological studies. Cenote Azul is a deep (>90 m) karst sinkhole in the Yucatan peninsula characterized by microbialites that develop along its wall and hydrogeochemistry defined by the saturation of carbonate, sulfate and calcium ions. In this study, high throughput sequence analysis of 16S rRNA genes allowed characterization of the prokaryotic communities associated with microbialites in a depth profile. The most represented phyla were Proteobacteria (23.6–30.1%), Planctomycetes (11.6–13.8%), Cyanobacteria (9.7–16.5%), Acidobacteria (6.1–8.3%), Rokubacteria (4.1–7.8%), Chloroflexi (3.3–4.4%), Nitrospirae (3.5–4.6%), Actinobacteria (2.6–5%) Bacteroidetes (1.7–4.1%) and Thaumarchaeota (7.5–11.1%). Phylogenetic distance analyses described two distinct clusters of microbialites: Shallow (5 and 10 m) and Deep (20 and 30 m). The dominant diversity at the phylum level of the prokaryotic community described in this system is similar to that of other microbialites from different environments, but differences are reported at the classification level of order, family and genus. The mineral composition of the Cenote Azul microbialites has calcite as the main constituent mineral (~97%). Finally, this work establishes a baseline on the presence of microbialites and its relation to depth in the sinkholes of the Yucatan peninsula and stimulates the monitoring of these communities as a tool for the conservation of sites with high tourism pressure.

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Community structure; karst system; microbialites; sinkholes; Yucatan peninsula

Introduction

Stromatolites constitute the oldest fossil record of life (~3500 MYA) dating back to the Archean eon (Grotzinger and Knoll 1999; Schopf 2006). Microbialites are rock-like structures, formed by microbial communities present in mineral-saturated water bodies. These communities have the physical capacity and metabolic ability to trap, bind and promote the precipitation of inorganic minerals (Burne and Moore 1987; Dupraz et al. 2011). Microbialites were abundant all over the planet during the Precambrian, participating in the oxidation of the atmosphere and the development of the biogeochemical cycles that allowed the evolution of eukaryotic life (Dupraz et al. 2009; Falkowski et al. 2008). Currently, the most extensive studies of marine microbialites are from Shark Bay in western Australia (Babilonia et al. 2018; Logan 1961) and Highborne Cay in Bahamas (Khodadad and Foster 2012; Reid et al. 1999), while lacustrine environments that harbor microbialites include Lake Van in Turkey (Kempe et al. 1991), Bacalar lagoon, Alchichica crater-lake and Cuatro Ciénegas in Mexico (Alcántara-Hernández et al. 2017; Beltrán et al. 2012; Centeno et al. 2012; Couradeau et al. 2011; Gischler et al. 2008; Valdespino-Castillo et al. 2018; Winsborough and Golubic 2007; Yanez-Montalvo et al. 2020), Pavilion Lake in Canada (Laval et al. 2000), among others (Chagas et al. 2016). The increase in studies of modern microbialites has allowed the exploration of the role of microbes in the formation and maintenance of these structures and to better understand the ancient record of life (Omelon et al. 2013).

Microbialite formation depends on the balance between physical, chemical and biological reactions (Dupraz et al. 2009), and the interaction of a wide variety of microbial metabolisms(Archaea/Bacteria) including phototrophs, methanogens, sulfur oxidizers, denitrifiers, and those involved in carbonate precipitation. In microbialites, microbes remain in a layer of exopolymeric substances (EPS) that provides adhesion, protection and adsorbed cations (Ca^{2+}, Mg^{2+}) (Braissant et al. 2007; Dupraz et al. 2004).

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The organic mineralization occurs within the EPS, oxygenic photosynthesis contributes to create an alkaline environment that promotes the formation of carbonate nucleation sites (Dupraz et al. 2009; Obst et al. 2009; Paulo and Dittrich 2013). In the same EPS matrix, processes such as anoxygenic photosynthesis and sulfate reduction are involved in the precipitation of minerals (Braissant et al. 2007). In contrast, processes such as sulfide oxidation or fermentation, promote the dissolution of carbonates (Dupraz and Visscher 2005). Microbialite accretion is related to the biological and metabolic balance of microbial metabolisms.

Characterization of biological attributes and diversity allows a better understanding of microbialite formation and their responses to the environment, which is useful when considering these communities as bioindicators of environmental disturbance and health (Lindsay et al. 2017; McDevitt-Irwin et al. 2017; Meziti et al. 2016; Yanez-Montalvo et al. 2020). The presence of microbialites in freshwater bodies represents an important opportunity for ecological studies. These are long-lived sessile microbial assemblages, and therefore, are exposed to changes caused by intense anthropogenic activities (the increase of greenhouse gases and temperature, habitat modification and incorporation of nutrients, among others) (Dupraz et al. 2011; Foster et al. 2019; Lindsay et al. 2017).

Lacustrine and marine microbialites around the world are mainly constituted by Proteobacteria, Cyanobacteria, Actinobacteria, Bacteroidetes and Planctomycetes (Breitbart et al. 2009; Centeno et al. 2012; Chagas et al. 2016; Suosaari et al. 2016; Warden et al. 2016; White et al. 2015, 2016; Yanez-Montalvo et al. 2020). In general, microbialites that develop in different environments have a similar composition at the phylum level, but a unique composition at the genus and species levels, in relation to biogeographic processes, environmental conditions and habitat modification (Centeno et al. 2012; Valdespino-Castillo et al. 2018; Yanez-Montalvo et al. 2020). Additionally, stromatolites that develop in depth gradients, such as Alchichica (Mexico) and Pavilion (Canada) lakes, the microbial composition includes an the significant abundance of Acidobacteria, Nitrospirae and Archaea, has been documented (Russell et al. 2014; Valdespino-Castillo et al. 2014; White et al. 2016).

The Yucatan peninsula (YP) in Mexico, is a vast limestone platform with karstic environments, formed during the Miocene-Pleistocene, composed of limestone, dolomite, and anhydrite (Perry et al. 2009; Bauer-Gottwein et al. 2011). Perry et al. (2002) divided the YP into six hydrogeochemical/physiographic regions based on its geological characteristics. The karstic nature of each region is characterized by exceptional permeability due to the dissolution of the rock and connection of groundwater that flows through systems, where caves, canals and sinkholes intervene (Bauer-Gottwein et al. 2011). Sinkholes, also called cenotes (in Mayan, ts'onot), are formed by processes involving the collapse of subterranean caverns, with an opening in the surface, coupled with the dissolution of the limestone platform by carbonic acid (Cervantes-Martínez et al. 2009; Gabriel et al. 2009; Schmitter-Soto et al. 2002). Sinkhole research in the YP has focused on the hydrochemistry of waters, connectivity and zooplankton composition (Schmitter-Soto et al. 2002; Pérez-Ceballos et al. 2012; Montes-Ortiz and Elías-Gutiérrez 2018). However, no investigation about the microbial community composition of microbialites.

The eastern evaporative region of the YP is very peculiar and presents connectivity processes, in terms of hydrology, biogeochemistry and ecology (Perry et al. 2002). The Coastal Transversal Corridor is a hydrodynamic model for southern Quintana Roo (QR) proposed by Hernández-Arana et al. (2015) as a strategy for research and conservation of interconnected ecosystems including coral reefs, coastal lagoons, mangrove forests, floodplains, cenotes and karst lakes. In addition, this region has different aquatic environments that harbor microbialites, including Chetumal Bay, Muyil lagoon, Chichancanab lagoon and Bacalar lagoon (Beltrán et al. 2012; Centeno et al. 2012; Gischler et al. 2008; Johnson et al. 2018; Rasmussen et al. 1993; Valdespino-Castillo et al. 2018; Yanez-Montalvo et al. 2020). Bacalar lagoon is one of the freshwater sites with the highest presence of microbialites in the world (Gischler et al. 2008). In Bacalar lagoon, microbialites are located in reef patches throughout the 40 km of the lagoon and show spatial differentiation along a north-south gradient (Yanez-Montalvo et al. 2020). The Bacalar lagoon system has four sinkholes: Brujas, Esmeralda, Cocalitos and Xulha, within the main water body (Gischler et al. 2008, 2011) and the Cenote Azul (CA), located, on the land side, at a distance of approximately 200 m. This research focuses on the CA sinkhole, which has particular hydrogeochemical and biological characteristics (Perry et al. 2002) and where, Hernández-Arana et al. (2015) reported microbialites at a depth of 18 m. In this study, we aimed to explore the prokaryotic composition of the microbialites from the CA sinkhole, following a depth gradient. We hypothesize that changes in the structure and microbial composition of microbialites will be mainly related to depth as seen in other environments (Águila 2018; Russell et al. 2014). To this aim, we followed the next strategy: (a) describe the hydrogeochemical parameters of the water column, (b) analyze the mineral composition of the microbialites, and (c) describe the composition and structure of the microbialites following a high-throughput sequencing approach of the V4 hypervariable region of the 16S rRNA gene.

Materials and methods

Study site

The CA is an open karst sinkhole, located in Quintana Roo in southeastern YP, Mexico (18°38′48″N, 88°24′42″W). It is close (<200 m) to Bacalar lagoon (Figure 1), has a circular shape, with a depth >90 m, and a diameter of ~100 m, making it one of the largest sinkholes in the region (Cervantes-Martínez et al. 2009; Perry et al. 2002). Cenote Azul is considered an oligotrophic, well-mixed water system, with no halocline, and an average annual temperature of 29.2 ± 0.9 °C (Cervantes-Martínez et al. 2002; Schmitter-Soto et al. 2002).

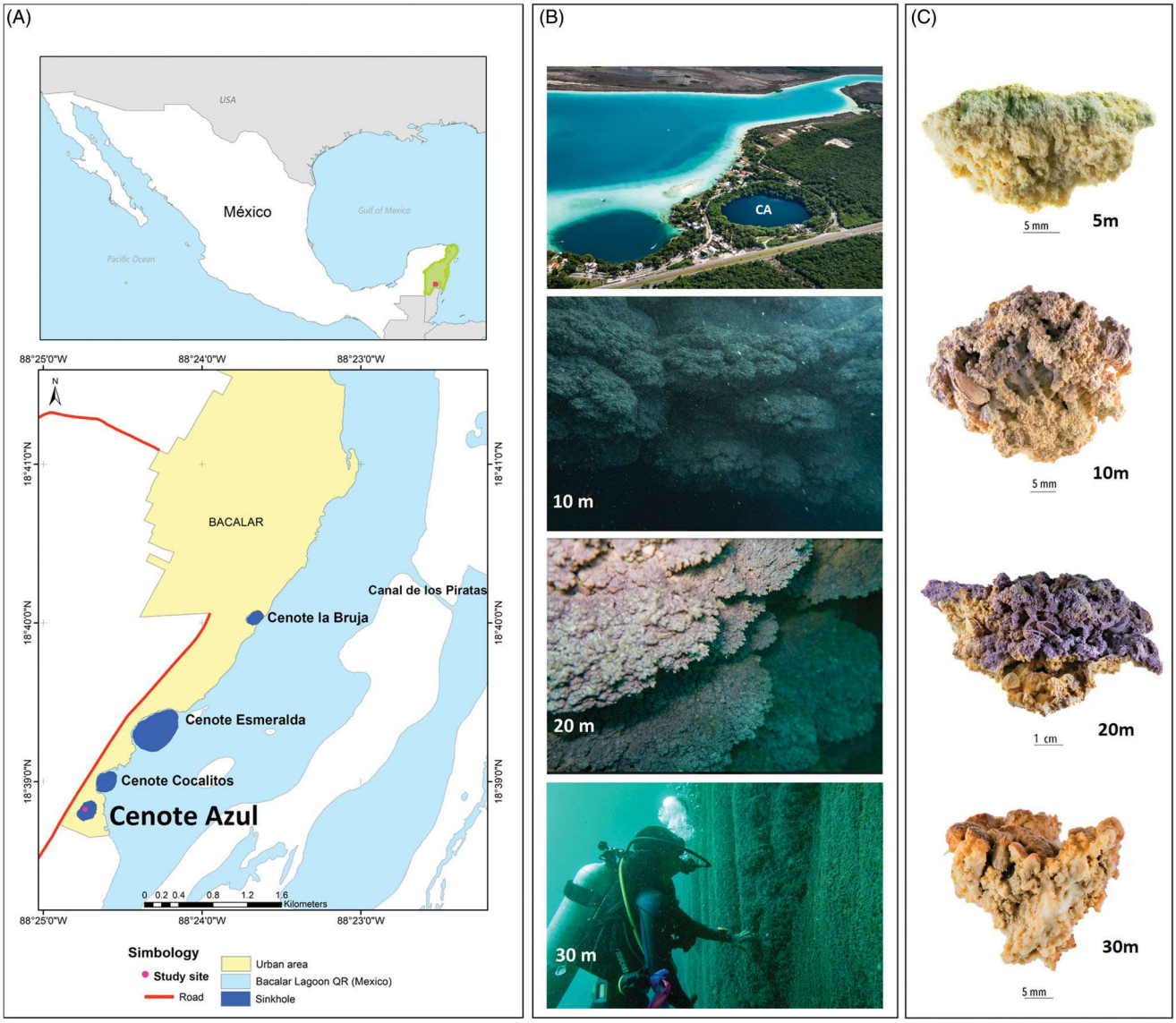

Figure 1. Geographical location of the sampling site. (a) Location of the study site and its separation in reference to Bacalar lagoon. (b) Panoramic view of the CA and photograph of the microbialites inside the sinkhole. (c) Photograph of microbialite fragments in the depth profile of the study.

Sample collection

Microbialite samples were obtained in a bathymetric profile, at 30, 20, 10 and 5 m using autonomous diving equipment. At each depth, the microbialites had similar physical characteristics including color and texture. Five microbialite fragments ($10 \times 5 \times 2$ cm) were collected in each depth, with separation of approximately one meter per sample, using a sterile chisel and a sledgehammer (Supplementary Figure 1). The fragments were placed individually in nets previously labeled for each depth. Samples were transferred to sterile containers and stored in refrigeration until arrival in the laboratory. Water samples (2.5 L) were collected at each depth with a Niskin bottle and used for genetic (1 L) and physicochemical characterization (1.5 L). All samples were placed in refrigeration at 4 °C for transport to the laboratory and then stored in a freezer at −80 °C (microbialites). The transfer time of the samples from the workstation to storage (−80 °C) was less than two hours. All microbialites were sampled under collector permit PPF/DGOPA-113/14. Field studies did not involve endangered or protected species.

Water chemistry analysis

Major cations and anions were analyzed in the Institute of Geology (UNAM, LANGEM-PT-LCL) by ion chromatography with conductometric detection on Waters 1525 binary HPLC pumps, autosampler (model No. 717) and conductivity detector (model No. 432). An IC-Pak Anion HR (Waters) stationary column was used with 5 µm amino packing. Anionic analysis used a mobile phase consisting of a mixture of acetonyl, butanol and a solution of gluconate/sodium borate in water with one mL/min flux rate. Major cations were run with a mobile phase consisting of dipicolinic acid and nitric acid (1.7 mM each) in water, with a flux

rate of 0.9 mL/min. Volume of sample inserted to the chromatograph was 10 µL. Temperature, pH, and conductivity were measured *in situ* with a multiparametric probe.

X-Ray diffraction (XRD)

All determinations were done in the X-Ray Diffraction Laboratory, UNAM. An EMPYREAN Diffractometer equipped with an Fe filter, a cobalt thin tube focus and PIXcel3D detector was used to analyze the mineral composition of the microbialites. XRD analysis conditions were as follows: samples were homogenized with an agate mortar, sieved through a mesh <75 µm, and measured using an aluminum sample-holder (non-oriented fractions). All measures were carried in the angular range of 2θ from 5° to 80° in step scanner mode with a "step scan" of 0.003° with a scan speed of 40 sec per step. The diffraction patterns were analyzed with the HighScore software (version 4.5) with reference patterns from the ICDDPDF-2 and ICSD databases.

Total DNA extraction and 16S rRNA amplification

Microbialite samples were extracted using the DNeasy PowerSoil® Kit (Qiagen) following the manufacturer's protocols. Duplicate samples for each depth were used for the extraction ($n = 10$ extraction/depth). Total DNA was eluted in a final volume of 30 µl molecular grade water and stored at −20 °C prior to PCR amplification. DNA was verified by gel electrophoresis and amplified (prokaryotic hypervariable region V4, 16S rRNA) using primers 515 F/806R, according to established protocols (Caporaso et al. 2011). PCR amplification conditions were performed in three independent reactions applied to each sample. All PCRs were run with the following program: 98 °C for 30 s followed by 35 cycles of 95 °C for 30 s, 52 °C for 40 s, and 72 °C for 90 s, with a final elongation step of 12 min at 72 °C, and kept at 4 °C. PCR products were pooled and purified with Ampliclean magnetic beads (NimaGen, NDL). The quantification of the purified amplicon library was performed with a QUBIT® fluorometer (Promega, USA). The final concentration of libraries per sample was 20 ng/µl and were sequenced on a 2 × 300 Illumina MiSeq platform (Yale Center for Genome Analysis, CT, USA).

Water from each sampling depth (1 Lt) was filtered into 0.22 µM DURAPORE (Millipore) membranes and total DNA was extracted following the protocol of the DNeasy PowerWater® Kit (Qiagen). All used membranes per depth were pooled together to obtain a representative composition. DNA was extracted, the 16S rRNA region V4 and amplified and sequenced as mentioned above.

Bioinformatic processing of illumina 16S rRNA sequences and biostatistical analysis

The sequences generated in this study (V4 region, 16S rRNA) belonging to 40 microbialite samples (10 samples/depth) and four water column samples have been submitted to the Sequence Read Archive (SRA, Leinonen et al. 2011) and deposited in GenBank under BioProject PRJNA630412. Raw sequences were imported and processed in QIIME2 (QIIME 2, v.2018.6) (Bolyen et al. 2019), sequences were clustered into ASVs and were assigned taxonomy using the SILVA database (release 132–99% OTUs, 515–806 region) (Quast et al. 2013). Based on quality plots, forward and reverse reads were truncated at their 3′ end at the 200 sequencing positions, respectively. Chimeric sequences were removed using the dada2 pipeline (Callahan et al. 2016). ASV's (amplicon sequence variants) were grouped to 100% sequence similarity. Sequences were aligned using MAFFT (Katoh and Standley 2013), and rooted trees were constructed using FastTree for analysis of phylogenetic diversity (Price et al. 2009).

Subsequently, all sequence data were analyzed in the R statistical environment (version 3.6.3), with Phyloseq R (McMurdie and Holmes 2013), ggplot2 (v 2.1.0) (Ginestet 2011), and vegan (v2.3-3) (Oksanen et al. 2016), ampvis2 (Andersen et al. 2018) and BiodiversityR (Kindt and Kindt 2019) packages were used for data visualization and statistical testing. ASVs representing less than 1000 sequences across the dataset, all singletons plus chloroplast and mitochondrial sequence reads were eliminated using customized R scripts.

The prokaryotic (Bacteria + Archaea) genetic composition of microbialites was described along the depth profile, using sequences representing >2% of relative abundance at the phylum level. The biological diversity used to determine the similarity in the community structure of microbialites was explored using the alpha diversity metrics (Shannon and Simpson, observed ASV's and Chao1). Beta diversity was analyzed using a matrix of community composition (ASV's matrix) to obtain the community turnover along the depth profile. The dissimilarity in community structure between samples (Beta diversity) was evaluated with a Principal Coordinates Analysis (PCoA) with weighted UniFrac distance metrics (Lozupone et al. 2011) and with the Local Contribution to Beta Diversity (LCBD) metrics proposed by Legendre and De Cáceres (2013). Venn diagrams were constructed using the software Venny 2.1 (Oliveros 2007–2015). To visually inspect the structure of the prokaryotic community in the depth profile, a heatmap was generated by pheatmap (R package 1.0.7) (Kolde and Kolde 2015). For heatmap analysis, normalized counts were log transformed with the DEseq2 package (Love et al. 2014). Additionally, the composition of prokaryotes in the water column was analyzed (Supplementary Figure 2) and the sequences were deposited in BioProject PRJNA631060.

Statistical analysis

Discrimination between prokaryotic community composition along the depth gradient were resolved with a Canonical Analysis of Principal Coordinates (Anderson and Robinson 2003; Anderson and Willis 2003) and their *a priori* classification according to the depth profile, based on Bray–Curtis dissimilarities and 999 permutations. All the indices for alpha diversity were compared using tests of

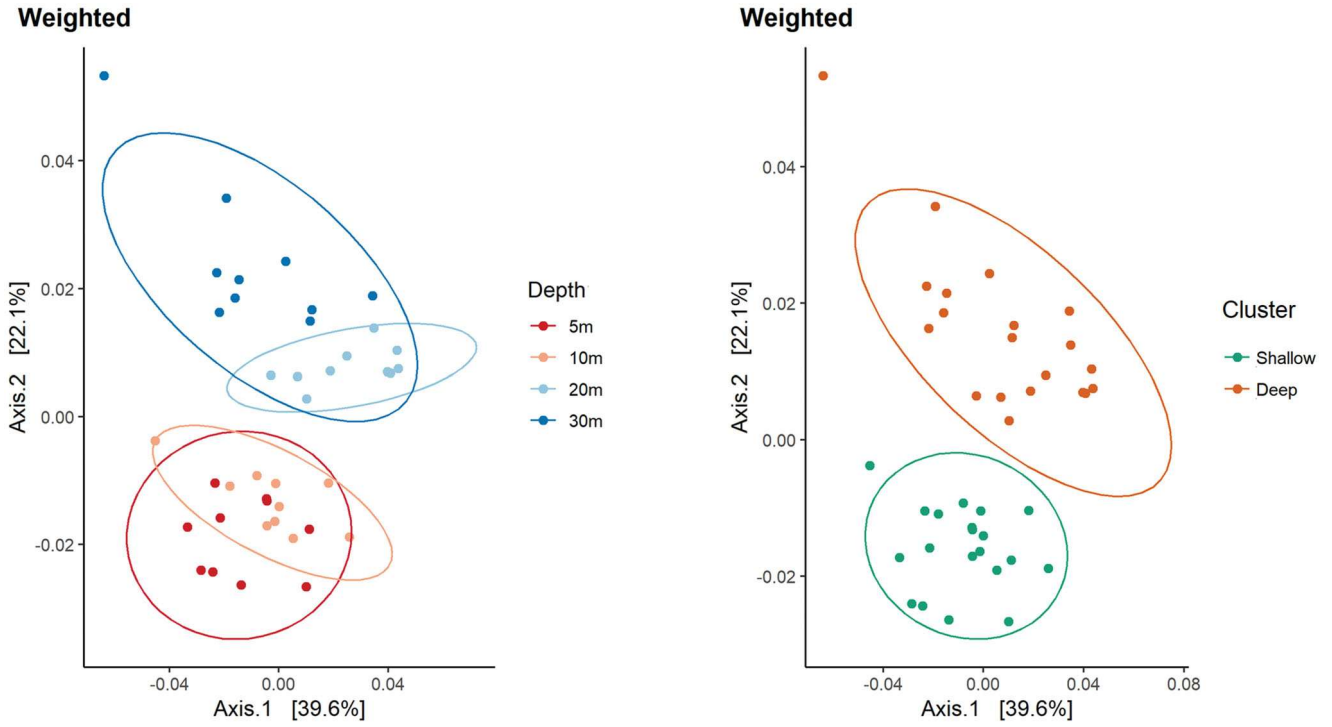

Figure 2. The analysis of the main coordinates (PCoA) based on the weighted UniFrac distances. (a) Microbialites in the depth profile. (b) Separation into two clusters of microbialites according to their phylogenetic assemblage.

Mann–Whitney–Wilcoxon. For beta-diversity, PERMANOVA analyses were performed using the Adonis function of the vegan package, based on the weighted UniFrac matrix. All correlations and tests with p-value < 0.05 were considered significant after 999 permutations.

Results

Hydrogeochemical characteristics of the Cenote Azul sinkhole

The physico-chemical parameters of the water column along the sampled depth profile are summarized in Supplementary Table 1. CA exhibited a homogeneous composition in all parameters with a range of values for pH (7.9–8.5), conductivity (2.31–2.29 µS/cm) and total dissolved solids (1.12–1.3 ppt) typical of the YP groundwater (Perry et al. 2002, 2012; Sánchez-Sánchez et al. 2015). The ions in the water column also had homogeneous values SO_4^{2-} $>HCO_3^-$ $>Cl^-$ $>NO_3^-$ and Ca^{2+} $>Mg^{2+}$ $>Na^+$ $>K^+$ (Supplementary Tables 2 and 3). Nitrates were only detected at 5 m (NO_3^- = 28.1 mg/L) along with the highest value for chlorine (95.2 mg/L). Some ions and compounds were not detected. For these, the following detection limit ranges were used: ammonium (LD = 0.2 mg/L), nitrites (LD = 2 mg/L), phosphates (LD = 0.2 mg/L), bromine (LD = 0.8 mg/L), fluorine (LD = 5 mg/L). No significant gradient in hydrogeochemical parameters was recorded.

Mineralogy of microbialites of Cenote Azul

The mineral composition of the microbialites in the depth profile are shown in Supplementary Table 4. Microbialites from 5 m were not as hard as the rest (10–30 m). Calcite made up 90–97% of the mineral composition of all microbialites. Traces of other minerals were found including quartz and hematite, with a smaller percentage of magnesite, while rhodochrosite was detected only at 10 m.

Diversity and structure of the prokaryotic community of the microbialites of Cenote Azul

An initial dataset of 7,080,965 16S rRNA sequences was recovered, where a total of 5,032,627 high-quality reads were obtained from the 40 samples after quality filtering, ranging from 15,458 to 420,654 with a mean of 127,002 reads per sample. A total of 19,306 ASVs were observed for all samples. Water column samples had an initial dataset of 223,336 16S rRNA sequences; a total of 91,288 high-quality reads were obtained from eight samples after quality filtering, ranging from 6,134 to 17,143 with a mean of 11,829 reads per sample. A total of 1,687 ASVs were registered for water column samples (Supplementary Figure 2), which are different in composition to microbialites.

Principal coordinate analysis (PCoAs) using weighted UniFrac distances were performed to investigate the community structure of the microbialites (Figure 2). Based on the PCoAs, the weighted UniFrac matrix explained up to 61.7% of the variation in genetic composition related to depth (Figure 2(a)) and forming two clusters: Shallow (5 and 10 m) and Deep (20 and 30 m) (Figure 2(b)). To statistically support the observed clustering of the prokaryotic diversity in the above PCoAs, the communities of the two profiles were examined with a PERMANOVA test using the weighted UniFrac matrix (Supplementary Table 5). The

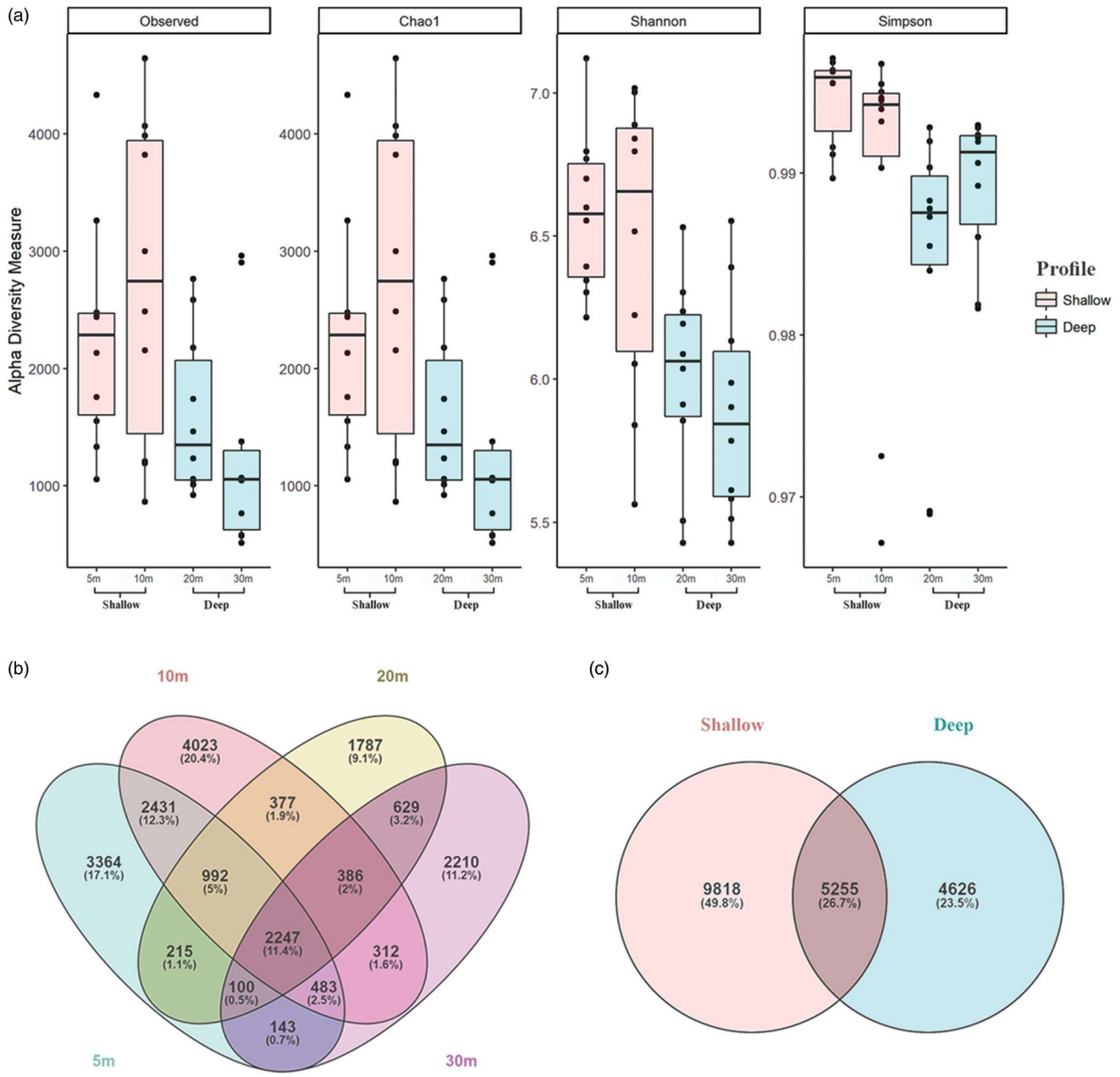

Figure 3. Diversity indices and the ASVs shared between the clusters. (a) Boxplot representation of the α-diversity and richness metrics for communities at depth profile and cluster. (b) Venn diagrams of unique and shared ASVs in the depth profile and (c) in depth cluster.

microbialites from the four sampled depths were distributed in high percentages for the first two components and gave significantly different results ($p < 0.05$), suggesting depth as an explanatory factor in the structure of the microbialite genetic diversity (Figure 2(a)). Notably, the cluster distribution between Shallow and Deep was also significant ($p < 0.05$) supporting these depth clusters, which also have different phylogenetic assemblages (Figure 2(b)).

A classification analysis (CAPdiscrim) based on the composition in the different sampling points (5,102,030 m) showed an *a priori* classification success of the samples, in significant proportions of 93% (Supplementary Table 5). The exploration of community diversity and distribution is presented in Figure 3. Shannon, Simpson, Observed and Chao1 indexes were calculated to estimate and compare richness and diversity in all microbialites (Figure 3(a)), which were significantly different ($p < 0.05$) between the Shallow and Deep clusters.

Beta diversity analysis showed a change in the community structure between Shallow and Deep clusters, first visualized in the PCoA based on the UniFrac matrix, and later corroborated through the LCBD index ($p < 0.05$). Additionally, the LCBD index showed that the most significant changes occur in the prokaryotic assemblages at depths of 5 and 30 m, $p < 0.05$. The microbialites sampled at 10 m showed the highest values of diversity and richness. Venn diagram allowed us to represent the core of ASVs, i.e., those ASVs that are shared in all microbialites, was represented by

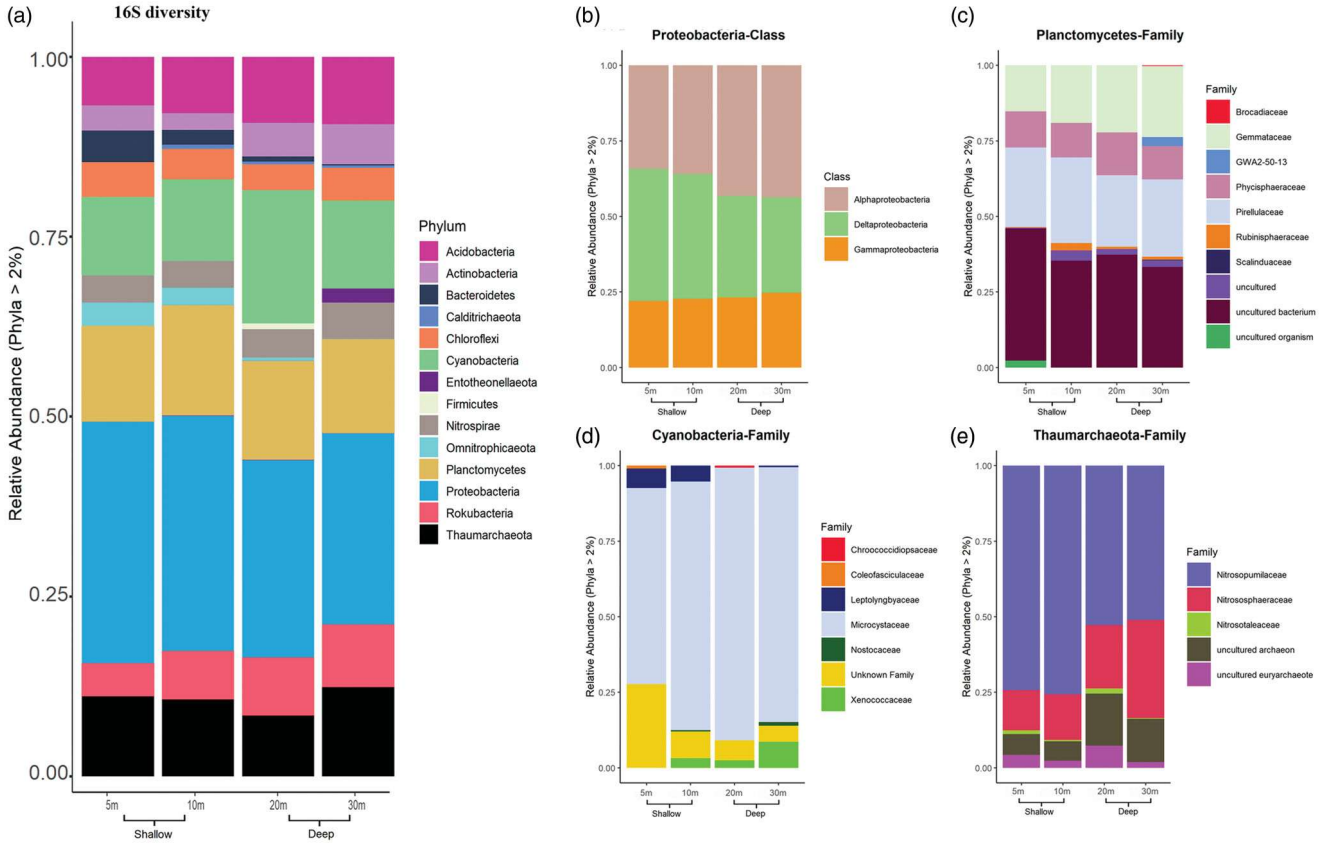

Figure 4. Distribution and relative abundance of the dominant prokaryotic communities from microbialites. Classified at the (a) phylum level; (b) Proteobacteria (class level). (c) Planctomycetes (family level). (d) Cyanobacteria (family level), (e) Thaumarchaeota (family level). All recovered sequences grouped in ASVs with a frequency higher than 2%.

11.4% (Figure 3(b)), where microbialites at 10 m have the highest percentage of shared ASVs (20.4%), while the lowest percentage is at 20 m (9.1%). Further, the ASVs representing the Shallow profile (49.8%) was higher than the deep profile and the core between clusters was 26.7% (Figure 3(c)).

A total of 54 different prokaryotic phyla constitute the microbiome associated with microbialites in CA. The core composition was represented (>2% abundance) by Proteobacteria (23.6–30.1%), Planctomycetes (11.6–13.8%), Cyanobacteria (9.7–16.5%), Acidobacteria (6.1–8.3%) Rokubacteria (4.1–7.8%), Chloroflexi (3.3–4.4%), Nitrospirae (3.5–4.6%), Actinobacteria and (2.6–5%) Bacteroidetes (1.7–4.1%) with Thaumarchaeota (7.5–11.1%) as the dominant archaea, all of them representing a total of 85.7% of the prokaryotic community (Figure 4). The core of the prokaryotic community composition is homogeneously distributed, where only some phyla show greater abundance, for example, Proteobacteria, Bacteroidetes and Omnitriophicaeota in the Shallow profile microbialites, and for the Deep microbialites including Cyanobacteria, Acidobacteria, Rokubacteria and Actinobacteria. The other phyla such as Thaumarchaeota, Planctomycetes and Chloroflexi are contributing similarly to both depth clusters.

Within Proteobacteria, Alpha (39.3%), Delta (37.6%) and Gamma (23.2%) were the dominant classes (Figure 4(b)). Planctomycetes groups a vast diversity, including previously uncultured organisms that constitute close to half the diversity within this phylum. The deepest microbialites (30 m) exhibit the presence of Brocadiaceae, which could suggest the relevance of anammox in the system (Figure 4(c)). Cyanobacteria showed dominance of Microcystaceae (approx 70%), followed by a previously undescribed family, which shall merit further investigation, and members of Xenococcaceae which increased with depth (Figure 4(d)). Another relevant component at the phylum level is the Thaumarchaeota (Archaea domain), in which Nitrosopumilaceae and Nitrososphaeraceae, represented up to 80% of the abundance (Figure 4(e)).

Prokaryotic community similarities

Changes in the structure of the prokaryotic community were represented in a hierarchical heat map (Figure 5(a)) that shows the differential abundances between the clusters (Shallow and Deep). A hierarchical dendrogram groups the samples in the depth profile and shows the grouping of the proposed clusters (Shallow and Deep). A mean sea-level line, based on the Caribbean sea-level curve proposed by Toscano and Macintyre (2003) was included to relate microbialites found in the depth profile to possible timelines of formation for these structures (Figure 5). The geomorphology of the microbialite wall in the CA sinkhole (Figure 5(b)) is represented based on the divers observations.

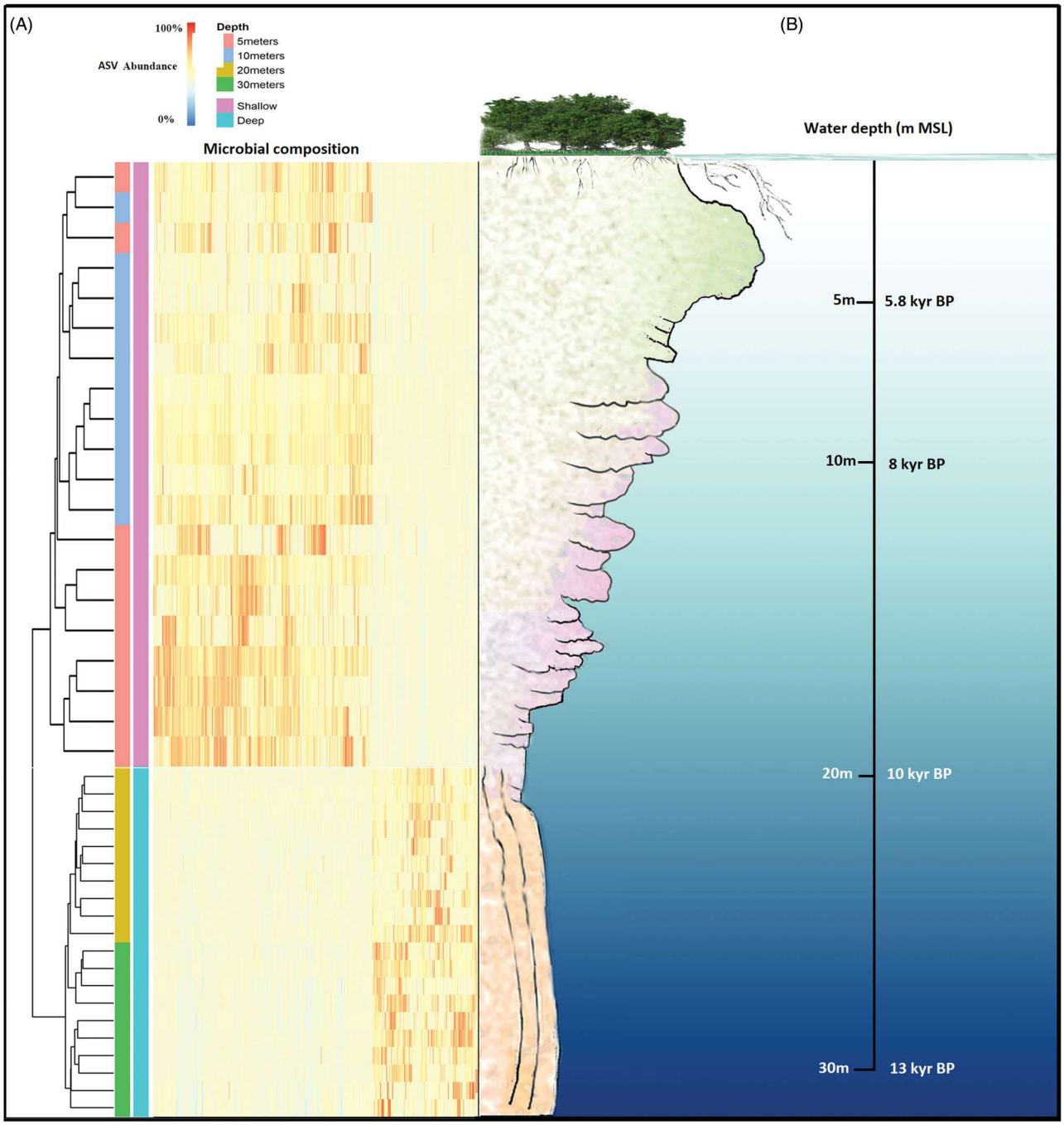

Figure 5. Structure of the prokaryotic community and the geomorphic representation of the wall of microbialites in the Cenote Azul sinkhole. (a) Heat map generated with DESeq2 that represents the community in the Cenote Azul sinkhole. The hierarchical dendrogram shows the differentiation of the community in the four sampling sites (5 m, 10 m, 20 m and 30 m) and the two depth profiles (Shallow and Deep). (b) Geomorphic representation of the microbialite wall and the distribution of the prokaryotic community. The line representing mean sea level was generated based on the local curve of Caribbean sea-level from Toscano and Macintyre (2003).

Discussion

This is the first report on the microbialites that develop in the Cenote Azul (CA) sinkhole providing a baseline on microbialite research in the region that opens the opportunity to monitor an extreme karst system, located in an area of accelerated tourism growth. This study corroborated the presence of a wall of microbialites in the CA (Hernández-Arana et al. 2015), a karstic sinkhole, which extends >90 m depth and found that the prokaryotic communities of the microbialites are phylogenetically associated in two clusters designated as Shallow (5 and 10 m) and Deep (20 and 30 m). While these microbialites depicted a depth related gradient, as noted in the hypothesis, additionally we suggest that differences in microbial assemblages in CA may be associated with local impacts of natural and anthropogenic surface processes, as well as, with the geomorphology, landscape evolution and the hydrological history of sea level in the southeast of the YP.

CA is a unique system for the study of microbialites along a depth gradient, with dissolved oxygen and

deuterium concentrations near the meteoric water line, creating slow evaporation processes (Perry et al. 2002, 2009). The hydrogeochemical variables indicate disconnection with the local coastal aquifer suggesting this sinkhole is recharged from a remote area as far as Esmeralda and Chichancanab lagoons (Perry et al. 2002). Saturation of sulfate ions, bicarbonates, calcium, and the unusual presence of chlorine, coincide with those reported for the water systems in the area, including Bacalar Lagoon (Castro-Contreras et al. 2014; Perry et al. 2012).

Calcite ($CaCO_3$) constitutes 90–97% of the mineral composition of microbialites, corroborating previous studies, where microbialites, in the southern region of QR, are reported to be composed mostly by calcium carbonate (Valdespino-Castillo et al. 2018; Yanez-Montalvo et al. 2020). In the YP, it is common for water bodies such as cenotes (sinkholes), lagoons and the aquifer to be saturated with bicarbonate ions as a consequence of the processes of dissolution of the limestone (Perry et al. 2002). Valdespino-Castillo et al. (2018) suggest through SEM observations coupled to SR-FTIR imaging that calcite-rich microbialites in Bacalar lagoon are associated with organic structures formed of lipid and protein amides that deposit in the organomineral fabrics. The mineral composition of the CA sinkhole analyzed does not differ from the microbialites in Bacalar lagoon, which have the highest content of C_{org} reported for these structures in the YP. Further research is required to better understand the dynamics of carbonate precipitation and formation in depth microbialites. Calcite, the main mineral of CA microbialites, occurs through the precipitation of calcium carbonate, which requires supersaturation of the carbonate ion in the system, availability of Ca^{2+} ion and the appearance of nucleation centers in the EPS, produced mainly by Cyanobacteria (Benzerara et al. 2014; Dupraz et al. 2009; Dupraz and Visscher 2005; Obst et al. 2009). Other bacterial phyla such as Chloroflexi, Alpha-, Delta-proteobacteria and Planctomycetes present in CA microbialites, have been described in microbialites and are postulated to promote the precipitation of carbonate minerals through photosynthesis (oxygenic and anoxygenic) and sulfate reduction (Bundeleva et al. 2012; Gallagher et al. 2012; Visscher et al. 2000).

The main phyla reported as constituents of CA microbialites (Proteobacteria, Planctomycetes, Cyanobacteria, Thaumarchaeota, Rokubacteria, Chloroflexi, Nitrospirae, Actinobacteria and Bacteroidetes) (Figure 4) are similar to those reported around the world for both marine: Highborne Cay, Bahamas (Baumgartner et al. 2009), Shark Bay, Australia (Suosaari et al. 2016) and freshwater microbialites: Cuatro Ciénegas, Mexico (Souza et al. 2012), Salar de Atacama, Chile (Farías et al. 2014), as well as those reported in depth such as Lake Van, Turkey (López-García et al. 2005), Pavilion lake, Canada (Russell et al. 2014), Lake Alchichica, Mexico (Centeno et al. 2012; Couradeau et al. 2011), including the microbialite reef that develops in Bacalar lagoon (Centeno et al. 2012; Johnson et al. 2018; Valdespino-Castillo et al. 2018; Yanez-Montalvo et al. 2020). Although the spatial arrangement based on phylogenetic distance (weighted UniFrac) indicates that there are two large clusters (Shallow and Deep), when we analyze their composition at higher taxonomic levels, these differences are not appreciable, thus suggesting that their composition is similar at the phylum level, but the greatest differences are found at the genus and species level (Figure 4), this idea has been discussed previously (Centeno et al. 2012; De Anda et al. 2018; Lindsay et al. 2017; Yanez-Montalvo et al. 2020). In this study, the CA does not present an environmental gradient along the depth profile related to hydrogeochemistry, but previous research has suggested that light availability and temperature decline rapidly after 10 m depth in YP open sinkhole systems (Cervantes-Martínez et al. 2002; Schmitter-Soto et al. 2002).

In microbialites, oxygenated photosynthesis by cyanobacteria is a common metabolic process for carbonate precipitation in freshwater and marine environments (Chagas et al. 2016). Cyanobacteria, both filamentous and unicellular, have been found to be more abundant in thrombolytic-type microbialites (Chagas et al. 2016). Thrombolites are the main types of microbialites reported for Bacalar lagoon (Beltrán et al. 2012; Centeno et al. 2012; Johnson et al. 2018; Valdespino-Castillo et al. 2018; Yanez-Montalvo et al. 2020) and this study. CA microbialites have a strong component of unicellular colonial Microscyteceae (Figure 4(d)). Two sequences of this family, (uncultured) are among the 10 most abundant genus-level taxa (Supplementary Figure 3). These sequences may belong to an uncultured *Cyanothece* sp. or *Aphanothece* sp., which were observed abundantly by optical microscopy as endoliths in all microbialite samples. Microscyteceae are reported to be efficient calcifiers (Bundeleva et al. 2014). Other families such as Chroococcidiospidaceae are tolerant to high amounts of UV radiation and extreme temperatures (Das and Singh 2017; Lacap-Bugler et al. 2017) and were found in the Deep microbialite cluster. Coleofasciculaceae, a family within Oscillatoriales, was found only in Shallow samples, along with a high percentage of unclassified sequences that could be affiliated to filamentous Synechococcales which were confirmed with microscopy. Leptolyngbyaceae was more abundant in the Shallow CA-microbialites, while Pleurocapsales were more abundant in the Deep microbialites. Pleurocapsales have been reported to be important for carbonate mineral precipitation and microbialite formation in some oligotrophic lake systems (Gérard et al. 2013; Power et al. 2011). *Scytonema* sp., was also common in Shallow samples. This distribution pattern of species of the family Leptolyngbyaceae in Shallow microbialites and the change toward species of the family Xenococcaceae in the Deep profiles, is a phenomenon reported in microbialites from Lake Alchichica (Águila 2018; Saghaï et al. 2015) and Lake Pavilion (Russell et al. 2014).

Cyanobacteria and Proteobacteria are the dominant phyla in microbialites (Baumgartner et al. 2009; Foster et al. 2009; Khodadad and Foster 2012), and together comprised approximately 50% of the prokaryotic diversity in CA microbialites, regardless of depth. Delta- (41–43%) and Alpha-proteobacteria (43%) are the most abundant classes,

in Shallow and Deep profiles, respectively, while Gammaproteobacteria (22–24%) are distributed homogeneously, these groups were also dominant in Pavilion Lake microbialites, a similar karst system (Russell et al. 2014). Certainly, Cyanobacteria and Proteobacteria are the major known diazotrophs in these communities, where the potential for N_2-fixation becomes relevant to sustain these complex microbial assemblages (Alcántara-Hernández et al. 2017; Beltrán et al. 2012). Bacalar lagoon microbialites are known to fix N_2 during the day, suggesting the relevance of cyanobacterial heterocyst-forming filamentous colonies (Beltrán et al. 2012). CA microbialites harbor Nostocales corresponding to *Fischerella* sp., but probably many other N_2-fixers are part of these assemblages.

Alpha-proteobacteria are a group of bacteria with a high metabolic diversity (Havemann and Foster 2008; Ley et al. 2006). Alphaproteobacteria with anoxygenic and heterotrophic phototrophic metabolisms are among the most abundant in microbialites (Gérard et al. 2013). For CA, Rhizobiales, non-sulfur purple photoheterotrophic bacteria, were very abundant (Supplementary Figure 4). This group has been reported to play an important role in Highborne Cay and Shark Bay microbialites (Foster and Green 2011). Alpha-proteobacteria have also been studied for the P-assimilation potential in microbialites, in conjunction with Acidobacteria (Valdespino-Castillo et al. 2014), another common constituent of CA microbialites. Another relevant anoxygenic phototroph, Chloroflexi (Shih et al. 2017) was a constant component of microbialites along all sampled depths in CA microbialites.

Further, Delta-proteobacteria, host the group of sulfate-reducing bacteria (SRB), which reduce sulfate to sulfur, oxidize organic carbon to bicarbonate, and contribute to form an alkaline environment within microbialite fabrics, which in turn is conducive to carbonate mineral precipitation (Baumgartner et al. 2006; Couradeau et al. 2011). Deltaproteobacteria were present in all CA microbiates. Sulfur-cycling Deltaproteobacteria are also typically associated with microbialites and their surrounding sediments (Warden et al. 2016; White et al. 2016). Sulfate reducing bacteria are observed in close proximity to cyanobacteria in oxic portions of hypersaline mats in Guerrero Negro Lagoon, Baja California, Mexico (Baumgartner et al. 2006; Canfield and Des Marais 1991), and in the Bahamas (Reid et al. 2000) suggesting tight biogeochemical cycling between these two groups of organisms (Visscher et al. 2000; Visscher and Stolz 2005). The most abundant Deltaproteobacteria in CA microbialites included several NB1-j, Myxococcales, Desulfarculales and Syntrophobacterales (Supplementary Figure 4). Most sulfate reducers are found in the Deltaproteobacteria class and additionally, some Firmicutes, Nitrospirae, and Archaea are known as sulfate-reducers (Loy et al. 2002; Muyzer and Stams 2008).

In the CA microbialites, distribution of the abundance of the Gammaproteobacteria is independent of the depth profile (Figure 4(b)). Bacteroidetes and sulfate-reducing (Deltaproteobacteria) are reported in microbialites as well as Alpha-proteobacteria and Planctomycetes (Centeno et al. 2012; Couradeau et al. 2011). Betaproteobacteria, Delta- and Gamma-proteobacteria, Actinobacteria, Acidobacteria, Nitrospirae, and Flavobacteria are observed in Pavilion Lake microbialites (Russell et al. 2014). Cuatro Ciénegas microbialites also harbor populations of Delta- and Gamma-proteobacteria and Nitrospirae (Breitbart et al. 2009; Nitti et al. 2012), another group with possible relevance to N-cycling. Nitrospirae, a phylum involved in the nitrification process, was a constant component of CA microbialites. So far, we do not know if the *Nitrospira*, found in great abundance in the microbialites of CA (Figure 4), has the potential to do complete nitrification (Comammox), which will be clarified when a metagenomic approach is followed in this system (Ehrich et al. 1995; Koch et al. 2019). *Nitrospira* were also found along the depth gradient, constituting approx. 25% of accounted diversity. Planctomycetes were represented by approx. 50% of previously undescribed groups. We found an abundance of families that have been described as having interesting metabolisms and in-depth sample studies, such as Gemmataceae, a family of aerobic chemo-organotrophs (Kulichevskaya et al. 2020), Pirellulaceae, which are ammonia oxidizers and Phycisphaeraceae are the main components (Kellogg 2019; Mohamed et al. 2010). Interestingly, Brocadiaceae, reported as a family with known anammox bacteria (Pereira et al. 2017), were detected in the microbialites from 30 m.

Thaumarchaeota, the most abundant archaea known on Earth, includes all recognized archaeal ammonia oxidizers (Kimble et al. 2018). This archaeal phylum is distributed in all the depths of the CA, and its main families (Nitrosopumilaceae, Nitrososphaeraceae), have been recorded as nitrifying groups (Wong et al. 2015). This phylum has been reported in oligotrophic aquatic environments such as caves and also in microbialites in Lake Salda (Turkey) and thrombolites in Highborne Cay, Bahamas (Balci et al. 2020; Mobberley et al. 2015). Interestingly Rokubacteria was found in all samples, increasing their abundances toward 30 m. Rokubacteria is a recently described group, with a potential role in methane oxidation, reported to be found in soils, the rhizosphere, volcanic muds, oil wells, and aquifers (Chernov et al. 2019; Kroeger et al. 2018). They have small cells with large genomes with a high percentage of GC and with general metabolic strategies (mixotrophic) in oligotrophic environments; they also have a high genomic heterogeneity between individuals (Becraft et al. 2017).

CA is a deep sinkhole (>90 m), and we do not know how much deeper microbialites develop along its wall. Similar systems, such as Pavilion lake (Canada), Alchichica crater lake (Mexico) and Lake Van (Turkey) have already reported the presence of microbialites in depths up to 45 m (Águila 2018; López-García et al. 2005; Russell et al. 2014). Interestingly, CA microbialites were different in composition and abundance with the presence of groups such as Acidobacteria, Nitrospirae, Rokubacteria and Thaumarchaeota; the genetic diversity was higher for CA microbialites ($H' = 5.9–6.5$), compared to those of Bacalar lagoon ($H' = 3.3–5.7$), a system separated to CA by 200 m

(Yanez-Montalvo et al. 2020). The particularity of CA in comparison to Bacalar lagoon was manifested in a comparative study of zooplankton, where it showed the presence of species exclusive to CA, and concluded that these systems had low connectivity, despite their proximity (Montes-Ortiz and Elías-Gutiérrez 2018).

The phylogenetic separation of the prokaryotic communities along the depth gradient found in CA microbialites might be related to the sea-level variation that YP has experienced (Figure 5). The current structure of YP was reached in the Pleistocene era (López-Ramos 1975; Torrescano-Valle and Folan 2015); during the last glacial maximum (LGM), and different eustatic changes have been experienced at spatial and temporal scales, where the sea-level was lower than present (about 130 m) (Back and Hanshaw 1970; Ward and Halley 1985). Several studies have described the possible evolution of sea-level in the Caribbean during the Holocene, their measurements were based on approximately 737 points covering a period of 12 ky BP, taken from samples of mangrove peat, microbial mats, beach rock and corals (Cuevas et al. 2008; Gabriel et al. 2009; Khan et al. 2017). The Holocene records of sea-level have been highly variable, depending on the coastal environment features and its relation to sinkholes. Numerous works have described coastal environmental changes that influence sea-level in sinkholes in northern and southern QR including the Rio Hondo region and Chetumal Bay, near the Belizean border (Torrescano and Islebe 2006; Gabriel et al. 2009; Aragón-Moreno et al. 2018). The works carried out for the QR area agree with those indicated in the calibrated sea-level curve of Toscano and Macintyre (2003). Based on sea level changes and environmental coastal interactions with sinkholes which indicates that sea-level 10 ky BP was around −25 to −30 m, and may indicate that the microbialites that develop >30 m in the CA are older (Deep profile) than those in the Shallow cluster. The communities at 30 m have had to adapt to the changing ecological conditions, bathymetry and light intensity and hydrogeochemical changes throughout the Holocene period, but also the accretion rate of these communities have balanced with the sea-level changes as we can tell due to the continuous microbialite structure along the depth gradient.

In this study of microbialites occurring in the CA, most of the variables measured in the water column were homogeneous. Only in the most surficial sample (5 m), where the microbialites were more friable and green color (Figure 1), the environmental conditions varied by the presence of the nitrate and an increase in chlorine ions. The presence of these ions is in concentrations comparable to Bacalar lagoon, and their values may be associated with natural runoff and anthropogenic activities in the region. The depth of 10 m has more solid microbialites and lavender color, it is the maximum depth where the Secchi disk is observed. Previous research has suggested that the availability of light and temperature decreases rapidly after 10 m depth in YP open-sink systems (Schmitter-Soto et al. 2002; Cervantes-Martínez et al. 2002). The available light and other environmental conditions can make the 10 m niche conducive to a more transient microbial assembly, since at this depth the highest number of ASVs were detected.

As a whole, this study proposes that the Shallow profile (5 and 10 m) is a dynamic ecosystem of environmental variables, the microbial assemblies at this depth gradient present greater biological interactions and environmental forces. Interestingly, the Deep profile (20 and 30 m), is a habitat with a stable dynamism, the mean alpha diversity, richness and particulate ASVs were lower compared to the Shallow profile (Figure 3). We consider that a prokaryotic community acclimated to the natural and historical conditions of the site is maintained in this range, constituting an extreme niche due to its oligotrophic conditions, hydrogeochemistry and presenting practically no recent anthropogenic alterations. It is recognized that microbial assemblages from extreme environments contain community members that may represent autochthonous or indigenous species, adapted to the set of physical, chemical and biological parameters of the site (Panikov 2010).

Microbialites in the CA sinkhole support a diversity of prokaryotic associated with the biogeochemical cycles of N, C, S and P. We consider that the good health status (based on diversity and species composition) of the microbialites in the CA responds to the current water quality. The model of microbialites as bioindicators of microbial communities becomes relevant in systems such as CA, which are in regions with intense human activities including tourism, lack of wastewater treatment and agriculture based on an intense use of synthetic fertilizer compounds. Generating the basic knowledge on the prokaryotic composition associated with CA microbialites (in southern QR) provides a guideline for future work focused on their metabolic potential and how these communities respond to changing temporalities, anthropogenic regional pressures and global climate change, which will allow the development of effective strategies for their management and sustainability.

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